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Chitosan as a non-viral co-transfection system in a cystic fibrosis cell line

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Abstract

Successful gene therapy requires the development of suitable vehicles for the selective and efficient delivery of genes to specific target cells at the expense of minimal toxicity. In this work, we investigated a non-viral gene delivery system based on chitosan (CS) to specifically address cystic fibrosis (CF). Thus, electrostatic self-assembled CS-pEGFP and CS-pEGFP-siRNA complexes were prepared from high-pure fully characterized CS (Mw ~20 kDa and degree of acetylation ~30%). The average diameter of positively-charged complexes (i.e. ζ ~ +25 mV) was ~200 nm. The complexes were found relatively stable over 14 h in OptiMEM. Cell viability study did not show any significant cytotoxic effect of the CS-based complexes in a human bronchial cystic fibrosis cell line (s). We evaluated the transfection efficiency of this cell line with both CS-pEGFP and co-transfected with CS-pEGFP-siRNA complexes at (N/P) charge ratio of 12. We reported an increase in the fluorescence intensity of CS-pEGFP and a reduction in the cells co-transfected with CS-pEGFP-siRNA. This study shows proof-of-principle that co-transfection with chitosan might be an effective delivery system in a human CF cell line. It also offers a potential alternative to further develop therapeutic strategies for inherited disease treatments, such as CF.

1. Introduction

Chitosan is the main derivative of chitin, the second most abundant polysaccharide in nature. It is a linear biodegradable polysaccharide composed of randomly distributed β(1–4)-linked D-glucosamine and N-acetylglucosamine units (Ravi Kumar, 2000; Rinaudo, 2006). The relative proportion of positive charges provided by the protonation of the glucosamine units under slightly acidic conditions and the molecular weight of chitosan play an important role in the development of new applications (Grenha et al., 2010; Tan, 1998). Chitosan exhibits several properties that makes it in an interesting material for pharmaceutical formulations. It induces low cytotoxicity, is biocompatible, biodegradable, and mucoadhesive (Rinaudo, 2006; Younes and Rinaudo, 2015; Menchicchi et al. 2014). These properties along with its polycationic character make of chitosan a potential unique candidate as a gene delivery system. The first report on using chitosan to complex DNA and evaluate it as a non-viral delivery system for a plasmid dates from 1995 (Mumper et al., 1995). Driven by electrostatic interactions, chitosan-pDNA complexes have been used for transfection of mammalian cells both in vitro and in vivo (Koping-Hoggard et al., 2001; Romøren et al., 2003; Vauthier et al., 2014). Nevertheless, results of transfection efficiency using chitosan-based systems are strongly dependent on chitosan properties (e.g., molecular weight and the relative amount of N-acetylglucosamine units, namely degree of acetylation (DA)) (Lavertu et al., 2006; Santos-Carballal et al., 2015; Strand et al., 2005). Chitosan has been reported as a suitable candidate for transmucosal administration of drugs (Grenha et al., 2010). In addition, it has been observed that after intratracheal administration, the complexes using CS were found in the mid-airways, and transgene expression was observed in epithelial cells (Koping-Hoggard et al., 2001). In general, gene therapy, based on the use of chitosan as a non-viral vector, has been extensively considered in the last decade or so (Gomes et al., 2014).

Gene therapy may lead to new strategies to address life-threatening respiratory diseases such as cystic fibrosis (CF). CF is the most lethal inherited disease in the Caucasian population characterized by chronic airway inflammation (Jennings et al., 2014). The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Kerem et al., 1989), which encodes for a protein that, among different functions, includes the cAMP-dependent chloride channel. CFTR is expressed in the epithelia of several exocrine tissues such as airways, lung, pancreas, liver, intestine, vas deferens, and sweat gland/duct (Welsh and Smith, 1993). The impaired CFTR protein would lead to alterations in the transport of ions and homeostasis across epithelial barriers (Cantin et al., 2015). Subsequently, it causes sticky mucous secretions that impede mucociliary clearance (Boucher, 2007). The consequences are chronic inflammation and recurrent bacterial infection (Davis, 2006), leading to the progressive destruction of the lung tissue. Altogether, the pulmonary disease accounts for the main cause of mortality in CF (Gibson et al., 2000). Therefore, the correction of the defective CFTR gene, offers to be the most attractive solution for this disease. Gene therapy focused in the use of viral carriers has been widely studied in CF treatments due to the high transfection efficiency reported (Conese et al., 2011). However, the use of viruses as vectors raises many concerns regarding possible immune response, its biosafety and severe inflammation after long periods of administration (Griesenbach and Alton, 2012). Therefore, non-viral vectors have emerged as a safer alternative (Armstrong et al., 2014) and only few researches have addressed chitosan as a potential gene delivery vector for CF (McKiernan et al., 2013; Nydert et al., 2008).

The aim of this study is to investigate the potential of chitosan-based self-assembled electrostatic complexes as a transfecting strategy towards human airways epithelial cells. To this end, we designed a co-transfection approach based on a reporter plasmid enhanced green fluorescent protein (pEGFP) and its knockdown siRNA sequence and evaluated its efficacy in a cystic fibrosis bronchial epithelial cell line (CFBE41o−). To the best of our knowledge, this

is the first report using chitosan as a carrier to simultaneously deliver two functional nucleic acids. In general, this study seeks the potential use of chitosan as a transfection reagent for human airways epithelium.

2. Materials and methods

2.1 Preparation of complexes

Ultra-pure biomedical grade chitosan used to prepare the complexes was provided by HMC+ (Halle, Germany; Code 70/5 Product No. 24200, Batch No. 212-170614-01; DA = 30%, Mw = 20 kDa based on the manufacturer's specifications). The chitosan was stoichiometrically dissolved in HCl (5% stoichiometric excess of equivalent D-glucosamine of chitosan) overnight at room temperature to a stock concentration of 5 mg/mL, and then diluted with milliQ water to reach the desired concentration. A series of complexes were prepared at different charge (N/P) ratios, (defined as the molar ratio of amine to phosphate groups) by mixing the chitosan working solutions with a constant amount of pEGFP-C1 (1 µg) or pEGFP-C1 (1 µg)/siRNA (2.5 pmol/cm^2) (Table 1). The mixtures were incubated for 30 min at room temperature to form the self-assembled complexes.

Table 1. Composition of the chitosan-nucleoitide complexes of varying molar charge ratios

N/P Ratio[a]	pEGFP-C1 (nmol)[b]	siRNA (nmol)[b]	Total (nmol)[c]	Chitosan (nmol)[d]
0.1	6.10	--	6.10	0.610
5	6.10	--	6.10	30.5
8	6.10	--	6.10	48.8
12	6.10	--	6.10	73.2
0.1	6.10	0.11	6.21	0.621
5	6.10	0.11	6.21	31.1
8	6.10	0.11	6.21	49.7
12	6.10	0.11	6.21	74.6

[a] Molar ratio of equivalent charges of $-NH_3^+/-PO_4^-$

[b] Equivalent concentration of $-PO_4^-$ from nucleic acid

[a] Total equivalent concentration of $-NH_3^+$ and $-PO_4^-$ from chitosan and nucleic acids, respectively.

[a] Equivalent concentration of $-NH_3^+$ from chitosan

2.2 Size distribution and zeta potential of complexes

The size distribution of the CS-nucleoitide complexes was determined by dynamic light scattering with non-invasive back scattering (DLS-NIBS) at an angle of 173° with an automatic attenuator setting. The zeta potential (ζ) was determined from the electrophoretic mobility by mixed-laser Doppler electrophoresis and phase analysis light scattering (M3-PALS), using the well-known Henry's equation and Smoluchowski's approximation as reported in our previous studies (Menchicchi et al., 2015). Both parameters were measured using a Malvern Zetasizer NANO-ZS (Malvern Instruments, Worcestershire, UK) equipped with a 4 mW He/Ne laser beam (λ = 633 nm).

2.3 Gel retardation assay

The binding strength of pEGFP-C1 and siRNA with CS was determined by agarose gel electrophoresis method. Complexes prepared with different N/P charge ratios as described above, ranging from 0.1 to 12, were loaded onto 1.5 % agarose gel in 0.5 x TBE buffer supplemented with 1.25 µL of ethidium bromide (10 mg/mL) and electrophoresed at 128 V for 40 min. Finally, the DNA bands were visualized using a UV illuminator BioDocAnalyze System (Biometra, Göttingen, Germany).

2.4 Stability of complexes

The stability of the complexes was assessed by diluting the previously described quantities (Table 1) with 100 µL of OptiMEM (Life Technologies) and subsequently by incubating them during 14 h at 37 °C. The stability was evaluated by measuring the evolution of the hydrodynamic ratio, determined as described above.

2.5 Cell studies

2.5.1 Metabolic capability (MTT assay)

Evaluation of cytotoxicity was studied by the MTT assay. Briefly, CFBE41o- cells were seeded in 96-well plates at a density of 10,000 cells/well and incubated for 24 h at 37°C, 5% CO_2. The complexes with chitosan were prepared under the same conditions used for transfection experiments and incubated for 30 min at 37°C. Cells were washed twice with MEM serum-free medium. The different treatments were applied to the cells and incubated them for 4 h at 37°C and 5% CO_2. Cell proliferation and viability were determined by measuring dehydrogenase activity. We added 25 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/mL) to each well containing 100 µL of fresh MEM serum-

free medium. Cells were incubated for an additional 4 h at 37°C and 5% CO_2 to allow the formation of a purple formazan salt. The medium was replaced with 100 µL dimethylsulfoxide and the plates were incubated for a further 15 min at 37°C and 5% CO_2. Absorbance was measured at 570 nm using a Micro Plate Reader (SAFIRE II, Tecan Group Ltd., Männedorf, Switzerland). Cell viability was calculated according to the following equation:

$$Cell\ viability\ (\%) = \left(A_{570\ (sample)} / A_{570(control)}\right) \cdot 100$$

where $A_{570\ (sample)}$ represents the absorbance measurement from the treated well with complexes and $A_{570(control)}$ represents the absorbance measurement from the control wells treated only with medium.

2.5.2 Transfection efficiency of complexes

To test the transfection efficiency in the cystic fibrosis bronchial epithelial cell line (CFBE41o-), pEGFP-C1 plasmid was used to introduce the reporter gene GFP. For control experiments, pEGFP-C1/GFP-small interfering siRNA, H_2O and non-transfected cells were used. Cells were seeded at of density of 0.5 x 10^5 per cover slip (diameter 12 mm) 3 days before transfection and cultivated in Eagle´s Minimal Essential Medium with L-glutamine (MEM) in addition to 10% fetal calf serum, 1% penicillin/streptomycin and 1% L-glutamine. On the day of transfection, all cells were grown close to confluence and ca. 3 h before transfection, they were cultivated in MEM without serum. Thus, cells were transfected with 1 µg of pEGFP-C1 or co-transfected with 2.5 pmol/cm^2 of GFP-specific siRNA or a respective amount of water. Cells were fixed after 24 h of incubation with 0.05 % glutaraldehyde in HEPES buffer for 10 min at 37°C in an incubator. Thereafter, the cells were washed once with HEPES buffer and subsequently with 1x PBS. Autofluorescence due to the presence of aldehyde groups from the glutaraldehyde was quenched by the addition of sodium borohydride. The solution was

removed and fresh sodium borohydride was applied again under the same conditions. Therefore, 2-3 wash steps were carried out with 1x PBS. Finally, the cover slips were washed once with Millipore H_2O, dripped and were placed top down in the object slides (Roth; Karlsruhe; Germany). Before use the object slides, a drop of Dako Fluorescent Mounting Medium (Dako; Glostrup; Denmark) was applied. After 24 h the transfection efficiency was determined by the analysis of the fluorescence intensities (λ_{ex} = 488 nm; λ_{em} = 509 nm) using a fluorescence microscope LSM 510 META (Carl Zeiss, Oberkochen, Germany). Images were recorded using AxioCamMRm and the LSM 510 4.2 SP1 software (Carl Zeiss, Oberkochen, Germany). For comparisons of total fluorescence intensities, exposure time was manually adjusted to 1 ms.

Analysis of fluorescence intensity data

Fluorescence intensities analyses were performed using ImageJ, version 1.41. The intensity of red, green and blue channels (RGB) was measured for each image. The value of the average ((R+G+B)/3) was compared between images. The average of non-transfected cells was set to 100% and the transfected average was normalized as a multiple of the non-transfected value.

Statistical analysis

Data are expressed as the arithmetic mean ± SD. Statistical analysis was carried out using GraphPad Software Prism v6 (San Diego, USA). MTT assays were statistically analysed using non-parametric tests using the Kruskal-Wallis test. The statistical analysis of the transfection assays data was done using Tukey multiple comparison test with a single pooled variance. Differences were considered statistically significant when $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***). All biological experiments were conducted at least in triplicate and with at least three technical replicates per experiment.

3. Results

3.1 Physicochemical characterization

Complexes were prepared using a stock CS solution containing a 5% stoichiometric excess of HCl and stock solutions of pEGFP-C1 and GFP-siRNA. Complexes with different N/P ratios (0.1, 5, 8 and 12) were formed and characterized in terms of their size, polydispersity and surface charge (zeta potential). In addition, the stability of the complexes was also studied.

3.1.1 Size distribution and zeta potential of complexes

The studies using DLS-NIBS, reveal information about the average size hydrodynamic diameter of the complexes assuming that they have a spherical shape. Figure 1 shows the average size diameter and polydispersity index for complexes formed either with CS-pEGFP or CS-siRNA-pEGFP. In both cases, the negatively charged complexes (i.e., N/P < 1.0) grow in size compared to positively charged systems. Complexes of CS-pEGFP with positive charge ratio (i.e., N/P > 1.0) showed an average value of ~200 nm and this value is independent of the amount of CS added (Figure 1a). By contrast, positively charged complexes of CS-siRNA-pEGFP present an average size diameter of 200- 320 nm (Figure 1b), which tends to grow with the amount of CS added. The values of polydispersity index (PDI) in all the cases are around 0.2, which indicates the formation of complexes with a monomodal distribution of particle size for both CS-pEGFP and CS-siRNA-pEGFP systems. Figure 2 shows the zeta potential of the complexes, which varies from -20 to +25 mV. For positively charged complexes, the systems were saturated and no further addition of CS increases the zeta potential.

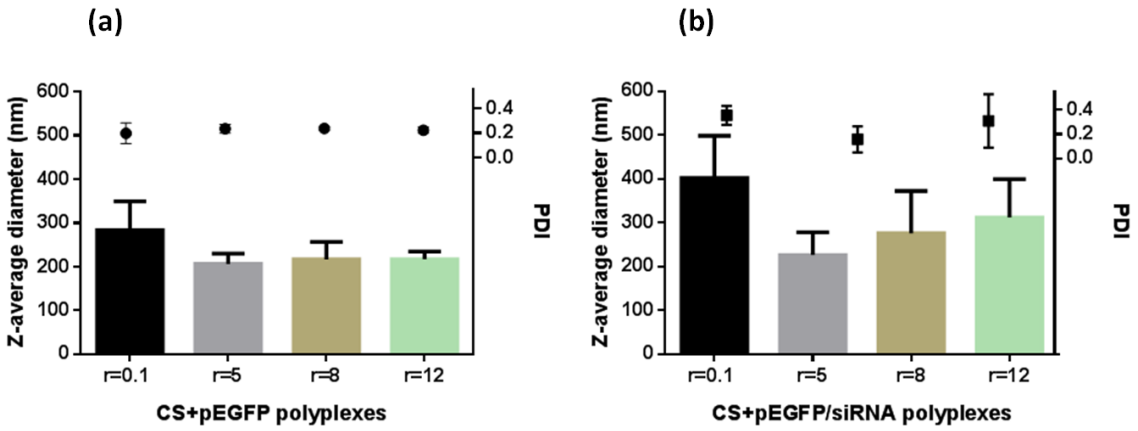

Figure 1. Variation of the Z-average size hydrodynamic diameter (nm) and polydispersity index (PDI of complexes formed with CS and pEGFP (a), and pEGFP-siRNA (b), formed at different N/P ratios (r= 0.1, 5, 8, 12). The values represented are the mean averages ± SD of three independent experiments.

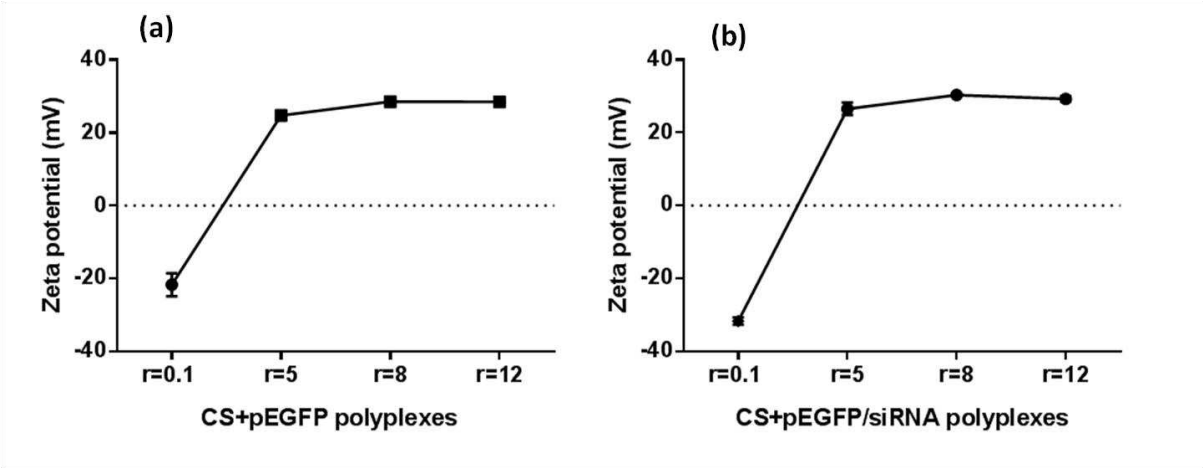

Figure 2. Variation of the zeta potential (mV) of complexes formed with CS and pEGFP (a) and pEGFP-siRNA (b) at varying N/P ratios (r= 0.1, 5, 8, 12). The values represented are the mean ± SD of three independent experiments.

3.1.2 Gel retardation assay

The binding strength of CS to pEGFP and pEGFP-siRNA and the influence of the composition given by the different N/P charge ratios was confirmed by gel retardation assay. Figure 3 shows

an agarose gel loaded with the various CS-nucleic acid complexes. Inspection of the gels shows that in the negatively charged complexes (i.e., N/P < 1.0) between CS-pEGFP (lane 2) and CS-pEGFP-siRNA (lane 6), the unbound nucleic acids migrate according with their electrophoretic mobility in the free form. Positively charged complexes (CS-pEGFP: lanes 3-5 and CS-pEGFP-siRNA: lanes 7-9), in turn, smeared bands are observed, which evidences that the electrophoretic mobility of the nucleic acids was retarded upon complexing with CS. The results are diagnostic that at positive N/P ratios CS complexes strongly with pEGFP and pEGFP-siRNA.

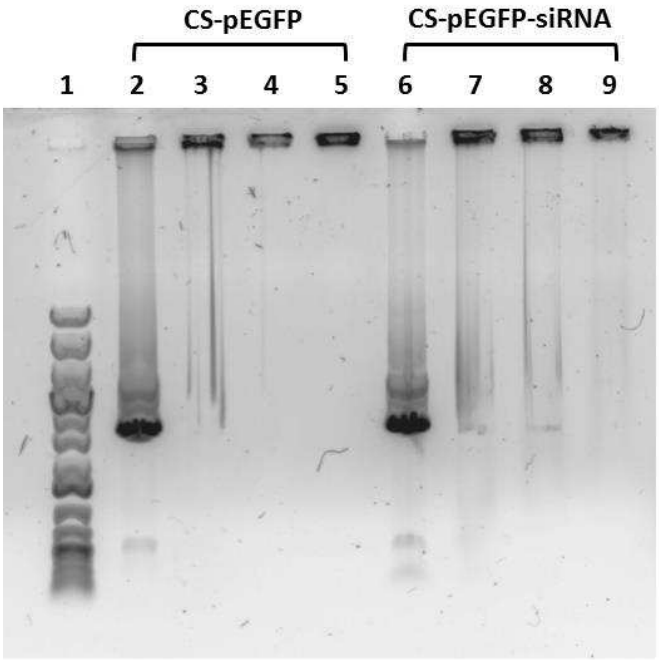

Figure 3. Agarose gel (1.5%) electrophoresis retardation assay of CS-pEGFP (lane 2-5) and CS-pEGFP-siRNA (lane 6-9) at different N/P ratios (r=0.1, 5, 8, 12). Marker used in lane 1 GeneRuler^{TM} 1kb.

3.3 Stability in biological media

The stability of different complexes during incubation in Opti-MEM containing HEPES and mannitol to reach hypertonic (580 mM) conditions needed before dilution upon applying the transfection treatments was studied by DLS. Evolution of particle size distribution curves was analyzed after initial incubation at 37 °C for 30 min and 14 h. Opti-MEM added with HEPES and mannitol is recommended as a suitable transfection medium for Novafect® (a CS-based commercial transfecting agent) from by Novamatrix (Sandvika, Norway). At the same time, it is also well known that Opti-MEM is a commonly used medium to transfect epithelial cells. Figure 4 shows the size distribution curves of CS - pEGFP (Fig 4a) and CS - pGFP-siRNA (Fig 4b) complexes after 30 min (black line) and 14 h (red line) of incubation. For all negatively charged complexes (N/P=0.1) it is observed a multimodal size distribution curves. Notice that he population with the greatest size distribution exceeds 1000 nm after 30 min of incubation. This was followed by aggregation at longer incubation times that prevented to record subsequent DLS measurements. The rapid onset of aggregation in these systems is diagnostic of their low colloidal stability. On the other hand, notice that in the positively charged complexes of CS-pEGFP of N/P=5 and 8, after 30 min incubation, two distinct populations of particles with Gaussian distributions were observed, namely, one with Z-average diameter of ~40 nm and a much larger one of ~500 nm. Both populations increased, to ~100 and ~1000 nm, respectively, upon incubation during 14 h, diagnostic that even when their size grew, they remained fairly stable. A similar trend was observed in the case of the complexes of CS-pEGFP of highest CS content (N/P=12), though the shape of the larger predominant peaks was hardly Gaussian, thus reflecting, a lower stability than the systems of lower N/P stoichiometry. In the case of the positively charged (N/P=5, 8 and 12) systems comprising the two nucleic acids (CS-pEGFP-siRNA), also two populations were observed, namely a predominant one with Z-average size of ~600 and a minor one of very small size <1 nm. Upon 14 h of incubation, the

original populations observed initially after 30 min, persisted in the three cases. Interestingly, for the systems of N/P=8, a third, proportionally smaller population, appeared at ~60 nm. In turn, at N/P=12 the two original populations persisted and although the Z-average diameter of the largest peak increased to ~1000 nm, it revealed a closer to Gaussian distribution. No visible aggregation was observed for none of the positively charged systems. Further experiments were performed using positively charged complexes with N/P of 12 due to its higher stability.

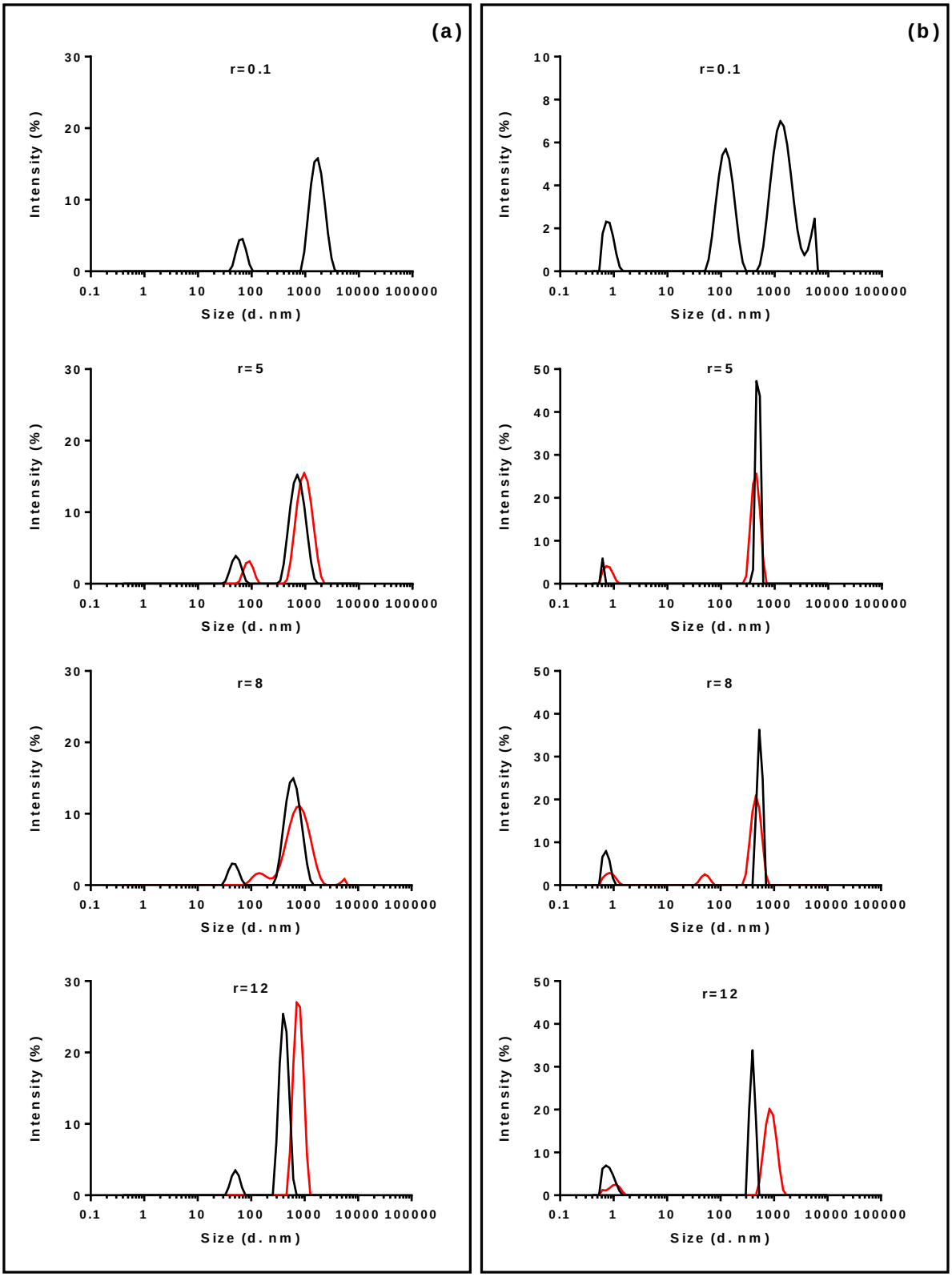

Figure 4. Stability of the complexes after initial incubation for 30 min (black line) and after 14 h (red line) in Opti-MEM containing HEPES and mannitol at 37 °C. a) CS-pEGFP complexes and b) CS-pEGFP-siRNA complexes.

3.4 Metabolic capability (MTT assay)

The principle of the MTT assay is based on the ability of the mitochondrial dehydrogenase enzyme to cleave the tetrazolium ring of MTT and transform it into dark blue insoluble formazan, which accumulates in living cells and can subsequently be quantified by a colorimetric assay. Thus, MTT it is an assay of metabolic competence of viable cells, the number is directly proportional to the concentration of formazan created. This formazan is then solubilized in a suitable solvent and absorbance is measured (Mosmann, 1983). MTT assay was performed to determine cell viability after 4 h of incubation at 37 °C (Figure 5). Results from the MTT assay demonstrated that neither CS alone nor CS-nucleic acid complexes induced any cytotoxic effect towards CFBE41o- cells. By contrast, the complexes formed with the transfecting reagent Lipofectamine showed a significant statistical decrease of the cell viability (>60%).

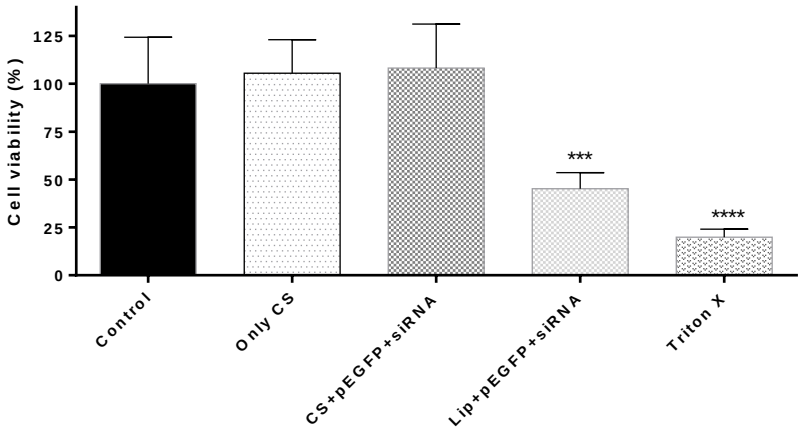

Figure 5. Cell viability of the CFBE41o- cells determined by MTT assay following incubation for 4 h at 37°C. Cell viability was expressed relative to the control of untreated cells. Positive controls were cells treated with Lipofectamine and Triton X. Data is expressed as mean ± SD of three biological independent experiments. Statistical analysis using Kruskal-Wallis test for non-parametrical distribution were used (*** $p \leq 0.001$; ****$p \leq 0.0001$).

3.5 Transfection efficiency

CFBE41o- cells were transfected with the reporter gene encoding for green fluorescence protein (GFP), as a model plasmid to evaluate the transfection efficiency of chitosan as potential nanocarrier for human airway epithelial cells. Co-transfection of CS with both nucleic acids pEGFP and siRNA was also tested. We used the commercial reagent Lipofectamine as a control for transfection and co-transfection assays.

Figure 6 shows representative fluorescence images of pEGFP-transfected CFBE41o- cells with CS and Lipofectamine. Qualitative increase in the fluorescence signal was observed for both transfecting agents after 24 h of transfection. At the same time, we studied the co-transfection of pEGFP-siRNA and it was observed a drastic reduction in the fluorescence signal, due to the knockdown of the pEGFP by the specific binding of siRNA to pEGFP. These results evidence that the cells were efficiently co-transfected with the CS-based complexes.

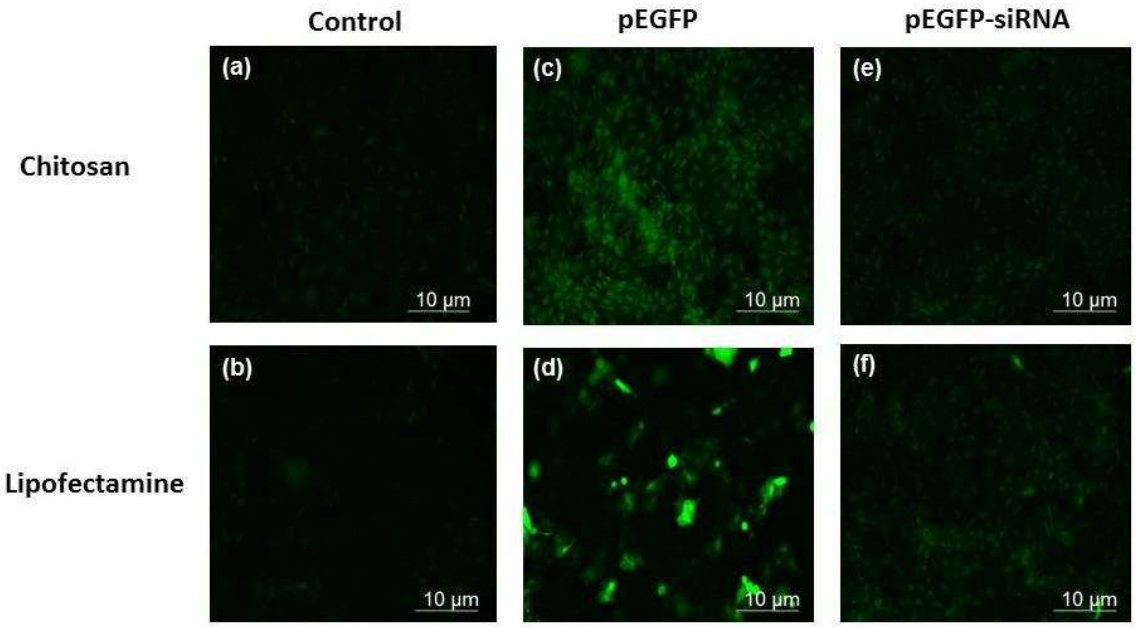

Figure 6. Representative fluorescence microscopy images of transfected CFBE41o- cells with chitosan at N/P ratio of 12 and Lipofectamine. GFP expression after 24 h of transfection: Control cells (a, b); CS-pEGFP (c); Lipofectamine-pEGFP (d); CS-pEGFP-siRNA (e); Lipofectamine-pEGFP-siRNA (f).

The intensity of the fluorescence signal is used as an indicator of transfection efficiency and the statistical evaluation of the fluorescence intensities is shown in Figure 7. The evaluation of the fluorescence intensities demonstrates that cells transfected with pEGFP, either with CS or Lipofectamine, exhibited significant increase in fluorescence ($p \leq 0.0001$) as compared to the control ones (non-transfected cells) after 24 h. In addition, we did not find any statistical significant difference between CS and Lipofectamine. Furthermore, we observed a significantly ($p \leq 0.05$) more efficient knockdown of the pEGFP after co-transfection with siRNA using CS than when Lipofectamine was used.

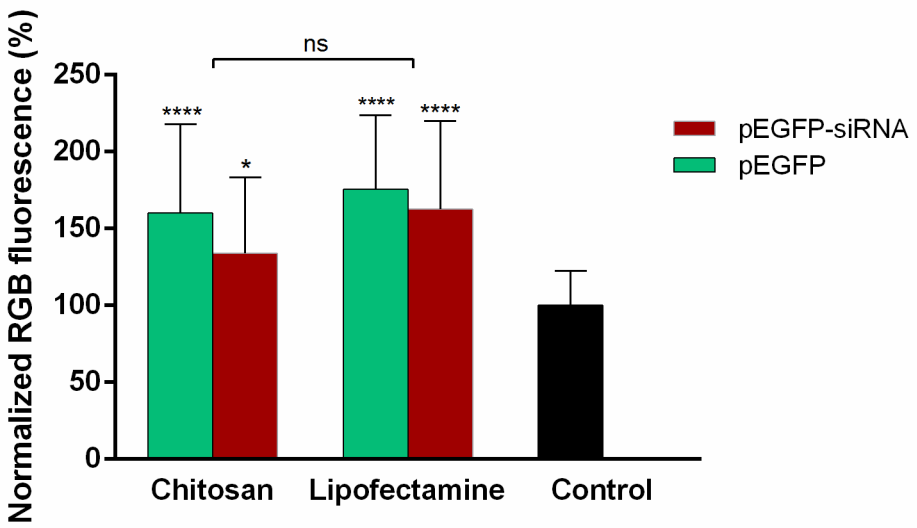

Figure 7. Normalized fluorescence intensities of the CFBE41o- cells transfected with CS-pEGFP and CS-pEGFP-siRNA complexes using a N/P ratio of 12 (Opti-MEM, 37°C, 24 h). Negative control was non-transfected cells and positive control were cells transfected with Lipofectamine. Transfection data were normalized to negative control. Data is expressed as mean ± SD of three biological independent experiments. Statistical comparisons were made using Tukey´s multiple comparisons test (* $p \leq 0.05$; ****$p \leq 0.0001$; ns: non significance).

4. Discussion

Chitosan compasses several beneficial characteristics that accounts for its potential use as a suitable advanced drug and gene delivery system. Among the main features that this biopolymer convenes is that it is a well-established biocompatible and biodegradable natural polymer. Besides, chitosan is able to establish ionic, hydrogen, and hydrophobic bonding with negatively charged chains of mucin, the structural component of mucus fluids (Menchicchi et al., 2014). All these properties, place chitosan as a potential alternative to administrate drugs to or through the lung. There are reports showing the effectiveness of chitosan increasing lung retention of drugs (Grenha et al., 2005; Li and Birchall, 2006; Teijeiro-Osorio et al., 2009). Another advantage of its cationic nature, is that chitosan can be used as non-viral vector for gene therapy due to the formation of complexes with negatively charged pDNA, RNA, siRNA and microRNA (Santos-Carballal et al., 2015). Therapy for monogenetic hereditable diseases,

such as cystic fibrosis, calls for a new strategy based on non-viral gene vectors. The use of viral vectors in CF gene therapy has shown inflammatory and immunogenic response. Therefore, chitosan-based system could be an alternative and stable formulation to deliver CFTR in a long-term and repeated manner.

In this study, we aimed to use chitosan as a safe and efficient non-viral vector to transfect a human airway cystic fibrosis cell line. We gained proof-of-principle on the reporter gene enhanced green fluorescence protein (pEGFP) and its knockdown by the specific siRNA sequence. To the best of our knowledge, this is the first study that accounts for the use of chitosan to complex and transfect concomitantly a tandem system of nucleic acids, namely pEGFP and its silencing RNA.

The airways are relatively hard epithelial barriers to overcome as they are protected by a viscoleastic layer of mucus. Mucoadhesive CS particles could efficiently bind to the mucus surface and therefore, prolong the residence time while favoring the possibility of uptake by the epithelial cells (Menchicchi et al., 2015) . To this end, the molecular weight and the degree of acetylation of chitosan are relevant on the stability and transfection efficiency of chitosan complexes (Mao et al., 2010). The physicochemical and stability characterization of CS-nucleic acid complexes is essential to understand and optimize their functional behavior at molecular and cellular level. In the present study, self-assembled electrostatic complexes between CS-pEGFP, and CS-pEGFP-siRNA, have been obtained spontaneously by mixing CS and the nucleic acid in aqueous solution. The Z-average size of CS-pEGFP and CS-pEGFP-siRNA systems comprised by a stoichiometric charge excess of the nucleic acid were ~300 and ~400 nm, respectively. When there was a stoichiometric excess of the CS component (N/P > 1.0), the average size diameter decreased to less than ~300 nm. The overall size range of CS-pEGFP complexes is in concordance with previous reports (Lavertu et al., 2006; Liu et al., 2005; Mao et al., 2010). The presence of siRNA does have an influence on the average particle

size, thus forming CS-pEGFP-siRNA complexes 100 nm larger than CS-pEGFP complexes. This is the expected consequence of either the formation of a thicker shell or else an overall more expanded structure, hence less densely packed. It has been described that an increase in the concentration of nucleic acid increases the complexes size (MacLaughlin et al., 1998; Romøren et al., 2003). The selection of a specific chitosan (DA = 30%; 20 kDa) was based on already conducted studies of different CS binding to miRNA (Santos-Carballal et al., 2015) and this CS has also shown to bind to mucin and decrease its viscosity (Menchicchi et al., 2015). Chitosan with an intermediate value of DA (30 %) has been reported to adopt a more flexible conformation like coiled shape (Novoa-Carballal et al., 2013; Santos-Carballal et al., 2015). This may offer the conformational adaptation to CS necessary to complex efficiency the nucleic acids. This is consistent with our previous studies on CS-mucin systems (Menchicchi et al., 2014). Moreover, chitosans with a Mw ~ 20 kDa have been shown to bind and protect completely the nucleic acid from enzymatic degradation (Köping-Höggård et al., 2004).

The zeta potential reached a stable value of +25 mV for all positively charged complexes. This is the expected consequence of an excess of protonated amino groups of chitosan that did not take part in the neutralization with negatively charged nucleic acids. This positive zeta potential is required to facilitate the interaction with the negatively charged cell membrane, promote internalization and efficient transfection (Puras et al., 2013).

The condensation, protection and release of pEGFP by chitosan were qualitative evaluated by means of an agarose gel electrophoresis retardation assay. Only the positively charged complexes showed to retain the nucleic acids in the well. Likewise, CS-pEGFP-siRNA complexes obtained by the same procedure led to similar results, thus revealing that the presence of siRNA does not interfere in the binding efficiency of the plasmid.

Results from the stability studies after incubation in cell culture medium (Opti-MEM) showed that positively charged complexes were relatively stable after 14 h of incubation at 37°C, with

a noticeable increase in size and polydispersity. Despite this, the complexes were stable against aggregation. By contrast, complexes with a defect of positive charges are destabilized in presence of Opti-MEM. In general, there is a dearth of information about the stability of complexes in cell culture medium, despite being an essential parameter for further in vitro applications. As it was described in our materials section, the Opti-MEM has moderate saline content. The presence of specific ions might allow relative stabilization of the complexes due to hydration forces as found in previous studies on chitosan-based nanocapsules (Goycoolea et al., 2012; Santander-Ortega et al., 2011)(ref. Santander-Ortega et al 2011, Goycoolea et al. 2011). It is claimed that ions from the medium could accumulate in the proximity of the hydrophilic surface sites of the complexes. Subsequently, this effect will produce short-range repulsive hydration forces, which would lead the stabilization of the complexes (López-León et al., 2008; Santander-Ortega et al., 2011). The extension of this hydration forces is highly dependent on the DA of the chitosan used and on the type of ion. This will determine the hydrophilicity of the complexes and therefore, the amount of counter ions needed for repulsive forces. It is known that chitosans with higher DA enhance the hydrophilicity of the surface and stability, even at low ionic concentration (Santander-Ortega et al., 2011). Hence, the relative high DA of the chitosan sample used in the present studies (DA=30%) may explain the stability of the complexes.

Chitosan of varying characteristics are generally biocompatible, and induce low cytotoxicity in various type of cell lines(Chae et al., 2005; Kean and Thanou, 2010). We tested the effect on cell viability induced by chitosan and complexes of chitosan with pEGFP or with pEGFP-siRNA in comparison with the corresponding ones formed by Lipofectamine, the commonly used lipotransfection agent. The data did not show evidence of cytotoxicity after 4 h of incubation with the systems based on chitosan. Similar studies using chitosan (Santos-Carballal et al., 2015) and chitosan derivatives (Hu et al., 2006) in comparison with Lipofectamine-based

systems are found in the literature, where chitosan did not exhibit cytotoxic effects and Lipofectamine reduces significantly the cell viability (Corsi et al., 2003; Sato et al., 2001; Thanou et al., 2002). In our previous study (Santos-Carballal et al., 2015), we evaluated the cytotoxity of CS-microRNA complexes of varying N/P ratio formed by CS of different Mw and DA after 6 and 24 h. No differences were observed between the two elapsed times. None of these complexes induced a reduction in mitochondrial activity. Hence, in the present study, we evaluated the effect of the complexes only after 3 h and consider unnecessary to extend the study to 24 h. This evidence, makes chitosan-based complexes a promising non-viral gene delivery systems in the treatment of pulmonary diseases.

In a further step we evaluated the in vitro transfection efficiency for CS complexes after 24 h in CFBE41o-. Previously, we have found successful pEGFP expression and its knockdown in co-transfection with siRNA using Lipofectamine in this CF cell line after 24 h (Bangel-Ruland et al., 2013). In the present study, transfection efficiency to CFBE41o- cells showed a significant increase, either using CS or commercial Lipofectamine, as compared to negative controls (non-transfected cells) (Figure 7). In the case of CS complexes, this efficiency could be attributed to the electrostatic interaction of the complexes with the negatively charged cell membrane. Endocytosed complexes are believed to release the nucleic acids based on the proton sponge effect hypothesis (Freeman et al., 2013). This hypothesis proposes that endosomes are acidified and chitosan promotes the active transport of protons due to the presence of NH_2 groups at D-glucosamine residues. The accumulation of protons in the vesicle must be balanced by an influx of counter ions (Cl^-), which produces osmotic swelling, burst of the late endosome and release of the cargo (Pack et al., 2005; Richard et al., 2013). Chitosan may undergo biodegradation by enzymes present in the endosomal/lysosomal vesicles. The release of nucleic acids will start immediately after endocytosis of the chitosan complexes (Kean and Thanou, 2010).

Although the transfection efficiency with chitosan was somewhat lower than the obtained with Lipofectamine, we did not find statistic significant differences between them. Interestingly, such high transfection efficiency has never been reported before using CS as transfection agent for a CF cell line. Indeed, in previous studies by Nydert and coworkers, it was shown a poor transfection efficiency for CFBE41o- cells using CS (DA=0.5%; ~6kDa) as non-viral vector (Nydert et al., 2008). They documented the difficulties associated to in vitro transfect with CS a highly differentiated cell line like CFBE41o- (Nydert et al., 2008). Moreover, it has been suggested that in vitro transfection efficiency is cell-type dependent (Corsi et al., 2003; Erbacher et al., 1998). In addition, we obtained evidence that CS-based systems were capable of co-transfection and to knockdown the co-administered pEGFP (Figure 7). The knockdown achieved by the CS-based complexes was more than 2-fold efficient (27%) than the observed in the cells co-transfected with Lipofectamine-based systems (13%), making chitosan not only a suitable vector for the transfection airway epithelial cells but also a promising carrier for co-transfection procedures.

5. Conclusions

Lung gene therapy based on the use of chitosan as non-viral vector is not yet widely explored. In this study we demonstrated the feasibility and efficiency of chitosan as a co-transfection reagent for a cystic fibrosis human bronchial epithelial cell line. We prepared and characterized complexes based on chitosan with a molecular weight of 20 kDa and DA of 12% using pEGFP and pEGFP-siRNA. We found that chitosan is able to bind to two different nucleic acids with equal affinity, as revealed by gel retardation assay. The average size diameter of positively charged complexes varied from 200 – 300 nm, whereas negatively charged complexes increased in size up to 400 nm. Complexes prepared with an excess of amino groups were stable over 14 h of incubation in Opti-MEM. In vitro studies showed that chitosan do not

exhibit any cytotoxic effect on CFBE41o- cells. Successful transfection efficiency was achieved when cells were transfected either with CS-pEGFP or co-transfected with CS-pEGFP-siRNA at N/P charge ratio of 12. To the best of our knowledge, chitosan has never been shown as a potential carrier able to transport concomitantly two different nucleic acids, as we have shown in the present study for pEGFP and its specific knockdown siRNA. A possible interpretation to the high efficiency found for chitosan as a co-transfection agent may lie in the particular molecular architecture of the complexes incorporating pDNA and siRNA. Further studies are necessary to elucidate in greater detail the biophysical properties of these systems at molecular level using high resolution scattering techniques, such as synchrotron SAXS and high resolution fluorescence microscopy. The used chitosan complexes are biotechnologically suitable transfecting systems for gene therapy purposes. Pulmonary diseases, like CF, have unsuccessfully addressed strategies to delivery CFTR in lung tissue. Problems associated with high toxicity, poor transfection efficiency or immunogenic problems of the vectors so far evaluated are important limitations that need to be overcome. Indeed, the identification of an optimal vector for gene therapy in CF is a major challenge. Viral vectors, although substantially used in gene therapy research, have been reported to induce strong immune responses, whereas the use of non-viral vectors has emerged as a promising alternative, since they are less immunogenic. In the present study we provide proof-of-principle on the use of chitosan, as a natural non-toxic vector, able to co-transfect a well-established model cell line for CF, reaching comparable levels to those achieved using lipid-based systems. Further in vivo studies should be directed to deliver the CFTR gene condensed by chitosan in lung.

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