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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 Opti-MEM. Cell viability study did not show any significant cytotoxic effect of the CS-based complexes in a human bronchial cystic fibrosis cell line (CFBE41o-). 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 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., 2013). 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.,

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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-nucleotide 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.
[c] Total equivalent concentration of $-NH_3^{+}$ and $-PO_4^{-}$ from chitosan and nucleic acids, respectively.
[d] Equivalent concentration of $-NH_3^{+}$ from chitosan.

2.2. Size distribution and zeta potential of complexes

The size distribution of the CS-nucleotide 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 × 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 previous described quantities (Table 1) with 100 μL of Opti-MEM (Life Technologies) and subsequently incubating them during 14 h at 37 °C. The stability was evaluated by measuring the evolution of the hydrodynamic radius, determined as described above.

2.5. Cell studies

2.5.1. Cell line

CFBE41o- cells were provided by Dr. Dieter Gruenert (Department of Otolaryngology- Head and Neck Surgery, University of California, San Francisco, CA, USA). These cells are derived from a CF bronchus and are homozygous for the most common mutation F508del contributing to CF. The cells were immortalized using the pSVori plasmid that contains a replication-deficient simian virus 40 (SV40) genome (Gruenert et al., 2004, 1988). Cells were grown as previously reported (Bangel-Ruland et al., 2013) in Eagle's Minimal Essential Medium with L-glutamine (MEM) in addition to 10% (v/v) fetal calf serum (FCS), 1% penicillin (10.000 U/mL)/streptomycin (10 mg/mL) and 1% L-glutamine at 37 °C in 5% CO_2 and 95% air.

2.5.2. 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