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TPP-dendrimer nanocarriers for siRNA delivery to the pulmonary epithelium and their dry powder and metered-dose inhaler formulations

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ABSTRACT

The regulation of genes utilizing the RNA interference (RNAi) mechanism via the delivery of synthetic siRNA has great potential in the treatment of a variety of lung diseases. However, the delivery of siRNA to the lungs is challenging due to the poor bioavailability of siRNA when delivered intraveneously, and difficulty in formulating and maintaining the activity of free siRNA when delivered directly to the lungs using inhalation devices. The use of non-viral vectors such as cationic dendrimers can help enhance the stability of siRNA and its delivery to the cell cytosol. Therefore, in this work, we investigate the ability of a triphenylphosphonium (TPP) modified generation 4 poly(amidoamine) (PAMAM) dendrimer ($G4NH_2$-TPP) to enhance the *in vitro* transfection efficiency of siRNA in a model of the pulmonary epithelium and their aerosol formulations in pressurized metered dose inhalers (pMDIs) and dry powder inhalers (DPIs). Complexes of siRNA and $G4NH_2$-TPP were prepared with varying TPP densities and increasing N/P ratios. The complexation efficiency was modulated by the presence of the TPP on the dendrimer surface, allowing for a looser complexation compared to unmodified dendrimer as determined by gel electrophoresis and polyanion competition assay. An increase in TPP density and N/P ratio led to an increase in the *in vitro* gene knockdown of stably green fluorescent protein (eGFP) expressing lung alveolar epithelial (A549) cells. $G4NH_2$-12TPP dendriplexes ($G4NH_2$ PAMAM dendrimers containing 12 TPP molecules on the surface complexed with siRNA) at N/P ratio 30 showed the highest *in vitro* gene knockdown efficiency. To assess the potential of TPP-dendriplexes for pulmonary use, we also developed micron particle technologies for both pMDIs and DPIs and determined their aerosol characteristics utilizing an Andersen Cascade Impactor (ACI). Mannitol microparticles encapsulating 12TPP-dendriplexes were shown to be effective in producing aerosols suitable for deep lung deposition for both pMDI formulations (fine particle fraction of 50–53%) and DPI formulations (fine particle fraction of 39%) with no impact on the *in vitro* gene knockdown efficiency of the siRNA. This work demonstrates the potential benefits of utilizing TPP-conjugated dendrimers in the formation of dendriplexes for siRNA delivery to the pulmonary epithelium and their aerosol formulation for local delivery to the lungs using portable inhalers.

Abbreviations: RNAi, RNA interference; siRNA, short interfering RNA; PAMAM, poly(amidoamine); $G4NH_2$, generation 4 amine-terminated PAMAM dendrimer; TPP, triphenylphosphonium((3carboxypropyl)triphenylphosphonium bromide); pMDIs, pressurized metered dose inhalers; HFAs, hydrofluoroalkanes; DPI, dry powder inhaler; ACI, Anderson cascade impactor; GFP, green fluorescent protein; LS, light scattering; RF, respirable fraction; FPF, fine particle fraction; MMAD, mean mass aerodynamic diameter; GSD, geometric standard deviation; ED, emitted dose; SEM, scanning electron microscopy.

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1. Introduction

Synthetic small interfering RNA (siRNA) is a promising therapeutic for the treatment of a variety of lung diseases including asthma (Xie et al., 2016), chronic obstructive pulmonary disease (COPD), (De Backer et al., 2015a) cystic fibrosis, (Bangel-Ruland et al., 2015) viral infections, (Zhang et al., 2005; Li et al., 2005; Liang et al., 2015) pulmonary tuberculosis, (Man et al., 2016) lung cancer, (Conde et al., 2013; Guo et al., 2015) and also for the treatment of the so called "non-druggable" diseases (Soutschek et al., 2004). Despite recent developments and the many promising applications of siRNA therapeutics, there are still many challenges that hinder the efficient and safe use of siRNA to treat pulmonary disorders including the formulation of siRNA and their vectors for efficient local lung delivery, (Conti et al., 2014; Chow and Lam, 2015) and the extra and intracellular barriers that exist for the transport of bioactive siRNA to the cytoplasm of relevant cells in the lungs (De Backer et al., 2015b; Lam et al., 2012).

Non-viral vectors such as cationic polymers represent one of the promising approaches for the efficient delivery of siRNA (Günther et al., 2011). Amine-terminated, poly(amidoamine) (PAMAM) dendrimers have been widely investigated as gene delivery vectors including carriers of siRNA (Hu et al., 2016), and their use in oral inhalation to the lungs (Conti et al., 2014; Nasr et al., 2014; Zhong and da Rocha, 2016). PAMAM dendrimers are hyperbranched polymers with uniform structure and size and with multifunctional modifiable surface groups (Kurtoglu, 2010). Amine-terminated PAMAM dendrimers carry a positive surface charge due their protonatable surface primary amines and can thus serve to induce the formation of complexes with anionic siRNA via electrostatic interactions (Hu et al., 2016; Jensen et al., 2011). Such nanoscale structures, termed dendriplexes, have been shown to promote cellular internalization of siRNA. However, gene knockdown efficiency is relatively low, and cytotoxicity profiles in models of the pulmonary epithelium are not very favorable (Conti et al., 2014; Lam et al., 2012).

In order to increase the transfection efficiency and biocompatibility of PAMAM dendrimers, a variety of modifications to the dendrimer surface have been explored (Yang et al., 2015). Of these modifications, the use of a triphenylphosphonim (TPP) ion has been recently reported via direct conjugation to PAMAM dendrimers (Biswas, 2012; Bielski et al., 2015; Wang, 2014). TPP is a delocalized lipophilic cation and well-known mitochondrial-targeting agent (Murphy, 2008). The conjugation of TPP to PAMAM dendrimers enhances mitochondrial targeting, as well as internalization and accumulation of dendrimers into cells while exhibiting relatively low cytotoxicity (Biswas, 2012; Bielski et al., 2015). TPP conjugation has led to enhanced delivery of DNA/dendrimer complexes *in vitro* and their improved transfection ability (Wang, 2014). Therefore, the modification of TPP to amine-terminated PAMAM dendrimers holds great promise in enhancing the transfection ability and biocompatibility of dendrimers for siRNA delivery to the lung tissue.

Oral inhalation (OI) is promising route for local siRNA delivery to the lungs as it avoids systemic degradation (Merkel et al., 2014) and poor lung targeting associated with i.v. administration (Durcan et al., 2008). Pressurized metered dose inhalers (pMDIs) and dry powder inhalers (DPIs) are the two most widely used portable inhalation devices (Chow and Lam, 2015; Labiris and Dolovich, 2003). In a pMDI, the therapeutic particles are suspended in the propellant (hydrofluoroalkanes – HFAs) (Chow and Lam, 2015). The propellant aerosolizes the therapeutic for inhalation when the device is actuated (Chow and Lam, 2015; Labiris and Dolovich, 2003). DPIs allow for the inhalation of dry powders as an aerosol cloud upon breath actuation (Chow and Lam, 2015). In order to achieve successful delivery of siRNA to the lungs using such portable inhalers, the nanoscale dendriplexes must be formulated into particles that form aerosols with optimum aerosol diameters within the range of 1–5 μm. This process must be done without compromising the biological activity of siRNA (Lam et al., 2012). We have previously reported successful use of spray drying technique with sugar excipients to formulate dendriplexes into suitable micron-sized particles that results in optimum aerosol sizes when formulated in pMDIs (Conti et al., 2014). The use of spray drying can also be easily extended for preparation of DPI formulations as well, and has been shown to work successfully to formulate micron particles of PLGA-siRNA nanoparticles via spray drying with sugar excipients (Jensen et al., 2012).

Considering the challenges and opportunities stated above, the goal of this study was twofold: (i) to design a PAMAM-based dendrimer conjugate that led to improvements in gene knockdown efficiency in an *in vitro* model of the pulmonary epithelium when compared to the unmodified amine-terminated counterpart, (ii) and to develop efficient strategies for the formulation of such dendriplexes in portable oral inhalation devices. We conjugated generation 4, amine-terminated PAMAM dendrimers ($G4NH_2$) with increasing TPP densities (0, 4, 8, 12 TPP/dendrimer). Complexes of $G4NH_2$-TPP dendrimers with siRNA ($G4NH_2$-TPP-dendriplexes) at various N/P ratios were prepared and characterized. The gene knockdown efficiency and toxicity of these $G4NH_2$-TPP-dendriplexes (simply, TPP-dendriplexes) was tested in an *in vitro* model of the pulmonary, namely stably-transfected, green fluorescent protein (GFP) expressing A549 cells. The most effective TPP-dendriplex system was selected to be formulated in portable OI devices. Micron-sized particles of the TPP-dendriplex were prepared using mannitol as an excipient and spray dried. The aerosol characteristics of the mannitol-TPP-dendriplex particles formulated in pMDIs and DPIs were assessed using an Anderson Cascade Impactor (ACI). This study demonstrates the successful use of TPP-targeted PAMAM dendrimers as vectors for siRNA delivery for a model of the pulmonary epithelium, and their formulations using pMDIs and DPIs for direct and noninvasive siRNA delivery to the lungs.

2. Materials and methods

2.1. Materials

Generation four, amine-terminated, poly(amidoamine) (PAMAM) dendrimer ($G4NH_2$) in methanol at 9.8% w/w was purchased from Dendritech Inc. (Midland, MI). Double-stranded Dicer substrate siRNA targeting eGFP ((+) siRNA) and double stranded respective mismatch as a negative control ((−) siRNA) was obtained from Integrated DNA Technologies (Leuven, Belgium) (Conti et al., 2014). Dimethyl sulfoxide (DMSO) anhydrous (Acros), *N*-Hydroxysuccinimide (NHS) (Acros), agarose, sodium chloride (NaCl), potassium chloride (KCl), potassium phosphate, monobasic, anhydrous (KH_2PO_4), potassium hydroxide (KOH) agarose, and (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) were purchased from Thermo Fischer Scientific (Rockford, IL). Sodium phosphate, dibasic, anhydrous (Na_2HPO_4) was purchased from EMD Chemicals, Inc. (Gibbston, NJ). (3-Carboxypropyl) triphenylphosphonium bromide (TPP), P-toluenesolfonic acid (p-TSA), *D*-Mannitol (98%), and heparin sodium salt (194 U/mg) were purchased from Sigma Aldrich (St. Louis, MO). *N*-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) was purchased from Advanced ChemTech Inc. (Louisville, KY). Diethylpyrocarbonate (DEPC) and ethylenediaminetetraacetic acid (EDTA) (pH 8, 0.5 M, sterile) were acquired from Amresco (Solon, OH, United States). Dymel 227 ea/P hydrofluoroalkane (HFA227) propellant was a gift from DuPoint (Fort Worth, Texas, United