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# Cellular internalization and transport of biodegradable polyester dendrimers on a model of the pulmonary epithelium and their formulation in pressurized metered-dose inhalers

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ABSTRACT

The purpose of this study was to evaluate the effect of generation and surface PEG*ylation* of degradable polyester-based dendrimers nanocarriers on their interactions with an *in vitro* model of the pulmonary epithelium as well as to assess the ability to formulate such carriers in propellant-based, portable oral-inhalation devices to determine their potential for local and systemic delivery of drugs to and through the lungs. Hydroxyl (-OH) terminated polyester dendrimers of generation 3 and 4 (G3, and G4) were synthesized using a divergent approach. G4 was surface-modified with PEG (1,000 Da). All dendrimers and their building blocks were determined to be highly compatible with the model pulmonary epithelium, with toxicity profiles much more favorable than non-degradable polyamidoamine dendrimers (PAMAM). The transport of the species from the apical to basolateral side across polarized Calu-3 monolayers showed to be generation and surface-chemistry (PEG*ylation*) dependent. The extent of the transport is modulated by their interaction with the polarized epithelium and their transient opening of the tight junctions. G3 was the one most efficiently internalized by the epithelium, and had a small impact on the integrity of the monolayer. On the other hand, the PEG*ylated* G4 was the one least internalized by the polarized epithelium, and at the same time had a more pronounced transient impact on the cellular junctions, resulting in more efficient transport across the cell monolayer. PEG*ylation* of the dendrimer surface played other roles as well. PEG*ylation* modulated the degradation profile of the dendrimer, slowing the process in a step-wise fashion – first the PEG layer is shed and then the dendrimer starts degrading. PEG*ylation* also helped increase the solvation of the nanocarriers by the hydro-fluoroalkane propellant used in pressurized metered-dose inhalers, resulting in formulations with excellent dispersibility and aerosol quality (deep lung deposition of 88.5%), despite their very small geometric diameter. The combined *in vitro* and formulation performance results shown here demonstrated that degradable, modified polyester dendrimers may serve as a valuable platform that can be tailored to target the lung tissue for treating local diseases, or the circulation, using the lungs as pathway to the bloodstream.



## 1. Introduction

Pulmonary delivery is not only the most sensible route for the regional administration of drugs to the lungs to treat lung diseases (Tewes et al., 2016), but also holds tremendous potential as a non-invasive route for the systemic administration of therapeutics *through the lungs* (Kunda et al., 2013; Ray et al., 2015). Some of the advantages of using oral inhalation for targeting systemic circulation include the large surface area and the thin barrier of the pulmonary epithelium, its low proteolytic activity and avoidance of the first pass effect. Such characteristics may provide opportunities to enhance drug bioavailability and thus therapeutic efficacy at reduced dosages when compared to alternative routes of administration (Agu and Ugwoke, 2011; Nasr et al., 2012; Patton and Byron, 2007). Pressurized metered-dose

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inhalers (pMDIs) are relevant in the context of pulmonary drug delivery as they are the least expensive portable aerosol devices available in the market and are easy to use and highly compliant, being also relevant in the context of pulmonary delivery for children and seniors (Myrdal et al., 2014; Smyth, 2003; Stein et al., 2014). However, there are still many difficulties in the development of hydrofluoroalkane (HFA)-based pMDI formulations, with the extremely low solubility of most drugs of interest in these semi-fluorinated propellants being a particularly challenging formulation problem (Wu et al., 2008a).

The potential to combine new formulation strategies for local lung delivery with recent advances seen in nanomaterials for biomedical applications is expected to provide even greater opportunities for the development of innovative strategies to treat relevant lung diseases such as COPD (Ghosh et al., 2015), asthma (Davis et al., 2015; Patel et al., 2014) and lung metastasis (Landesman-Milo et al., 2015; Wood et al., 2014), as well as systemic ailments (Animikh et al., 2015; Bharatwaj et al., 2015; Gandhimathi et al., 2015; Syed Sarim et al., 2014; Vasiliu et al., 2014). Dendrimer nanocarriers (DNCs)(García-Gallego et al., 2015; Hourani and Kakkar, 2010) are of particular interest given their high monodispersity, flexibility in terms of the chemistry of the building blocks, and availability of a large number of surface groups that can be functionalized with ligands to help modulate their interaction with the physiological environment, as well as the conjugation of therapeutics through bonds that may promote the temporal and spatial control of drug release (Caminade and Turrin, 2014; Perumal et al., 2008; Zou et al., 2015). Polyester-based DNCs offer an opportunity to alleviate toxicity issues that may be associated with non-biodegradable dendrimers, including accumulation in relevant tissues, as polyesters degrade in the physiological environment and can thus be more readily cleared (Leiro et al., 2015). While several groups have recently discussed the interaction of dendrimers with *in vitro* an *in vivo* models of the pulmonary epithelium (Bai and Ahsan, 2009; Bharatwaj et al., 2014, 2015; Perumal et al., 2008; Ryan et al., 2016), much fewer reports can be found on the formulation of dendrimers in pMDIs (Bharatwaj et al., 2014; Conti et al., 2014; Zhong and da Rocha, 2016), and this is the first work to address the formulation of a polyester-based dendrimer in pMDIs and their interaction with a model of the pulmonary epithelium.

Considering the challenges and opportunities above, the goal of this study was to investigate the interaction of degradable polyester-dendrimers with a model of the pulmonary epithelium, and the impact of the generation and surface chemistry of the dendrimer conjugates on their behavior. The influence of the dendrimer chemistry on their formulation in HFA-based pMDIs was also investigated. Generation 3 and 4 (G3, G4) polyester dendrimers (GXTMPOH) were synthesized and fully characterized. PEG*ylation* (1,000 Da) of G4TMPOH was also explored, which has relevance in terms of both the interaction of the conjugates with the model pulmonary epithelium and their formulation in pMDIs, as will be explained later. The degradation of the various materials was evaluated under physiologically relevant pH values (extracellular and lysosomal). The toxicity of the conjugates and of their building blocks was evaluated on Calu-3 cells, one of the most widely used polarizable models of the pulmonary epithelium. Permeability studies of the conjugates across polarized Calu-3 monolayers and synthetic mucus were performed by following their transport from the apical to the basolateral side with the help of a fluorescent probe conjugated to the dendrimers (FITC). Cellular uptake (rate and extent), also on polarized monolayers, was followed by flow cytometry. Physical stability and aerosol quality of the conjugates were investigated through visual observations and with an Anderson Cascade Impactor (ACI).

## 2. Materials

Trimethylolpropane (TMP), 2,2-bis(hydroxymethyl)propionic acid (bis-MPA), 2,2-dimethoxypropane, *p*-toluenesulfonic acid monohydrate (*p*-TSA), 4-(dimethylamino)pyridine (DMAP), dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), poly(ethylene glycol) monomethyl ether (mPEG, $M_n$ 1,000 Da), succinic anhydride, triethylamine (TEA), Dowex® 50W-X2, silica gel for chromatography (pore size 60 Å, 220–440 mesh particle size), 2,5-dihydroxybenzoic acid (DHB), Triton-X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Dibutyltin dilaurate (DBTL) was obtained from Alfa Aesar (Ward Hill, MA). All HPLC grade solvents utilized were obtained from EMD Millipore (Billerica, MA). Fluorescein isothiocyanate (FITC), sodium chloride (NaCl), phosphate buffered saline (PBS, 10X), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Thermo Fischer Scientific (Rockford, IL). All chemicals were used as received unless otherwise specified. Spectra/Por cellulose ester membrane dialysis tubing was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Deuterated DMSO (DMSO-$d_6$) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Hank's Balanced Salt Solution (1X HBSS) supplemented with 0.01 M HEPES was prepared according a recipe provided by Irvine Scientific (Santa Anna, CA). Deionized (DI) water (resistivity of 18.2 MΩ cm) was obtained from NANOpure Diamond UV ultrapure water system (Barnstead International, Lake Balboa, CA). Amicon Ultra-15 Centrifugal Filters (MWCO 10,000 Da) were purchased from EMD Millipore (Billerica, MA). Dulbecco's modified Eagle's medium (1X) high glucose (DMEM), penicillin-streptomycin (AB), Trypan Blue (0.4%), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Life Technologies (Grand Island, NY). Fetal Bovine Serum (FBS, nonheat inactivated) was purchased from Atlanta Biologicals (Flowery Branch, GA). Trypsin-EDTA (1X) (Corning), tissue culture flasks (Greiner BioOne, 25/75 $cm^2$), 24 (Corning Costar) and 96 well plates (Greiner BioOne), Transwell® Inserts (polyester membrane, Corning, 0.33 $cm^2$, 0.4 μm pore size) were purchased from VWR International. Human bronchial epithelial cell line Calu-3 (HTB-55) was purchased from ATCC (Manassas, VA). BCA protein assay was purchased from Pierce (Evanston, IL). All glassware was washed thoroughly in water and dried under vacuum prior to usage.

## 3. Methods

### 3.1. Synthesis of GXTMPOH

#### 3.1.1. General esterification procedure for the synthesis of GXTMPOH

Bis-MPA was first protected (Ihre et al., 1998) and then used for the production of the correspondent anhydride, (Gillies and Fréchet, 2002) as described elsewhere (Malkoch et al., 2002). TMP (2.0 g, 14.9 mmol – 44.7 mmol of –OH group, 1 eqv.) and DMAP (0.8 g, 6.7 mmol, 0.15 eqv.) were dissolved in 18 mL (5 eqv.) of pyridine at room temperature. A solution of acetonide-bis-MPA anhydride (19.19 g, 58.1 mmol, 1.3 eqv.) in 55 mL of $CH_2Cl_2$ was added to the reaction. The solution was stirred for 48 h at room temperature. The excess anhydride was quenched with 3–5 mL of water under vigorous stirring overnight. The reaction was then diluted with 500 mL of $CH_2Cl_2$ and extracted with 10%w/w of $NaHSO_4$ (3 × 50 mL), 10%w/w of $Na_2CO_3$ (3 × 50 mL), and brine (1 × 50 mL). The organic phase was dried ($MgSO_4$), filtered, concentrated, and purified by liquid column chromatography on silica gel, eluting with hexane and gradually increasing the polarity to 40:60 EtOAc:hexane, to give a yellow oil.