Dendrimer Nanocarriers for Transport Modulation

Across Models of the Pulmonary Epithelium

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Supporting Information

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S1. Synthesis and Characterization of Carboxylic acid-terminated mPEGs (cmPEG). Purified mPEG (0.018 mmol) and potassium tert-butoxide (0.089 mol) were dissolved in 150 mL of tert-butanol at 40°C. Ethyl bromoacetate (0.045 mol) was subsequently slowly added to this solution. The contents were reacted for a period of 2h, after which the tert-butanol was evaporated. The residue recovered was dissolved in 1M sodium hydroxide (to hydrolyze the ester), and the solution was allowed to stand at room temperature for 2h, after which the pH of the solution was adjusted to 2 using 4N HCl. The cmPEG obtained was extracted into chloroform (200 mL), and the extract was washed with water, and dried over anhydrous MgSO₄. The resulting product was characterized using $^1$H-NMR (Varian 400 mHz), and MALDI-TOF (Ultraflex, Bruker Daltonics). The sample for MALDI analysis was prepared by mixing 2 μl of 1 mg·ml⁻¹ solution of the PEG compound in a 1:1 (v/v) acetonitrile-water mixture with an equal volume of 2,5-dihydrobenzoicacid (DHB) matrix (10 mg·ml⁻¹ in the same solvent mixture, 1:1 AcN:water). 2 μl of this mixture was spotted and evaporated to dryness on a stainless steel MALDI target plate (MTP 384, Bruker Daltonics). A mass spectrum was generated in the linear mode using a nitrogen laser at 20 kV. The mass of the compound was determined by comparing the resulting spectra against a compendium of calibrated peptide standards using FlexAnalysis™ software (Bruker Daltonics).

S2. Conjugation of PEG grafts and FITC to G3-NH₂. A series of G3NH₂ with varying graft densities of PEG 1000 Da (G3NH₂-nPEG1000, where n is the number of PEG 1000 grafts) was prepared and characterized. Appropriate molar ratios of EDC and NHS, which depended on the desired extent of PEG surface density, were added to a stirred solution of G3NH₂ (150 mg, 21 μM) in anhydrous DMSO (3 mL), and the mixture was stirred for 4 hours. This was followed by the addition of appropriate molar ratios of cmPEG and FITC (dissolved in 1 mL DMSO) dropwise to the reaction under stirring. The reaction mixture was allowed to stir for a period of at least 4 days in case of low PEG graft density, and for up to two weeks for the higher PEG densities. Molar ratios of the individual reactants have been tabulated as Table S1. The contents from the flask were then transferred to a dialysis tube (Fisher, MWCO 12000 Da), and were dialyzed against DI water for 48h to remove any unreacted FITC, EDC, NHS and cmPEG. The final product was recovered by snap freezing the contents from the dialysis bag, and freeze drying the resulting product for 48h. Product yield was ca. 60%. Dendrimer conjugates were characterized by light scattering for size and zeta potential (ζ) using a Malvern Zetasizer, and by $^1$H-NMR, and MALDI-TOF. DLS was performed by dissolving 1.5 mg of the purified conjugate in HBSS and analyzing the resulting sample. The size reported here is an average of 4 runs with 10 counts for each run. For $^1$H-NMR 8 mg of the freeze dried G3NH₂-nPEG1000 was dissolved in d-DMSO (Cambridge Isotopes). The sample for MALDI-TOF analysis was prepared as described in the earlier section. Synthesis of FITC labeled G3NH₂ with no PEG was accomplished by reacting appropriate molar amounts of FITC dissolved in DMSO with G3NH₂ overnight in DMSO (5 mL). The resulting product was purified,
dialyzed (MWCO 1000 Da) against DI water for 48h, and the product was recovered by freeze drying.

**S3. Culture of Calu-3 Cells.** Calu-3 cells were plated in 75 cm² culture flasks in medium comprising of Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS and 2% antibiotic (AB, penicillin-streptomycin) solution. The medium was exchanged once every two days. Cells were split at a ratio of 2:3 when they attained ca. 90% confluence.

**S4. Cytotoxicity of G3NH₂-nPEG1000 conjugates.** MTT assay was prepared as per the protocol given by the manufacturer and stored at -20°C prior to use. Cells were plated at a density of 10,000 cells per well, in 96 well plates, in DMEM supplemented with 10% FBS and 2% AB. Cells were allowed to attach and grow for a period of 24h, after which they were washed thrice with 1X PBS, and replenished with serum-free medium containing the G3NH₂-nPEG1000 conjugates at varying concentrations. The cells were incubated in the conjugate laden media for a period of 24h, after which the medium was removed, the cells washed thrice with 1X PBS and incubated in MTT assay at 37°C for 4h, after which 75 µl of the media was removed and the wells were incubated in 50 µl of DMSO for 10 minutes in order to dissolve the insoluble formazan crystals. Cell monolayers were subjected to UV analysis at a wavelength of 540 nm using a plate reader (Molecular Devices, CA) to assess the extent of cell viability. Untreated cells were used as the control, and the total cell kill was determined by comparing the absorbance of the treated cells with their untreated counterparts. The results expressed here are cell viability averages of 5 wells for each concentration.

**S5. Electron Microscopy of Cell monolayers.** Briefly, the monolayers were fixed in a 1:1 ratio of culture medium and 2.5% glutaraldehyde (fixing solution) in DI water. The fixing medium was added to both the apical and basolateral sides of the insert, and the insert was gently rotated for 5 minutes. Subsequently, the medium-fixative mixture was aspirated and replaced with 100% fixing solution (2.5% glutaraldehyde) for 1 hour. The fixed cell monolayers were bathed in 1% (w/v) OsO₄ in water for 2 hours. The cells were then dehydrated by washing in a series of increasing gradients of ethanol (20, 40, 60, 80 and 100% ethanol for 8-10 minutes each) and lyophilized (LabConco FreeZone) for 36h. Cell monolayers thus prepared were carefully removed from the insert and mounted onto aluminum stubs using adhesive carbon tapes. They were sputter-coated (Ernst Fullam, NY) with gold for 30s, and imaged using an SEM (Hitachi S-2400) at 22kV.

**S6. Immunocytochemistry.** Select monolayers, with appropriate TEER values (values peaked and leveled) were sequestered and washed with PBS to remove traces of culture media. The washed cell monolayers were fixed in 4% paraformaldehyde (in PBS) for a period of 20 minutes. The fixed cells were washed thrice with PBS (5 minutes each time), and permeabilized with 0.2% triton X-100 in PBS for 30 minutes. The permeabilized monolayer was further washed with PBS and blocked using 6% BSA for 30 minutes to
This procedure was followed by incubation of the cells for 60 minutes in Rabbit anti ZO-1 antibody at 37°C. The cells exposed to the primary antibody were washed thrice with PBS, and were incubated for 60 min in Alexa Fluor 546 goat anti-rabbit IgG, the secondary antibody, at 37°C. The cell layer was washed thoroughly with PBS, and counterstained with DAPI (0.5 µg∙ml⁻¹ in DI water) for 30 minutes at room temperature. The cell culture support membranes from the Transwell® inserts were carefully removed and mounted onto glass slides using DPX mounting media, and sealed using cover slides. The slide was stored at 4°C overnight prior to observation under a fluorescence microscope (Zeiss Axiocam MR, Carl Zeiss) to visualize the presence of tight junctions.

**S7. Characterization of carboxy-methyl poly ethylene glycol (cmPEG).** cmPEG (MW 1000 Da) was synthesized according to method detailed in the literature. The first step in the process was the conversion of monomethoxy PEG (mPEG) to the carboxyl terminated PEG (cmPEG). An increase in the MW, with a distinct change in the MW distribution pattern as evidenced by the MALDI spectrum (Figure S1a) was observed. The conversion from mPEG to cmPEG was also confirmed by the appearance of a peak in the ¹H-NMR spectrum of cmPEG 4.126 ppm (CH₂ in mPEG), and the appearance of a peak in the FT-IR spectra at 1735 cm⁻¹, from the stretching band of the carbonyl moiety unique to cmPEG — Figure S1b, S1c. The compound sourced was purified and characterized using ¹H-NMR, MALDI-TOF and FT-IR spectrosopies. The results of the characterization are given in Figure S1. The formation of cmPEG can be corroborated by the absence of peak at 4.6 ppm attributed to the –OH peak of mPEG. The unique stretching band at 1735 cm⁻¹ (distinct to carbonyl moiety of cmPEG) in the FT-IR peak (Figure S1(b)) also confirms the conversion. MALDI-TOF analysis, shows an increase in MW of the mPEG from 1035 Da to 1201 Da with a distinct change the MW distribution also attributed to the formation of cmPEG (Figure S1 (c)).

**S8. Characterization of PEGylated G3NH₂.** FITC conjugation was accomplished through the formation of a thiourea bond between FITC and G3NH₂, and was performed in a one pot synthesis along with the cmPEG conjugation. Conjugation of cmPEG at various surface densities to G3NH₂ was accomplished through EDC-NHS chemistry, by coupling the –NH₂ group of the G3NH₂ to the –COOH on the cmPEG. The resulting products (G3NH₂-nPEG1000) were purified by dialysis of the contents of the reaction vessel against DI water. The G3NH₂-nPEG1000 were characterized using ¹H-NMR (DMSO-d6) and MALDI-TOF - Figures S2-S5. Various grafting density of PEG1000 was achieved by controlling the molar ratio of cmPEG to G3NH₂, and the reaction time. The singlet signal originating at 2.17 ppm in the ¹H-NMR spectra of the G3NH₂-nPEG1000 (Figure S2(a)-S5(a)) can be attributed to the methylene protons next to the carbonyl groups of the dendrimer. The presence of the PEG moiety can be ascertained by the presence of broad peaks occurring between 3.3 to 3.6 ppm, which is attributed to the protons of the...
CH₂CH₂O repeat unit, and the signal due to the terminal -CH₃ group of PEG at 3.23 ppm. The number of PEG moieties attached to the G3NH₂ surface was determined by comparing the integral values of the –CH₃ peak at 3.23 ppm, to that of the methylene peaks of the dendrimer at 2.18 ppm (set at 120 protons – internal standard). The multiplet peaks between 6.5-6.8 ppm can be attributed to the aromatic protons of FITC. From those results, the number of PEG1000 surface groups on the dendrimers conjugates was calculated to be 5, 13 and 25, and those of FITC were determined to be between 1.5-2.0.

All NMR spectra are shown In Figures s2(a) – 0 PEG, s3(a) – 5 PEG, s4(a) – 13 PEG and s5 (a) – 25 PEG. The presence of multiplets between 6 and 7 ppm can be attributed to the presence of aromatic protons of FITC. Singlet at 2.2 ppm is due to the presence of methylene protons of G3-NH₂ and they were set as an internal standard at 120 in order to estimate the extent of PEG conjugation. Broad peaks arising between 3.2 and 3.6 ppm can be traced to the CH₂CH₂O repeat unit of PEG. The singlet at 3.23, due to the presence of –CH₃ group of PEG was used to determine the number of tethered PEG molecules on the G3-NH₂ backbone.

The increase in MW of the prepared G3NH₂-nPEG1000 compounds was documented using MALDI TOF and the resulting spectra are given in the Figures s2(b) – 0 PEG, S3(b) – 5 PEG, S4(b) – 13 PEG and S5 (b) – 25 PEG. A gradual rise in MW with increase in PEG density is evident along with the change in the MW distribution when compared to bare G3NH₂ (Figure S2(b) –Inset). The MW of the commercial PAMAM dendrimer obtained from Sigma was determined to be 6882 Da, which was in good agreement with the value 6909 Da provided by the manufacturer. The average MW of the compound was determined from the major peak of the MALDI spectra. MW of G3NH₂-0PEG1000 was 7660 Da – an increase in MW of 778 Da when compared to pure G3-NH₂. This indicates the presence of 2 FITC molecules per dendrimer on average – each new FITC conjugate is 390 Da. The MW for the other G3NH₂-nPEG1000 was 11,403 Da, 18,160 Da and 34,015 Da, respectively. With the knowledge of the MW of cmPEG, also from MALDI, and that of the G3NH₂-nPEG1000, the number of PEG1000 surface groups was also determined from MALDI. Those results are also included in Table 1, and can be compared to the results from ¹H-NMR. The combination of NMR, MALDI and DLS results (discussed in the manuscript) confirm the presence of PEG on the dendrimer surface.

The size of G3NH₂ and G3NH₂-0PEG1000 (just FITC-conjugated PAMAM) was determined to be 2.9 and 3.6 nm, respectively. These values are in good agreement with those reported in the literature of 3.2 nm and 3.6 nm, respectively. Furthermore, a similar increase in size of ca. 0.6 nm has been reported when FITC was conjugated onto G4-PAMAM dendrimers. With the increase in PEG1000 surface density, a gradual increase in hydrodynamic diameter from 3.6 nm (0 PEG) to ca. 8 nm (25 PEG) was observed. While a direct comparison cannot be made, owing to a lack of literature pertaining the DLS analysis of G3NH₂-nPEG1000, several studies have been published reporting a similar
increase in size of dendrimers upon grafting their end groups with PEG of varying MWs.\textsuperscript{10-12} The increase in size reported here is observed to be within the size range reported in the case of G3-polylysine dendrimers (diameter of unmodified dendrimers ca. 2.8 nm) that were grafted with 32 surface groups of PEG750, with a reported size of ca. 6.7 nm.\textsuperscript{10} The increase in the size reported in this study is relatively high, perhaps due to the presence of longer PEG chains.\textsuperscript{13} In another study, PEG of MW 3400 Da was tethered to the surface of G3 NH\textsubscript{2} and DLS analysis reported a size increase of 3.4 nm for a PEG surface coverage of 3.4. Our results are also in line with the results reported for G3NH\textsubscript{2}–PEG2000, where a diameter of ca. 12 nm has been reported at 99\% surface coverage.\textsuperscript{6} This value is higher than what we observed, but this increase is to be expected given the higher density and MW of PEG grafted on the dendrimer surface in that case. In another study, G4NH\textsubscript{2} with 50\% PEG density and PEG with MW of 5000 Da was reported to have a size of ca. 13 nm,\textsuperscript{12} further reaffirming that the conjugates synthesized in this work seem to fall within the expected size range.
## Table S1. Molar ratios of reactants for each individual dendrimer construct.

| Compound          | Molar ratios (mmol) |
|-------------------|---------------------|
|                   | EDC  | NHS  | cmPEG |
| G3NH₂-0PEG1000    | 0    | 0    | 0     |
| G3NH₂-5PEG1000    | 0.23 | 0.25 | 0.36  |
| G3NH₂-13PEG1000   | 0.6  | 0.58 | 0.79  |
| G3NH₂-25PEG1000   | 1.1  | 1.4  | 1.5   |
**Figure S1.** (a) MALDI-TOF spectra of cmPEG obtained using DHB as a matrix. (inset) MALDI-TOF spectra of mPEG. (b) FT-IR spectra of mPEG and cmPEG obtained using a KBr crystal. The distinct stretching at 1735 cm⁻¹ is assigned to the C=O bond formed in the cmPEG. (c) ¹H-NMR spectra of cmPEG - CDCl₃ as the solvent. The appearance of the peak at 4.126 ppm indicates the conversion of mPEG to cmPEG.
Figure S2. (a) $^1$H-NMR and (b) MALDI-TOF spectra of FITC-conjugated G3NH$_2$-0PEG1000. The average number of FITC molecules conjugated here was 2. (inset) MALDI-TOF spectra of pure PAMAM. $^1$H-NMR in DMSO-$_d_6$.
Figure S3. (a) $^1$H-NMR and (b) MALDI-TOF spectra of G3NH$_2$-5PEG1000. The average number of FITC molecules conjugated here was 1.5. $^1$H-NMR in DMSO-$d_6$. 
Figure S4. (a) $^1$H-NMR and (b) MALDI-TOF spectra of G3NH$_2$-13PEG1000. The average number of FITC molecules conjugated here was 1.7. $^1$H-NMR in DMSO-$d_6$. 
Figure S5. (a) $^1$H-NMR and (b) MALDI-TOF spectra of G3NH$_2$-25PEG1000. The average number of FITC molecules conjugated here was 2. $^1$H-NMR in DMSO-$d_6$. 
Figure S6. Average molar transport of G3NH2-nPEG1000 transported from apical to basolateral side as a function of time.
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