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Effect of the Route of Administration and PEGylation of Poly(amidoamine) Dendrimers on Their Systemic and Lung Cellular Biodistribution

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Abstract

There are many opportunities in the development of oral inhalation (oi) formulations for the delivery of small molecule therapeutics and biologics to and through the lungs. Nanocarriers have the potential to play a key role in advancing oi technologies and pushing the boundary of the pulmonary delivery market. In this work we investigate the effect of the route of administration and PEGylation on the systemic and lung cellular biodistribution of generation 3, amino-terminated poly(amidoamine) (PAMAM) dendrimers (G3NH₂). Pharmacokinetic profiles show that the dendrimers reach their peak concentration in systemic circulation within a few hours after pulmonary delivery, independent of their chemistry (PEGylated or not), charge (+24 mV for G3NH₂ vs −3.7 mV for G3NH₂-24PEG1000), or size (5.1 nm for G3NH₂ and 9.9 nm for G3NH₂-24PEG1000). However, high density of surface modification with PEG enhances pulmonary absorption and the peak plasma concentration upon pulmonary delivery. The route of administration and PEGylation also significantly impact the whole body and local (lung cellular)

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Notes

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distribution of the dendrimers. While ca. 83% of G3NH₂ is found in the lungs upon pulmonary delivery at 6.5 h post administration, only 2% reached the lungs upon intravenous (iv) delivery. Moreover, no measurable concentration of either G3NH₂ or G3NH₂-24PEG1000 is found in the lymph nodes upon iv administration, while these are the tissues with the second highest mass distribution of dendrimers post pulmonary delivery. Dendrimer chemistry also significantly impacts the (cellular) distribution of the nanocarriers in the lung tissue. Upon pulmonary delivery, approximately 20% of the lung endothelial cells are seen to internalize G3NH₂-24PEG1000, compared to only 6% for G3NH₂. Conversely, G3NH₂ is more readily taken up by lung epithelial cells (35%) when compared to its PEGylated counterpart (24%). The results shown here suggest that both the pulmonary route of administration and dendrimer chemistry combined can be used to passively target tissues and cell populations of great interest, and can thus be used as guiding principles in the development of dendrimer-based drug delivery strategies in the treatment of medically relevant diseases including lung ailments as well as systemic disorders.

Keywords

pulmonary administration; dendrimers; lung cellular distribution; pharmacokinetics; systemic distribution; PEGylation

1 Introduction

Pulmonary administration is an attractive and effective route for the noninvasive delivery of small molecule drugs and biomacromolecules for the treatment of lung disorders such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis.^{1–6} Oral inhalation (oi) also has several advantages when compared to intravenous (iv) and noninvasive administration routes (e.g., nasal and oral) with respect to the systemic delivery of therapeutics (*through* the lungs), including the large total surface area available for drug absorption, the avoidance of first-pass metabolism, and the potentially rapid onset of therapeutic effect.^{7,8}

In spite of the many potential advantages in the local or systemic delivery of drugs to and through the lungs, there is a notably small number of commercial oi products, and only very few classes of drugs are formulated as oi products. Those include adrenocortical steroids (e.g., beclomethasone),⁹ bronchodilators (e.g., isoproterenol, metaproterenol, albuterol),¹⁰ antiallergics (e.g., cromoglicic acid),¹¹ and inhalable insulin (Afrezza).¹²

There are, therefore, many opportunities for further development in the pulmonary drug delivery market. Advances in the formulation of portable aerosol systems and the ability to develop innovative nanotechnologies capable of modulating the interaction between the therapeutic agents and the local physiological environment^{13–19} are both expected to support and accelerate the development of novel oi formulations.^{20–25}

Dendrimers represent a promising class of nanocarriers for the delivery of small molecule therapeutics and biomacromolecules.^{26–30} Dendrimers are hyperbranched polymers that are highly monodisperse and possess a high density of functionalizable surface groups.^{31–33} A particular class of dendrimers—poly(amidoamine) (PAMAM)—has received a lot of

attention in the literature, particularly those with aminoterminated (NH_2) surface groups. These dendrimers can be functionalized with therapeutics and other ligands and have the ability to efficiently gain access to the intracellular milieu.³⁴ Toxicity³⁵ and rapid clearance^{36,37} of unmodified NH_2 -terminated PAMAM dendrimers have somewhat reduced the excitement toward these carriers. However, surface modification of NH_2 -terminated dendrimers with polyethylene glycol (PEG)—PEGylation—has been shown to be one effective strategy in the development of dendrimer nanocarriers. The benefits of PEGylation include reduced toxicity,³⁵ improved pharmacokinetic (PK) profiles,^{36,37} and improved aqueous solubility, which is a major issue that arises upon conjugation of hydrophobic therapeutics such as doxorubicin.^{38,39}

The tissue distribution and PK of PEGylated PAMAM dendrimers have been extensively studied *in vivo* upon iv administration, and the results suggest significantly decreased toxicity,⁴⁰ prolonged systemic circulation,^{40,41} and effective accumulation in target tissues through passive⁴² and active targeting strategies.⁴³ On the other hand, few studies have focused on the systemic biodistribution of nanocarriers upon pulmonary administration.¹⁵ Some of the nanomaterials/nanocarriers investigated include gold nanoparticles,¹⁶ PEGylated polylysine dendrimers,¹⁷ PEGylated poly(ethylene imine),⁴⁴ diethylaminopropylamine-poly(vinyl alcohol)-poly-(lactide-*co*-glycolide) copolymer,⁴⁵ and polystyrene nanoparticles.¹³ Additionally, the local distribution of PAMAM dendrimers in the lungs (cellular distribution) and their systemic biodistribution upon pulmonary administration have not been reported yet. Such knowledge is of great relevance as it will help guide the design of dendrimer nanocarriers that can passively target different tissues (systemic delivery) and the various lung cell populations (local delivery). For example, knowledge on the distribution to lymph nodes may help us design improved vaccine delivery systems,⁴⁶ while alveolar macrophage targeting may lead to the development of new strategies for the treatment of pulmonary tuberculosis.^{47,48}

The goal of this work was to investigate the systemic and local biodistribution of NH_2 -terminated PAMAM dendrimers upon lung delivery, and the effect of PEGylation on their distribution profile. We have selected generation 3, NH_2 -terminated PAMAM (G3NH_2) and G3NH_2 modified with a high density of PEG 1000 Da for this study. The PK parameters of the bare dendrimer and highly PEGylated dendrimer were investigated *in vivo*, upon lung delivery, and benchmarked against the profiles obtained upon iv administration. Systemic biodistribution was qualitatively determined by *ex vivo* imaging, and quantitative characterization was achieved by the extraction and quantification of the dendrimers from the various tissues. The local distribution of dendrimers in different pulmonary cell populations was quantified using cell type specific staining in combination with flow cytometry. These results help us understand how the chemistry of such carrier systems may be used to passively target different tissues and cell populations, and thus serve as a guide for the design of new dendrimer-based nanocarriers for the spatially and temporally controlled delivery of therapeutics for the treatment of lung and systemic disorders upon oi administration.

2 Materials and Methods

2.1 Materials

Generation 3, amine-terminated, poly-(amido amine) (PAMAM) dendrimers (G3NH₂), with 32 –NH₂ surface groups and theoretical molecular weight of 6909, was purchased from Dendritech, Inc. (Midland, MI, USA). Cyanine 3 (Cy3) NHS ester was purchased from Lumiprobe Corporation (Hallandale Beach, FL, USA). Methoxy polyethylene glycol 1000 Da (PEG1000) succinimidyl ester (PEG1000-SC) was purchased from NANOCS Inc. (New York, NY, USA). Paraformaldehyde solution 4% in PBS, saponin, Dispase, and 10 kU heparin sodium salt from porcine intestinal mucosa were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium phosphate (dibasic, anhydrous) and sodium phosphate (monobasic, monohydrate) were purchased from EMD Chemicals, Inc. (Gibbstown, NJ, USA). Deuterated dimethyl sulfoxide (DMSO-*d*₆) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Rat anti-mouse CD16/CD32 monoclonal antibody (Fc blocker) was provided by Thermo Fisher (Waltham, MA, USA). PerCP-Cy5.5 labeled anti-CD45 (30-F11) and PE-labeled anti-CD31 were purchased from EBioscience (San Diego, CA, USA). A primary antiprosurfactant protein C antibody (pro-SPC) (1:100) and a primary anti-tubulin antibody (1:100) were obtained from abcam (Cambridge, U.K.). Alexa Fluor647 F(ab')₂ goat anti-mouse IgG secondary antibody (1:100) and pacific blue F(ab')₂ goat anti-mouse IgG secondary antibody (1:100) were purchased from Life Technologies (Grand Island, NY, USA). Falcon cell strainers with mesh size 100 μm were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Ultrapure deionized water (DI H₂O) was obtained with the Barnstead NANOpure DIamond System (D11911) from Thermo Fisher Scientific (Waltham, MA, USA). Amicon Ultra 15 centrifugal filters (MWCO = 3 kDa) were purchased from EMD Millipore (Billerica, MA, USA). Thin layer chromatography (TLC) silica gel 60 F₂₅₄ plastic sheet was purchased from Merck KGaA (Darmstadt, Germany). All reagents were used as received unless otherwise specified.

2.2 Methods

2.2.1 Cy3 Labeling of PAMAM Dendrimers (G3NH₂-3Cy3)—G3NH₂ (27.2 mg, 3.94 μmol) was dissolved in 3.0 mL of phosphate buffer solution (PBS, 0.2 M, pH 8.4). Cy3 NHS ester (7.29 mg, 12.35 μmol) was dissolved in 0.5 mL of anhydrous dimethyl sulfoxide (DMSO) and then added dropwise to the above PBS. The reaction mixture was stirred for 2 h at 4 °C and another 4 h at room temperature. The unreacted Cy3 was removed using a centrifugal filter device (MWCO = 3 kDa) until TLC showed no free Cy3 in the product. The product was lyophilized and then stored at 4 °C for further use. The resulting structure of the Cy3-labeled G3NH₂ (number of Cy3 conjugated to the dendrimer) was determined using proton nuclear magnetic resonance (¹H NMR) and matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF). ¹H NMR (DMSO-*d*₆, ppm) spectra and peak assignment and MALDI-TOF spectra are provided in the Supporting Information. The ratio of Cy3 to dendrimer was also determined using UV spectrometry. The UV spectra of Cy3-labeled dendrimer were recorded using a Varian Cary 50 UV–vis spectrometer (Agilent Technologies, Santa Clara, CA). The number (*n*) of Cy3 per PAMAM dendrimer molecule was calculated with the following equations:

$$C_{\text{Cy3}} = \frac{A}{\epsilon b}, \quad C_{\text{G3NH2}} = \frac{m - 476.13 \times 3A / \epsilon b}{6909 \times 3}, \quad n = \frac{c_{\text{Cy3}}}{c_{\text{G3NH2}}}$$

where C_{Cy3} (mmol/mL) is the molar concentration of Cy3 in G3NH2-3Cy3, in aqueous solution, C_{G3NH2} (mmol/mL) is the molar concentration of G3NH2 in G3NH2-3Cy3, also in aqueous solution, A is UV absorption of G3NH2-Cy3 at 555 nm (dimensionless quantity), ϵ is molar absorptivity of Cy3 at 555 nm ($\epsilon = 150,000 \text{ mL}\cdot\text{cm}/\text{mmol}$), b is the path length of the quartz cuvette ($b = 1 \text{ cm}$), m (mg) is the mass of G3NH2-3Cy3 used to prepare 3 mL of phosphate buffer solution (0.1 M) for UV measurement, 476.13 (mg/mmol) is molecular weight of Cy3 NHS ester excluding NHS functional group, 3 (mL) is the volume of buffer solution for UV measurement, and 6909 (mg/mmol) is molecular weight of G3NH2 as provided by Dendritech, Inc. The result was compared with the data from ^1H NMR and MALDI-TOF.

2.2.2 Synthesis of Highly PEGylated PAMAM Dendrimer

(G3NH2-24PEG1000-3Cy3)—The resulting Cy3-labeled G3NH2 (15.2 mg, 1.84 μmol) was dissolved in 3.0 mL of phosphate buffer (0.2 M pH 8.4). 1 mL of anhydrous *p*-dioxane of PEG1000-SC (56.2 mg, 55.2 μmol) was added to the above aqueous buffer solution. The reaction mixture was stirred for 2 h at 0 °C and another 4 h at room temperature. The unreacted PEG1000-SC was removed using a centrifugal filter device (MWCO = 3 kDa). The resulting PEGylated dendrimer was lyophilized and then stored at 4 °C for further use. ^1H NMR (DMSO- d_6 , ppm) spectra and peak assignment and MALDI-TOF spectra are provided in the Supporting Information.

2.2.3 Size and Surface Charge of the Conjugates—The hydrodynamic diameter (HD) and zeta potential (ζ) of bare and PEGylated dendrimer conjugates were measured using a Malvern NanoCS Zetasizer (Malvern Instruments; Worcestershire, U.K.). The sample (1.0 mg/mL) was dissolved in DI H₂O. The aqueous suspension was equilibrated for 120 s before measurement, and the Mitoschi model was used as a scattering model. The average values and standard errors of HD ($n = 14$) and ζ ($n = 50$) were statistically calculated using the built-in software.

2.2.4 Pulmonary Administration of the Dendrimers—Pulmonary delivery of the bare and PEGylated Cy3-labeled dendrimers was performed using the pharyngeal aspiration (P.A.) technique.¹³ Briefly, male balb/c mice (25 g, 10–12 weeks old) were deeply anesthetized with 2.5% v/v isoflurane/oxygen and then placed on a tilted board in a supine position. The tongue was held gently in extension while a 50 μL saline solution of G3NH2-24PEG1000-3Cy3 (4.09 mg/mL) or G3NH2-3Cy3 (1.03 mg/mL)—same total G3NH2 concentration—was gradually dripped in the pharynx region with a Hamilton900 series syringe (Hamilton Company, Reno, NV, USA). The tongue was continuously held until after a few breaths. As the whole solution was administered, the mice were left under anesthesia for another 2 min and returned to the cage for monitoring of rapid recovery. The P.A. technique has comparable effectiveness to intratracheal instillation (I.T.), while less invasive, and also allows for the delivery of high dosages.⁴⁹

2.2.5 Pharmacokinetics (PK) of the Administered Dendrimers—After the administration of the dendrimers, blood samples were collected at predetermined time points (0.25, 0.5, 1, 3.25, and 6.5 h). At each time point, 80 μL of blood was collected from the tail vein and mixed with 20 μL of heparin saline (10 U/mL). The mixture was centrifuged at 5000 rpm for 45 s, and the plasma obtained was pulsed into a flat bottom 96-well plate for Cy3 fluorescence determination using a BioTek Synergy HT multicode microplate reader (Winooski, VA, USA).

The PK parameters of α , β (hybrid constant of $C_{p(t)} = a e^{-\alpha t} + b e^{-\beta t}$ in 2-compartment pharmacokinetic model), and k (elimination rate constant) were determined from the respective slopes of the log [plasma concentration] versus time graphs assuming a 2-compartment model for G3NH2-Cy3 dendrimers and a 1-compartment model for G3NH2-24PEG1000-Cy3 dendrimers. Then adsorption rate constants (k_a) were calculated from the slope of the absorption values over the first hour using the method of residuals. The respective half-lives were then found by dividing 0.693 by α , β , k , or k_a , respectively. The C_{max} (peak concentration of dendrimers in plasma) and t_{max} (time at which C_{max} is observed) values were directly observed from concentration versus time profiles. For all study groups, linear regression fits were $R^2 > 0.940$. To analyze dendrimer uptake kinetics from the lung, k_a 's were calculated from data points over the first hour of exposure, making the assumption that no significant tissue distribution or systemic clearance occurs during this early period. Other calculated PK parameters are defined and listed in Table 2.

2.2.6 Ex Vivo Imaging of Excised Tissues—The excised tissues were visualized using a Carestream In Vivo Xtreme (Rochester, NY, USA) imaging system (excitation/emission: 555/571 nm). The exposure time was set to 30 s. Visible light and Cy3 fluorescence images were taken and overlaid using the manufacturer's software.

2.2.7 Systemic Biodistribution of the Administered Dendrimer Conjugates—Our previous work indicated that PAMAM dendrimers delivered via P.A. reached peak concentration in blood circulation before or at 6 h,³⁶ which may suggest that dendrimers are distributed systemically (different tissues) through transcytosis from the lungs and locally in lungs (various lung cell populations) at that time point. Additionally, an early terminal point was selected in favor of elucidating cellular distribution of dendrimer nanocarriers in the lungs as dendrimers gain relatively fast access to systemic circulation. Therefore, mice were sacrificed 6.5 h after the administration of the dendrimers. This time point is based on our previous experience where we measured the PK profile for bare G3NH2 in the same model and after using the same route.³⁶ The various tissues were excised and washed with saline, including axillary lymph nodes (ALN), brachial lymph nodes (BLN), cervical lymph nodes (CLN), mesenteric lymph nodes (MLN), thymus, brain, heart, liver, lungs, kidneys, stomach, and spleen. The residual saline was wiped off using a filter paper, and the excised tissues were immediately used for analysis. These studies were bench-marked by comparing the biodistribution upon iv through the tail vein. These mice were sacrificed, organs were harvested, and samples analyzed in the same manner as the P.A. study groups.

2.2.8 Quantification of Dendrimers in Tissues—Each excised tissue was fully homogenized using a Cole-Parmer LabGEN 7 series homogenizer (Vernon Hills, IL) in a 3

mL aqueous solution of 3 N sodium hydroxide (NaOH), and the dendrimers were then extracted with gentle shaking for 72 h at room temperature in darkness. The tissue homogenate suspension was centrifuged at 14000g for 10 min at 4 °C, with the extracted dendrimer conjugates residing in the supernatant. The supernatant (200 μ L) was carefully removed (avoid collecting tissue homogenate) and transferred to a black flat-bottom 96-well plate. The Cy3 fluorescence of the supernatant was measured with a BioTek Synergy HT multicode microplate reader (excitation/emission = 530 $\pm$ 30/590 $\pm$ 20 nm). The amount of dendrimers in each tissue was quantified according to established calibration curves (the mass concentration of G3NH2-3Cy3 or G3NH2-24PEG1000-3Cy3 based on the Cy3 fluorescence) in the corresponding tissues, which were obtained by spiking fresh tissues of unexposed mice with a series of predetermined concentrations of G3NH2-3Cy3 or G3NH2-24PEG1000-3Cy3 saline, and then the dendrimers were extracted and measured as described above. The quantification of dendrimer biodistribution based on such calibration curve provides for a strategy to account for losses due to absorption by tissues/cells, binding to cellular proteins, and others such as photobleaching of the Cy3 fluorescent probe.

2.2.9 Single Cell Staining for Pulmonary Cellular Biodistribution of the Dendrimer Nanocarriers—Pulmonary myeloid cells, alveolar epithelial cells, endothelial cells, and ciliated airway epithelial cells were stained by probe-labeled antibodies and then analyzed via flow cytometry following a reported method with modifications (See Supporting Information).⁵⁰ Briefly, the excised lungs were tapped gently and incubated with Dispase and DNase to break extracellular matrix. The resulting cell suspension was filtered with 100 μ m nylon cell strainers. The obtained cells were fixed with 4% paraformaldehyde solution and then incubated with 0.15% saponin buffer for permeabilization at 4 °C. Subsequently, the cells were incubated with Fc-block for 20 min and then stained with the probe-labeled antibodies against CD45 or CD31 or with the primary antibodies against pro-SPC and β -tubulin for another 25 min at 4 °C in darkness. Primary antibodies were then labeled with pacific blue-labeled secondary antibody for another 25 min at 4 °C in the dark. The stained cells were analyzed with a BD Bioscience BD LSR II Analyzer (San Jose, CA, USA). Data analysis was performed with TREESTAR FlowJo software (Ashland, OR, USA). Lung cells were sorted in two ways: those that contained and those that did not contain internalized dendrimer conjugates. Second, from those that contained dendrimer conjugates, the fraction of myeloid (CD45), (alveolar) epithelial (pro-SPC), endothelial (CD31), and ciliated airway (β -tubulin) cells was determined. Studies were performed for both G3NH2-3Cy3 and G3NH2-24PEG1000-3Cy3 conjugates, so that the effect of chemistry on the dendrimer cellular distribution could also be assessed.

2.2.10 Statistical Analysis—GraphPad Prism 5 was used to perform the statistical analysis. Data were compared using Student's *t* test. Means were considered significantly different if **p* value < 0.05.

3 Results and Discussion

3.1 Synthesis of PEGylated Dendrimers

Both Cy3 labeling and PEGylation were performed using a primary amine/NHS ester chemistry, resulting in the formation of an alkyl amide bond in each case, which is a bond known to be stable under both *in vitro* and also *in vivo* physiological conditions.⁵¹ The synthetic route is shown in Scheme 1.

The first step in the preparation of the conjugates was to label G3NH₂ with Cy3 before PEGylation to guarantee the same number of Cy3 molecules on both the non-PEGylated and PEGylated dendrimers. The ¹H NMR with peak assignments and the MALDI-TOF spectra are shown in the Supporting Information. The ¹H NMR peaks at 7.614–7.163, 6.476, 1.509, and 1.353 ppm indicated successful conjugation of Cy3 to G3NH₂. Similarly, the molecular weight shift in MALDI-TOF from 6900 to 8285 Da after Cy3 reaction also confirms the successful conjugation of Cy3. UV spectrometry was also used to quantify the ratio of conjugated Cy3 to dendrimer. As shown in Table 1, an average of ca. 3 Cy3 molecules was conjugated per dendrimer as determined (in close agreement) by ¹H NMR (3.3), MALDI-TOF (3.1), and UV spectrometry (2.8).

Subsequently, dendrimers with a high PEG density were prepared by reacting G3NH₂-3Cy3 with PEG1000-SC. The ¹H NMR peak at 4.02 ppm revealed the successful conjugation of PEG1000 to G3NH₂-3Cy3 with an amide bond. ¹H NMR and MALDI-TOF were also used for quantification of PEG1000 density, revealing an average of 23.9 and 24.5 PEG1000/dendrimer, respectively.

The size and surface charge of bare dendrimer and PEGylated dendrimer conjugates were determined by light scattering (LS). Compared to the bare dendrimer, the size of G3NH₂-3Cy3 increased slightly to 5.1 nm, while at high PEG density the HD of the dendrimer increased to nearly 10 nm, as summarized in Table 1. This increase may be attributed to the stretching of the densely packed layer of PEG1000.^{52,53} The surface charge of G3NH₂-3Cy3 was seen to be even more cationic than the bare dendrimer due to the introduction of tertiary amines of Cy3, which have a stronger electron donating capacity. Even though a primary amine from the dendrimer was used up in the conjugation of each Cy3 molecule, two tertiary amines were brought into the conjugate per Cy3.

In contrast, the surface charge of the dendrimer was reversed upon PEGylation, resulting in a slightly negative charge, as shown in Table 1. The change in ζ upon PEGylation was in good agreement with a previous report.³⁶ In addition to the disparate surface chemistry between the PEGylated and non-PEGylated dendrimers (e.g., PEGylation changes hydrophilicity, aqueous solubility, and hydrogen bonding of dendrimer nanocarriers), these two carriers are also different in two very important ways: their surface charge (one is positive and the other negative/neutral) and their size (PEGylated dendrimer is twice as big as non-PEGylated). The PEGylation of the nanocarriers is expected to prolong their residence in systemic circulation and reduce nanocarrier toxicity.^{36,37,54} The surface charge of the conjugates and their size are also expected to impact their transport across the extracellular barriers of

the lung tissue.^{15,36,55,56} Extracellular barriers include the epithelial layer itself if the target is systemic circulation, and whose gap junctions are in the order of 3 nm.^{57,58}

For simplicity, in the following discussion “Cy3” will no longer be mentioned when describing the dendrimer nomenclature: the dendrimers will be referred to simply as G3NH₂ or G3NH₂-24PEG1000.

3.2 Plasma Concentration Profiles of Dendrimer Conjugates Administered via Pulmonary and Iv Routes

The plasma concentration–time profiles of the bare dendrimer (G3NH₂) and highly PEGylated dendrimer (G3NH₂-24PEG1000) following pulmonary (P.A.) and intravenous (iv) administration are summarized in Figure 1.

We have reported the effect of the PEGylation density of G3NH₂ on the transport across models (*in vitro* and *in vivo*) of the pulmonary epithelium.³⁶ The results discussed next thus serve to some extent to corroborate to our previous work, and also to provide a link to both works, so that the conclusions obtained here and before can be linked, so as to develop a comprehensive body of knowledge, which is well founded on materials that behave similarly. Note, however, that the materials discussed here not only are newly synthesized dendrimers but also contain a different fluorescence probe, which is more suitable to *in vivo* imaging/biodistribution. We thus provide a short discussion of the results in this section.

The plasma concentration of dendrimer conjugates delivered via the pulmonary route (Figure 1a) was seen to increase soon after administration (0.25 h is the first time point for blood collection). For G3NH₂-24PEG1000, it leveled off at 3.25 h post administration (plasma concentration increased slightly from 3.25 to 6.5 h, $p = 0.138$), while for G3NH₂, a peak was reached at 3.25 h (plasma concentration at 6.5 h significantly lower than at 3.25 h). The plasma concentration of the PEGylated dendrimer was 6-fold higher than that of the bare dendrimer (peak concentration: $0.6 \pm 0.2 \mu\text{g/mL}$ vs $3.8 \pm 0.4 \mu\text{g/mL}$ or $2.1 \pm 0.6\%$ vs $13.2 \pm 1.7\%$ of overall dose). The plasma concentration of the G3NH₂ conjugates administered iv decreased quickly, reaching $1.1 \pm 0.3 \mu\text{g/mL}$ (3.8% of overall dose) at 6.5 h post administration as can be seen in Figure 1b. However, the plasma concentration of the highly PEGylated dendrimer G3NH₂-24PEG1000 decreased only slightly within the same time remaining at 76% of the delivered dose. At 6.5 h the plasma concentration of the PEGylated dendrimer was 19.5-fold higher than that of bare dendrimer ($21.6 \pm 1.5 \mu\text{g/mL}$ vs $1.1 \pm 0.3 \mu\text{g/mL}$). A similar plasma concentration–time profile has been reported for PEGylated polylysine dendrimers of 11 kDa, 22 kDa, and 78 kDa, upon delivery to the lungs via intratracheal instillation.¹⁷

The effect of PEGylation on plasma concentration is in line with previous results for PAMAM dendrimers and other polymeric nanoparticles.^{41,42,59} The plasma concentration and *in vivo* biodistribution of the dendrimer conjugates are mainly affected by its absorption, distribution, metabolism, and elimination.³⁷ Surface charge, size, and surface functionality of the dendrimer conjugates determine their interaction with serum proteins and blood cells, uptake into target and nontarget organs, and potential routes of elimination. It has been demonstrated that PEGylation can neutralize surface charge of cationic polymers^{43,60} and

reduce the binding affinity of proteins to nanocarriers,^{61–64} which attenuates plasma protein adsorption, opsonization, phagocytosis, and stimulation of immune cells.^{40,64–66} Therefore, the blood circulatory residence of PEGylated dendrimers is significantly prolonged compared to the non-PEGylated counterparts.

In contrast to iv administration, dendrimers administered via pulmonary route need to cross several extracellular barriers to enter the local/systemic blood circulation. These extracellular barriers include the fast-renewing mucus layer and ciliated epithelial cells on the airways, resident alveolar macrophages, lung surfactants, and the alveolar epithelial cells in the deep lungs. The significantly different plasma concentration profiles for G3NH₂ and G3NH₂-24PEG1000 suggest that their charge and/or size have a significant impact on how they interact with the extracellular barriers before they reach systemic circulation. We have previously observed that even though dendrimers (<10 nm) are much smaller than the mucus mesh size, which is ca. 100 nm,⁶⁷ they may be retained in the mucus layer depending on their charge. Cationic dendrimers interact with the mucus environment much more strongly and, thus, take longer to traverse the mucus layer compared to neutral/negatively charged dendrimers.^{36,67} The increase in size of the PEGylated dendrimers may in principle also have an effect as the tight junctions of the epithelial barriers strongly modulate the transport of molecules as a function of time. For example, upon lung delivery, biomacromolecules with sizes <40–50 kDa (HD: 5–6 nm) are known to peak in the systemic circulation in a matter of minutes,⁶⁸ while larger molecules peak in the systemic circulation over periods of hours, days, or weeks.⁶⁹ However, our previous *in vitro* results show that even highly PEGylated dendrimers can interact with the tight junctional proteins between the pulmonary epithelial cells, and thus modulate their way across the barrier (paracellular pathways), mooted the impact of their size.³⁶ This is at least true for the size range being investigated and discussed here.

The impact of the dendrimer size can perhaps more strongly affect the route of elimination upon reaching systemic circulation. The HD of the non-PEGylated dendrimer (5.1 ± 1.4 nm) is smaller than the size limit (ca. 6 nm) in which nanoparticles are quickly eliminated from blood circulation by the kidneys (glomerular filtration).⁷⁰ Meanwhile, those nanocarriers larger than 6 nm cannot be readily eliminated by the kidneys, but instead accumulate in liver and spleen through the mononuclear phagocyte system (MPS): a relatively slow process.^{71,72} However, the hydrophilicity imparted by the PEGylation reduces or delays MPS processing^{63,73} and prolongs systemic circulation.³⁶ Thus, once the dendrimers are absorbed systemically, the PEGylation prevents glomerular filtration (due to their size) and also reduces MPS processing (due to their hydrophilicity), as seen for example with the G3NH₂-24PEG1000 dendrimers (HD = 9.9 ± 3.6 nm).

3.3 PK Analysis of Dendrimer Conjugates Administered via Pulmonary and Iv Routes

Given that the quantitative biodistribution studies performed here require terminal time points, a full PK study was not performed, as it would require significantly more time points at the later stages. Therefore, the parameters of AUC, bioavailability (F), volume of distribution (V_D), and clearance (Cl) were not determined: significant amounts of dendrimer still remained in the animal (i.e., bloodstream and tissues) at the final time point of the

biodistribution study. However, other PK parameters were determined, and are summarized in Table 2.

Following iv administration of G3NH2 dendrimers, it was found that they followed 2-compartment kinetics, indicating that the dendrimers distribute outside of the systemic circulation ($t_{1/2\alpha} = 2.39 \pm 0.04$ h). PEGylation of the dendrimers likely prevents extensive tissue distribution, resulting in 1-compartment kinetics. Comparing the elimination half-lives of the two dendrimer constructs, it is evident that the G3NH2 dendrimers are much more rapidly cleared ($t_{1/2\beta} = 2.39 \pm 1.4$ h) than the G3NH2-24PEG1000 dendrimers ($t_{1/2} = 18.2 \pm 2.25$ h): at a rate 7.6-fold higher. This indicates a greatly extended circulation time of the G3NH2-24PEG1000 dendrimers. When considering the pulmonary administered dendrimers, the plasma concentrations for both dendrimer chemistries did not reach an elimination phase over the studied period (6.5 h). The “apparent” $C_{\max}$ of the G3NH2-24PEG1000 dendrimer was 6-fold higher than the G3NH2 dendrimer, and $t_{\max}$ was extended for G3NH2-24PEG1000. Absorption rate constants (k_a) for each formulation illustrated that the G3NH2-24PEG1000 dendrimer was absorbed much more quickly than the G3NH2 dendrimer (k_a values are 0.992 ± 0.095 h⁻¹ and 0.327 ± 0.177 h⁻¹, respectively). The faster absorption for G3NH2-24PEG1000 can also be echoed by its smaller half-life of absorption $t_{1/2ka}$ (2.12 ± 0.196 h). The G3NH2-24PEG1000 dendrimers have both an increased pulmonary absorption and increased circulation time resulting in significantly higher plasma concentrations after both pulmonary and iv administration.

3.4 Systemic Biodistribution of the Dendrimers Delivered via the Pulmonary and Intravenous (iv) Routes

The effect of the administration route and PEGylation on the systemic distribution of dendrimer conjugates was investigated. Mice were sacrificed 6.5 h after P.A. or iv administration, and the major organs were excised, including lymph nodes, thymus, heart, lungs, stomach, spleen, liver, and kidneys. *Ex vivo* imaging was used to qualitatively assess the biodistribution of the dendrimers in these tissues. The results, using representative tissues, are summarized in Figure 2.

Quantitative assessment of the concentration of the dendrimers was performed by fluorescence spectroscopy after recovering the Cy3-tagged dendrimers from the various tissues as discussed in Materials and Methods. The results, in terms of % of (total) dose, as a function of route of administration and dendrimer chemistry, are summarized in Figure 3. The results are also summarized in terms of the mass dendrimer/mass tissue: they are shown in Figure S4.

The results demonstrate that administration route and chemistry of the dendrimers have a significant impact on the dendrimer biodistribution. It can be observed that the amount of dendrimers found in the lungs was much higher when they were administered via P.A. than compared to iv delivery (compare Figures 3a and 3b and results in Figure 2), as expected. While $83.4 \pm 7.3\%$ of the administered dose for G3NH2 and $67.7 \pm 7.4\%$ of administered dose for G3NH2-24PEG1000 were present in the lungs 6.5 h post P.A. administration, only $1.6 \pm 0.5\%$ G3NH2 and $0.9 \pm 0.4\%$ G3NH2-24PEG1000 can be found in the lungs at the same time when injected iv. The lung retention upon P.A. delivery is also impacted by the

chemistry of the nanocarriers. The positively charged G3NH₂ is retained longer in the lungs, while the PEGylated dendrimer translocates to systemic circulation and leaves the lungs to a higher extent. A dense PEG coating layer reduces the surface charge of amine-terminated dendrimers (+24.5 mV to −3.7 mV in this case), is expected to reduce lung surfactant protein binding, and modulates the way dendrimer interacts with pulmonary epithelial cells. All these advantages favor PEGylated dendrimers to enter local/systemic circulation at a relatively faster rate. These results suggest the potential of the dendrimer nanocarriers for the targeting of lung diseases when combined with oral inhalation formulations as the surface chemistry of the dendrimers can be used to modulate the transport of therapeutics across the pulmonary epithelium. The potential in using such nanocarrier systems is supported by our recent results where we demonstrate that dendrimer nanocarriers can be formulated in portable oral inhalation devices,⁵⁵ and those can be used for the delivery of small molecules^{3,55} or biomacromolecules such as nucleic acids.^{1,29}

We also observed that the dendrimers administered via P.A. showed up in large quantities in the lymph nodes (LNs), while no/very little accumulation was observed upon iv administration. In fact, on a mass/tissue basis (Figure S4), the mass of conjugates found in the LN upon P.A. was second only to the lungs. It can also be observed that the chemistry of the conjugates plays a major role in terms of their biodistribution to the LNs upon P.A. delivery. The PEGylated dendrimer appeared in significantly greater concentrations in the brachial lymph nodes (BLN) and cervical lymph nodes (CLN) when compared to the non-PEGylated conjugates.

Charge effects on biodistribution have been reported for solid nanoparticles.^{15,71} Neutral (polystyrene-polyacrylate) and hydrophilic (PEG20 kDa) organic nanoparticles smaller than 38 nm and with PEG ligands were rapidly translocated to mediastinal lymph nodes, while anionic and cationic charged nanoparticles were readily bound to endogenous proteins in the lungs. When polystyrene nanoparticles ranging from 50 to 900 nm were administered to the lungs, the highest nanoparticle deposition was also detected in the lymph nodes 3 h after pulmonary administration.¹³

These results point to a strategy for passively targeting the lymph nodes with dendrimers upon delivery via P.A., which may prove relevant for the development of various dendrimer-based therapies, including pulmonary vaccination (e.g., influenza,⁷⁴ tuberculosis,² HPV infection⁷⁵) and diseases of lymphatic system such as metastasis (e.g., breast cancers⁷⁶).

No obvious fluorescence was found in the major organs responsible for elimination of the dendrimers such as liver, spleen, and kidneys at 6.5 h post administration when the dendrimers were delivered via P.A. In contrast, significant quantities of the conjugates were detected in liver, spleen, and kidneys when the conjugates were administered intravenously. In that case, greater concentrations of the non-PEGylated dendrimers were cleared/found in the kidneys ($32.8 \pm 8.8\%$), spleen ($5.3 \pm 2.5\%$), and liver ($7.0 \pm 3.7\%$) when compared to the PEGylated conjugates ($2.7 \pm 1.2\%$ in kidneys, $2.9 \pm 1.7\%$ in liver, and $2.7 \pm 1.6\%$ in spleen). These results are in excellent agreement with the PK results discussed above. In fact, the accumulation of bare dendrimer in the kidneys may be underestimated to some extent since previous studies showed that PAMAM dendrimers can be detected in urine 2 h after iv

injection.⁷⁷ The distribution of dendrimers in both kidneys and liver/spleen reveals that renal excretion and MPS are able to play roles in dendrimer elimination. Noticeably, the kidneys showed the greatest fluorescence in the case of bare dendrimer, demonstrating that the bare dendrimer is mainly eliminated renally due to its small size (5.1 nm).⁷² However, the PEGylated dendrimers showed no preferable accumulation site at 6.5 h.

3.5 Lung Cellular Biodistribution of the Dendrimer Conjugates Administered via Pulmonary Route

The internalization of the dendrimer conjugates in selected lung cell types upon P.A. was also assessed. Four typical pulmonary cell populations were selected to be tagged during the flow cytometry experiments, namely, myeloid, endothelial, alveolar epithelial, and airway ciliated epithelial cells. The characteristic receptors of these cells are CD45 on myeloid cells, CD31 on endothelial cells, lung pro-surfactant protein C (pro-SPC) on alveolar epithelial type II cells, and cilia layer on apical side of airway epithelial cells. Cilium is a microtubule-based cytoskeleton that is constructed by β -tubulin in combination with α -tubulin. Therefore, ciliated airway epithelium has much higher levels of β -tubulin than other cells do. The flow cytometry results are summarized in Figure 4: representative dot plots are shown in Figure S3.

The flow cytometry results indicate that G3NH2 was found internalized in $32.8 \pm 3.6\%$ of the pulmonary cells upon P.A. delivery, while the % of the cells that had internalized G3NH2-24PEG1000 was $27.9 \pm 2.7\%$ (inset in Figure 4). While no statistically significant difference was found when comparing the average for both groups, a lower percentage of internalization of the PEGylated dendrimer would be in agreement with the fact that PEGylation has been shown to decrease the rate and extent of internalization within this time frame: as shown *in vitro*.^{36,54} This trend also supports the observation that the PEGylated dendrimers are transported into systemic circulation faster (PK results) and are found at a lower concentration in the lungs (biodistribution results).

Myeloid cells, including monocytes, granulocytes, dendritic cells, and macrophages, were observed to internalize the largest fraction of dendrimers when compared to all other cells. While the specific cell subpopulation was not determined, these results suggest that such dendrimers would be able to target cells that are relevant in the treatment of medically important diseases such as tuberculosis,² and in vaccine delivery applications.⁷⁴ It is also observed that the cell populations interact differently with the different chemistries. For example, while 37.2% of the cells that internalized G3NH2 were myeloid type, the preference of myeloid cells was lower toward G3NH2-24PEG1000. As a matter of fact, the percentage of all cells (based on cell type) that had internalized G3NH2-24PEG1000 was lower than the percentage of cells that internalized G3NH2. This was not true for endothelial cells, where 20.4% contained G3NH2-24PEG1000, compared to only 6.0% for G3NH2. These results support the view that the PEGylated dendrimers can more efficiently transport across the epithelial barrier of the pulmonary epithelium, and gain access to the endothelial layer, where they are internalized, while at the same time being able to evade to some extent (in comparison to G3NH2) internalization by myeloid cells.

There are two main routes for the dendrimers to show up within the endothelial cells after pulmonary administration: (i) internalization by endothelial cells after apical-to-basolateral transport of dendrimers across the pulmonary epithelium upon lung delivery, and (ii) reabsorption of dendrimers by endothelial cells from the bloodstream after the dendrimers have gained access to systemic circulation via peripheral lung vasculature. While reabsorption is likely, we hypothesize that the first route is the major pathway for dendrimer internalization to the endothelial cells. It is understood that the rate and extent of internalization of nanocarriers with a given chemistry are proportional to the concentration and the time of exposure to cells. This means that once dendrimers enter systemic circulation, it is expected that they will have a lesser chance to be taken up by the pulmonary endothelial cells due to a relatively short exposure time due to fast flowing circulation and lower plasma concentration (Figure 1a), when compared to the more stagnant and greater concentration in the interstitial layer when the dendrimers are en route to systemic circulation. The systemic biodistribution results shown in Figure 2 further indicate that both dendrimers (PEGylated or not) behave similarly in that they are present in the central lung (low fluorescence in the peripheral lung) at 6.5 h post pulmonary administration.

In addition, the dendrimer nanocarriers can be significantly internalized by alveolar epithelial cells ($G3NH2 = 22.9 \pm 3.7\%$ and $G3NH2-24PEG1000 = 16.1 \pm 4.5\%$ of those cells internalizing dendrimer nanocarriers) and ciliated airway epithelium ($G3NH2 = 12.4 \pm 4.2\%$ and $G3NH2-24PEG1000 = 8.3 \pm 1.8\%$ of those that internalized dendrimer). No statistically significant difference was found between $G3NH2$ and $G3NH2-24PEG1000$ in either alveolar or airway epithelial cells. If both epithelial cells are considered together for statistical analysis, however, $G3NH2$ were internalized by epithelial cells (alveolar + airway) to a greater significant extent when compared to $G3NH2-24PEG1000$ ($35.4 \pm 4.5\%$ vs $24.3 \pm 3.8\%$; $*p = 0.0315$ obtained by Student's *t* test). These results show that highly positively charged bare dendrimers are entrapped by or interact with the epithelial layer to a larger extent than their PEGylated counterparts. The internalization of dendrimer nanocarriers by alveolar/airway epithelial cells implies the potential for drug delivery to the diseases that cause an acute/chronic dysfunctional respiratory tract and lungs as for example pneumonia (usually in alveolar sacs) and asthma (airways and lungs).

Combined, the biodistribution, PK, and lung cellular distribution results shown here provide us with clues that can help formulate a reasonable hypothesis as to why the dendrimer conjugates tend to accumulate in the lymph nodes upon P.A. While large particles (>500 nm) require the activation of antigen-presenting cells (e.g., dendritic cells, B cells) to be trafficked to lymph nodes,¹³ dendrimer nanocarriers with hydrodynamic diameters <10 nm are able to rapidly translocate across pulmonary epithelium from airway/alveolar luminal surface to septal interstitial fluids, followed by rapid accumulation to lymph nodes. This step does not require the activation of antigen-presenting cells (APCs). It is believed that the passive pathway may play a major role in targeting dendrimer nanocarriers to draining lymph nodes. Previous studies show that PEGylated inorganic nanoparticles or PEG nanoparticles with hydrodynamic diameters below 38 nm and neutral/negatively charged surface are able to efficiently drain into lymph nodes when delivered through pulmonary route.⁷⁸ Although APCs would rather phagocytize large particles than small ones, the abundance of APCs in the respiratory tract and lungs may also effectively take up smaller

carriers such as dendrimers through various endocytic pathways. Subsequently, the migratory macrophages and dendritic cells activated by the phagocytosis of dendrimer nanocarriers move to tracheobronchial and bronchiolar lymph nodes including bronchial-associated lymphatic tissue (BALT), and are eventually carried into draining lymph nodes. 14,79,80 Both a cellular-mediated transport and rapid translocation to the interstitium are relevant routes in the context of using dendrimers for drug delivery. For example, the capture by pulmonary APCs and transport to the lymph nodes may be relevant in the development of vaccine delivery systems, as we have shown in our recent work on the use of dendrimers for the delivery of subunit vaccines for chlamydia (dendrimers work as both carrier and adjuvant). Macrophage targeting would be relevant in the treatment of pulmonary tuberculosis, while the direct transport of the nanocarriers through migration and transport through interstitium could be useful in the treatment of relevant disease such as cancer. In addition, it seems that the draining of dendrimer nanocarriers to lymph nodes is also affected by their surface chemistry.

4 Conclusions

The results in this work show that both surface chemistry (PEGylated vs non-PEGylated) and the route of administration (pulmonary delivery vs intravenous injection) have a strong effect on the PK, systemic tissue biodistribution, and local (lung) cellular distribution of G3NH₂ dendrimers. PEGylated dendrimers are much more rapidly adsorbed into systemic circulation upon lung delivery (0.992 h^{-1}) when compared to the non-PEGylated counterpart (0.327 h^{-1}). At the same time, biodistribution results show that while 83% of G3NH₂ is found in the lungs 6.5 h post P.A. administration, PEGylation enhances clearance: ca. 68% of G3NH₂-24PEG1000 is found in the lungs at the same time point upon P.A. administration. PEGylation also significantly increases the percentage of lung endothelial cells that internalized dendrimers upon P.A. administration—20% contained G3NH₂-24PEG1000 vs 6% for G3NH₂—while decreasing the percentage internalized in the lung epithelium: 35% for G3NH₂ vs 24% for G3NH₂-24PEG1000. Combining these results suggests that PEGylation of G3NH₂ has the effect of enhancing dendrimer transport across extracellular pulmonary barriers and also across the pulmonary epithelium, thus more efficiently reaching the endothelial cells and systemic circulation. P.A. administration and PEGylation are seen to promote the passive targeting of dendrimers to lymph nodes. P.A. administration also led to ca. 52–75-fold increase in dendrimer concentration in the lung tissue compared with iv administration, when less than 1.6% of the dose reached the lungs. The results shown here suggest that both the pulmonary route of administration and dendrimer chemistry combined can be used to passively target tissues of great interest, and can thus be used as guiding principles in the development of dendrimer-based drug delivery strategies in the treatment of medically relevant diseases including lung ailments, pulmonary vaccination, and malignant metastases among others.

Supporting Information

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used

PAMAM	poly(amidoamine)
G3NH2	generation 3 amine-terminated PAMAM dendrimer
PEG1000	polyethylene glycol of molecular weight 1000 Da
G3NH2-3Cy3	G3NH2 dendrimer fluorescently labeled with 3 Cy3 molecules
G3NH2-24PEG1000-3Cy3	G3NH2 dendrimer with 24 PEG1000 and 3 Cy3 on surface
NHS	<i>N</i> -hydroxysuccinimide
Cy3	cyanide 3
DMSO	dimethyl sulfoxide
2,5-DHB	2,5-dihydroxybenzoic acid
MWCO	molecular weight cutoff
DI water	deionized water
PBS	phosphate buffer saline
¹H NMR	proton nuclear magnetic resonance
MALDI	matrix-assisted laser desorption/ionization
LS	light scattering
MW	molecular weight
HD	hydrodynamic diameter
MFI	median fluorescence intensity
PA	pharyngeal aspiration
iv	intravenous
LN	lymph node
ALN	axillary lymph node

BLN	brachial lymph node
MLN	mesenteric lymph node
CLN	cervical lymph node
pro-SPC	pro-surfactant protein C antibody

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Figure 1.

Plasma concentration (C_p) as a function of time after administration of G3NH2 and G3NH2-24PEG1000 via (a) pharyngeal aspiration (P.A.) and (b) intravenous injection (iv) ($n = 3$ per group). The statistical analysis was performed between G3NH2 and G3NH2-24PEG1000 with Student's t test (** $p < 0.01$, and *** $p < 0.001$).

Figure 2.

Ex vivo biodistribution of G3NH2 and G3NH2-24PEG1000 delivered via pharyngeal aspiration (P.A.) or iv, determined 6.5 h after administration ($n = 3$ per group). ALN: axillary lymph nodes. BLN: brachial lymph nodes. CLN: cervical lymph nodes. MLN: mesenteric lymph nodes. The tissues that are enlarged for better visualization in the figure are ALN, BLN, CLN, and MLN. Liver is shrunk instead for better visualization.

Figure 3.

In vivo biodistribution of G3NH2 and G3NH2-24PEG1000 delivered via (a) pharyngeal aspiration (P.A.) and (b) iv, 6.5 h after administration. The insets highlight the biodistribution of the dendrimer conjugates in the different lymph nodes; when they are all combined, they are represented in the main figure as LN. ALN: axillary lymph nodes. BLN: brachial lymph nodes. CLN: cervical lymph nodes. MLN: mesenteric lymph nodes. The statistical analysis was performed between G3NH2 and G3NH2-24PEG1000 with Student's *t* test (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). $n = 3$ per group. ID = initial dose.

Figure 4.

Lung cellular distribution studies upon lung delivery of the dendrimer conjugates. Breakdown in terms of cell type (out of those cells that had internalized dendrimer conjugates) and dendrimer chemistry (PEGylated or not). Inset: % of lung cells, out of all the cells in the tissue single cell suspensions, which had internalized dendrimer conjugates ($n = 3$ per group). Statistical analysis was performed with Student's t test ($*p < 0.05$). n.s.d = not significantly different.

Scheme 1. Schematic Diagram of the Synthesis of the Cy3-Labeled, PEGylated Dendrimer Conjugates^a

^aThe Cy3 terminology is dropped in Results and Discussion. For simplicity, the conjugates are referred to as G3NH2 and G3NH2-*m*PEG1000, where *m* is the number of PEG 1000 Da graft moieties; the number of Cy3 is the same for all conjugates; r.t. = room temperature.

Table 1
Characterization of the Cy3-Labeled, PEGylated Dendrimer Conjugates (G3NH2 and G3NH2-24PEG1000)^a

compd	MW (Da)	<i>m</i>		<i>n</i>			HD ± sd (nm)	PDI	ζ ± sd (mV)
		MALDI	NMR	MALDI	NMR	UV			
G3NH2 (as received)	6900	0	0	0	0	0	3.8 ± 1.3	0.17	+18.8 ± 5.0
G3NH2	8285	0	0	3.1	3.3	2.8	5.1 ± 1.4	0.20	+24.5 ± 6.9
G3NH2-24PEG1000	33312	24.5	23.9	3.1	3.3	2.8	9.9 ± 3.6	0.28	−3.7 ± 5.0

^aMolecular weight (MW), number of PEG1000 grafts (*m*), number of Cy3 per dendrimer (*n*), size (hydrodynamic diameter, HD), polydispersity (PDI), and zeta potential (ζ) as determined by MALDI, ¹H NMR, UV spectrometry, and light scattering (LS).

Table 2
Pharmacokinetic (PK) Parameters for the G3NH2 and G3NH2-24PEG1000 Conjugates as a Function of the Route of Administration: P.A. Pharyngeal Aspiration (P.A.) and Intravenous (Iv)^a

PK params ^b	G3NH2		G3NH2-24PEG1000	
	P.A.	iv	P.A.	iv
dose ($\mu\text{g/kg}$)	2163	2163	2127	2127
α (h^{-1})		1.42 ± 0.05		
$t_{1/2\alpha}$ (h)		2.39 ± 0.04		
β (h^{-1})		0.29 ± 0.12		
$t_{1/2\beta}$ (h)		2.39 ± 1.4		
k (h^{-1})				0.038 ± 0.04
$t_{1/2}$ (h)				18.2 ± 2.25
t_{max} (h)	3.25 ± 0.0		6.50 ± 0.0	
C_{max} ($\mu\text{g/mL}$)	0.61 ± 0.18		3.75 ± 0.48	
k_a (h^{-1})	0.327 ± 0.177		0.992 ± 0.095	
$t_{1/2ka}$ (h)	2.12 ± 0.196		0.7 ± 0.073	

^a 2-compartment analysis was applied to bare G3NH2 dendrimer; 1-compartment analysis was used for the G3NH2-24PEG1000 dendrimer.

^b Dose is the amount of dendrimers administered. α and β are called hybrid constant of $C_p(t) = a e^{-\alpha t} + b e^{-\beta t}$ in 2-compartment pharmacokinetic model which is characterized by an α distributive phase and a following β elimination phase. $t_{1/2\alpha}$ is half-life of α distributive phase, and $t_{1/2\beta}$ is half-life of β elimination phase. k is elimination constant. $t_{1/2}$ is the time required to reduce plasma concentration to half of its initial value. C_{max} is the peak plasma concentration after dendrimers are administered. t_{max} is the time at which C_{max} is observed. k_a is absorption rate constant. $t_{1/2ka}$ is half-life of absorption.