Published in final edited form as:

Bioconjug Chem. 2008 August ; 19(8): 1660–1672. doi:10.1021/bc700483s.

Systematic Investigation of Polyamidoamine Dendrimers Surface-Modified with Poly(ethylene glycol) for Drug Delivery Applications: Synthesis, Characterization, and Evaluation of Cytotoxicity

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Abstract

Surface-modification of amine-terminated polyamidoamine (PAMAM) dendrimers by poly(ethylene glycol) (PEG) groups generally enhances water-solubility and biocompatibility for drug delivery applications. In order to provide guidelines for designing appropriate dendritic scaffolds, a series of G3 PAMAM-PEG dendrimer conjugates was synthesized by varying the number of PEG attachments and chain length (shorter PEG₅₅₀ and PEG₇₅₀ and longer PEG₂₀₀₀). Each conjugate was purified by size exclusion chromatography (SEC) and the molecular weight (MW) was determined by ¹H NMR integration and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). NOESY experiments performed in D₂O on selected structures suggested no penetration of PEG chains to the central PAMAM domain, regardless of chain length and degree of substitution. CHO cell cultures exposed to PAMAM-PEG derivatives ($\leq 1 \mu\text{M}$) showed a relatively high cell viability. Generally, increasing the degree of PEG substitution reduced cytotoxicity. Moreover, compared to G3 PAMAM dendrimers that were *N*-acetylated to varying degrees, a lower degree of surface substitution with PEG was needed for a similar cell viability. Interestingly, when longer PEG₂₀₀₀ was fully incorporated on the surface, cell viability was reduced at higher concentrations (32 μM), suggesting increased toxicity potentially by forming intermolecular aggregates. A similar observation was made for anionic carboxylate G5.5 PAMAM dendrimer at the same dendrimer concentration. Our findings suggest that a lower degree of peripheral substitution with shorter PEG chains may suffice for these PAMAM-PEG conjugates to serve as efficient universal scaffolds for drug delivery, particularly valuable in relation to targeting or other ligand-receptor interactions.

INTRODUCTION

Synthetic macromolecules are often employed as drug carriers to improve overall pharmacokinetic properties of monomeric drugs and to enhance their therapeutic effects (1, 2). For instance, one of the most successful tumor targeting approaches greatly benefits from therapeutics with macromolecules by implementing their ability to readily extravasate from

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Supporting Information Available: ¹H NMR and MALDI-MS spectra, a complete list of cytotoxicity values, and selected images of cell cultures containing dendrimers used for cytotoxicity experiments. This material is available free of charge via the Internet at <http://pubs.acs.org/BC>.

the leaky tumor blood vessels and accumulate in the tumor interstitium through the enhanced permeability and retention (EPR) effect (3). As originally proposed by Ringsdorf and others, synthetic macromolecular carriers facilitate the incorporation of various functional units such as solubility-enhancers, targeting units, and visualizing groups, in addition to the particular drug moieties of interest (4). Unfortunately, these carriers may suffer from an elevated toxicity and immunogenicity (i.e., low biocompatibility). Accordingly, additional modification of the structure might be necessary for a synthetic macromolecular drug delivery system to minimize undesirable properties for practical applications.

The dendrimer, one of the latest additions to the polymer family, has a globular shape and a relatively predictable size as represented by the hydrodynamic volume in a given solvent (5–8). Indeed, the virtue of using these (nearly) monodisperse dendrimers as drug carriers over the conventional polymeric agents relies heavily on their robust shape and controllable size and physical properties—to result in consistent biological effects—that can be attained by routine organic synthesis (9–18). Characteristics of dendritic structures, including toxicity, interactions with foreign objects (e.g., cells, opsonins), routes for cellular uptake, and intracellular fate will most likely be governed by the imparted surface groups (19–21). In contrast, such properties of conventional polymeric carriers may vary depending on their preferred folding pattern in a particular environment. Thus, a judicious choice of surface groups is crucial to optimize the pharmacological effects of a dendrimer-based drug delivery system.

Linear poly(ethylene glycol) (PEG) dissolves in water and most organic solvents and manifests crystalline properties in the solid-state. Its strong but neutral hydrophilic nature without any significant toxic effect has found many applications in drug delivery as a structural modifier (22–25). In general, attachment of PEG (i.e., PEGylation) improves water-solubility, reduces toxicity, decreases enzymatic degradation, and increases the in vivo half-lives of small-molecule drugs. A possible reduction in drug potency due to the sterics imparted by a long flexible PEG chain can be compensated by a reduced renal elimination rate.

Numerous reports described examples of attaching PEG to dendrimers through different types of *bond* formations to create hybrids of various *geometries*, amongst which the application for drug delivery has been most prevalent (26): i) by forming either covalent (27–33) or electrostatic bonds (34) between a dendrimer and PEG groups; ii) by attaching either linear PEG derivatives to the dendrimer periphery (i.e., unimolecular micelle) (29,32,35), or mono-/multi-functional PEG derivatives to one or more core units of dendrons to form linear/branched-dendritic block copolymers (27,31,33,36–43). Physical properties of these PEG-dendrimer conjugates were often dependent on the weight contribution of each block and the solvent used, occasionally exhibiting semi-crystalline morphologies by phase-segregation (37,40,42,43). Some examples involving self-assembly of amphiphilic PEG-dendritic block copolymers allowed the controlled release of electrostatically bound (27,44) or/and hydrophobically encapsulated (32,45–47) therapeutic agents. Intriguingly, a fully surface-PEGylated ($M_n = 2000$) dendrimer with a basic interior efficiently retained and slowly released hydrophobic anticancer drugs with acidic functionalities, in aqueous medium of a low ionic strength (32). Alternatively, when ligands are covalently attached (i.e., activation without chemical cleavage) to the termini of a dendrimer, the neighboring surficial PEG chains may impose a substantial steric barrier to impede the direct accessibility of ligands to their receptors. Therefore, strategies to covalently connect ligands to these dendrimer conjugates involved presenting them at the surface through peripheral PEG groups as long spacers (28,48,49) or degradable linkages (50–53). However, it is noteworthy that hydrophobic molecules were not well-encapsulated into a dendrimer when most of the periphery was derivatized by shorter PEG chains ($M_n = 550/750$), suggesting a relatively loose cavity (i.e., a low steric barrier) (32,54).

Despite their known structural defects (55), poly(amidoamine) (PAMAM) dendrimers have been widely used for biomedical applications due to their commercial availability and relatively biocompatible nature (56). Each layer (i.e., generation) of PAMAM dendrimer is formed by a two-step procedure of double Michael addition with methyl acrylate followed by the chain extension with ethylenediamine, to present amide and tertiary amine functionalities in the interior. Of many PAMAM variations, the popularity of the amine-terminated PAMAM dendrimer, especially for oligonucleotide delivery (27,30,33,57–62), may arise from its unique cationic properties—a pH-dependent two-stage swelling behavior in aqueous solutions (62, 63). Under physiological conditions, the peripheral amino groups of PAMAM dendrimers are predominantly charged—the pK_a values of the terminal primary amine and the internal tertiary amine are 6.9 and 3.9, respectively. This is advantageous to form a charge complex with anionic drugs to allow entry into the cell mainly by endocytosis and their release under lysosomal pH conditions (20,21). Unfortunately, these polycationic PAMAM dendrimers are toxic (19,29, 64–66), and various strategies have been applied to conceal the terminal amino groups. Partial acetylation of the PAMAM surface, where a fraction of the toxic amino groups was left uncovered to achieve desired properties, affected water-solubility and reduced the hydrodynamic volume (67–69). Partial conversion into lauroyl end groups increased membrane permeability and reduced cytotoxicity (29,65). However, this modification may increase hydrophobicity and promote aggregation in water through the attached aliphatic chains. Other examples, such as modifying into small alkyl alcohol groups, reduced the cytotoxicity and maintained water-solubility (70,71). Overall, PAMAM surface modification by relatively small functional groups required complex tuning of the stoichiometry for each appended functional moiety to achieve both the desired physicochemical and pharmacological properties. With the recent success of gene delivery across the blood brain barrier (72), a PAMAM scaffold with peripheral PEG modifications may still be among the safest and versatile dendritic drug carriers. Here, the synthesis, characterization, and evaluation of cytotoxicity of a series of third generation (G3) PAMAM-PEG conjugates are explored in the context of drug delivery applications. PEG groups were attached to the surfaces of amine-terminated PAMAM dendrimers by varying their size (i.e., $M_n = 550, 750, \text{ and } 2000$) and number of attachments. Each PAMAM-PEG conjugate was characterized by NMR and mass spectrometry. The cytotoxicity of each PAMAM-PEG dendrimer was evaluated in Chinese Hamster Ovary (CHO) cell cultures, which was compared with the cytotoxicity of acetylated G3 PAMAM structures and the commercial anionic PAMAM dendrimers of different generations.

EXPERIMENTAL PROCEDURES

Materials and Methods

Glassware was oven-dried and cooled in a desiccator before use. All reactions were carried out under a dry nitrogen atmosphere. Solvents were purchased as anhydrous grade and used without further purification. Suppliers of the commercial compounds are listed as follows: amine-terminated G3 PAMAM dendrimer and carboxylate-terminated PAMAM dendrimers of G2.5, G3.5, and G5.5 all with the ethylenediamine as an initiator core (8), poly(ethylene glycol) methyl ether ($M_n = 550, 750, \text{ and } 2,000$), acetic anhydride (Ac_2O), 4-nitrophenyl chloroformate, triethylamine, *N,N*-diisopropylethylamine (DIEA), dimethyl sulfoxide (DMSO), methanol (MeOH), and chloroform (CHCl_3) were purchased from Aldrich; *N,N*-dimethylformamide (DMF) and tetrahydrofuran (THF) were purchased from Acros; DMSO- d_6 , chloroform- d (CDCl_3), and D_2O were purchased from Cambridge Isotope Laboratories.

Preparative SEC was performed on Bio-Beads® S-X1 beads (BIO-RAD, MW operating range from 600–14,000 Da), 200–400 mesh, with DMF (Aldrich 99.8%, anhydrous) as an eluent at ambient pressure.

NMR spectra were recorded on either a Varian Inova 300 or a Bruker DRX-600 spectrometer at 25.0 °C under an optimized parameter setting for each sample, unless otherwise mentioned. ^1H NMR chemical shifts were measured relative to the residual solvent peak at 2.50 ppm in DMSO- d_6 , at 7.26 ppm in CDCl_3 , and at 4.80 ppm in D_2O . ^{13}C NMR chemical shifts were measured relative to the residual solvent peak at 39.51 ppm in DMSO- d_6 and at 77.23 ppm in CDCl_3 . Complete NMR peak assignments were made possible with 2D COSY and NOESY experiments. For dendrimer conjugates, integrals were reported only for the peaks clearly resolved (i.e., with a relatively good baseline-separation) in the ^1H NMR spectra. Detailed methods for NMR analysis including peak labeling and assignments, integration, determination of the stoichiometry, and the estimation of average MWs for dendrimer conjugates are described in the Supporting Information.

The electrospray ionization (ESI) MS experiments were performed on a Waters LCT Premier mass spectrometer at the Mass Spectrometry Facility, NIDDK, NIH. MALDI-TOF MS experiments were performed on an Applied Biosystems Voyager-DE STR spectrometer at the Mass Spectrometry Laboratory, University of Illinois. 2,5-Dihydroxybenzoic acid (DHB) or 2,4,6-trihydroxyacetophenone (THAP) was used as the matrix for the MALDI samples. Average MWs determined by MALDI are listed in Table 1 and Table 2.

General Procedure for Acetylation of PAMAM G3 Dendrimer

To a 2.68 mM DMSO solution of PAMAM G3 **1** was added slowly the corresponding amount of Ac_2O in DMSO (10%, v/v) with stirring. Reaction was continued to stir for >24 h. Ca. 50 μL of each reaction mixture was taken, dried *in vacuo* for >2 h, and dissolved in 650–700 μL of DMSO- d_6 to determine the degree of acetylation by ^1H NMR. Later it was found that the stoichiometric control of acetylation reaction was better achieved, if methanol was removed from commercial PAMAM dendrimer **1** *in vacuo*, then the dried sample was dissolved in DMSO- d_6 (ca. 1 mM), and the corresponding amount of Ac_2O (ca. 1 M in DMSO- d_6) was slowly added to the dendrimer solution. The reaction was stirred for 20–24 h, and the NMR spectrum was readily obtained by diluting an aliquot of the reaction mixture with DMSO- d_6 .

Acetylated PAMAM Dendrimers 2 and 3

G3 PAMAM dendrimer **1** (2.68 mM, 5.40 mL, 14.5 μmol) was treated with Ac_2O (10% (v/v); 220 μL , ca. 233 μmol for **2**; 330 μL , ca. 349 μmol for **3**) in DMSO (total volume: 6.00 mL) and stirred for 40 h to yield a colorless glassy solid. ^1H NMR (600 MHz, DMSO- d_6) **2**: δ 8.15–7.81 (m, 73.18H, $\text{NH}_{\text{G}0}$, $\text{NH}_{\text{G}1}$, $\text{NH}_{\text{G}2}$, $\text{NH}_{\text{G}3}$, and NH_{Ac}), 3.09–3.07 (m, 179.31H, H_d , H_f , H_{fAc} , and H_{gAc}), 2.64–2.60 (m, 153.29H, H_b and H_g), 2.42 (m, 62.74H, H_e and H_a), 2.19–2.18 (m, 120.00H, H_c), 1.88 (s, 24.69H, CH_3CO^-), 1.79 (s, 40.60H, H_h); **3**: δ 8.14–7.81 (m, 75.79H, $\text{NH}_{\text{G}0}$, $\text{NH}_{\text{G}1}$, $\text{NH}_{\text{G}2}$, $\text{NH}_{\text{G}3}$, and NH_{Ac}), 3.09–3.07 (m, 187.24H, H_d , H_f , H_{fAc} , and H_{gAc}), 2.65–2.61 (m, 138.75H, H_b and H_g), 2.42 (m, 62.77H, H_e and H_a), 2.19–2.18 (m, 120.00H, H_c), 1.89 (s, 30.78H, CH_3CO^-), 1.79 (s, 59.22H, H_h).

Acetylated PAMAM Dendrimer 4

G3 PAMAM dendrimer **1** dried *in vacuo* (20.7 mg, 3.00 μmol) was dissolved in 1.50 mL of DMSO- d_6 and treated with Ac_2O (13.7 μL , 145 μmol). The reaction was stirred for 24 h and the ^1H NMR of the crude mixture was taken. Additionally, a portion of reaction mixture was purified by SEC (H 39 cm $\times$ O.D. 3.0 cm) in DMF to give **4** as a colorless glassy solid, and its ^1H NMR was acquired. ^1H NMR (600 MHz, DMSO- d_6) δ 7.94 (s, $\text{NH}_{\text{G}3}$), 7.88 (s, 35.05H, NH_{Ac}), 7.80 (br s, 26.41H, $\text{NH}_{\text{G}0}$, $\text{NH}_{\text{G}1}$, and $\text{NH}_{\text{G}2}$), 3.09–3.07 (m, 189.64H, H_d , H_{fAc} , and H_{gAc}), 2.65 (m, 116.29H, H_b), 2.42 (m, 59.60H, H_e and H_a), 2.18 (m, 120.00H, H_c), 1.79 (s, 96.34H, H_h).

General Procedure for Synthesis of PEG carbonate **7**

PEG carbonate **7** was prepared following the modified procedure of Kojima et al (32). To a mixture of poly(ethylene glycol) monomethyl ether **5** and 4-nitrophenylchloroformate (2 equiv) in THF, was added triethylamine or DIEA (2 equiv). The reaction was stirred at room temperature for >5 d. Solvent was removed under reduced pressure, and the crude mixture was loaded on a SEC column for purification. The SEC column fractions were collected in small portions, and those verified to contain only the desired compound by ^1H NMR were combined. Purification by SEC was repeated, if necessary.

PEG Carbonate **7a**

The reaction of poly(ethylene glycol) monomethyl ether **5a** ($M_n = 550$, 0.500 mL, 0.990 mmol), 4-nitrophenylchloroformate (398 mg, 1.92 mmol), and triethylamine (0.280 mL, 2.01 mmol) in THF (28 mL) gave the activated PEG carbonate **7a** as a sticky yellowish solid. ^1H NMR (300 MHz, CDCl_3) δ 8.26 (d, 2H, $J = 9.5$ Hz, H-3 of *p*-nitrophenol), 7.38 (d, 2H, $J = 9.1$ Hz, H-2 of *p*-nitrophenol), 4.42 (m, 2H, $\text{OCH}_2\text{CH}_2\text{OCO}$), 3.79 (m, 2H, $\text{OCH}_2\text{CH}_2\text{OCO}$), 3.72-3.52 (m, 55H, satellites $J = 70.1$ Hz, $\text{OCH}_2\text{CH}_2\text{O}$ and $\text{OCH}_2\text{CH}_2\text{O}$), 3.36 (s, 3H, CH_3O); ^{13}C NMR (75 MHz, CDCl_3) δ 155.7, 152.6, 145.5, 125.5, 122.0, 72.1, 70.9, 70.7, 68.8, 68.5, 59.2; HRMS (ESI) Calcd for $\text{C}_{32}\text{H}_{59}\text{N}_2\text{O}_{17}$ ($m = 12$, $\text{M} + \text{NH}_4^+$): 743.3814, Found: 743.3785.

PEG Carbonate **7b**

The reaction of poly(ethylene glycol) monomethyl ether **5b** ($M_n = 750$, 793 mg, 1.06 mmol), 4-nitrophenylchloroformate (438 mg, 2.11 mmol), and DIEA (0.370 mL, 2.12 mmol) in THF (40 mL) gave the activated PEG carbonate **7b** as a sticky yellowish solid. *The desired compound was contaminated with an inert PEG derivative, which was removed in the next step. The contaminant is suspected to be a methyl carbonate derivative of **7b** which has formed by the methanol (ca. 1 mL) added at the end of the reaction to quench the activity of excess 4-nitrophenylchloroformate. Methanol was not added for the other two reactions to make **7a** and **7c**. ^1H NMR (300 MHz, CDCl_3) δ 8.27 (d, 2H, $J = 9.2$ Hz, H-3 of *p*-nitrophenol), 7.38 (d, 2H, $J = 9.4$ Hz, H-2 of *p*-nitrophenol), 4.43 (m, 2H, $\text{OCH}_2\text{CH}_2\text{OCO}$), 3.80 (m, 2H, $\text{OCH}_2\text{CH}_2\text{OCO}$), 3.72-3.53 (m, *126H, satellites $J = 70.8$ Hz, $\text{OCH}_2\text{CH}_2\text{O}$ and $\text{OCH}_2\text{CH}_2\text{O}$), 3.37 (s, 3H, CH_3O); ^{13}C NMR (75 MHz, CDCl_3) δ 125.5, 122.0, 72.1, 70.9, 70.8, 68.8, 68.5, 61.9, 59.2; HRMS (ESI) Calcd for $\text{C}_{40}\text{H}_{75}\text{N}_2\text{O}_{21}$ ($m = 16$, $\text{M} + \text{NH}_4^+$): 919.4862, Found: 919.4877.

PEG Carbonate **7c**

The reaction of poly(ethylene glycol) monomethyl ether **5c** ($M_n = 2000$, 2.00 g, 1.00 mmol), 4-nitrophenylchloroformate (404 mg, 1.95 mmol), and triethylamine (0.280 mL, 2.01 mmol) in THF (100 mL) gave the activated PEG carbonate **7c** as a pale yellow solid. ^1H NMR (600 MHz, CDCl_3) δ 8.27 (d, 2H, $J = 9.0$ Hz, H-3 of *p*-nitrophenol), 7.38 (d, 2H, $J = 9.2$ Hz, H-2 of *p*-nitrophenol), 4.43 (m, 2H, $\text{OCH}_2\text{CH}_2\text{OCO}$), 3.80 (m, 2H, $\text{OCH}_2\text{CH}_2\text{OCO}$), 3.69-3.53 (m, 186H, satellites $J = 70.4$ Hz, $\text{OCH}_2\text{CH}_2\text{O}$ and $\text{OCH}_2\text{CH}_2\text{O}$), 3.37 (s, 3H, CH_3O); ^{13}C NMR (75 MHz, CDCl_3) δ 125.5, 122.0, 72.1, 70.8, 68.8, 68.5, 60.1; HRMS (ESI) Calcd for $\text{C}_{98}\text{H}_{187}\text{N}_2\text{O}_{50}\text{Na}$ ($m = 45$, $\text{M} + \text{Na}^+$): 2201.2019, Found: 2201.1978.

General Procedure for Synthesis of PAMAM-PEG conjugates

The commercial G3 PAMAM dendrimer **1** (30–90 μL) was dried *in vacuo* to remove methanol and was weighed (50 μL gave ca. 9 mg, Aldrich). The dried dendrimer **1** was dissolved in DMSO and the corresponding amount of the activated PEG carbonate **7** was added slowly either as a solution (for PEG₅₅₀ and PEG₇₅₀) in DMSO or as a solid (for PEG₂₀₀₀). The final concentration of the dendrimer solution was ca. 1.3–1.5 mM and the reaction was stirred at room temperature for ≥ 4 d. The crude mixture was loaded directly on a SEC column and the

fractions containing the desired product were identified by ^1H NMR. The first and last SEC fractions confirmed to contain minor amounts of the desired dendrimer by NMR were eliminated deliberately to reduce the polydispersity of the PAMAM-PEG dendrimer conjugates. In general, the yield of the each reaction calculated based on the NMR-determined MW (Table 2) was nearly quantitative.

PAMAM-PEG₅₅₀ Dendrimer Conjugate 8

To a stirred solution of G3 PAMAM dendrimer **1** (16.62 mg, 2.41 μmol) in DMSO (1.33 mL), was added PEG carbonate **7a** (6.88 mg, 9.62 μmol) in DMSO (275 μL). The mixture was continued to stir for 4 d and the crude mixture was loaded on a SEC column (H 38 cm $\times$ O.D. 4.5 cm) to isolate the desired dendrimer conjugate **8** (17.8 mg). ^1H NMR (600 MHz, DMSO-*d*₆) δ 8.16-7.83 (m, 57.62H, NH_{G0} , NH_{G1} , NH_{G2} , and NH_{G3}), 7.24 (br s, 3.77H, NH_{PEG} of major isomer), 6.83 (br s, 0.23H, NH_{PEG} of minor isomer), 4.03 (t, 8.47H, $J = 4.5$ Hz, H_{h}), 3.62-3.39 (m, 203.76H, satellites $J = 70.6$ Hz, H_{i} , H_{j} , and H_{k}), 3.24 (s, H_{l}), 3.09-3.05 (m, H_{d} , H_{f} , H_{fPEG} , and H_{gPEG}), 2.65, 2.57 (m, 157.75H, H_{b} and H_{g}), 2.43 (m, 58.97H, H_{e} and H_{a}), 2.19 (m, 120.00H, H_{c}).

PAMAM-PEG₅₅₀ Dendrimer Conjugate 9

To a stirred solution of G3 PAMAM dendrimer **1** (15.7 mg, 2.27 μmol) in DMSO (1.08 mL), was added PEG carbonate **7a** (13.0 mg, 18.2 μmol) in DMSO (520 μL). The mixture was continued to stir for 4 d and the crude mixture was loaded on a SEC column (H 43 cm $\times$ O.D. 4.5 cm) to isolate the desired dendrimer conjugate **9** (21.2 mg). ^1H NMR (600 MHz, DMSO-*d*₆) δ 8.06-7.84 (m, 56.29H, NH_{G0} , NH_{G1} , NH_{G2} , and NH_{G3}), 7.24 (br s, 6.88H, NH_{PEG} of major isomer), 6.83 (br s, 0.54H, NH_{PEG} of minor isomer), 4.03 (t, 13.87H, $J = 4.7$ Hz, H_{h}), 3.62-3.38 (m, 338.47H, satellites $J = 70.9$ Hz, H_{i} , H_{j} , and H_{k}), 3.24 (s, 25.79H, H_{l}), 3.08-3.04 (m, 144.64H, H_{d} , H_{f} , H_{fPEG} , and H_{gPEG}), 2.64, 2.56 (m, 149.14H, H_{b} and H_{g}), 2.42 (m, 59.52H, H_{e} and H_{a}), 2.19 (m, 120.00H, H_{c}).

PAMAM-PEG₅₅₀ Dendrimer Conjugate 10

To a stirred solution of G3 PAMAM dendrimer **1** (9.88 mg, 1.43 μmol) in DMSO (950 μL), was added PEG carbonate **7a** (16.5 mg, 23.1 μmol) in DMSO (150 μL). The mixture was continued to stir for 13 d and the crude mixture was loaded on a SEC column (H 38 cm $\times$ O.D. 3 cm) to isolate the desired dendrimer conjugate **10** (18.6 mg). ^1H NMR (600 MHz, DMSO-*d*₆) δ 7.97-7.86 (m, 60.53H, NH_{G0} , NH_{G1} , NH_{G2} , and NH_{G3}), 7.27 (br s, 13.07H, NH_{PEG} of major isomer), 6.85 (br s, 1.26H, NH_{PEG} of minor isomer), 4.03 (t, 27.31H, $J = 4.1$ Hz, H_{h}), 3.62-3.38 (m, 649.88H, satellites $J = 70.7$ Hz, H_{i} , H_{j} , and H_{k}), 3.23 (s, 42.76H, H_{l}), 3.08-3.01 (m, 158.09H, H_{d} , H_{f} , H_{fPEG} , and H_{gPEG}), 2.64, 2.54 (m, 144.01H, H_{b} and H_{g}), 2.42 (m, 57.85H, H_{e} and H_{a}), 2.19 (m, 120.00H, H_{c}); ^1H NMR (600 MHz, D₂O) δ 4.20, 4.16 (m, 28.00H, H_{h} , two isomers), 3.77-3.53 (m, 691.77H, H_{i} , H_{j} , and H_{k}), 3.34 (s, 46.20H, H_{l}), 3.25-3.20 (m, 153.89H, H_{d} , H_{f} , H_{fPEG} , and H_{gPEG}), 2.78 (m, 145.01H, H_{b} and H_{g}), 2.58 (m, 60.70H, H_{e} and H_{a}), 2.40-2.37 (m, 120.00H, H_{c}).

PAMAM-PEG₅₅₀ Dendrimer Conjugate 11

To a stirred solution of G3 PAMAM dendrimer **1** (5.62 mg, 0.813 μmol) in DMSO (210 μL), was added PEG carbonate **7a** (37.4 mg, 52.3 μmol) in DMSO (340 μL). The mixture was continued to stir for 23 d and the crude mixture was loaded on a SEC column (H 38 cm $\times$ O.D. 3 cm) to isolate the desired dendrimer conjugate **11** (14.7 mg). ^1H NMR (600 MHz, DMSO-*d*₆) δ 7.94 (s, 33.53H, NH_{G3}), 7.79 (br s, 28.10H, NH_{G0} , NH_{G1} , and NH_{G2}), 7.23 (s, 30.00H, NH_{PEG} of major isomer), 6.80 (br s, 2.60H, NH_{PEG} of minor isomer), 4.03 (t, 63.13H, $J = 4.1$ Hz, H_{h}), 3.61-3.38 (m, 1540.42H, satellites $J = 69.0$ Hz, H_{i} , H_{j} , and H_{k}), 3.23 (s, 99.29H, H_{l}),

3.08-3.00 (m, 231.53H, H_d, H_{fPEG}, and H_{gPEG}), 2.64 (m, 121.74H, H_b), 2.41 (m, 58.91H, H_e and H_a), 2.17 (m, 120.00H, H_c).

PAMAM-PEG₇₅₀ Dendrimer Conjugate 12

To a stirred solution of G3 PAMAM dendrimer **1** (11.3 mg, 1.64 μ mol) in DMSO (960 μ L), was added PEG carbonate **7b** (24.2 mg, 26.4 μ mol) in DMSO (140 μ L). The mixture was continued to stir for 23 d and the crude mixture was loaded on a SEC column (H 43 cm $\times$ O.D. 4.5 cm) to isolate the desired dendrimer conjugate **12** (18.3 mg). ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.08-7.86 (m, 59.30H, NH_{G0}, NH_{G1}, NH_{G2}, and NH_{G3}), 7.26 (br s, 8.56H, NH_{PEG} of major isomer), 6.85 (br s, 0.51H, NH_{PEG} of minor isomer), 4.03 (t, 17.80H, J = 4.1 Hz, H_h), 3.62-3.38 (m, 551.24H, satellites J = 71.1 Hz, H_i, H_j, and H_k), 3.23 (s, 29.99H, H_l), 3.08-3.00 (m, 157.03H, H_d, H_f, H_{fPEG}, and H_{gPEG}), 2.64, 2.54 (m, 142.08H, H_b and H_g), 2.42 (m, 59.26H, H_e and H_a), 2.19 (m, 120.00H, H_c).

PAMAM-PEG₇₅₀ Dendrimer Conjugate 13

To a stirred solution of G3 PAMAM dendrimer **1** (5.63 mg, 0.815 μ mol) in DMSO (270 μ L), was added PEG carbonate **7b** (48.3 mg, 52.8 μ mol) in DMSO (280 μ L). The mixture was continued to stir for 13 d and the crude mixture was loaded on a SEC column (H 38 cm $\times$ O.D. 4.5 cm) to isolate the desired dendrimer conjugate **13** (16.8 mg). ¹H NMR (600 MHz, DMSO-*d*₆) δ 7.94 (s, 34.61H, NH_{G3}), 7.79 (br s, 28.15H, NH_{G0}, NH_{G1}, and NH_{G2}), 7.23 (s, 29.94H, NH_{PEG} of major isomer), 6.81 (br s, 2.56H, NH_{PEG} of minor isomer), 4.03 (t, 63.72H, J = 4.2 Hz, H_h), 3.62-3.38 (m, 1949.61H, satellites J = 70.4 Hz, H_i, H_j, and H_k), 3.23 (s, 96.44H, H_l), 3.08-3.00 (m, 207.73H, H_d, H_f, H_{fPEG}, and H_{gPEG}), 2.64 (m, 121.55H, H_b), 2.41 (m, 59.85H, H_e and H_a), 2.17 (m, 120.00H, H_c).

PAMAM-PEG₂₀₀₀ Dendrimer Conjugate 14

A mixture of G3 PAMAM dendrimer **1** (17.0 mg, 2.45 μ mol) and PEG carbonate **7c** (21.2 mg, 9.79 μ mol) in DMSO (1.6 mL) was continued to stir for 5 d. The crude mixture was loaded on a SEC column (H 38 cm $\times$ O.D. 4.5 cm) to isolate the desired dendrimer conjugate **14** (35.0 mg). ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.04-7.84 (m, 54.97H, NH_{G0}, NH_{G1}, NH_{G2}, and NH_{G3}), 7.24 (br s, 3.68H, NH_{PEG} of major isomer), 6.83 (br s, 0.19H, NH_{PEG} of minor isomer), 4.03 (t, 8.45H, J = 4.6 Hz, H_h), 3.62-3.39 (m, 712.46H, satellites J = 70.8 Hz, H_i, H_j, and H_k), 3.24 (s, H_l), 3.09-3.01 (m, 136.94H, H_d, H_f, H_{fPEG}, and H_{gPEG}), 2.64, 2.55 (m, 155.18H, H_b and H_g), 2.42 (m, 57.90H, H_e and H_a), 2.19 (m, 120.00H, H_c); ¹H NMR (600 MHz, D₂O) δ 4.20, 4.16 (m, 8.87H, H_h, two isomers), 3.78-3.54 (m, 773.85H, H_i, H_j, and H_k), 3.34 (s, 16.59H, H_l), 3.27-3.20 (m, 132.80H, H_d, H_f, H_{fPEG}, and H_{gPEG}), 2.78-2.72 (m, 158.64H, H_b and H_g), 2.58 (m, 61.43H, H_e and H_a), 2.40-2.38 (m, 120.00H, H_c).

PAMAM-PEG₂₀₀₀ Dendrimer Conjugate 15

A mixture of G3 PAMAM dendrimer **1** (15.6 mg, 2.25 μ mol) and PEG carbonate **7c** (38.9 mg, 18.0 μ mol) in DMSO (1.6 mL) was continued to stir for 5 d. The crude mixture was loaded on a SEC column (H 43 cm $\times$ O.D. 4.5 cm) to isolate the desired dendrimer conjugate **15** (50.1 mg). ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.02-7.83 (m, 59.93H, NH_{G0}, NH_{G1}, NH_{G2}, and NH_{G3}), 7.23 (br s, 7.63H, NH_{PEG} of major isomer), 6.82 (br s, 0.66H, NH_{PEG} of minor isomer), 4.03 (t, 15.03H, J = 4.3 Hz, H_h), 3.62-3.39 (m, 1406.73H, satellites J = 70.7 Hz, H_i, H_j, and H_k), 3.24 (s, 30.32H, H_l), 3.09-3.00 (m, 149.47H, H_d, H_f, H_{fPEG}, and H_{gPEG}), 2.64, 2.55 (m, 158.48H, H_b and H_g), 2.42 (m, 58.93H, H_e and H_a), 2.19 (m, 120.00H, H_c).

PAMAM-PEG₂₀₀₀ Dendrimer Conjugate 16

A mixture of G3 PAMAM dendrimer **1** (11.2 mg, 1.62 μ mol) and PEG carbonate **7c** (56.2 mg, 26.0 μ mol) in DMSO (1.1 mL) was continued to stir for 25 d. The crude mixture was loaded

on a SEC column (H 43 cm $\times$ O.D. 4.5 cm) to isolate the desired dendrimer conjugate **16** (53.1 mg). ^1H NMR (600 MHz, DMSO- d_6) δ 7.98–7.83 (m, 66.70H, NH_{G0} , NH_{G1} , NH_{G2} , and NH_{G3}), 7.25 (br s, 15.80H, NH_{PEG} of major isomer), 6.83 (br s, 1.23H, NH_{PEG} of minor isomer), 4.03 (br s, 32.67H, H_{h}), 3.62–3.38 (m, 3002.14H, satellites $J = 70.7$ Hz, H_{i} , H_{j} , and H_{k}), 3.23 (s, 59.22H, H_{l}), 3.09–3.00 (m, 166.13H, H_{d} , H_{f} , H_{fPEG} , and H_{gPEG}), 2.64, 2.55 (m, 134.95H, H_{b} and H_{g}), 2.41 (m, 58.40H, H_{e} and H_{a}), 2.18 (m, 120.00H, H_{c}).

PAMAM-PEG₂₀₀₀ Dendrimer Conjugate 17

A mixture of G3 PAMAM dendrimer **1** (5.64 mg, 0.816 μmol) and PEG carbonate **7c** (113 mg, 52.2 μmol) in DMSO (550 μL) was continued to stir for 13 d. The crude mixture was loaded on a SEC column (H 43 cm $\times$ O.D. 4.5 cm) to isolate the desired dendrimer conjugate **17** (12.5 mg). ^1H NMR (600 MHz, DMSO- d_6) δ 7.93 (s, 35.70H, NH_{G3}), 7.79 (br s, 28.60H, NH_{G0} , NH_{G1} , and NH_{G2}), 7.22 (s, 29.53H, NH_{PEG} of major isomer), 6.80 (br s, 2.88H, NH_{PEG} of minor isomer), 4.03 (br s, 64.23H, H_{h}), 3.62–3.38 (m, 5633.29H, satellites $J = 70.8$ Hz, H_{i} , H_{j} , and H_{k}), 3.23 (s, 100.03H, H_{l}), 3.07–3.00 (m, 289.19H, H_{d} , H_{fPEG} , and H_{gPEG}), 2.63 (m, 121.87H, H_{b}), 2.41 (m, 58.47H, H_{e} and H_{a}), 2.17 (m, 120.00H, H_{c}).

Cytotoxicity Assays

Typically a stock solution of dendrimer derivative was prepared by dissolving 0.5 μmol of a vacuum-dried solid sample in 50 μL of DMSO (10 mM solution). Dendrimers **2** and **3** used for cytotoxicity studies were not purified by SEC, in order not to disrupt the average MWs. Thus, samples **2** and **3** contained some acetic acid, and used as reaction mixtures after drying *in vacuo* extensively. All other synthesized PAMAM-PEG dendrimer conjugates were purified by SEC. To ensure the dissolution, each 50 μL dendrimer samples in DMSO was heated at 80 $^{\circ}\text{C}$ for 30 min, and then allowed to cool to room temperature. A partial gelation appeared with dendrimer conjugate **8** (see Results and Discussions), and thus the actual concentration of assay samples of **8** can be lower. 5 mL of DMEM/F12 media (Mediatech Inc.) containing 10% fetal bovine serum and antibiotics was added to this 50 μL solution to make 1% (v/v) DMSO as a total content, which was then heated at 37 $^{\circ}\text{C}$ for another 30 min to ensure the homogeneity. Any further dilutions used the media supplemented with 1% (v/v) DMSO, which was shown in control experiments not to affect the cell growth.

Serial dilutions were carried out to prepare samples of the following concentrations: 0.32, 1.0, 3.2, 10, and 32 μM for dendrimers **1–4** and **8–17**; 1.0, 3.2, 10, and 32 μM for dendrimers **18**, **19**, and **20**. 1.6 mL of each dilution was added to a sixwell plate, and 30,000 cells were seeded per well. A well containing the 1.6 mL of media with 1% (v/v) DMSO was prepared simultaneously as a control along with each dendrimer series, which was seeded with the same number of cells. Two plates for each dendrimer compound were prepared so that one plate could be used for cell counting and the other plate could be used for hematoxylin staining. The cells grew for a period of 5 d, when the control well was 90% confluent. Subsequently, the media was aspirated and 1 mL of phosphate buffer saline (PBS) was added to each well and then removed. To count the cells, the cells were detached with 0.2 mL of trypsin and diluted with 2 mL of media (without the DMSO). The cell density in each well was measured using a hemocytometer to determine the effect of the added dendrimer derivative on cell survival, as an indication of cytotoxicity. Each well was homogenized and three counts were made to determine the accuracy. Thus, the percent cell survival is determined by normalizing each cell count against the value obtained from the corresponding control and is reported as mean $\pm$ standard error. For the hematoxylin staining, cells were fixed with methanol for 10 min. After PBS wash ($\times 3$) for 5 min each, the cells were stained with 4 g/L hematoxylin (containing 35.2 g/L aluminum sulfate and 0.4 g/L sodium iodate) for 10 min. The cells were washed ($\times 3$) with PBS for 5 min, allowed to air-dry, and then treated with glycerol. The image was visualized using a Zeiss bright-field microscope (73).

RESULTS AND DISCUSSION

Acetylation of PAMAM Dendrimers

We began our studies by preparing acetylated G3 PAMAM dendrimers. Partial acetylation of PAMAM dendrimers has been commonly applied as a way to enhance water-solubility and to reduce cytotoxicity of amine-terminated PAMAM dendrimers for drug delivery applications. Recent studies suggested that the partial acetylation altered the surface properties of the PAMAM dendrimer and led to a more compact structure, allowing it to better expose the attached ligands—and thus improved targeting—by suppressing the potential of backfolding (67,69). In general, commercial PAMAM dendrimers are somewhat heterogeneous, displaying a distribution of structures (i.e., defects), mainly caused by incomplete coupling and purification in each step of the divergent layer growth. Accordingly, we performed simple acetylation reactions to understand the stoichiometry involving heterogeneity and to establish the characterization method based on the ^1H NMR integration. Furthermore, these partially acetylated PAMAM dendrimers may serve as controls to compare with the PEGylated PAMAM dendrimers of similar degrees of substitutions for their cytotoxic effects.

Dendrimer conjugates were synthesized from G3 PAMAM dendrimer **1** (Figure 1) with the ethylenediamine as an initiator core (8, Scheme 1). Initially, a stock solution of PAMAM dendrimer **1** in DMSO was prepared. In order to accurately determine the concentration of the stock solution, six individual batches containing the same volume of PAMAM stock solution, treated with different amounts of acetic anhydride in DMSO, were subjected to analysis of ^1H NMR integrals. Here, addition of organic base was not necessary for the acetylation reaction in DMSO, possibly due to the self-neutralizing effect of PAMAM dendrimers, which can form an ionic complex with acetic acid, the by-product, at either the remaining peripheral amine ($\text{p}K_a$ 6.9) or the tertiary amine ($\text{p}K_a$ 3.9) in the interior (62). Next, the PAMAM dendrimer in DMSO was treated with ca. 16, 24, or 48 equivalents of acetic anhydride. Typically, when the peripheral amino groups of PAMAM dendrimers were acetylated by acetic anhydride, a singlet corresponding to the methyl of acetamide appeared at 1.79 ppm (“h”, Figure S1, Supporting Information) in deuterated DMSO- d_6 , and a methyl peak from acetate anion was found at ca. 1.90 ppm which disappeared upon purification. When the integrals were normalized against the methylene peak of PAMAM at 2.18 ppm (“c”, 120 H), the internal standard of PAMAM for ^1H NMR integration, these dendrimers were found to contain 14, 20, and 32 acetamide groups on average, respectively (**2**, **3**, and **4**, Table 1). In fact, prior attempts to exhaustively acetylate the peripheral amino groups of PAMAM G3 dendrimers indicated that the number of terminal amino groups was close to the theoretical value of 32 by this normalization method based on the internal standard “c” after purification by SEC. Thus, 32 peripheral groups were assumed for PAMAM G3 dendrimers for the remainder of our structural analysis based on the ^1H NMR integration. Interestingly, when a portion of either of the partially acetylated PAMAM dendrimer reaction mixtures, **2** or **3**, was passed through a SEC column in DMF, the average number of acetamide groups shifted toward higher values by ^1H NMR integration, whereas the value for the fully acetylated PAMAM **4** remained the same. This may have resulted from the poorer solubility of PAMAM dendrimers with lower degree of acetylation in DMF, reducing the relative recovery after SEC compared to the recovery of dendrimers bearing more acetamide groups in the same batch.

Various matrices and conditions were attempted to obtain the mass spectra of the acetylated PAMAM dendrimers by MALDI (see Supporting Information). As reported previously, MALDI spectra obtained with either DHB or THAP as a matrix generally gave the best results for our analysis (74). A relatively broad major peak was observed for each acetylated dendrimer, **2**, **3**, or **4**, spanning up to the mass range corresponding to the fully acetylated PAMAM. In either matrix, the overall pattern of the peak distribution was more or less the same between these three acetylated dendrimers. A secondary broad peak region corresponding

to the half-size of the desired MW was detected in the MALDI spectra of acetylated dendrimers, **2**, **3**, and **4**, as well as for the commercial PAMAM **1**. This half-size peak may have originated from the fragmentation near the tertiary amine of the core (75) and/or may indeed represent G2 PAMAM derivatives which were formed by the reagents carried over to the next step without removal in the commercial PAMAM synthesis. The average MWs of **2**, **3**, and **4** determined by MALDI in either matrix were lower than those determined by ^1H NMR (Table 1). Unlike the analysis based on ^1H NMR, average MWs estimated by MALDI slightly varied depending on the specific conditions applied (e.g., sample preparation, scanned mass range, laser intensity, etc.) or by the chemical nature of samples affecting fragmentation pattern and the tendency to form matrix/salt adducts. Overall, the MALDI-estimated average MWs increased (except for **4** with DHB matrix) in both matrices as the degree of acetylation increased from **1** to **4**.

Synthesis, Purification, and Characterization of PAMAM-PEG Conjugates

In order to systematically study the influence of PEG, relative to its size and abundance on reducing the cytotoxic effect of PAMAM dendrimers, conjugates were prepared starting from three different lengths of monomethyl PEG ether **5** (i.e., M_n 550, 750, and 2000, Scheme 2) by varying the degree of PEGylation. Preparation of the activated PEG carbonate **7** followed the modified procedure of Kojima et al. (32). As reported previously, contamination of the commercial monomethyl PEG ether by its diol derivative produced a mixture of mono- and di-activated PEG carbonates (23,76). Di-activated PEG carbonate analog of **7** (structure not shown) may result in unwanted intra- and inter-molecular cross-links, and thus a tedious and cumbersome purification was necessary to remove these species by SEC in DMF. SEC fractions verified to contain only the desired PEG derivative **7** by ^1H NMR were combined and were used for the next step. The average MW of each PEG derivative **7** was determined based on the analysis of ^1H NMR and MS. Here, each purification carried out by SEC slightly shifted the distribution of PEG derivative **7** to affect the average MW. Strangely, even after repeated purification, analysis of **7b** (from PEG₇₅₀) by ^1H NMR integration indicated the number of repeat units to be approximately twice the anticipated value. Despite the suggested contamination, the mass spectrum of **7b** displayed the desired peak distribution as a major entity, and thus **7b** was used for next step (Figure S7, Supporting Information). Unlike **7a** or **7c**, conjugation of **7b** to PAMAM indeed created some discrepancies in stoichiometry (*vide infra*); however, the contaminant was successfully removed by SEC at this later step without causing any further contamination.

Next, G3 PAMAM dendrimer **1** was treated with different amounts of PEG carbonate **7** (Scheme 3). Stoichiometry of the conjugation was generally well-managed when methanol was removed *in vacuo* from the commercial PAMAM G3 dendrimer **1**, and then the corresponding amount of the activated PEG **7** was added relative to the mass of dry PAMAM **1**. Preparation of ten different PAMAM-PEG derivatives was planned by adding: 4, 8, 16, and 64 equivalents of **7a** (from PEG₅₅₀); 16 and 65 equivalents of **7b** (from PEG₇₅₀); 4, 8, 16, and 64 equivalents of **7c** (from PEG₂₀₀₀). Reaction was generally performed in the concentration range of 1.3–1.5 mM per dendrimer, and upon addition of **7**, the colorless reaction mixture instantly turned an intense yellow color, indicating the appearance of *p*-nitrophenolate species. After stirring for ≥ 4 d, the reaction mixture was loaded on a SEC column with DMF as an eluent, and the desired fractions were combined after careful analyses of ^1H NMR spectra. Here, the first and last SEC fractions confirmed to contain minor amounts of the desired dendrimer by NMR were eliminated deliberately. This was intended to reduce the polydispersity and thus to achieve more reliable biological effects by restricting the range of structural dissimilarity in the distribution, which is a limitation of the partial derivatization method commonly applied in PAMAM dendrimer chemistry.

The stoichiometry of PAMAM-PEG conjugates was established by ^1H NMR integration in $\text{DMSO-}d_6$ (Table 2, Figure 2 and Figure 3). Detailed methods used for the analysis of NMR data are described in the Supporting Information. In summary, PAMAM dendrimer conjugates were characterized to contain: 4 (**8**), 7 (**9**), 14 (**10**), and 32 (**11**) of PEG_{550} chains; 9 (**12**), and 32 (**13**) of PEG_{750} chains; 4 (**14**), 8 (**15**), 17 (**16**), and 32 (**17**) of PEG_{2000} chains. Except for the PEG_{750} derivative **12**, which was prepared from a contaminated PEG derivative **7b** (*vide supra*), stoichiometric control of the conjugation reaction was elaborately executed as planned. In addition, the NMR-based MW estimation of PAMAM-PEG derivatives in $\text{DMSO-}d_6$ is summarized in Table 2. Alternatively, selected structures **10** and **14** were characterized similarly by ^1H NMR in D_2O , to give nearly identical results (Figure S4, Supporting Information).

Overall, these PAMAM-PEG conjugates were hygroscopic and exhibited relatively good water-solubility except for the dendrimer **8**, which was substituted with four short chains of PEG_{550} . Surprisingly, a severe irreversible gelation occurred for a portion of compound **8**, hampering any further usage of the batch. Gelation phenomena from amine-terminated PAMAM dendrimers were noticed previously, especially with lower substitution, and neither sonication nor the treatment with various organic solvents, water, acid/base, or heat restored to the solution state (77).

Next, to help predict the solution conformation of PAMAM-PEG conjugates under physiological conditions, NOESY experiments were carried out in D_2O (Figures S5 and S6, Supporting Information). Dendrimer **10** with multiple numbers of short PEG chains (14PEG_{550}) and dendrimer **14** substituted with fewer numbers of long PEG chains (4PEG_{2000}) were chosen to explore the influence of PEG chain length and population on the overall geometry in solution. No NOE cross-peaks were observed from either structure between peaks from PAMAM and PEG regions in D_2O . This strongly suggests that in water, the terminal hydrophilic PEG groups are entirely segregated from the central PAMAM domain (i.e., no backfolding) regardless of PEG chain length studied here. Thus, the geometry of PAMAM-PEG conjugates in aqueous media may closely resemble that of the phase-separated micelle. Indeed, the concept of a dendritic/hyperbranched “unimolecular micelle” with hydrophilic end groups was introduced earlier mainly for the entrapment of small hydrophobic molecules (35,54,78–82).

MALDI mass spectra of PAMAM-PEG conjugates were obtained using 2,5-dihydroxybenzoic acid (DHB) and 2,4,6-trihydroxyacetophene (THAP) as matrices (Figure 4, Supporting Information). Generally, the desired peaks were better resolved when the MALDI scan range was narrowed. Average MWs were calculated from the mass range encompassing the desired major peak. Again, the peaks corresponding to the half-size of the desired MW were detected in all cases. In certain cases, these half-size peaks were partially incorporated to the mass range for MW calculation due to the slight overlap, to result in further underestimation of the desired, especially when the expected MWs of conjugates were relatively lower. Overall, when the half-size peak was not included, MALDI underestimated the MW of PAMAM-PEG conjugates by 7–18% compared to the MW determined by NMR. Again, broadening of peaks may have originated from random fragmentation under applied MALDI conditions (e.g., between the carbon-nitrogen bond at the interior tertiary amine), structural defects from the commercial starting material, or by forming matrix/salt adducts (74). Interestingly, MALDI of conjugates derivatized with a fewer numbers of longer PEG_{2000} chains, **14** and **15**, displayed individual broad peaks separated by ca. 2,000 Da, corresponding to PAMAM dendrimers with increasing numbers of PEG substitutions. MALDI-estimated average MWs of the PAMAM-PEG dendrimer conjugates in each matrix are listed in Table 2.

In Vitro Cytotoxicity of PAMAM Dendrimer Derivatives

Evaluation of the cytotoxicity of PAMAM dendrimer derivatives with various surface modifications has been reported. A relevant study compared the cytotoxicity and hemolytic potential of the melamine-based dendrimers each bearing cationic (amine, guanidine), anionic (carboxylate, sulfonate, phosphonate), or neutral (PEG) hydrophilic surface group (83). Unfortunately, only limited systematic studies were performed to date along these lines that may provide guidelines to estimate the proper degree of peripheral substitutions needed when a particular functional group is used. Here, we examined the cytotoxic effects of dendrimer derivatives with several commonly used end groups in PAMAM-based drug delivery—acetamide, carboxylate, and PEG. Our systematic approach included varying the degree of terminal substitution for each functionality (acetamide, PEG), the size of PEG chains (PEG₅₅₀, PEG₇₅₀, and PEG₂₀₀₀), and the generation of dendrimer (carboxylate). We investigated near the micromolar concentration range where somewhat marked differences in cytotoxicity between studied dendrimers were elicited. CHO cells were chosen as our target which were often used in our laboratory for studies on G protein-coupled receptors (74 and references therein).

First, the cytotoxicity of PAMAM dendrimers with acetylated peripheries was examined (Figure 5A). Dendrimer **2** carried 14 acetamide groups (ca. 44% substitution) as analyzed by ¹H NMR integration method, dendrimer **3** had 20 acetamide groups (ca. 63% substitution), and dendrimer **4** was fully acetylated (100% substitution) leaving no free primary amino groups at the periphery. As expected, dendrimers with a higher degree of acetylation showed less toxicity. For instance, fully acetylated dendrimer **4** exhibited ca. 75% cell survival at 32 μ M (the highest concentration tested), whereas the unsubstituted PAMAM **1** and a nearly half-acetylated dendrimer **2** showed only ca. 5% cell survival. All dendrimer derivatives including the commercial PAMAM **1** exhibited >75% cell survival at ≤ 1 μ M under the applied assay conditions. Recently, a similar systematic study was reported on acetylated G2 and G4 PAMAM dendrimers, which manifested concentration-dependent cytotoxic effects in Caco-2 cell cultures (84).

Next, our synthesized PAMAM-PEG dendrimer conjugates **8–17** were subjected to cytotoxicity evaluation under the same conditions in CHO cell cultures (Figures 5B and 5C). Generally, the cytotoxic effects of PAMAM-PEG conjugates decreased with increasing numbers of peripheral substitutions with respect to the same PEG chain length (i.e., PEG₅₅₀, PEG₇₅₀, or PEG₂₀₀₀). Nearly no cytotoxic effects were observed up to 1 μ M concentration with dendrimers having a lower degree of PEG-substitution (22–28%), **9**, **12**, and **15**, which all exhibited similar cytotoxic values at higher concentrations within the permitted error range. Despite the limited experimental trials, when the cytotoxicity was compared between fully substituted PAMAM-PEG dendrimers, **11**, **13**, and **17** (Figure 1), interestingly, only **17** with the longest PEG groups showed a sudden drop in cell survival rate at the highest concentration of 32 μ M. This dendrimer **17** was more toxic at 32 μ M than the less substituted analogues, **15** and **16**, of the same chain length (PEG₂₀₀₀). A previous report proposed the possibility of the intermolecular agglomeration for fully substituted PAMAM conjugates with longer PEG chains (PEG₂₀₀₀ or PEG₅₀₀₀) at higher concentrations, deterring efficient encapsulation of small hydrophobic molecules (54). Similarly, this potential agglomeration of dendrimer **17** may negatively affect cell viability at higher concentrations.

The cytotoxicity of PAMAM-PEG conjugates were then compared with that of acetylated dendrimers. At the highest concentration studied (32 μ M), partially PEGylated dendrimers (**8–10**, **12**, and **14–16**) with 13–53% peripheral substitution were generally less toxic (28–53% cell survival) regardless of their chain length, compared to the partially acetylated dendrimers **2** and **3** (44–63% peripheral substitution, 5–28% cell survival). More specifically, the cytotoxicity profile of nearly half-substituted PAMAM-PEG derivatives, **10** and **16**, was more

or less the same over the entire concentration range studied, suggesting negligible effects of chain length (PEG₅₅₀ vs. PEG₂₀₀₀). However, when these two medium-range PEG-substitutions were compared to acetylated PAMAM **2** with 14 acetyl groups, cytotoxicity was significantly lower at $\geq 10 \mu\text{M}$ (49–64% cell survival for **10** and **16**; 5–31% cell survival for **2**). On the other hand, except for the dendrimer **17** with longer PEG chains, cell survival rates of fully substituted and relatively nontoxic dendrimers **4** (acetamide), **11** (PEG₅₅₀), and **13** (PEG₇₅₀) were similar. Taken together, PAMAM dendrimer with a lower degree (ca. 25% or less) of short PEG-substitutions may substantially reduce the cytotoxicity of amine-terminated PAMAM dendrimers at a micromolar concentration range with good water-solubility. In contrast, the smaller acetamide groups may require a higher degree of surface-masking to achieve similar cell viability, limiting the number of available peripheral amino groups for further attachments of other functional moieties for drug delivery applications (e.g., drugs, targeting units, markers, etc.). Furthermore, water-solubility of the final dendrimer with a partially acetylated surface may be more governed by the physical properties of these other appended moieties compared to the PEGylated surface, requiring additional fine-tuning of the stoichiometry.

Carboxylate-terminated anionic PAMAM dendrimers possess excellent water-solubility. However, these derivatives have been used less frequently for drug delivery compared to the amine-terminated PAMAM dendrimers. In the same manner, we evaluated the cytotoxicity of commercial G2.5 (**18**, Figure 1), G3.5 (**19**), and G5.5 (**20**) PAMAM dendrimers with the ethylenediamine as an initiator core, which contain 32, 64, and 256 carboxylate end groups, respectively, in theory (Figure 5D). Essentially, no cytotoxic effect was observed from lower generation dendrimers, **18** and **19**, at all concentrations studied. Interestingly, for G5.5 PAMAM **20**, a sudden increase in cytotoxicity was observed at the highest concentrations (32 μM). Similar to the result obtained for dendrimer **17**, this relatively large dendrimer **20** with multiple hydrophilic end groups may aggregate intermolecularly (or alone) to display an increased level of cellular toxicity at elevated concentrations. Thus, for carboxylate PAMAM series, usage of G5.5 or higher generations may be limited to lower concentrations ($\leq 10 \mu\text{M}$) for drug delivery applications.

CONCLUSION

Attachment of PEG chains to macromolecular therapeutics generally alters the surface properties, leading them to achieve excellent water-solubility and biocompatibility. PAMAM dendrimers are frequently used for dendrimer-based drug delivery applications due to their known relative biocompatibility and commercial availability. PEGylation has been applied to PAMAM dendrimers as a way to reduce toxicity of their amine termini and to offer a sufficient steric barrier for the efficient encapsulation of a drug or gene. Despite its advantageous effects, overcrowding the surface of these carriers by longer PEG chains may cause intermolecular aggregation, increase cytotoxicity, and prohibit intracellular drug release by deterring the uptake process (9). An estimation of minimally required PEG substitution is crucial when other functional moieties are appended on the PAMAM surface, especially when targeting or other ligand-receptor interaction is involved. Accordingly, to provide guidelines in designing PAMAM-based drug delivery agents, a series of PAMAM-PEG conjugates were prepared varying the degree of substitution and PEG chain length. Each dendrimer was purified by SEC and characterized by NMR and MALDI. A careful analysis of ¹H NMR integrals allowed the complete characterization of PAMAM-PEG conjugates for MW determination. NOESY experiments in D₂O confirmed the absence of backfolding of the peripheral PEG regardless of its size and population on the PAMAM surface, suggesting a micellar geometry.

The cytotoxicity of PAMAM-PEG derivatives was evaluated in CHO cell cultures. Compared to the acetylated G3 PAMAM dendrimers, a lower degree of surface substitution was needed

when PEG was present in order to achieve similar cell viability. Our systematic investigation indicated that a relatively low degree of surface-modification (ca. 25% or less) by shorter PEG chains (PEG₅₅₀/PEG₇₅₀) may significantly reduce the cytotoxicity of amine-terminated PAMAM dendrimers while maintaining good water-solubility.

In summary, PAMAM-PEG dendrimer conjugates may serve as universal scaffolds to build efficient and more versatile drug carriers. Current findings led us to further explore the influence of PEG chain length and number of attachments on eliciting potential pharmacological effects of ligands attached to the dendrimer surface involving receptor interactions, which will be reported in a separate manuscript.

ACKNOWLEDGMENTS

This research was supported in part by the Intramural Research Program of the NIH, NIDDK. We thank Dr. Haijun Yao at the Mass Spectrometry Laboratory of the University of Illinois, for numerous attempts to obtain MALDI spectra of our PAMAM dendrimer derivatives. We are grateful to Rick Dreyfuss at ORS, NIH, who helped us to obtain the images for the cytotoxicity results. Y.K. thanks the Can-Fite Biopharma for financial support.

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Figure 1. Structures of PAMAM dendrimer derivatives with homogeneous end groups: a commercial G3 PAMAM (**1**), acetylated G3 PAMAM (**4**), PEGylated G3 PAMAM (**11** from PEG₅₅₀, **13** from PEG₇₅₀, and **17** from PEG₂₀₀₀), and a commercial G2.5 PAMAM with carboxylate terminal groups (**18**).

Figure 2. ¹H NMR spectra of PAMAM-PEG dendrimer conjugates (A) **8**, (B) **9**, (C) **10**, (D) **11**, (E) **12**, and (F) **13** in DMSO-*d*₆.

Figure 3. ^1H NMR spectra of PAMAM-PEG dendrimer conjugates (A) **14**, (B) **15**, (C) **16**, and (D) **17** in $\text{DMSO}-d_6$.

Figure 4. MALDI-TOF MS spectra of PAMAM-PEG dendrimer conjugates using DHB as a matrix: (A) 8; (B) 9; (C) 10; (D) 11; (E) 12; (F) 13; (G) 14; (H) 15; (I) 16; (J) 17.

Figure 5.

Cytotoxicity of dendrimers in CHO cell cultures: (A) acetylated G3 PAMAM dendrimers, G3 PAMAM dendrimers derivatized with (B) shorter PEG chains (PEG₅₅₀/PEG₇₅₀) or (C) a longer PEG chain (PEG₂₀₀₀), and (D) anionic carboxylate PAMAM dendrimers (G2.5, G3.5, and G5.5). Each dendrimer sample was prepared using DMEM/F12 media (Mediatech, Inc.) containing 10% fetal bovine serum and antibiotics with 1% (v/v) DMSO. 30,000 cells were seeded per six-well plate containing dendrimer media, and the cells were counted after a 5 d-incubation. Cell survival is reported by normalizing the cell counts to the value obtained from a control well with 1% (v/v) DMSO, which did not contain the dendrimer. Cell survival is reported as mean $\pm$ standard error.

Scheme 1.

Scheme 2.

Scheme 3.

Table 1
Structural analysis of acetylated PAMAM G3 dendrimers by ¹H NMR and MALDI MS.

compd	NMR		MALDI			
	no. of cetamide	MW ^d	M _n ^b	DHB matrix M _w ^c	PDI ^d	THAP matrix M _w ^c
1	0	6909	5772	5909	1.02	6085
2	14	7497	7161	7313	1.02	7125
3	20	7750	7219	7390	1.02	7225
4	32	8254	7056	7256	1.03	7351

^aPAMAM dendrimer **1** used for calculation here was assumed to have 32 peripheral amino groups and no structural defects.

^bNumber-average molar mass.

^cWeight-average molar mass.

^dPolydispersity index.

Table 2Structural analysis of PAMAM-PEG conjugates by ^1H NMR and MALDI MS.

cmpd	M_n of 5 ^a	NMR ^b		MALDI						
		<i>m</i>	<i>n</i>	MW ^c	DHB matrix			THAP matrix		
					M_n ^d	M_w ^e	PDI ^f	M_n ^d	M_w ^e	PDI ^f
8	550	13	4	9432	6896 ^g	7433 ^g	1.08 ^g	7835 ^g	8091 ^g	1.03 ^g
9	550	12	7	11016	9035 ^g	9536 ^g	1.06 ^g	10385 ^g	10857 ^g	1.05 ^g
10	550	12	14	15122	13330	14009	1.05	12335	12823	1.04
11	550	12	32	25682	22039	22973	1.04	21441	22348	1.04
12	750	16	9	13775	11432	12098	1.06	9879 ^g	10709 ^g	1.08 ^g
13	750	16	32	31321	24780	25588	1.03	25743	26202	1.02
14	2000	44	4	14894	10514 ^g	12329 ^g	1.17 ^g	10947 ^g	12533 ^g	1.14 ^g
15	2000	45	8	23232	19266	20019	1.04	18479 ^g	19069 ^g	1.03 ^g
16	2000	45	17	41596	35167	36284	1.03	33054	34486	1.04
17	2000	44	32	70792	58947	60400	1.02	57626	60062	1.04

^a M_n of PEG monomethylether **5** originally used to prepare the corresponding carbonate precursor **7**. M_n values were taken from the Aldrich bottle.^b Based on ^1H NMR integration determined in DMSO-*d*₆.^c PAMAM dendrimer **1** used for calculation here was assumed to have 32 peripheral amino groups and no structural defects.^d Number-average molar mass.^e Weight-average molar mass.^f Polydispersity index.^g Mass range selected for the average MW calculations contained (a part of) the peak region corresponding to the half-size of each desired compound.