



NIH Public Access

Author Manuscript

Biomacromolecules. Author manuscript; available in PMC 2013 March 05.

Published in final edited form as:

Biomacromolecules. 2010 June 14; 11(6): 1544–1563. doi:10.1021/bm100186b.

Amino acid-functionalized dendrimers with heterobifunctional chemoselective peripheral groups for drug delivery applications

RS Navath^{†,‡,§,Γ}, AR Menjoge^{†,‡,§,Γ}, B Wang^{†,‡,§}, R Romero[§], S Kannan^{¶,§}, and RM Kannan^{†,‡,§,*}

[†]Department of Chemical Engineering and Material Science, Wayne State University

[‡]Department of Biomedical Engineering, Wayne State University

[§]Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, and Department of Health and Human Services (NICHD/NIH/DHHS)

[¶]Children's Hospital of Michigan, Wayne State University

Abstract

Dendrimers have emerged as multifunctional carriers for targeted drug delivery, gene delivery and imaging. Improving the functional versatility at the surface for carrying multiple conjugation reactions is becoming vital. Typically, generation four polyamidoamine (G4-PAMAM) dendrimers bear ~64 symmetrical end groups, often requiring different spacers to conjugate various functional groups (drugs and targeting moieties), increasing the synthetic steps. In the present study, a simple one-step synthesis to convert each symmetrical end group of G4-PAMAM dendrimers into two reactive, distinct orthogonal and chemoselective groups is described. A near-complete end-capping of the dendrimers (87–93%) with amino acids results in heterobifunctional G4-PAMAM dendrimers bearing a very high (~110) diverse peripheral end groups (OH+NHBoc, OH+COOMe, SH+NHBoc, and COOH+NHBoc). Postfunctionalization ability of these dendrimers was evaluated. The heterobifunctional groups at the dendrimer periphery could be chemoselectively conjugated to multiple moieties such as drugs (indomethacin and dexamethasone) and drugs and imaging agents (dexamethasone and FITC). These conjugations could be achieved in immediate succession without functional group conversions, eliminating the additional elaborate synthetic steps traditionally required to append specific linkers. Furthermore, one of the two functional handles at periphery was used to develop *in situ* forming hydrogels, whereas the other handle could be used for conjugating the drugs (e.g., dexamethasone). The heterobifunctional dendrimers with either “NH₂ or SH (thiopyridyl protected form)” terminations showed *in situ* hydrogel formation by cross-linking with *N*-hydroxysuccinimide or thiol-terminated multiarm polyethylene glycol (20 kDa). The choice of amino acids as versatile linkers would enable biocompatible dendrimer scaffolds for use in drug delivery. Ζ-potential measurements showed drastic lowering of the charge on G4-PAMAM-NH₂ dendrimers by end-capping with amino acids, whereas in the case of neutral G4-PAMAM-OH dendrimers, the charge did not increase or decrease substantially. The *in vitro* cytotoxicity and hemolysis assay showed that the heterobifunctional dendrimers were noncytotoxic in the 100 ng/mL to 1 mg/mL concentration range. With this study, we demonstrate the development of biocompatible dendrimers bearing multiple orthogonal surface groups, enabling the attachment of drugs, imaging agents, and gel formation using minimal synthetic steps.

*Corresponding Author: Department of Chemical Engineering and Material Science, Wayne State University, Detroit, Michigan, 48202, rkannan.wsu@gmail.com.

†These authors contributed equally to this manuscript.

INTRODUCTION

Dendrimers are a class of well-defined nanostructured macromolecules with narrow polydispersity and a multivalent surface amenable for further modifications.^{1,2} Dendrimers are extensively and continually investigated for biomedical applications such as gene therapy, drug delivery, and bioimaging purposes.^{3–8} As nanocarriers, dendrimers have the versatility to allow conjugation, complexation, and encapsulation of multifunctional moieties.^{9–13} The functional groups on the periphery of dendrimer act as highly accessible handles for drug or other functional group attachments.^{3,14} Because the functionalities of the drugs and ligands are diverse, there is a need to explore multiple functional group presentations at the dendrimer surface.¹⁵ Adding diverse functional moieties (drugs or imaging agents) onto a single dendrimer is difficult because all peripheral groups of the symmetric dendrimer have the same reactivity.² A suitable linker or spacer is required to react with the surface functionality of dendrimer, which offers the flexibility to link multiple moieties¹⁶ such as drugs, imaging,^{17,18} or targeting agents.¹⁹

Functionalization of dendrimers has enabled several end objectives such as reduction in cytotoxicity,^{20,21} targeted drug delivery,^{4,22} formation of hydrogels,²³ increase in plasma residence time, imaging *in vivo* biodegradation,²⁴ or potentially any combination of these.² For example, modification of G4 dendrimers with 19, 29 and 46 molecules of phenylalanine resulted in improved gene transfection ability, whereas modification with 64 molecules of phenylalanine resulted in poorly soluble compounds with a loss in DNA complexing ability.²⁵ A more widespread use of cationic dendrimers in drug and gene delivery is hindered by their cytotoxicity. PEGylation and acetylation are highly successful approaches in overcoming the cytotoxicity of amine-terminated dendrimers,^{26–30} but the higher degree of amine neutralization compromises its gene slicing efficiency.^{28,29} Ideally, the dendrimer surface modification should therefore be such that several end objectives are met without compromising on any attributes and yet having chemically reactive groups suitable for modifications to attach drug or targeting moieties. There is a need to develop new methodologies for synthesis of functionalized dendrimers, which involve fewer reaction steps, achieve high yields, are compatible with a variety of functional groups, and occur under mild reaction conditions offering clean and efficient synthesis.^{31,32}

To make dendrimers as efficient delivery vectors, apart from multivalency, there is a need to have unique orthogonal end groups for chemoselective surface modifications and multifunctionalization. The concept of heterobifunctional dendrimers has been previously described.^{10,32–35} In typical, producing a well-defined end product requires elaborate steps^{10,35} and could be expensive for scale-up in drug delivery applications, potentially limiting their commercial availability to PAMAM, DAB, phosphorus PMMH, and 2,2-bis(methylol)propionic acid (bis-MPA) dendrimers.³¹ There have been a few reports on the synthesis of dendrimers bearing different asymmetric groups at the periphery.^{2,31,33,36,37} Previously, melamine dendrimers with orthogonal reactive groups on surface comprising 4 hydroxyl groups, 4 hydroxyl groups masked as *tert*-butyldiphenylsilyl ether, and 16 *tert*-butoxycarbonyl protected amines were synthesized in eight total steps with a 55% overall yield.³⁸ Melamine dendrimers with one or two *tert*-butoxycarbonyl protected amines were synthesized in eight total steps with a 55% overall yield.³⁸ Melamine dendrimers with one or two *tert*-butoxycarbonyl-protected amines on the periphery were prepared by orthogonal and convergent synthesis in five to six linear steps (11 or 12 total steps) with 40% overall yield, and these unique sites could be deprotected and subjected to additional chemistry.³⁹ Catalytic peptide dendrimers with diverse surface functionality were synthesized by (A) alternating amino acids with (B) symmetrical branching diaminoacids in iterations of A2B or (A²A¹)₂ dendrons.^{40,41} An efficient method to synthesize dendrimers with orthogonal peripheral groups is to grow a symmetric dendrimer in bulk and then tune its periphery for

the desired application.² However, this process requires that the subsequent differentiation and coupling steps be minimal in number and efficient in reactivity.²

Functionalization of the peripheral groups of dendrimers is a fruitful and convenient strategy for developing novel functional material for biomedical applications, and ways to simplify the synthesis toward achieving would be beneficial.⁴² For biomedical and drug delivery applications of dendrimers, there is a need to develop these scaffolds with materials that are biocompatible or generally recognized as safe by the U.S. Food and Drug Administration (FDA), such that their metabolites are nontoxic. Because dendrimers offer multivalency, one of the advantages is to use the functional handles to append diverse functional groups such as different drug molecules and imaging agents. However, these functional groups bear different reactive groups, and to append these on dendrimers, there is a need to use several synthetic steps for the attachment of specific linkers or spacer molecules. Therefore, dendrimers with biocompatible orthogonal groups, which facilitate chemoselective attachment of these functional groups in minimal synthetic steps would be desirable.

The objective of the present study was to modify the multivalent symmetrical terminal groups of G4-PAMAM dendrimers into multifunctional groups using biocompatible materials. In the present study, we demonstrate that “near complete” peripheral modification of dendrimers containing two reactive, distinct, orthogonal, and chemoselective groups can be achieved in a one-step, one-pot reaction. A facile and efficient synthetic methodology converting the 64 (theoretical) symmetrical end groups of G4-PAMAM dendrimer into diverse functional handles (~110) is described. A small library of representative dendrimers having diverse and high density of peripheral functional groups has been developed. We envisage that multifunctional multivalent end groups can be used for varied applications: (i) chemoselective conjugation of two different drugs in immediate succession, (ii) conjugation of drug and fluorescent imaging agent, (iii) *in situ* hydrogel formation using one functional handle while using the second for drug conjugation, (iv) attaching targeting ligands, and (v) enhanced solubility.

MATERIALS AND METHODS

Materials

Ethylenediamine-core poly(amidoamine) (PAMAM) dendrimers (diagnostic grade generation four with OH, NH₂ groups, and generation 3.5 with -COOH end groups) were purchased from Dendritech. Other reagents were obtained from assorted vendors in the highest quality available; these include serine (Aldrich), cysteine (Aldrich), Boc-aspartic acid (Aldrich), BOc-Cys-OH (Aldrich), cysteine (Aldrich), Boc-aspartic acid (Aldrich), Boc-Cys-OH (Aldrich), *N*-hydroxysuccinimide (Aldrich), benzotriazol-1-yl-oxytritypyrrolidinophosphonium hexafluorophosphate (PyBop, Aldrich), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC·CH₂, Aldrich), 4-dimethylaminopyridine (DMAP, Aldrich), diisopropyl ethylamine (Aldrich), dimethyl sulfoxide (DMSO, Aldrich), trifluoroacetic acid (Aldrich), dimethylformamide (DMF, Aldrich), ethanol (Aldrich), acetonitrile (Aldrich), phosphate buffer saline (PBS, pH 7.4, Aldrich), and Spectra/Por dialysis membrane (M_w cutoff 1000).

Methods

NMR Analysis—All ¹H NMR and ¹³C NMR spectra were recorded on 400 MHz. The nMR chemical shifts are reported in ppm and calibrated against DMSO-*d*₆ (δ 2.48).

MALDI-TOF MS—Matrix-assisted laser desorption ionization (MALDI) mass spectra were acquired using a Bruker Ultraflex with a 384 well sample plate, reflectron, and PSD

fragmentation capability. Spectra of dendrimers were obtained using a 2,5-dihydroxybenzoic acid (DHB) matrix. Samples were prepared by mixing dilute solutions (~0.1 mM) of the analyte in acetonitrile/water (0.1 TFA) with approximately equal volumes of a 0.1 M solution of DHB in acetonitrile/water (0.1 TFA), followed by deposition of 1 μ L of sample solution onto a 384-well aluminum plate and air drying. Horse heart myoglobin (MW 16 952 g mol⁻¹), bovine serum albumin (MW 66 431 g mol⁻¹), and cytochrome C (MW 12 361 g mol⁻¹) were used as external standards. Positive ion mass spectra were acquired in linear mode, and the ions were generated by using a nitrogen lase (337 nm) pulsed at 3 hz with a pulse width of 3 ns. Ions were accelerated at 19 to 20 000 V and amplified using a discrete dynode multiplier. Spectra (100 to 200) were summed into a LeCroy LSA 1000 high-speed signal digitizer. All data processing was performed using Bruker XMass/XTOF V 5.0.2. molecular mass data, and polydispersities of the broad peaks were calculated by using the Polymer Module included in the software package. The peaks were analyzed using the continuous mode. Delta values were set at minimum levels. The peak width at half height is evaluated to determine the overall sample homogeneity. Samples that deviate significantly from a normal distribution function give significantly broader peaks than those that are observed for the compounds described here.

Reverse-Phase HPLC—HPLC characterization of bifunctional dendrimers and dendrimer conjugates was carried out with a Waters HPLC instrument equipped with two pumps, an autosampler, and dual UV detector interfaced to Empower 2 software. The mobile phase used was acetonitrile/water (pH 2.25) both containing 0.14% TFA. The water phase was freshly prepared, and both phases were filtered and degassed prior to use. A Supelco discovery BIO wide-pore C5 HPLC column (5 μ m particle size, 25 cm length, 4.6 mm I.D.) equipped with two C5 supelguard cartridges (5 μ m particle size, 2 cm length, 4.0 mm I.D.) was used for characterization of the conjugates. The gradient method was used for analysis of water/acetonitrile 100:0 to 60:40 in 25 min, followed by returning to initial conditions in 15 min. The flow rate was 1 mL/min. The UV absorbance detector was used at wavelenghtts of 210 nm. For the FITC-labeled compound, we used a fluorescence detector at $\lambda_{\text{ex}} = 495 \text{ nm}/\lambda_{\text{em}} = 521 \text{ nm}$.

Dynamic Light Scattering and ζ Potential—Dynamic light scattering (DLS) and a ζ -potential analyses were performed using a Malvern Instruments Zetasizer Nano ZEN3600 instrument (Westborough, MA) with reproducibility being verified by collection and comparison of sequential measurements. Bifunctional dendrimer and PAMAM dendrimer samples were prepared using PBS pH 7.4. DLS measurements were performed at 90° scattering angle at 37°C. Z-average sizes of three sequential measurements were collected and analyzed. ζ -potential measurements were collected at 25°C, and the Z-average potentials following three sequential measurements were collected and analyzed.

Cell Culture—Human lung carcinoma A549 cells were obtained from ATCC and maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS). These cells were cultured at 37°C with 5% CO₂, and the media were replaced at 2-day intervals.

Cell Cytotoxicity Assay—A549 (passage 16) cells were seeded in a 96-well plate at 10⁴/well. After 24 h, cells were exposed to various concentrations of new compounds (1 μ g/mL to 1 mg/mL) in serum-free medium for 24 h. The highest concentration (1 mg/mL) of compounds (**4**, **7**, **11**, **14**, and **17**) tested was 0.053, 0.051, 0.062, 0.052, and 0.052 mM, respectively. Controls were carried out with medium alone. Cytotoxic effect was determined using MTT. The proportion of viable cells in the treated group was compared with that of the control.

Hemolysis Assay—Rabbit blood (5 mL) was drawn from the artery of ear and collected in a BD vacutainer tube containing heparin as coagulant. The blood samples were centrifuged for 10 min at 900 rpm, and the serum fraction was removed. The red blood cells were washed with freshly prepared cold 0.9% saline three times. After saline was removed, the volume was raised to whole blood with PBS and the RBCs were further diluted 1 to 10 with PBS. The 200 μ L of diluted red blood cells was treated with test compound solutions (800 μ L) in the concentration range of 1 μ g/mL to 10 mg/mL. Trimeton X-10 (1%) was used as a positive control, and PBS alone was used as negative control. The samples were incubated at 37°C for 3 h and mixed by inversion every 30 min. The samples were centrifuged at 13 000 rpm for 5 min. The supernatant was collected, and its absorbance was measured at 540 nm using a UV-vis spectrophotometer. The percentage of hemolysis was calculated with the formula:

$$\% \text{ hemolysis} = [(OD \text{ of sample}) - (OD \text{ of blank})] / (OD \text{ of positive control}) \times 100\%$$

EXPERIMENTAL PROCEDURES

General Method for Synthesis of Compounds of 3 and 6

Boc-AA-NHS (**2**, **5**) was prepared using a previously reported method.⁴³ G4-PAMAM-NH₂ (**1**) (1 equiv) and Boc-AA-NHS (2 equiv) were dissolved in DMSO/DMF (4:1, 25 mL) followed by the addition of DIEA (2 equiv) under a nitrogen atmosphere. The reaction mixture was stirred for 24 h at room temperature (r.t.) and purified to obtain the product (Table 1).

General Method for Purification

The crude product obtained from the reaction mixtures was purified by dialysis using spectrapor dialysis membranes (MW cut-off 1000 Da) against DMSO by changing solvent every 8 h three times to remove byproduct and the excess of reactants; after dialysis, the solvent was removed under lyophilization to get pure compounds in good yields (Table 1).

General Method for Deprotection of tert-butoxycarbonyl(Boc)

Groups—Boc-protected compounds (**3**, **6**, **13**, **16**, and **28**) were treated with TFA/DCM (50:50% v/v, 10 mL) solution in a round-bottomed flask stirring for 10 min. Post deprotection, the solution was neutralized (pH 7.0) using a 1N NaOH solution and purified to obtain the product.

General Method for Coupling Reaction for Synthesis of Compounds **10**, **13**, **16**, and **28**

Dendrimer (1 equiv) was dissolved in DMSO/DMF (4:1, 12 mL), and DMAP (2 equiv) was added to this solution, followed by the addition of EDC (2 equiv) under the nitrogen atmosphere; the reaction was allowed to proceed for 24 h at r.t. After completion of reaction, the product was purified by dialysis using the purification method described above.

Synthesis of G3.5-PAMAM-CO-NH-Ser-OH (**11**)

Hydrolysis of methyl ester was carried out by the addition of G3.5-PAMAM-CO-Ser (OH)-OMe (**10**) (100 mg, 0.005 mol) to LiOH (5 mg, 2.26 mol) solution in THF/H₂O (9:1 10 mL). The reaction was carried out for 5 h, after completion of reaction, the product was purified by dialysis using the purification method described above (Table 1).

Synthesis of G4-PAMAM-O-CO-Asp-(CO-Dex)-NH₂ (20)

G4-PAMAM-O-CO-Asp-(COOH)-NHBoc (**16**) (200 mg, 0.0105 mol) was dissolved in DMSO/DMF (3:1, 20 mL) solution. EDC (506 mg, 2.64 mol), DMAP (160 mg, 1.31 mol), and dexamethasone (**15**) (520 mg, 1.32 mol) were added to this solution, and the reaction was allowed to proceed at room temperature for 12 h under a nitrogen atmosphere. After completion of the reaction, crude product was purified by using the purification method described above. Post Boc deprotection, the compound was subjected to dialysis by using the purification method described above (Table 1).

Synthesis of G4-PAMAM-O-Asp (CO-Dex)-Ind (22)

Indomethacin (**21**) (249 mg, 0.69 mol) was dissolved in DMSO/DMF (3:1, 20 mL), EDC (133 mg, 0.69 mol) and DMAP (85 mg, 0.69 mol) were added to it, and the reaction mixture was stirred under a nitrogen atmosphere. After 15 min, G4-PAMAM-O-CO-Asp (CO-Dex)-NH₂ (**20**) (80 mg, 0.0036 mol) was added to it. The reaction was continued at room temperature for 15 h. After completion of the reaction, crude product was purified by dialysis, as described in the purification method above to get G4-PAMAM-O-Asp(CO-Dex)-Ind (**22**) pure compound. Apart from dexamethasone protons listed for (**20**), the ¹H NMR of compound (**22**) shows the appearance of protons corresponding to indomethacin (Table 1).

Synthesis of G4-PAMAM-O-CO-Asp(CO-Dex)-NH-FITC (24)

G4-PAMAM-O-CO-Asp-(CO-Dex)-NH₂ (**20**) (100 mg, 0.0038 mol) was dissolved in DMSO (10 mL), FITC (**23**) (17.6 mg, 0.045 mol) was added to it under stirring, and the reaction was allowed to proceed for 12 h in dark. After completion of the reaction, crude product was purified by dialysis, as described in the purification method above to get (**24**) pure compound. Apart from dexamethasone protons listed for (**23**), the ¹H NMR of compound (**24**) shows the appearance of protons corresponding to FITC (Table 1).

Synthesis of Boc-Cys (S-TP)-OH (27)

Boc-Cys(*S*-TP)-OH (**27**) was prepared by reaction of 2,2'-dithiodipyridine (7.96 g, 36 mol) with Boc-Cys-OH (4 g, 18 mol) in a mixture of methanol and water (1:1, 40 mL). The reaction was stirred for 24 h at room temperature. Upon completion of the reaction (monitored by TLC), the methanol was removed in vacuo, and the residue was recrystallized with acetone and petroleum ether to give the pure product as a white solid (Table 1).

Synthesis of G4-PAMAM-O-CO-Cys(S-TP)-NHBoc (28)

G4-PAMAM-OH (**12**) (500 mg, 0.036 mol) was dissolved in DMSO/DMF (3:1), and Boc-Cys (*S*-TP)-OH (**27**) (1484 mg, 4.48 mol) was added under stirring. DMAP (273 mg, 2.23 mol) and EDC (856 mg, 4.48 mol) were added to this solution, and the reaction was allowed to proceed overnight for 18 h under a nitrogen atmosphere. The product was purified by dialysis, as described in the purification method above to get pure compound (**28**) (Table 1).

RESULTS AND DISCUSSION

Design

A library of dendrimer having heterobifunctional groups at periphery, amenable for further modifications, is described (Table 2). An important consideration was to attain a robust, simple, and synthetic approach to attain near-complete surface modification with amino acids to yield heterobifunctional end terminations on a biocompatible dendrimer scaffold (Figure 1). Past reports on the synthesis of bifunctional dendrimers involve multiple steps.^{10,31} A simple one-pot synthesis to achieve orthogonal and chemoselective end groups

by complete end-capping of the G4 PAMAM dendrimers with amino acids is shown in Schemes 1–4 and 6. The present concept demonstrates that 110 heterobifunctional end groups can be achieved on a generation four (G4) dendrimer (Figure 1) without going to the next generation (G5) dendrimers. This is significant because it is well-known that dendrimers exhibit generation-dependent cytotoxicity.⁴ PEGylation of G5 and G6 PAMAM-NH₂ dendrimers significantly reduced its hemolytic activity but paradoxically compromised its transfection ability.⁴ Our approach to the choice of the materials to design these heterobifunctional dendrimers was based on developing biocompatible dendrimer scaffolds for drug delivery applications and also retaining the reactivity of terminal groups for drug conjugation. All reactions were monitored by MALDI-TOF MS analysis to ensure complete substitution, and the purity of compounds was analyzed by NMR, HPLC, and MALDI-TOF MS analysis. One of the advantages of the present system, having multiple diverse functional handles on periphery, is the ease of conjugating different drugs along with imaging agents and targeting ligands without the need of additional synthetic steps to attach specific spacer or linker molecules. Furthermore, the repetitive protection or deprotection steps were not needed in the present synthetic methodology, and a single-step deprotection postsynthesis was enough to achieve the distinct peripheral functional groups. The feasibility of the concept and the chemoselective and orthogonal nature of these new heterobifunctional dendrimers was demonstrated by conjugation of (1) two drugs, viz. dexamethasone and indomethacin, and (2) indomethacin and imaging agent (FITC) on the aspartic acid surface-modified PAMAM dendrimer. Therefore, two different moieties were added in immediate succession without any functional group conversions owing to the orthogonal peripheral groups. Additionally, of the two diverse functional handles on the heterobifunctional dendrimers, one of the functional handles was selectively used for *in situ* hydrogel formation, whereas the second functional handle was used for conjugating drug and imaging agents.

Synthesis of Heterobifunctional G4 PAMAM Dendrimers

To achieve heterobifunctional G4-PAMAM dendrimer with high density of amine and hydroxyl functional groups at the periphery of (**4**), we reacted the symmetrical terminal “amine” groups of G4-PAMAM dendrimer (**1**) with acid terminal of Boc-Ser-NHS (**2**). This was a straightforward coupling reaction that converted the symmetrical peripheral amines (~64 theoretically) of G4-PAMAM-NH₂ (**1**) dendrimer into a total of ~116 heterobifunctional groups on the periphery bearing 58 of “Box-amine” and 58 of “hydroxyl” functionalities, respectively (Scheme 1), in a one-step reaction. The coupling reaction between G4-PAMAM-NH₂ dendrimer (**1**) and Boc-Ser-NHS (**2**) with amine terminations of G4-PAMAM-NH₂ (**1**), it is expected that the product will consist of Boc-Ser residues conjugated at the G4-PAMAM-NH₂ (**1**) by amide bond; the reaction was monitored by MALDI-TOF MS analysis to ensure complete substitution. The product so obtained was purified by dialysis using DMSO to remove the excess of unreacted Boc-Ser-NHS and other byproducts. The appearance of characteristic signals of Boc-serine in the ¹H NMR spectrum at 1.38 (s, 9H, Boc), 2.10–2.22 (br.s, OH), 3.22–3.38 (m, 1H, CH₂), 4.50–4.58 (m, 1H, CH₂), 4.80–4.90 (m, 1H, CH), 6.50–6.60 (m, 1H, NH amide), and 7.78–7.97 (br.d, NH interior dendrimer amide) ppm of G4-PAMAM-NHCO-Ser(OH)-NH₂ (**3**) (Figure 2) indicate that the desired product is obtained. The integral ratio of the amide protons of PAMAM-NH-CO-Ser(OH)-NH₂ at 7.78–7.97 ppm to the two methylene protons of serine at 3.22–3.38 (m, 1H, CH₂) and 4.50–4.58 (m, 1H, CH₂) ppm suggests that each G4-PAMAM-NH₂ dendrimer contains ~58 Boc-serine molecules attached. The MALDI-TOF-MS analysis of G4-PAMAM –NH-CO-Ser(OH)-NH₂ (**3**) shows the appearance of molecular mass peak at 24.5 kDa (Figure 2). For G4-PAMAM dendrimer, the measured molecular weights (13.7 kDa) were lower than the theoretical value (14.21 kDa). Similar results for mass spectrum of PAMAM dendrimers were observed in the past and were

attributed to their structural defects.⁴⁴ The increase in mass (~10.8 kDa) from 13.7 kDa of unfunctionalized G4-PAMAM-NH₂ to 24.5 kDa for G4-PAMAM-NH-CO-Ser(OH)-NHBoc (**3**) suggests attachment of 58 Boc-serine molecules because the molecular weight of serine is 205 Da. The MALDI spectrum obtained for (**3**) is broad, and this peak broadening could be attributed to the starting material and not to the reaction sequence. Previously, the broad MALDI peaks for PAMAM starting compounds and their highly functionalized derivatives have been ascribed to structural defects in starting compounds.^{45–48} The spectra obtained by us for various compounds seem to show similar peak broadening. The MALDI data further support the NMR data, which showed attachment of 58 molecules. The conversion of symmetrical terminal amines of G4-PAMAM to heterobifunctional “OH” and “NHBoc” terminal groups was 93%. Previously, 84% surface modification with sugars on G4-PAMAM dendrimer was reported,⁴⁹ and our results show that a high degree of surface functionalization has occurred. The PAMAM dendrimers have some structural defects in the starting compounds themselves, and these are known to preclude the complete conversion.⁴⁹ The HPLC chromatogram (210 nm) shows a single peak corresponding to G4-PAMAM-NH-CO-Ser(OH)-NHBoc (**3**), suggesting relatively good purity of the product (Figure 2).

Achieving “near complete” attachment of the Boc-serine moieties on the dendrimer was challenging. There is always a possibility that conjugation of bulky molecules is impeded because of steric hindrance. When we compared the attachment of cysteine with one protecting group (**5a**) and two protecting groups (**27**) to G4-PAMAM-OH (**12**), a drastic reduction in the number of cysteine (**27**) attached to dendrimer was observed. This shows that the presence of thiopyridyl and *tert*-butoxy-carbonyl protecting groups makes the cysteine (**27**) molecule bulky and hence causes steric hindrance leading to a lower number of cysteines (**27**) attached to the dendrimer vis a vis cysteine (**5a**) with one protecting group. A single broad peak was observed in the MALDI-TOF MS spectrum (Figure 2), and the peak corresponding to dendrimer (starting compound) at ~13.7 kDa was not observed in this spectrum, indicating that the peak at 24.5 kDa belongs to G4-PAMAM-Ser-(OH)-NHBoc (**3**) compound. Furthermore, the spectrum did not show multiple peaks, indicating the absence of other byproducts. The ¹H NMR spectra and MALDI-TOF MS for G4-PAMAM-Ser-(OH)-NHBoc dendrimer (**3**) collectively suggest the attachment of 58 molecules of Boc-Serine of G4-PAMAM-NH₂. Our evaluations suggested that the two-fold excess of serine was sufficient to achieve “near”-complete end-capping of the G4-PAMAM-NH₂ dendrimer (**1**) to provide G4-PAMAM-Ser-(OH)-NHBoc dendrimer (**3**). The above compound so obtained was further used to get amine terminations at the periphery attained by global deprotection of the *tert*-butoxycarbonyl (Boc) groups using trifluoroacetic acid (Scheme 1), and the resulting heterobifunctional dendrimer (**4**) can be then utilized in a variety of subsequent conjugation reactions. The characteristic signals of *tert*-butyl groups appearing at 1.30 (s, 9H) in the ¹H NMR spectrum of (**4**) disappear on deprotection, but the other peaks corresponding to serine are seen in ¹H NMR spectrum at δ 1.0–1.19 (m, 2H, NH₂), 1.80–1.98 (br.s, 1H, OH), 4.21–4.26 (s, 1H), 8.0–8.15 (br.d from amide NH), and 8.30–8.60 (br.d, amide NH) of G4-PAMAM-NH-CO-Ser(OH)-NH₂ (**4**) (Figure 3) confirm the desired product. The integral ratio of the amide protons of PAMAM-NH-CO-Ser(OH)-NHBoc at 8.30–8.60 ppm to the two methylene protons of serine at 3.50–3.68 (m, 2H, CH₂) ppm suggests that each PAMAM-NH₂ dendrimer contains ~58 serine molecule attached. After deprotection, the molecular weight decreased from 24.5 kDa for (**3**) to 18.7 kDa for (**4**) (Figure 3). The mass of G4-PAMAM-NH₂ dendrimer is 13.7 kDa, and this increase (5 kDa) to 18.7 kDa corresponds to 58 molecules of serine attached because the molecular weight of serine is 105 Da. The HPLC chromatogram (210 nm) shows a single peak corresponding to G4-PAMAM-NH-CO-Ser(OH)-NH₂ (**4**), confirming the purity of the product (Figure 3). The diverse end groups so obtained are amenable for postfunctionalization modifications or reactions. The high density of diverse end groups is achieved through a choice of the end

termination of parent scaffold and the reacting amino acid. We explored different permutations of the end group functionality of the dendrimers and several amino acids to develop a library of heterobifunctional dendrimers (Tables 2 and 3).

Other heterobifunctional dendrimers (**6, 10, 13, 16, 28**) bearing “SH + NHBoc”, “COOMe + OH” “COOH + NHBoc” and “NHBoc + S-TP” terminal groups were synthesized by reacting G4-PAMAM-NH₂ dendrimer (**1**), G3.5-PAMAM-COOH dendrimer (**8**), and G4-PAMAM-OH (**12**) with Boc-Cys-NHD (**5**), and Boc-Ser-OMe (**9**), Boc-Cys(SH)-OH (**5a**), Boc-Asp-OH (**15**), Boc-Cys(S-TP)-OH (**27**) respectively (Schemes 2–4 and 6). The compounds **3, 6, 13, 16**, and **28** on global deprotection of Boc groups with trifluoroacetic acid and dichloromethane gave **4, 7, 14, 29**, and compound **10**, on hydrolysis of methyl ester with lithium hydroxide in tetrahydrofuran/water (THF/H₂O) gave compound **11**. Table 2 gives the PAMAM dendrimer scaffold and the respective amino acids used to functionalize the periphery. The percent conversion and number of amino acids attached to the dendrimer are given in Table 3. The ¹H NMR and MALDI TOF MS spectra for these compounds are provided in the Supporting Information. In the past, it has been reported that attachment of 64 groups of phenylalanine on G4 dendrimer resulted in a significant reduction in the water solubility of the compound.²⁵ In the present study, all compounds with significantly high end-group modifications of G4-PAMAM dendrimers with amino acids resulted in highly water soluble compounds (**4, 7, 11, 14**, and **17**).

Postfunctionalization Reactions of Heterobifunctional Dendrimers Bearing “COOH” and “NH₂” Terminations

The orthogonal and chemoselective nature of the peripheral end groups in the heterobifunctional dendrimers was demonstrated by conjugation of (i) two drugs vis. dexamethasone and indomethacin and (ii) indomethacin as well as an imaging agent (FITC) on the aspartic acid surface modified PAMAM dendrimer (**16**) (Scheme 4). In addition, the *in situ* hydrogel formation using only one functional handle of the heterobifunctional dendrimer is demonstrated, whereas the second functional handle is used for drug conjugation, as shown in Scheme 5.

A heterobifunctional G4-PAMAM dendrimer bearing “carboxylic” and “Boc-amine” peripheral terminations to facilitate the diverse postfunctionalization reaction was obtained by reaction by reacting the “hydroxyls” of G4-PAMAM-OH (**12**) with Boc-aspartic acid (**15**). The coupling reaction of G4-PAMAM-OH dendrimer (**12**) to Boc-aspartic acid (**15**) was carried out using EDC/DMAP (Scheme 4) to obtain G4-PAMAM-O-Asp(COOH)-NHBoc (**16**). Because aspartic acid has two carboxylic acid groups, there is a possibility of obtaining either an α or β product, but in either case, it would yield “COOH and NHBoc” terminal groups. MALDI-TOF MS of the functionalized dendrimer (**16**) reveals the mass peak at 25.7 kDa, as seen from the mass spectrum (Figure 4). The molecular weight of Boc-aspartic acid is 233 Da and thus the increase from ~14 kDa for G4-PAMAM-OH (**12**) to 25.7 kDa corresponds to 56 molecules of Boc-aspartic acid (**15**) attached to G4-PAMAM-O-Asp(COOH)-NHBoc (**16**). The appearance of characteristic signals of Boc-Asp-OH in the ¹H NMR spectrum at 1.30 (s, 9H), 2.10–2.20 (m, 2H), 4.50–4.60 (br.s, 1H), and 7.19–7.24 (br.s, amide NH) of G4-PAMAM-O-CO-Asp(COOH)-NHBoc (**16**) (Figure 5) confirms the desired product. The integral ratio of the amide protons of PAMAM-O-CO-Asp(COOH)-NHBoc at 7.70–8.05 (br.d, amide NH) to the two methylene protons of Boc-Asp-OH 2.10–2.20 (m, 2H) suggests that each PAMAM-OH dendrimer contains ~56 molecules of Boc-Asp-OH. We estimate the extent of surface functionalization at 90% by taking the average of the MALDI-TOF MS and NMR data and the purity of the compound obtained by RP-HPLC (Figure 6A). On repeating the synthetic procedure several times, we observed that 56 molecules could be conjugated, rather than the theoretical ~64 that are

available, resulting in a total of 112 end functionalities (56 + 56 each). The structural defects in the starting compounds themselves (PAMAM dendrimers) could contribute to the observed effect, as described previously.⁴⁹ Both MALDI-TOF MS and NMR analyses do not account for the small structural imperfections.⁴⁹ Our results show a high degree of conversion (90%) of “OH” terminal groups into “COOH and Boc-NH” groups. In addition to carboxylic groups, the amine terminations at the periphery are attained by global deprotection of the *tert*-butoxycarbonyl (Boc) groups using trifloroacetic acid (Scheme 4), and the resulting heterobifunctional dendrimer (**17**) can be then utilized in a variety of subsequent conjugation reactions. The characteristic signals of *tert*-butyl groups appearing at 1.30 (s, 9H) in ¹H NMR spectrum of (**17**) disappear on deprotection, but the other peaks corresponding to aspartic acid are seen at 2.20–2.38 (m, 2H), 4.22–4.31 (br.s, 1H), 7.96–8.10 (br.s, amide NH), and 8.10–30 (br.d, amide, NH). After deprotection, the molecular weight decreased from 25.7 kDa for (**16**) to 18.9 kDa for (**17**) (Figure 3); because the molecular weight of aspartic acid is 133 Da, the mass of **15** corresponds to 46 molecules of the aspartic acid appended on the dendrimer, thereby yielding a total of 92 end functionalities (46 + 46 each). Yet there is a merit in this scaffold because it has a high density of diverse end groups as compared with the G4-PAMAM-OH dendrimer.

To test if the terminal groups are amendable to further modification, G4-PAMAM-O-Asp(COOH)-NHBoc (**16**) was reacted with dexamethasone (**18**) (a widely used steroidal anti-inflammatory drug), involving the carboxylic end groups on dendrimer to link the drug by ester bond using EDC/DMAP as coupling reagents (Scheme 4). Compound (**19**) was used without further characterization to get amine terminations at the periphery by global deprotection of the *tert*-butoxycarbonyl (Boc) groups using trifluoroacetic acid (Scheme 4), and the resulting (**20**) can then be utilized in a variety of subsequent conjugation reactions. The formation of G4-PAMAM-O-Asp(CO-Dex)-NH₂ (**20**) conjugate was validated by ¹H NMR analysis. The appearance of dexamethasone methyl protons as 0.75 (s, 3H), 0.82 (s, 3H), and 1.45 (s, 3H) and double-bond protons at 6.01 (s, 1H), 6.23 (d, 1H), and 7.30 (d, 1H), confirms the conjugation between G4-PAMAM-O-Asp(COOH)-NHBoc (**16**) and dexamethasone (**18**) (Figure 5). The attachment of multiple copies of dexamethasone to G4-PAMAM-O-Asp(COOH)-NHBoc (**16**) dendrimers was determined by MALDI-TOF MS and by RP-HPLC (Figure 6C). The attachment of dexamethasone, followed by Boc deprotection shifted the mass of G4-PAMAM-O-Asp(CO-Dex)-NH₂ dendrimer from 25.7 to 21.9 kDa (Figure 4). Dexamethasone (**18**) has a molecular weight of 392 Da; therefore, the incremental mass corresponds to an average of eight molecules of dexamethasone molecules per dendrimer (number attained from three independent experiments).

To examine this further, the possibility of the conjugation of a second drug to the heterobifunctional dendrimer (**20**) in immediate succession, the G4-PAMAM-O-Asp-(CO-Dex)-NH₂ (**20**) was reacted with indomethacin (**21**) without the need to attach additional spacer or linker molecules. Indomethacin was chosen because it is an important anti-inflammatory drug. The conjugation was carried out in the presence of EDC/DMAP as coupling reagents (Scheme 4). The ¹H NMR analysis shows that the aromatic protons corresponding to indomethacin appear at 2.10–2.30 (m, 3H, CH₃), 3.62–3.80 (m, 5H, -OCH₃, -CO-CH₂-), 6.60–6.79 (m, 2H, Ar), 6.63–7.04 (m, 2H, Ar), and 7.60–7.70 (m, 3H, Ar) confirming the conjugation of indomethacin to G4-PAMAM-O-Asp(CO-Dex)-NH₂ (**20**) to yield G4-PAMAM-O-Asp(CO-Dex)-NH-Ind (**22**) (Figure 5). The purified dendrimer conjugate was subjected to MALDI-TOF-MS analysis, and the obtained mass exhibited an increase from 21.9 (for **20**) to 30.1 kDa (for **22**), as expected (Figure 4). The purity of the compound was confirmed by RP-HPLC (Figure 6E). The increase in molecular weight corresponds to an average of 24 indomethacin molecules per dendrimer molecule because indomethacin has a molecular mass of 357 Da, suggesting an overall 36% loading of dexamethasone and indomethacin (number attained from three independent experiments).

Our ^1H NMR analysis showed that any undesired side products were not observed, suggesting the clean attachment of two drugs (both dexamethasone and indomethacin). The drug loading was deliberately kept to lower levels for maintaining the solubility of the final conjugates in water.

Polymeric scaffolds used in drug delivery are often tagged with imaging agents and radionucleotides to investigate their distribution pattern in vitro and in vivo. This attachment could be direct on to the scaffold or mediated through an appropriate linking chemistry, which may at times require a suitable spacer molecule.^{9,17} In the present study, the carboxylic terminations G4-PAMAM-O-Asp-(CO-Dex)-NH₂ (**20**) were consumed for esterification with dexamethasone (**18**), but the presence of Boc-amine groups bestowed flexibility to explore after Boc deprotection for direct attachment of fluorescent imaging dye (FITC) (**23**) by thiourea bond. We demonstrate the ability of the heterobifunctional dendrimers to attach to drug and an imaging agent in immediate succession without any further modification, thereby excluding the additional synthetic steps to append a suitable spacer to the dendrimer scaffold. G4-PAMAM-O-Asp-(CO-Dex)-NH₂ (**20**) conjugate was tagged with FITC (**23**) (Scheme 4) in one step by adding FITC (**23**) to a solution of G4-PAMAM-O-Asp-(CO-Dex)-NH₂ (**20**) in DMSO, and the reaction was stirred at room temperature in dark. The FITC-labeled G4-PAMAM-O-Asp(CO-Dex)-(NH-FITC) (**24**) was purified by dialysis using spectrapor membrane (cutoff 1000 Da) against DMSO in the dark. The dialyzed product was dried under vacuum to obtain the conjugate (**24**). Purity of G4-PAMAM-O-Asp(CO-Dex)-NH-FITC (**24**) conjugate was confirmed by HPLC using a fluorescence detector ($\lambda_{\text{ex}} = 495 \text{ nm}/\lambda_{\text{em}} = 521 \text{ nm}$) (data not shown). Furthermore, the appearance of aromatic protons at 6.57–6.62 (d, 6H, Ar) and 6.63–6.70 (s, 3H, Ar) in the ^1H NMR spectrum indicates the attachment of FITC, and the integral ratio of amide protons of G4-PAMAM-O-Asp(CO-Dex)-NH₂ (**20**) appearing at 8.10–8.30 ppm to the aromatic protons at 6.57–6.62 and 6.63–6.70 suggests the attachment of six molecules of FITC in G4-PAMAM-O-Asp(CO-Dex)-(NH-FITC) conjugate (**24**) (Supporting Information, Figure S24). The MALDI-TOF MS of G4-PAMAM-O-Asp-(CO-Dex)-NH₂ (**20**) showed a mass of 21.9 kDa, and a further increase in mass to 23.2 kDa affirmed the attachment of six molecules of FITC (data not shown).

The presence of two functional handles led us to develop in situ forming hydrogels using only one of the functional handles for chemical reaction forming the gel, whereas the other handle can be used for conjugating the drugs (Scheme 5). The ability of the NH₂ groups of G4-PAMAM-O-Asp-(CO-Dex)-NH₂ (**20**) for hydrogel formation was tested by its reaction with *N*-hydroxy-succinimide-terminated 8-arm-PEG polymer (**25**), and blue dextran (M_w 5000) was physically entrapped in this gel. Hydrogel formation was determined by the “inverted tube method”, and hydrogels were considered to have formed once the solution ceased to flow from the inverted tube (Figure 7). The gelation times for these hydrogels ranged 30–50 s, and these open new vistas for the drug delivery application of these heterobifunctional dendrimers. Of the amine and the COOH terminal groups of G3-PAMAM-O-Asp-(CO-Dex)-NH₂ (**20**), the COOH groups were involved in the conjugation of drug dexamethasone (**18**) by ester linkage, whereas the NH₂ groups were involved in gel formation by amide linkages on reaction with *N*-hydroxy-succinimide-terminated 8-arm-PEG (**25**) polymer (Scheme 5). This provides a new approach to the design of hydrogels where the rate of drug release can be further slowed because the drug release involves two steps: (i) release from covalent linkage of dendrimer after the degradation or hydrolysis of the bond and (ii) diffusion of the drug from the hydrogel. Different concentrations of the polymer solution were tested in the stoichiometric ratio 1:1, and the gel formation was observed at 3, 5, and 8% w/w. Furthermore, FITC (**23**) was attached to a few NH₂ groups (Three end groups) of the G4-PAMAM-O-Asp-CO-Dex)-NH₂ (**20**), and this dendrimer also formed hydrogel by amide linkages on reactions with *N*-hydroxy-succinimide-terminated 8-

arm-PEG polymer (**25**); this gel is shown in Figure 7. The SEM image shows the gel network formed by reaction of PEG-NHS (**25**) with G4-PAMAM-O-Asp-(CO-Dex)-NH₂ (**20**) and G4-PAMAM-O-Asp-(CO-Dex)-NH-FITC (**24**).

The above conjugation reactions show the ability of robust postfunctionalization modifications in these heterobifunctional dendrimers, a key measure of synthetic efficiency that further indicates that these peripheral end groups exhibit chemoselectivity based on their asymmetric or orthogonal nature. With these results, we demonstrate that we could achieve a large number of asymmetric end groups (~112) on G4-PAMAM dendrimer as compared with 64 symmetric end groups available traditionally in a one-step one-pot reaction. Dexamethasone and indomethacin were conjugated to G4-PAMAM-O-Asp(COOH)-NHBoc (**16**) using an ester and after Boc deprotection amide linkage, respectively. Furthermore, we were able to attach FITC and dexamethasone to G4-PAMAM-O-Asp(COOH)-NHBoc by thiourea and ester linkage, respectively. The in situ gelling hydrogels with the ability to entrap physically and covalently attach the drugs was demonstrated. The diverse nature of these heterobifunctional groups on dendrimers additionally confers the flexibility to append several functional groups in immediate succession without the need to append a specific linker, all contributing the drastic reduction in the synthetic and purification steps.

In Situ Hydrogel Formation by Cross-Linking of Heterobifunctional Dendrimers Bearing “S-TP” and “NH₂” Terminations

Hydrogels have been used as vehicles for sustained drug delivery. The rich end functionalities prepared using the current approach could enable a new class of multifunctional hydrogels. We present a new approach where a dendrimer-based degradable hydrogel comprising a redox-sensitive bond is disclosed. The hydroxyl-terminated G4-PAMAM-OH dendrimer (**12**) was end-capped with Boc-Cys(*S*-thiopyridyl)-OH (**27**) to yield 68% heterobifunctional end groups comprising 42 thiol-protected and 42 amine terminations in protected form (Scheme 6). The thiol groups in Boc-cysteine were protected using 2-aldrithiol before modifying the dendrimer to yield G4-PAMAM-O-CO-Cys(*S*-thiopyridyl)-NHBoc (**28**). The thiol protection reaction was carried out under mild reaction conditions using methanol/water as solvent at room temperature for 24 h. The product was obtained by recrystallization in acetone and hexane. The *tert*-butoxycarbonyl (Boc) protecting groups were removed by using trifluoroacetic acid in dichloromethane to yield amine functionality. G4-PAMAM-O-CO-Cys(*S*-thiopyridyl)-NH₂ (**29**) so obtained was mixed with the solution of 8-arm-polyethylene glycol with thiol terminations (Ratio 1:4 w/v respectively), resulting in in situ forming hydrogel. This reaction is simple and occurs in physiological pH 7.4 phosphate buffer saline by the formation of disulfide cross-links (Scheme 6). The gelation time for these gels was <45 s, suggesting a relatively rapid disulfide cross-link reaction. Over a period of time (20 days) under physiological (pH 7.4, 37 °C) conditions, these gels undergo a gel to sol transformation, suggesting the degradation or breakdown of the gels. It has been reported that disulfide exchange reactions occur slowly under physiological conditions, which contribute to the degradation of the hydrogel.¹² With these studies, we demonstrate the potential of these heterobifunctional dendrimers for in situ forming hydrogels. These hydrogels can be further explored for physical encapsulation of drug or covalent linkage of drug to the other functional group for providing sustained release of drugs in a similar way as that disclosed for the gels formed between G4-PAMAM-Asp-(CO-Dex)-NH₂ (**20**) with PEG-NHS (**25**). An interesting feature of this reaction is that both the *tert*-butoxycarbonyl and thiopyridyl groups are orthogonal in nature, and under the acidic conditions, trifluoroacetic acid in dichloromethane used for deprotection of *tert*-butoxycarbonyl groups, the thiopyridyl groups are extremely stable. With the introduction of two protectin groups, in cysteine, we observed that the total number of copies of cysteine

attached drastically reduced to 40 (64.5% conversion) as compared with other amino acids used without protecting groups or with single protecting group showing 87–93% conversion. The ¹H NMR, MALDI-TOF MS characterization of G4-PAMAM-O-CO-Cys(*S*-thiopyridyl)-NH_{Boc} (**28**) and its *tert*-butoxycarbonyl deprotection are provided in the Supporting Information.

Particle Size and ζ Potential of Heterobifunctional Dendrimers

The impact of the surface modification of the dendrimers on particle size, ζ potential, blood retention, and in vivo organ distribution has been previously reported.⁴ The surface modification of the dendrimers with amino acids on an average increased the particle size by 1 to 2 nm, as seen from Table 4. The end-capping of the cationic G4-PAMAM-NH₂ dendrimer with serine and cysteine resulted in a drastic reduction in the ζ potential from +11.5 to –1.83 and 4.80 mV, respectively. The interesting part is that both of these constructs retain an equal number of surface NH₂ termini as compared with unmodified G4-PAMAM-NH₂, yet they exhibit reduced charge and are therefore expected to reduce the cytotoxicity. The hydroxyl-terminated G4-PAMAM dendrimers are nontoxic because of the neutral surface charge,⁵⁰ and end-capping it with aspartic acid and cysteine did not increase the charge significantly (Table 4). Again, both of these constructs have NH₂ termini in addition to other groups, yet they exhibit low charge. By end-capping of the hydroxyl-terminated dendrimer, we could attain hydrogels and attachment of several functional groups without eliciting the cytotoxicity. The carboxylic-acid-terminated dendrimer end-capped with serine exhibited unexpectedly high ζ potential (+8.83 mV). The increase in ζ potential was consistent with the increased cytotoxicity of this construct, and it would need further investigation to understand the underlying reasons for the observed results.

Hemolytic and In Vitro Cytotoxicity

Red blood cell (RBC) lysis is a simple quantitative measure of hemoglobin (Hb) release widely used to study polymer-membrane interaction.⁵⁰ Both cationic and anionic PAMAM dendrimers at 1 mg/mL concentration and exposure of 1 h induce marked morphological changes evidenced by clumping of RBCs.^{50,51} Our study showed a zero hemolysis and 100% hemolysis on incubating the RBCs with PBS (negative control) and 1% Triton X-100 (hemolytic agent-positive control), respectively. The G4-PAMAM-NH₂ dendrimer did not exhibit significant hemolysis up to 100 μ g/mL concentration and exhibited ~10% hemolysis at 1 mg/mL on exposure for 3 h. Previous studies using rat blood cells had shown a hemolysis of ~15% at 1 mg/mL concentration of G4-PAMAM-NH₂, and our values were slightly lower than those reported.⁵¹ All of the heterobifunctional dendrimers synthesized were nonhemolytic in the concentration range 1–100 μ g/mL on exposure for 3 h (Figure 8). At concentration of 1 mg/mL and exposure for 3 h, about 1.5 to 3.0% was observed for all compounds except the G3.5-PAMAM-CO-Ser(OH)-COOH, which showed a hemolysis of 5% in 3 h. In comparison with the G4-PAMAM-NH₂ dendrimer, the new compounds synthesized exhibited lower hemolytic activity. Consistent with the hemolysis study, the in vitro cytotoxicity study showed that the new compounds were nontoxic (Figure 8) in the concentration range 10–100 μ g/mL, and few (**14,17**) were nontoxic even at 1 mg/mL concentrations.

Dendrimer cytotoxicity is strongly influenced by the nature of surface group, and dendrimer bearing NH₂ termini display concentration- and generation-dependent cytotoxicity.⁵⁰ The amine-terminated dendrimers are known to exhibit cytotoxicity due to high cationic charge, whereas the hydroxyl dendrimers are noncytotoxic because of the neutral surface charge. The G4-PAMAM-NH₂ dendrimers exhibited cytotoxicity at 5 μ g/mL concentration after 5 h of exposure to B16F10.⁵¹ The cell viability fell to <10 % for the V79 chinese hamster lung fibroblasts cells after 24 h of exposure to PAMAM dendrimers generations G3 (1nM), G5

(10 mM), and G7 (100 nM). Our previous study showed that G4-PAMAM-OH and G3.5-PAMAM-COOH were nontoxic to A549 cells at concentrations of 10–1000 µg/mL, whereas G4-PAMAM-NH₂ dendrimer exhibited severe toxicity at 1000 µg/mL.⁵² These results were consistent with those reported by Duncan et al.⁵⁰

We evaluated the cytotoxicity of all the heterobifunctional dendrimers using human lung carcinoma cells (A549) with clinical implications for dendrimer use in cancer therapy.^{7,11} Amine-terminated G4-PAMAM dendrimer is known to be cytotoxic,^{50,51} and hence we evaluated and compared the cytotoxicity of compounds (**4,7,11,14**, and **17**) synthesized with G4-PAMAM-NH₂. MTT assay showed that the compounds G4-OH-Cys-(SH)-NH₂ and G4-OH-Asp-(COOH)-NH₂ were not toxic to A549 cells in the concentration range of 1 µg/mL to 1 mg/mL after 24 h of exposure (Figure 9). The G4-PAMAM-NH₂ dendrimer showed 60% cell viability at 100 µg/mL concentration and was highly cytotoxic at 1 mg/mL concentration. Our results are similar to those reported for G4-PAMAM-NH₂ dendrimer for A549 cells.⁵³ It is interesting to note that both of these compounds have NH₂ termini as one functional handle, yet they were nontoxic at higher concentrations (in contrast with G4-PAMAM-NH₂ dendrimers with the amine end groups) and retained the behavior similar to that of the neutral nontoxic G4-PAMAM-OH dendrimers. This was consistent with the ζ-potential measurements for both of these compounds, which showed that the ζ potential did not increase significantly from that of G4-PAMAM-NH₂ with serine and cysteine, the compounds G4-PAMAM-NH-Ser(OH)-NH₂ and G4-0PAMAM-NH-Cys(SH)-NH₂ were nontoxic at concentrations of 1–100 µg/mL after 24 h. Both compounds had NH₂ termini after surface modification, yet they were nontoxic at the concentrations evaluated. Furthermore, >40% of cells were viable after exposure to high concentration of 1 mg/mL for 24 h for these two compounds, which is indicative of marked reduction in cytotoxicity when compared with G4-PAMAM-NH₂ alone. It has been reported that COOH-terminated dendrimers G1.5 to 9.5 are nontoxic up to 5 mg/mL concentrations to B16F10, CCRF, and HepG2.⁵⁰ We observed that on modifying G3.5-PAMAM-COOH with serine, the compound was nontoxic at 1–10 µg/mL concentration but exhibited marked toxicity at 100 µg/mL, and this can be expected because this compound exhibited a very high ζ potential, +8.83 mV. The underlying reasons for the unexpectedly higher charge and cytotoxicity for this compound need further investigation, which is beyond the scope of this manuscript. From these results, the most promising candidates for the probable application in drug delivery appear to be G4-OH-Cys-(SH)-NH₂ and G4-OH-Asp-(COOH)-NH₂, which showed postfunctionalization ability and applicability in hydrogel formation and as carriers for multiple functional groups such as drug and imaging agents.

We demonstrate that by appropriate choice of G4-PAMAM dendrimer end groups (NH₂, OH, COOH) and amino acids for surface modifications, a library of multivalent, multifunctional dendrimers bearing OH and NH₂, COOH and NH₂, NH₂ and SH, and COOH and OH at the peripheries can be achieved. Similar conversions can be made on other generations of PAMAM dendrimers (G2-G10). Surface modification of poly(propyleneimine) dendrimer by amino acids (alanine, leucine, phenylalanine, threonine, tyrosine) was reported,⁵⁴ but the dendrimers in the present investigation yield distinct functionalities from those previously reported. High yields were achieved in the coupling reactions because an excess of amino acid could be used, and the unreacted material could later be removed by a simple dialysis process. Conversions 70–93% of heterobifunctional dendrimers were obtained by carrying out the reactions under mild conditions such as water/dimethylsulfoxide/dimethyl formamide or in some cases dimethylsulfoxide/dimethyl formamide. The orthogonal peripheral handles of the resulting dendrimers are available for the eventual attachment of drugs, imaging agents, or radiolabels and for the biological evaluation of these carriers. One of the key features is the use of biocompatible amino acids used to achieve these diverse end functionalities. The objective was to use compounds that

would not elicit undesirable interactions of dendrimers with cell surfaces, enzymes, and proteins in the blood serum. Furthermore, the degree of drug loading could be easily adjusted. The amino acids are known to provide catalytic pockets for the enzymatic cleavage,⁵⁵ and hence the byproducts obtained by the cleavage of drug products are expected to be nontoxic. By decorating the dendrimer periphery with amino acid motifs, enhanced solubility, reduced cytotoxicity, and reduced hemolytic toxicity could be achieved, while retaining the chemoselective reactivity and functional flexibility to conjugate drugs and/or imaging agents.

CONCLUSIONS

We have designed and synthesized a library of G4-PAMAM dendrimers of a different framework that bears very high heterobifunctional peripheral groups (110) using an efficient, one-step, scalable synthetic methodology. Our approach in choosing the materials to design these heterobifunctional dendrimers was based on developing biocompatible dendrimer scaffolds for drug delivery applications using varied amino acids for end-capping the dendrimers. The bifunctional dendrimers of the present study are amenable for efficient postfunctionalization reactions. We also demonstrate that these dendrimers were chemoselectively conjugated to two different drugs, viz. dexamethasone and indomethacin, and a drug and fluorescence imaging agent, viz. dexamethasone and FITC, each involving distinct linking chemistry: ester and amide and ester and thiourea linkages, respectively. Because the peripheral groups are orthogonal in nature, they are individually accessible to two different moieties in immediate succession without the need for any functional group conversions. This approach also eliminated the need to append a specific linker/spacer molecule to attach more than one functional group, thereby reducing the synthetic and purification steps. “Near quantitative” conversions of heterobifunctional dendrimer were obtained by carrying out the reactions under mild conditions, and the excess of unreacted amino acid was later removed by a simple dialysis process. The cytotoxicity and hemolytic toxicity of the dendrimers was reduced for the heterobifunctional dendrimers obtained by decorating the periphery with amino acids. The merits of this synthetic approach are: (1) near complete peripheral modification was achieved to yield 110 end functionalities, (2) diverse peripheral groups are obtained, (3) the synthesis is one- or two- step without elaborate alternating protection and deprotection steps, (4) the compounds are highly water-soluble, (5) different moieties can be appended in succession (orthogonal and chemoselective nature), (6) in situ forming hydrogels were developed using these heterobifunctional dendrimers, (7) the compounds used for surface modification are nontoxic, and (8) the cytotoxicity of the dendrimers is overcome, making these dendrimers a suitable carrier for drug delivery. The amino acid spacers are significantly more biocompatible than the typical spacers used in conjugation reactions and are more likely to gain FDA approval.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the Perinatology Research Branch, Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services (NICHD/NH/DHHS), and the Pediatric Critical Care Scientist Development Program.

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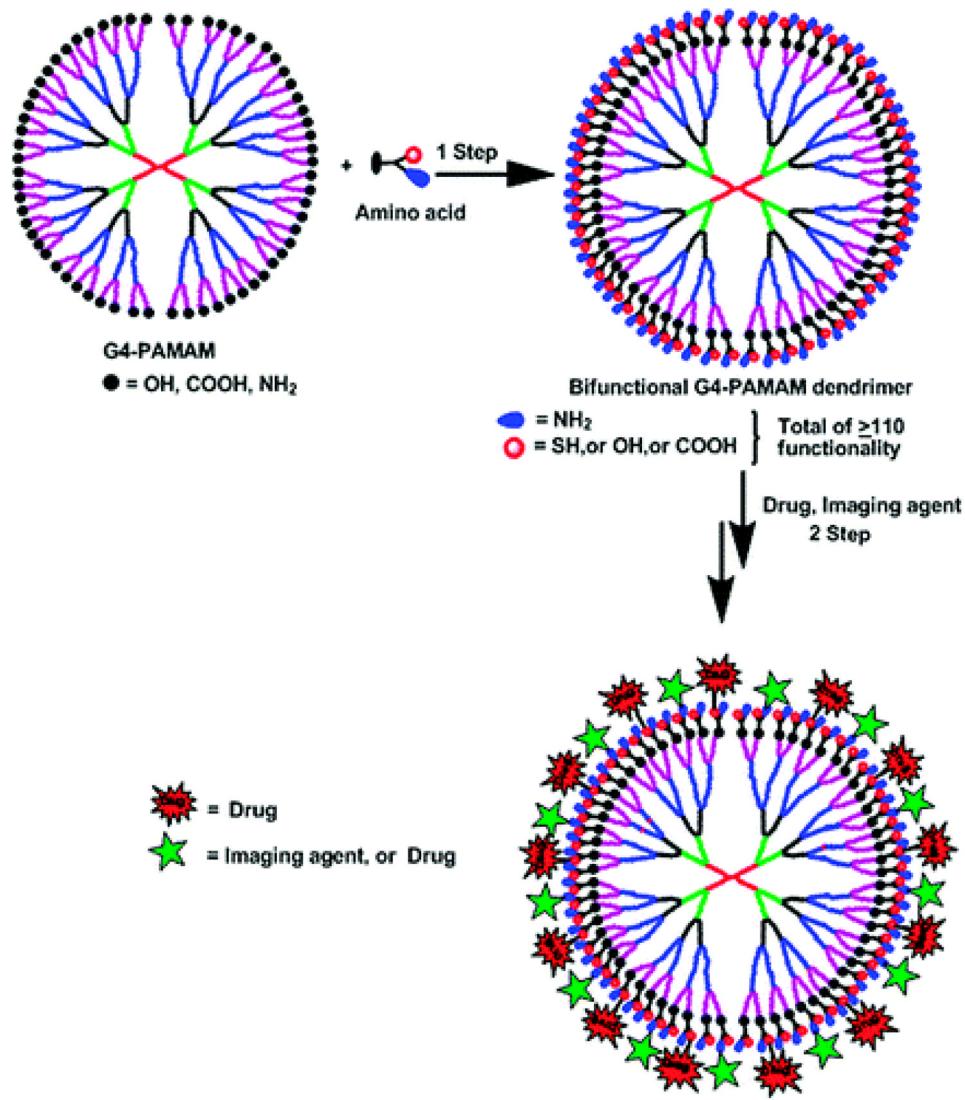


Figure 1.

Schematic representation of bifunctional dendrimer and its postfunctionalization in immediate succession.

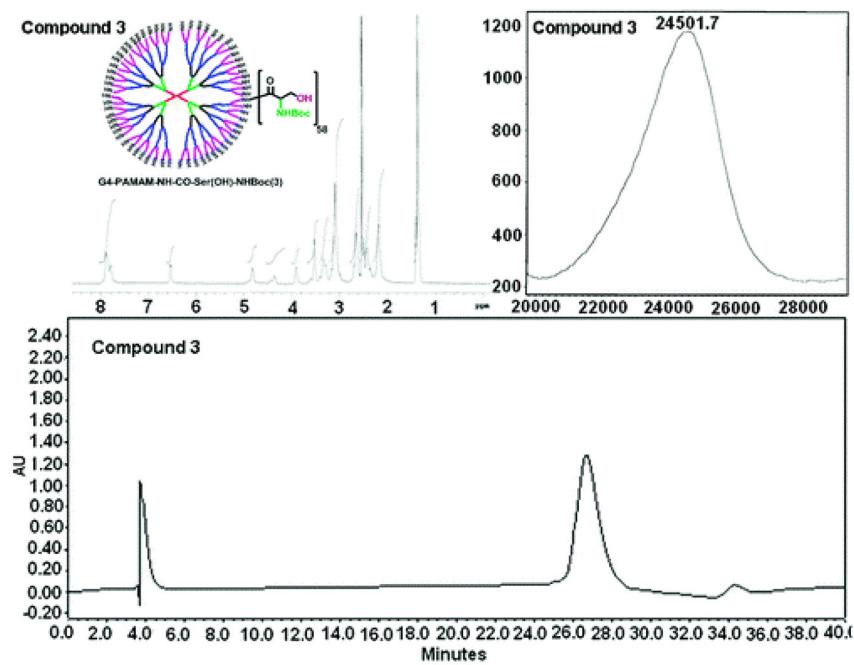


Figure 2.

¹H NMR, MALDI TOF MS spectrum, and HPLC chromatogram for G4-PAMAM-NH-CO-Ser(OH)-NHBoc (3).

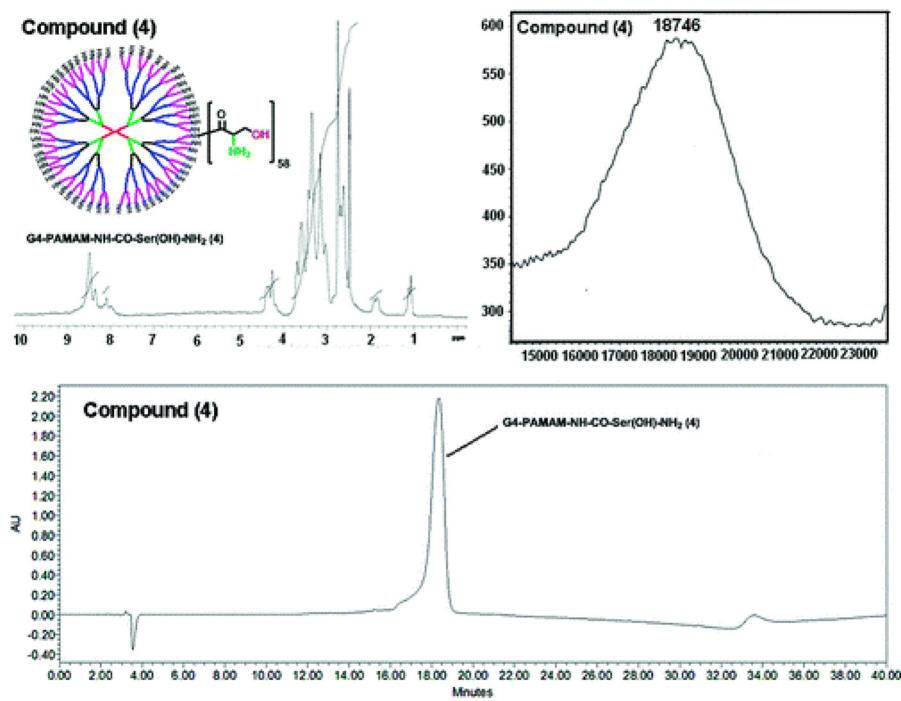


Figure 3.

^1H NMR, MALDI TOF MS spectrum, and HPLC chromatogram for G4-PAMAM-NH-CO-Ser(OH)-NH₂ (**4**).

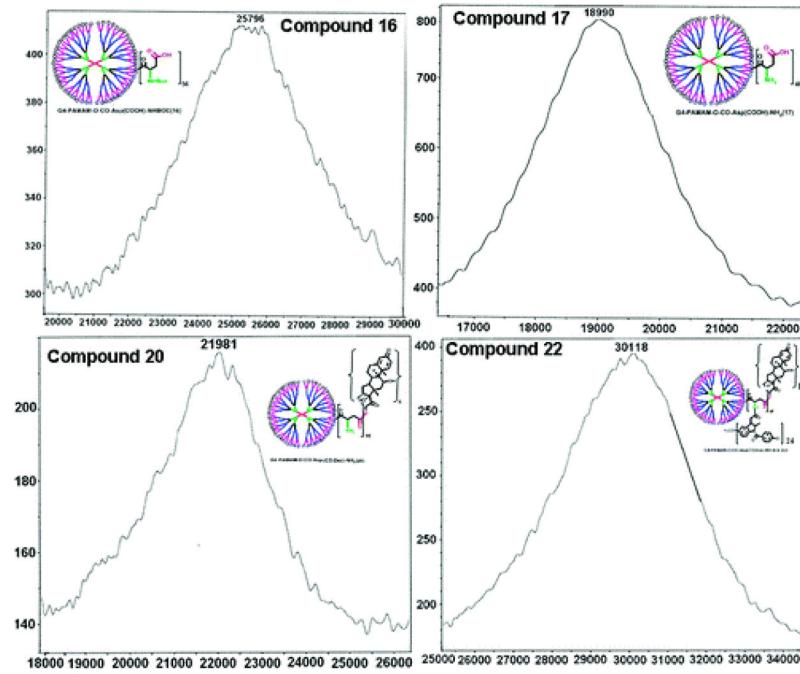
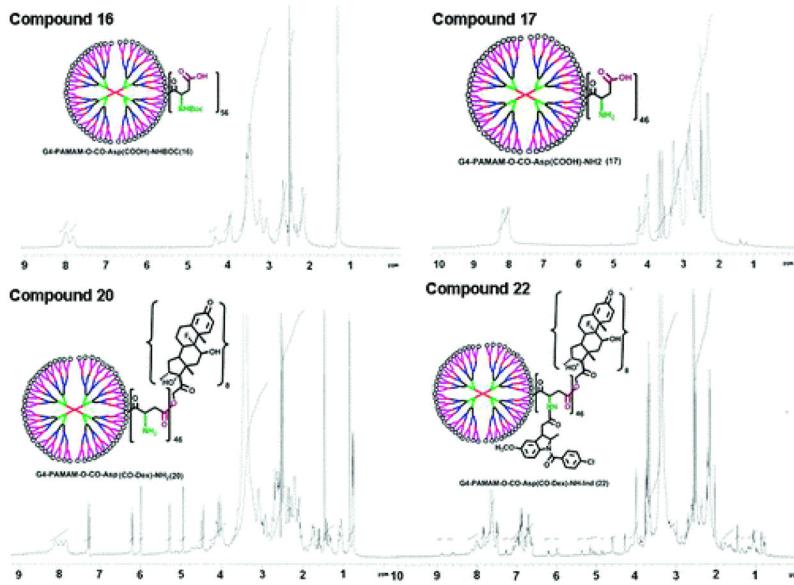


Figure 4.

The MALDI TOF MS spectra for G4-PAMAM-O-Asp(COOH)-NH_{Boc} (**16**) showing mass of 25.7 kDa. On deprotection of Boc groups, G4-PAMAM-O-Asp(COOH)-NH₂ (**17**) dendrimer showed a mass of 18.9 kDa. The conjugation of dexamethasone to (**17**) after Boc deprotection increases the mass to 21.9 kDa. Furthermore, the attachment of indomethacin on (**20**) increases the mass to 30.1 kDa on the formation of G4-PAMAM-O-Asp(CO-Dex)-NH-Ind (**22**).

**Figure 5.**

^1H NMR spectra for G4-PAMAM-O-Asp(COOH)-NHBOC (**16**), after deprotection of *tert*-butoxycarbonyl groups G4-PAMAM-O-Asp(COOH)-NH₂ (**17**), the conjugation of dexamethasone to give G4-PAMAM-O-Asp(CO-Dex)-NH₂ (**20**), and after the attachment of indomethacin G4-PAMAM-O-Asp(CO-Dex)-NH-Ind (**22**).

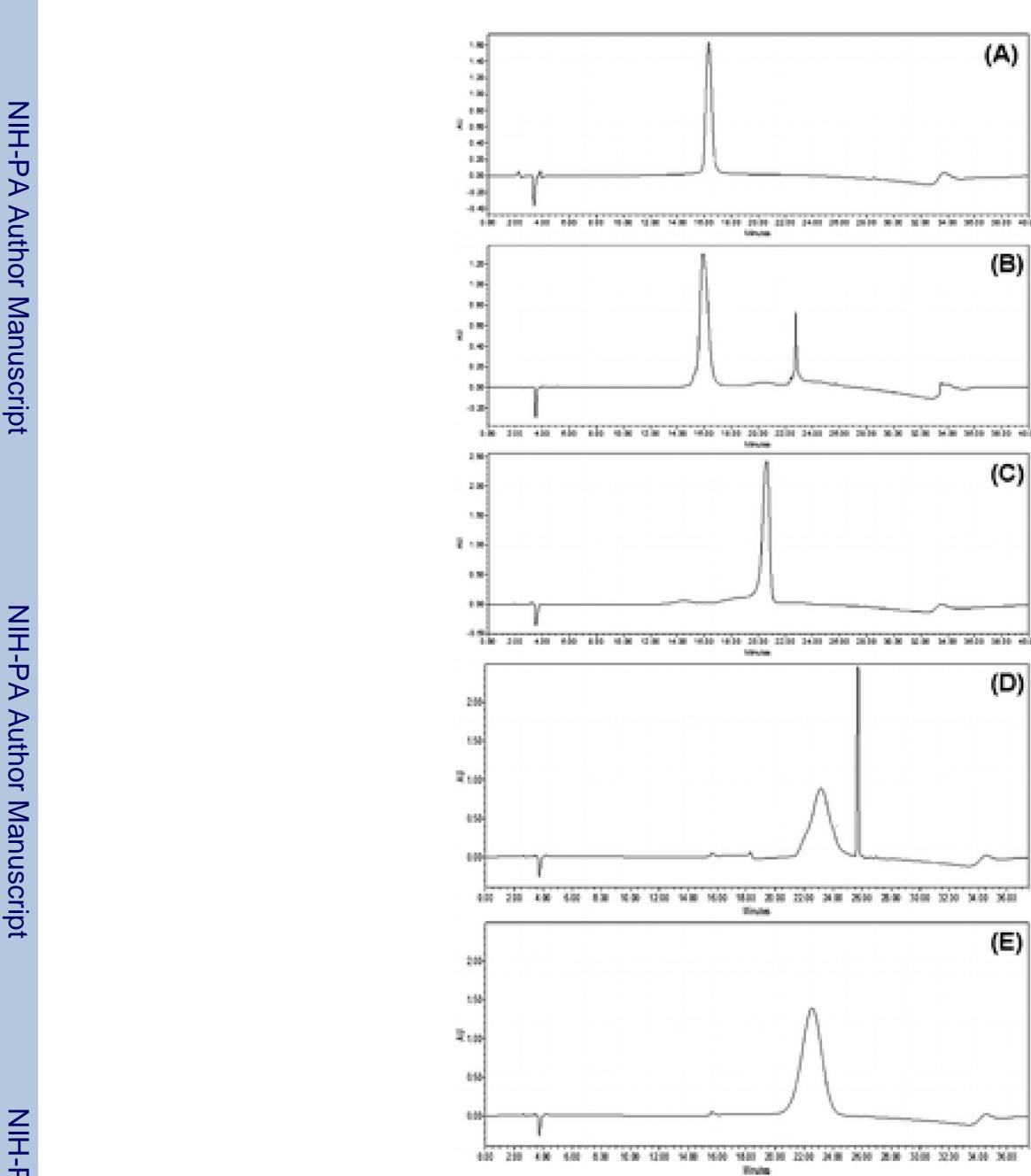
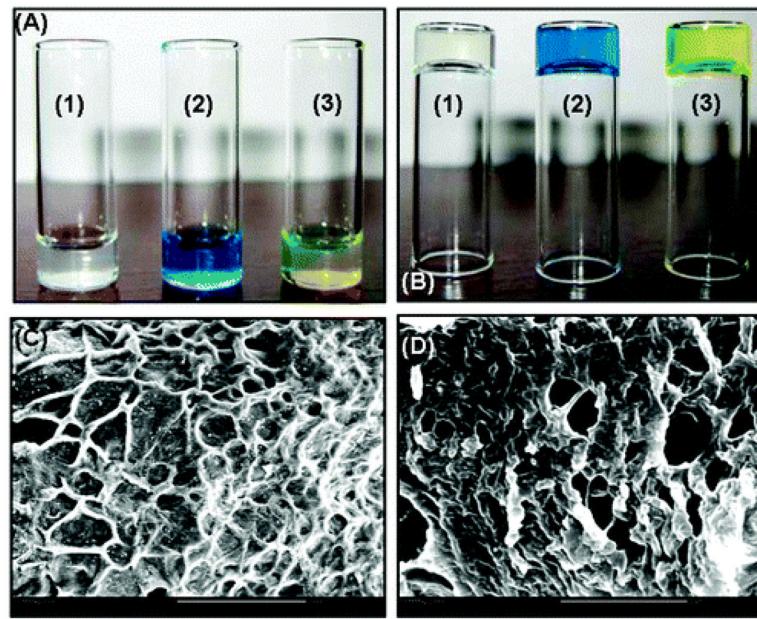


Figure 6.
HPLC chromatograms absorbance at 210 nm (arbitrary AU units) for G4-PAMAM-Asp-(COOH)-NHBoc and its postfunctionalization products. (A) G4-PAMAM-Asp-(COOH)-NHBoc showing retention time 16.5 min. (B) G4-PAMAM-Asp-(COOH)-NHBoc spiked with dexamethasone; the dexamethasone appears at 22.8 min. (C) G4-PAMAM-Asp-(CO-Dex)-NH₂ showing retention time of 20.5. (D) G4-PAMAM-Asp-(CO-Dex)-NH-Ind spiked with indomethacin; the unconjugated indomethacin appears at 25.8 min. G4-PAMAM-Asp-(CO-Dex)-NH-Ind appears at 22.7 min.

**Figure 7.**

In situ gel formation by cross-linking of G4-PAMAM-Asp-(CO-Dex)-NH₂ (**20**) with *N*-hydroxy-succinimide-terminated PEG (PEG-NHS) (**25**). The gel (3) formed by reaction of “NH₂” groups of G4-PAMAM-Asp-(COO-Dex)-NH₂ (**20**) with PEG-NHS (**25**) (colorless), whereas the “COOH” groups are used for conjugating dexamethasone by ester linkage. The hydrogel (2) physically entrapping blue dextran is seen in blue, whereas the hydrogel (3) formed by linking FITC to few NH₂ groups of G4-PAMAM-Asp-(CO-Dex)-NH₂ (**20**) while the remaining NH₂ groups cross-link by formation of amide bond on reaction with PEG-NHS is yellow. The SEM image shows the gel network (in 200 μm) for the (C) dexamethasone-conjugated and (D) FITC-conjugated dendrimer G4-PAMAM-Asp-(CO-Dex)-NH₂ cross-linked with PEG-NHS.

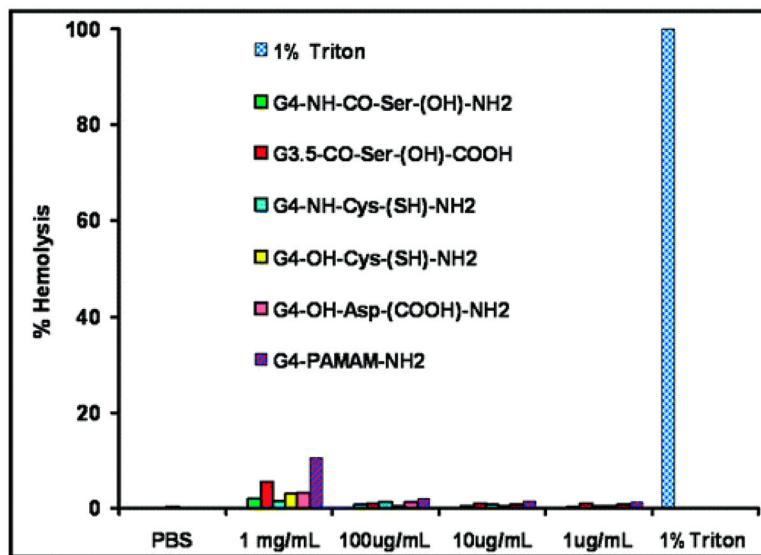


Figure 8.

In vitro hemolytic activity of new heterobifunctional dendrimers.

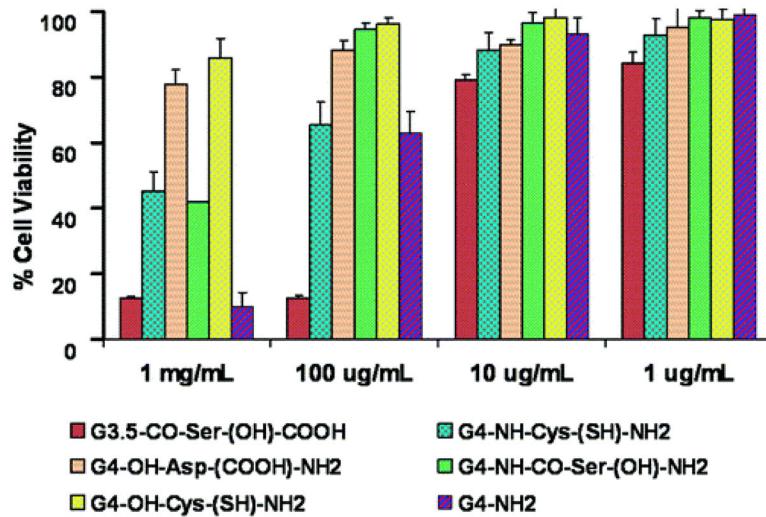


Figure 9.

In vitro cytotoxicity of new bifunctional dendrimers in the A549 cell line.

Characterization of Synthesized Compounds Using NMR and MALDI

Table 1

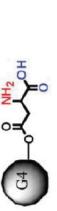
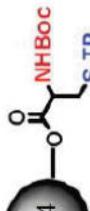
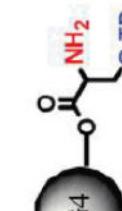
Name of compound	¹ H NMR in (DMSO- <i>d</i> ₆ , 400 MHz)	¹³ C NMR in (DMSO- <i>d</i> ₆ , 100 MHz) 6	MALDI (Da)	yield (%)
G4-PAMAM-NH-CO-Ser(OH)-NHBoc (3)	1.38 (s, 9H, Boc), 2.10–2.22 (br.s, OH), 3.22–3.38 (m, 1H, CH2), 4.50–4.58 (m, 1H, CH2) 4.80–4.90 (m, 1H, CH) 6.50–6.60 (m, 1H, NH amide), 7.78–7.97 (br.d, NH interior dendrimer amide)		24 501	75
G4-PAMAM-NH-CO-Cys(SH)-NHBoc (4)	1.0–1.19 (m, 2H, NH2), 1.80–1.98 (br.s, 2H, OH), 4.21–4.26 (s, 1H), 8.0–8.15 (br.d from amide NH), 8.30–8.6 (br.d, amide NH)		18 747	89
G4-PAMAM-NH-CO-Ser(OH)-NH ₂ (5)	1.35 (s, 9H, Boc), 2.10–2.20 (br.s, SH), 3.25–3.40 (m, 2H, CH2), 3.95–4.20 (m, 1H, CH), 7.80–8.20 (br.d, 1H, dendrimer interior amide), 8.22–8.45 (br.s, 1H, amide)		25 807	77
G4-PAMAM-NH-CO-Cys(SH)-NH ₂ (7)	1.0–1.23 (m, NH2), 1.78–2.00 (br.s, 2H, OH), 3.60–3.75 (m, 1H, -CH-for cysteine), 7.97–8.10 (br.s, amide NH from cysteine), 9.80–10.10 (br.m, NH from dendrimer interior amide)		19 365	88
G3.5-PAMAM-CO-NH-Ser-OMe (10)	3.60 (S, 3H, COOME), 6.62–3.75 (m, 2H, CH2), 4.30–4.58 (m, 1H, CH), 7.77–7.95 (br.d, NH), 8.37–8.41 (s, 1H, amide)		17 209	78
G3.5-PAMAM-CO-NH-Ser-OH (11)	1.40–1.50 (m, 2H, NH2), 1.92–2.05 (br.s, 1H, OH), 3.33–3.42 (br.s, 1H, -CH, Serine), 8.15–8.40 (br.d, amide NH), 8.75–8.90 (br.s, amide NH)		15 959	88
G4-PAMAM-O-CO-Cys(SH)-NHBoc (13)	1.25 (br.s, 1H from cysteine SH), 1.35 (br.s, 9H, <i>tert</i> -butyloxycarbonyl from cysteine), 2.10–2.25 (br.s, 1H, -SH from cysteine), 4.55–4.75 (br.d, -CH-from cysteine), 7.80–8.10 (br.d, NH from dendrimer interior amide), 8.20–8.30 (br.s, NH from cysteine amide)	28.59, 28.78, 33.86, 37.51, 38.02, 42.12, 50.23, 52.80, 54.19, 56.94, 60.55, 66.50, 79.76, 108.10, 143.20, 155.90, 156.69, 169.61, 172.01, 172.32, 172.56	25 068	80
G4-PAMAM-O-CO-Cys(SH)-NH ₂ (14)	2.12–2.24 (m, 2H, -CH ₂ - cysteine), 4.70–4.78 (m, 1H, -CH-, cysteine), 7.76–7.89 (br.d, NH from dendrimer interior amide), 7.91 (br.s, NH from cysteine amide)		19 262	87
G4-PAMAM-O-CO-Asp(COOH)-NHBoc (16)	1.30 (s, 9H), 4.28–4.38 (br.s, 1H), 7.75–7.90 (br.s, amide NH), 7.92–8.10 (br.d, amide NH)	28.77, 33.46, 37.39, 38.13, 50.04, 50.76, 52.79, 63.76, 79.08, 79.12, 95.10, 155.91, 170.66, 171.82, 172.23	25 740	80
G4-PAMAM-O-CO-Asp(COOH)-NH ₂ (17)	4.22–4.35 (br.s, 1H), 7.96–8.10 (br.s, amide NH), 8.10–30 (br.d, amide NH)		18 990	90
G4-PAMAM-O-CO-Asp(Co-Dex)-NH ₂ (20)	0.75 (s, 3H), 0.82 (s, 3H), 1.0–1.10 (m, 2H), 1.280–1.36 (d, 2H), 1.38–1.41(d, 2H), 1.45 (s, 3H), 4.45–4.50 (d, 1H), 4.92 (s, 1H), 5.24 (s, 1H), 6.01 (s, 1H), 6.23 (d, 1H), 7.30 (d, 1H)	15.98, 17.33, 23.55, 23.61, 27.94, 30.95, 32.68, 33.75, 34.21, 34.41, 35.55, 36.60, 42.10, 43.95, 48.12, 48.75, 50.15, 52.70, 60.56, 66.94, 71.18, 71.55, 90.84, 94.71, 101.06, 102.80,	21 981	90

Name of compound	¹ H NMR in (DMSO- <i>d</i> ₆ , 400 MHz)	¹³ C NMR in (DMSO- <i>d</i> ₆ , 100 MHz) 6	MALDI (Da)	yield (%)
G4-PAMAM-O-Asp(CO-Dex)-Ind (22)	2.10–2.30 (m, 3H, CH ₃), 3.62–3.80 (m, 5H, -OCH ₃ , -CO-CH ₂ -), 6.60–7.9 (m, 2H, Ar), 6.83–7.04 (m, 2H, Ar), 7.60–7.70 (m, 3H, Ar)	124.80, 129.67, 153.53, 167.80, 186.0	30118	78
G4-PAMAM-O-CO-Asp(CO-Dex)-NH-FTIC (24)	6.57–6.62 (d, 6H, Ar), 6.63–6.70 (s, 3H Ar)			85
Boc-Cys(<i>S</i> -TP)-OH (27)	1.40 (s, 9H, <i>tert</i> -butyloxycarbonyl), 2.49 (solvent DMSO- <i>d</i> ₆), 3.0–3.20 (m, 2H, -CH ₂ -), 3.33 (H ₂ O peak in DMSO- <i>d</i> ₆), 4.10–4.20 (m, 1H, -CH ₂ -), 7.25–7.30 (m, 1H, Ar), 7.35–7.40 (m, 1H, Ar), 7.78–7.88 (m, 2H, NH amide, and Ar), 8.42–8.52 (m, 1H, Ar), 12.88 (s, 1H, COOH)	28.83, 53.42, 79.03, 94.69, 119.96, 121.95, 138.48, 150.29, 172.86		70
G4-PAMAM-O-CO-Cys(<i>S</i> -TP)-NHBoc (28)	1.38 (s, 9H, from cysteine <i>tert</i> -butoxy carbonyl), 4.0–4.10 (m, 2H, -CH ₂ -, from cysteine), 4.60–4.70 (m, 1H, -CH ₂ , from cysteine), 6.70–7.77 (m, 1H, Ar), 7.0–7.18 (br.d, 1H, NH amide), 7.25–7.35 (m, 1H, Ar), 7.38–7.45 (m, 1H, Ar), 7.60–7.68 (m, 1H, Ar), 8.15–8.24 (m, 1H, amide NH)		26846	78
G4-PAMAM-O-CO-Cys(<i>S</i> -TP)-NH ₂ (29)	1.82–1.97 (m, 2H, -CH ₂ -, from cysteine), 4.62–4.70 (m, 1H, -CH ₂ -, from cysteine), 7.60–7.64 (d, 1H, Ar), 7.66–7.75 (m, 1H, Ar), 7.77–7.84 (m, 1H, Ar), 7.89–7.96 (m, 1H, NH amide), 8.19–8.25 (m, 1H, Ar), 8.40–8.52 (m, 1H, NH amide)	25594		50

Library of Amino Acid Surface-Modified Dendrimers

Table 2

Dendrimer generation and end group	Amino Acid	Deprotection/Hydrolysis	Peripheral Functionality	Structure
G4-PAMAM-NH ₂ (1)	Boc-Ser-OH	--	OH, NHBoc	
G4-PAMAM-NH ₂ (1)	Boc-Ser-OH	Boc	OH, NH ₂	
G4-PAMAM-NH ₂ (1)	Boc-Cys-OH	--	SH, NHBoc	
G4-PAMAM-OH (12)	Boc-Cys-OH	Boc	SH, NH ₂	
G3.5-PAMAM-COOH (8)	H-Ser-OMe	--	OH, COOME	
G3.5-PAMAM-COOH (8)	H-Ser-OMe	Me	OH, COOH	
G4-PAMAM-OH (12)	Boc-Cys-OH	--	SH, NHBoc	
G4-PAMAM-OH (12)	Boc-Cys-OH	Boc	SH, NH ₂	
G4-PAMAM-OH (12)	Boc-Asp-OH	--	NHBoc,	

Dendrimer generation and end group	Amino Acid	Deprotection/Hydrolysis	Peripheral Functionality	Structure
G4-PAMAM-OH (12)	Boc-Asp-OH	Boc	NH ₂ , COOH	
G4-PAMAM-OH (12)	Boc-Cys(S-TP)-OH	--	NHBoc, S-TP	
G4-PAMAM-OH (12)	Boc-Cys(S-TP)-OH	Boc	SH ₂ , S-TP	

Molecular Weight Estimation of Amino Acid Functionalized Dendrimers

Navath et al.

Page 31

Table 3

Name of the compound	MW	No. of amino acids attached	Total heterofunctional peripheral groups	% conversion	Purity of compound (%)	Solubility aqueous/DMSO
G4-PAMAM-NH-CO-Ser(OH)-NH-Boc (3)	24.5 kDa	58	116 (58+ 58)	93	98	DMSO soluble
G4-PAMAM-NH-CO-Ser(OH)-NH ₂ (4)	18.7 kDa	58	116 (58 + 58)	93	96	H ₂ O soluble
G4-PAMAM-NH-CO-Cys(SH)-NH-Boc (6)	24.8 kDa	55	110 (55 + 55)	89	96	DMSO soluble
G4-PAMAM-NH-CO-Cys(SH)-NH ₂ (7)	19.3 kDa	55	110 (55 + 55)	89	95	H ₂ O soluble
G3.5-PAMAM-CO-NH-Ser(OH)-COOMe (10)	17.2 kDa	57	114 (57 + 57)	92	97	DMSO soluble
G3.5-PAMAM-CO-NH-Ser(OH)-COOH (11)	15.9 kDa	57	114 (57 + 57)	92	96	H ₂ O soluble
G4-PAMAM-O-CO-Cys(SH)-NH-Boc (13)	25.0 kDa	56	112 (56 + 56)	90	96	DMSO soluble
G4-PAMAM-O-CO-Cys(SH)-NH ₂ (14)	19.2 kDa	46	92 (46 + 46)	74	98	H ₂ O soluble
G4-PAMAM-O-CO-Asp(COOH)-NH-Boc (16)	25.7 kDa	56	112 (56 + 56)	90	95	DMSO soluble
G4-PAMAM-O-CO-Asp(COOH)-NH ₂ (17)	18.99 kDa	46	92 (46 + 46)	74	98	H ₂ O soluble
G4-PAMAM-O-CO-Cys(S-TP)-NH-Boc (28)	26.8 kDa	42	84 (42 + 42)	68	97	DMSO soluble
G4-PAMAM-O-CO-Cys(S-TP)-NH ₂ (29)	25.5 kDa	40	80 (40 + 40)	64.5	95	DMSO soluble

Particle Size and ζ Potential of Heterobifunctional Dendrimers

Name of the sample	Sample particle diameter (nm)	ζ potential (mV)
G4-PAMAM-NH ₂	4.70	+11.5
G3.5-PAMAM-COOH	4.20	-9.30
G4-PAMAM-OH	4.78	-2.10
G4-PAMAM-NH-Ser(OH)-NH ₂	5.65	-1.83
G4-PAMAM-NH-Cys(SH)-NH ₂	6.21	+4.80
G3.5-PAMAM-CO-Ser(OH)-COOH	6.56	+8.83
G4-PAMAM-OH-Cys(SH)-NH ₂	6.01	+3.60
G4-PAMAM-Asp(COOH)-NH ₂	5.59	+1.51

Table 4