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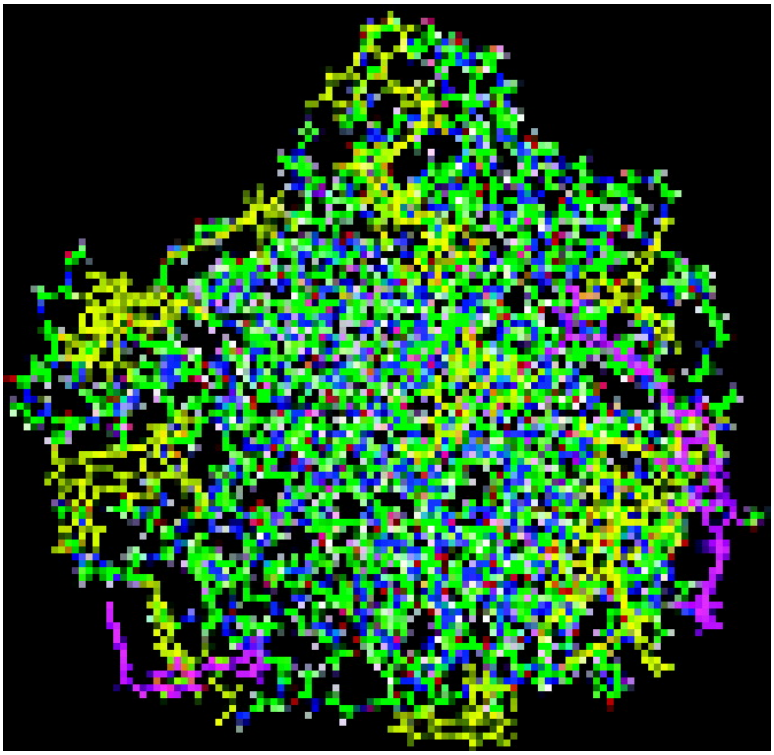

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Articles

Poly(amidoamine) Dendrimer-Based Multifunctional Engineered Nanodevice for Cancer Therapy

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Multifunctional cancer therapeutic nanodevices have been designed and synthesized using the poly(amidoamine) (PAMAM) dendrimer as a carrier. Partial acetylation of the generation 5 (G5) PAMAM dendrimer was utilized to neutralize a fraction of the primary amino groups, provide enhanced solubility of the dendrimer during the conjugation reaction of fluorescein isothiocyanate (FITC) (in dimethyl sulfoxide (DMSO)), and prevent nonspecific targeting interactions (in vitro and in vivo) during delivery. The remaining nonacetylated primary amino groups were utilized for conjugation of the functional molecules fluorescein isothiocyanate (FITC, an imaging agent), folic acid (FA, targets overexpressed folate receptors on specific cancer cells), and methotrexate (MTX, chemotherapeutic drug). The appropriate control nanodevices have been synthesized as well. The G5 PAMAM dendrimer molecular weight and number of primary amino groups were determined by gel permeation chromatography (GPC) and potentiometric titration for stoichiometric design of ensuing conjugation reactions. Additionally, dendrimer conjugates were characterized by multiple analytical methods including GPC, nuclear magnetic resonance spectroscopy (NMR), high performance liquid chromatography (HPLC), and UV spectroscopy. The fully characterized nanodevices can be used for the targeted delivery of chemotherapeutic and imaging agents to specific cancer cells. Here, we present a more extensive investigation of our previously reported synthesis of this material with improvements directed toward scale-up synthesis and clinical trials (*Pharm. Res*. **2002**, *19* (9), 1310−1316).

Introduction

The application of poly(amidoamine) (PAMAM) dendrimers for cancer treatment has great potential and is under critical investigation, as these macromolecules serve as targeted drug carriers, delivery agents, and imaging agents in human systems.[2−6] The characteristic nontoxicity of PAMAM dendrimers to biological systems makes their biocompatibility much greater than that of many other materials currently researched for use as controlled, chemotherapeutic drug delivery systems.[7] The multifunctionality and biocompatibility of dendrimer-based nanodevices are crucial for the development of targeted drug delivery technology.

PAMAM dendrimers are highly branched macromolecules composed of an ethylenediamine (EDA) initiator core with four dendron arms radiating from it. Repetitive reaction sequences comprised of exhaustive Michael addition of methyl acrylate (MA) and condensation (amidation) of the resulting ester with large excesses of EDA produce each successive generation.[8] The generation number is determined by the extent of the reactions performed, beginning at G0, with the latter generations labeled sequentially in half- or whole-number increments, depending on the termination of the reaction sequence. The generation number corresponds with the exponent to which the mathematical series describing the theoretical structure of the dendrimer is raised.[3,8,9] Each successive reaction theoretically doubles the number of surface amino groups, which can be activated for conjugation of various functional groups. PAMAM dendrimers of G7 and lower are generally used within human and animal systems as multifunctional carriers, as they closely mimic the sizes of biomolecules.[10]

Because of the tertiary amino groups present in the interior of the structure and the primary amino groups present on the surface, PAMAM dendrimers are pH responsive and have been used as nonspecifically targeted controlled drug delivery systems triggered at low pH.[11,12] Partial acetylation, however, can be used to neutralize the dendrimer surface, preventing side reactions and nonspecific targeting from occurring during device delivery while also increasing the solubility of the dendrimer. The remaining nonacetylated primary amino groups can then be used for the attachment of various functional molecules including targeting agents, imaging agents, and therapeutic drugs. Through conjugation of the functional molecule folic acid, which targets the overexpressed folate receptors found in many types of cancer cells, and partial or full acetylation of the dendrimer, *site-specific* targeted drug delivery by the dendritic device can be achieved.[13−15] The multivalency of the PAMAM dendrimer also facilitates the addition

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of functional molecules to the dendrimer surface.[11,16] The ability to enhance the PAMAM dendrimer structure through terminal group modification, combined with the enhanced solubility of the conjugated device and the nanoscale dimensions of these macromolecules, makes these dendrimers highly desirable for use in engineered nanodevices suitable for gene therapy, protein-receptors, catalysts, and drug delivery.[17−20]

In this study, fluorescein isothiocyanate (FITC), folic acid (FA), and methotrexate (MTX) were conjugated to the G5 PAMAM dendrimer. The dendritic device synthesized was capable of targeting the overexpressed membrane-associated folate receptors (folate binding proteins) of specific cancer cells with FA and inducing cellular cytotoxicity (submitted separately). MTX is known to prevent cell proliferation and to induce apoptosis in some cells through a variety of mechanisms.[21] Additionally, MTX was conjugated to the dendrimer through an ester bond instead of through an amide bond (synthesized as a control device), which had been used previously by Fréchet and colleagues as an MTX−dendrimer linker.[22] On the basis of preliminary research presented,[1] we sought to present improved synthetic procedures directed toward scale-up synthesis, with solubility and purification issues addressed.

Experimental Procedure

Materials. The G5 PAMAM dendrimer was synthesized and characterized at the Center for Biologic Nanotechnology, University of Michigan. Methanol (MeOH, HPLC grade), acetic anhydride (99%), triethylamine (99.5%), dimethyl sulfoxide (DMSO, 99.9%), FITC (90%), glycidol (racemic form, 96%), dimethylformamide (DMF, 99.8%), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide HCl (EDC, 98%), citric acid (99.5%), sodium azide (99.99%), D_2O, NaCl, and volumetric solutions (0.1 M HCl and 0.1 M NaOH) for potentiometric titration were purchased from Aldrich Co. and used as received. The methotrexate (99+%) and FA (98%) were from Sigma. The Spectra/Por dialysis membrane (MWCO 3500), Millipor Centricon ultrafiltration membrane (YM-10), and phosphate buffer saline (PBS, pH = 7.4) were from Fisher.

Potentiometric Titration. Titration was carried out manually using a Mettler Toledo MP230 pH meter and a MicroComb pH electrode at room temperature, 23 ± 1 °C. A 10 mL solution of 0.1 N NaCl was added to a precisely weighed 100 mg portion of PAMAM dendrimer to shield amine group interactions. Titration was performed with 0.1028 N HCl, and 0.1009 N NaOH was used for back-titration. The numbers of primary and tertiary amines were determined by back-titration.

Gel Permeation Chromatography (GPC). GPC experiments were performed on an Alliance Waters 2690 separation module equipped with a 2487 dual wavelength UV absorbance detector (Waters Corporation), a Wyatt Dawn DSP laser photometer, and an Optilab DSP interferometric refractometer (Wyatt Technology Corporation) and with TosoHaas TSK-Gel Guard PHW 06762 (75 mm × 7.5 mm, 12 μm), G 2000 PW 05761 (300 mm × 7.5 mm, 10 μm), G 3000 PW 05762 (300 mm × 7.5 mm, 10 μm), and G 4000 PW (300 mm × 7.5 mm, 17 μm) columns. Column temperature was maintained at 25 ± 0.1 °C with a Waters temperature control module. The isocratic mobile phase was 0.1 M citric acid and 0.025 wt % sodium azide, pH 2.74, at a flow rate of 1 mL/min. The sample concentration was 10 mg/5 mL with an injection volume of 100 μL. The weight average molecular weight, M_w, has been accurately determined by GPC, and the number average molecular weight, M_n, was calculated with Astra 4.7 software (Wyatt Technology Corporation) based on the molecular weight distribution.

Nuclear Magnetic Resonance Spectroscopy. ^1H spectra were taken in D_2O and were used to provide integration values for structural analysis by means of a Bruker AVANCE DRX 500 instrument.

UV Spectrophotometry. UV spectra were recorded using a Perkin-Elmer UV/vis Spectrometer Lambda 20 and Lambda 20 software, in PBS.

Reverse Phase High Performance Liquid Chromatography. The ion-pairing reverse phase high performance liquid chromatography (RP-HPLC) system consisted of a System GOLD 126 solvent module, a model 507 autosampler equipped with a 100 μL loop, and a model 166 UV detector (Beckman Coulter, Fullerton, CA). A Phenomenex (Torrance, CA) Jupiter C5 silica based HPLC column (250 mm × 4.6 mm, 300 Å) was used for the separation of analytes. Two Phenomenex Widepore C5 guard columns (4 mm × 3 mm) were also installed upstream of the HPLC column. The mobile phase for elution of PAMAM dendrimers was a linear gradient beginning with 90:10 water/acetonitrile (ACN) at a flow rate of 1 mL/min, reaching 50:50 after 30 min. Trifluoroacetic acid (TFA) at 0.14 wt % concentration in water as well as in ACN was used as a counterion to make the dendrimer−conjugate surfaces hydrophobic. The conjugates were dissolved in the mobile phase (90:10 water/ACN). The injection volume in each case was 50 μL with a sample concentration of approximately 1 mg/mL, and the detection of eluted samples was performed at 210, 242, or 280 nm. The analysis was performed using Beckman's System GOLD *Nouveau* software. Characterization of each device and all intermediates has been performed through the use of UV, HPLC, NMR, and GPC.

Syntheses. The synthetic scheme for production of dendritic devices is given in Figure 1a. Figure 1b depicts the chemical structure of G5-Ac^3(82)-FITC-FA-OH-MTX^e, represented as structure **9** in Figure 1a.

1. G5 Carrier. The PAMAM G5 dendrimer was synthesized and characterized at the Center for Biologic Nanotechnology, University of Michigan. The number average molecular weight was found to be 26 380 g/mol by GPC, and the number average number of primary amino groups was determined through potentiometric titration to be 110.

2. G5-Ac^3(82). 2.38696 g (8.997×10^{-5} mol) of G5 PAMAM dendrimer (M_n = 26 380 g/mol by GPC, number of primary amines = 110 by potentiometric titration) in 160 mL of absolute MeOH was reacted with 679.1 μL (7.198×10^{-3} mol) of acetic anhydride in the presence of 1.254 mL (8.997×10^{-3} mol, 25% molar excess) of triethylamine. After intensive dialysis in deionized (DI) water and lyophilization, 2.51147 g (93.4%) of G5-Ac^3(82) product was obtained. The average number of acetyl groups (82) was determined based on ^1H NMR calibration.[23]

3. G5-Ac^3(82)-FITC. 1.16106 g (3.884×10^{-5} mol) of G5-Ac^3(82) partially acetylated PAMAM (MW = 29 880 g/mol by GPC) in 110 mL of absolute DMSO was allowed to react with 0.08394 g (90% pure) (1.94×10^{-4} mol) of FITC under nitrogen overnight. After intensive dialysis in DI water and lyophilization, 1.10781 g (89.58%) of G5-Ac^3(82)-FITC product was isolated. Further purification was achieved by membrane filtration (used PBS buffer and DI water).

4. G5-Ac^3(82)-FITC-OH. 0.20882 g (6.51×10^{-6} mol) of G5-Ac^3(82)-FITC reacted with 19.9 μL (2.99×10^{-4} mol) of glycidol (racemic) in 150 mL of DI water. Two glycidol molecules were calculated for each remaining primary amino group. The reaction mixture was stirred vigorously for 3 h at room temperature. After intensive dialysis in DI water for 2 days and lyophilization, the yield of the G5-Ac^3(82)-FITC-OH product was 0.18666 g (84.85%).

5. G5-Ac^3(82)-FITC-OH-MTX^e. 0.02354 g of MTX (5.18×10^{-5} mol) was allowed to react with 0.13269 g (6.92×10^{-4} mol) of EDC in 27 mL of DMF and 9 mL of DMSO for 1 h at room temperature with vigorous stirring. This solution was added dropwise to 150 mL of DI water solution containing 0.09112 g (2.72×10^{-6} mol) of G5-Ac^3(82)-FITC-OH. The reaction mixture was vigorously stirred for 3 days at room temperature. After intense dialysis in DI water and lyophilization, the yield of the targeted molecule G5-Ac^3(82)-FITC-OH-

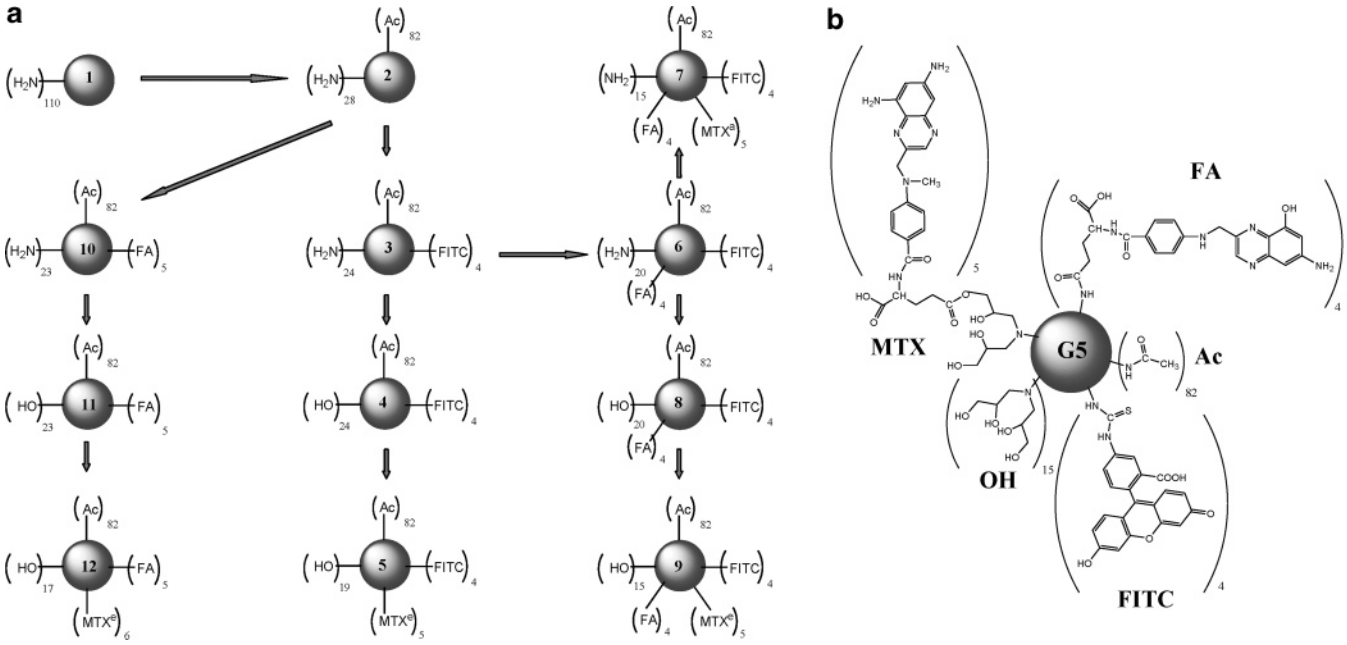

Figure 1. (a) Synthetic scheme for multifunctional PAMAM dendritic devices. The order of syntheses is as follows: (1) G5 carrier, (2) G5-Ac^3(82), (3) G5-Ac^3(82)-FITC, (4) G5-Ac^3(82)-FITC-OH, (5) G5-Ac^3(82)-FITC-OH-MTX^e, (6) G5-Ac^3(82)-FITC-FA, (7) G5-Ac^1(82)-FITC-FA-MTX^a, (8) G5-Ac^3(82)-FITC-FA-OH, (9) G5-Ac^3(82)-FITC-FA-OH-MTX^e, (10) G5-Ac^2(82)-FA, (11) G5-Ac^2(82)-FA-OH, (12) G5-Ac^2(82)-FA-OH-MTX^e. (Note: The superscripts e and a signify ester and amide bonds, respectively. The superscripts indicated in Ac^1, Ac^2, and Ac^3 are utilized to differentiate sets of acetylation reactions.) Analytical results have shown high reproducibility (not shown here). (b) Chemical structure of G5-Ac^3(82)-FITC-FA-OH-MTX^e.

MTX^e was 0.08268 g (73.5%). Further purification was accomplished by membrane filtration (used PBS buffer and DI water).

6. G5-Ac^3(82)-FITC-FA. 0.03756 g (8.51×10^{-5} mol) of FA (MW = 441.4 g/mol) reacted with 0.23394 g (1.22×10^{-3} mol) of EDC (1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide HCl; MW = 191.71 g/mol) in a mixture of 27 mL of dry DMF and 9 mL of dry DMSO under a nitrogen atmosphere for 1 h. This organic reaction mixture was added dropwise to the DI water solution (100 mL) of 0.49597 g (1.55×10^{-5} mol; MW = 31 250 g/mol) of G5-Ac^3(82)-FITC. The reaction mixture was vigorously stirred for 2 days. After dialysis and lyophilization the weight of the G5-Ac^3(82)-FITC-FA was 0.5202 g (98.1%). Further purification was carried out by membrane filtration (used PBS buffer and DI water).

7. G5-Ac^1(82)-FITC-FA-MTX^a. 0.00989 g (2.1763×10^{-5} mol) of MTX (MW = 454.45 g/mol) was allowed to react with 0.05933 g (3.0948×10^{-4} mol) of EDC in a mixture of 66 mL of dry DMF and 22 mL of dry DMSO under a nitrogen atmosphere for 1 h. This organic reaction mixture was added dropwise to the DI water solution (260 mL) of 0.09254 g (2.7051×10^{-6} mol; MW = 34 710 g/mol by GPC) of G5-Ac^1(82)-FITC-FA-NH_2. The solution was vigorously stirred for 2 days. After dialysis in DI water and lyophilization, the weight of G5-Ac^1(82)-FITC-FA-MTX^a was 0.09503 g (96.5%). Further purification was attained through membrane filtration (using PBS buffer and DI water). This trifunctional device will serve as a control compound for in vitro cytotoxicity studies.

8. G5-Ac^3(82)-FITC-FA-OH. 0.29597 g (8.63×10^{-6} mol) of G5-Ac^3(82)-FITC-FA partially acetylated PAMAM dendrimer conjugate (MW = 34 710 g/mol by GPC) in 200 mL of DI water was reacted with 20.6 μL (3.1×10^{-4} mol, 25% molar excess) of glycidol (MW = 74.08 g/mol) for 3 h. After intensive dialysis in DI water, lyophilization, and repeated membrane filtration, 0.27787 g (90.35%) of fully glycidylated G5-Ac^3(82)-FITC-FA-OH product was isolated. Nonspecific uptake was not observed in the in vitro study (see Part II of this research for the uptake study).[25]

9. G5-Ac^3(82)-FITC-FA-OH-MTX^e. 0.03848 g (8.4674×10^{-5} mol) of MTX (MW = 454.45 g/mol) was reacted with 0.22547 g (1.176×10^{-3} mol) of EDC in a mixture of 54 mL of dry DMF and 18 mL of dry DMSO under a nitrogen atmosphere for 1 h. This organic reaction mixture was added dropwise to the DI water solution (240 mL) of 0.16393 g (4.6339×10^{-6} mol; MW = 36 820 g/mol) of G5-Ac^3(82)-FITC-FA-OH. The solution was vigorously stirred for 3 days. After dialysis in DI water, repeated membrane filtration (using PBS and DI water), and lyophilization, the weight of G5-Ac^3(82)-FITC-FA-MTX^e was 0.18205 g (90.88%).

10. G5-Ac^2(82)-FA. FA was attached to G5-Ac^2(82) in two consecutive reactions. 0.03278 g (7.426×10^{-5} mol) of FA was allowed to react with a 14-fold excess of EDC (0.19979 g, 1.042 $\times 10^{-3}$ mol) in a solvent mixture of 24 mL of DMF and 8 mL of DMSO at room temperature, and then, this FA-active ester solution was added dropwise to an aqueous solution of the partially acetylated product G5-Ac^2(82) (0.40366 g, 1.347 $\times 10^{-5}$ mol) in 90 mL of water. After dialysis in DI water, repeated membrane filtration (using PBS and DI water), and lyophilization, the product weight was 0.41791 g (96.7%). The number of FA molecules was determined by UV spectroscopy. As an additional characterization, no free FA was observed with a gel permeation column equipped with a UV detector or by agarose gel.

11. G5-Ac^2(82)-FA-OH. 0.21174 g (6.60×10^{-6} mol) of the monofunctional dendritic device, G5-Ac^2(82)-FA, reacted with 20.1 μL (3.04×10^{-4} mol) of glycidol in 154 mL of DI water under vigorous stirring for 3 h. After dialysis in DI water and lyophilization, the glycidylated monofunctional device having hydroxyl groups on the surface (yield: 0.20302 g, 91.05%) participated in the conjugation reaction with methotrexate.

12. G5-Ac^2(82)-FA-OH-MTX^e. In a solvent mixture of 27 mL of DMF and 9 mL of DMSO, 0.02459 g (5.41×10^{-5} mol) of MTX and 0.14315 g (7.46×10^{-4} mol) of EDC were allowed to react under nitrogen at room temperature for 1 h. The reaction mixture was vigorously stirred. The MTX-active ester solution was added dropwise to the 0.09975 g (2.95×10^{-6} mol) of the monofunctional dendritic device, having hydroxyl groups on the surface, in 150 mL of DI water, and this reaction mixture was stirred at room temperature for 3 days. After dialysis in DI water, repeated membrane filtration (using PBS and DI water), and lyophilization, this bifunctional device G5-Ac^2(82)-FA-OH-MTX^e (yield: 0.11544 g, 93.9%) was characterized and tested in vitro[25] and in vivo.[26]

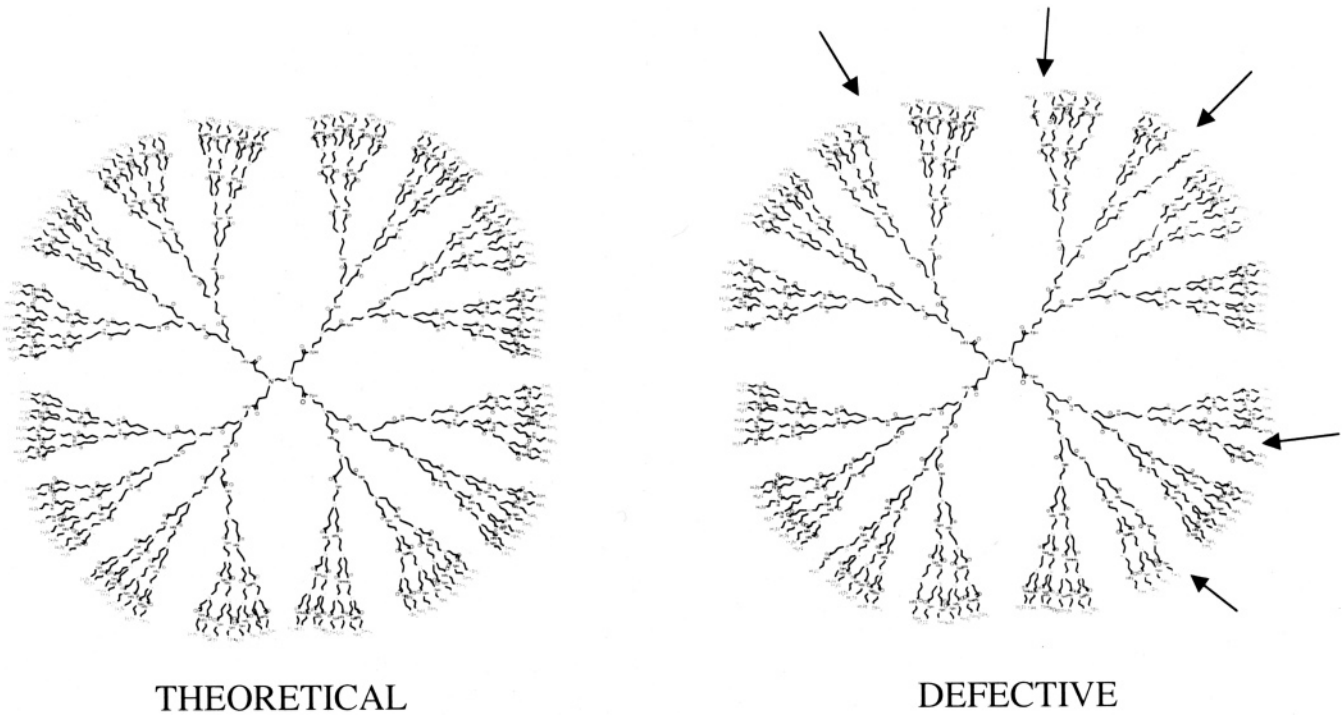

Figure 2. Theoretical and defective chemical structures of the G5 PAMAM dendrimer (missing arms are indicated by arrows).

Results and Discussion

The PAMAM dendrimer used in this study has been synthesized at the Center of Biologic Nanotechnology at the University of Michigan through use of GMP scale-up synthesis. Full characterization has been made through use of GPC, HPLC, ^{1}H and ^{13}C NMR, PAGE, potentiometric titration, and MALDI-TOF. Additional characterization has been published elsewhere.[24] Determination of molecular weight and the number of primary amino groups were fundamental in designing reactions resulting in the synthesis of a precise conjugate structure.

By possessing the ability to synthesize a stable, unique conjugate structure capable of targeted drug delivery and release within the targeted cell(s), molecular engineering affords us the capability of synthesizing complex yet *well-defined* devices, which is a key principle of targeted drug delivery technology. Figure 2 presents the theoretical and defective chemical structures of the G5 PAMAM dendrimer. Side reactions such as bridging and synthesis of fewer arms per generation combine to produce a slightly defective experimental G5 PAMAM dendrimer structure. The defective chemical structure of a G5 PAMAM dendrimer exhibits missing arms especially for higher generations (3, 4, and 5). Precise characterization of the PAMAM dendrimer platform allows for the design of reaction sequences with stoichiometry suitable for synthesis of engineered nanodevices. Molecular engineering principles are used to attach functional molecules to the terminal primary amino groups of the PAMAM dendrimer surface. The conjugated molecules may enhance drug delivery through targeted, controlled release in response to enzymatic biochemical mechanisms.

1. Acetylation of the Dendrimer. The acetylation reaction is the first requisite step in the synthesis of dendritic devices. Enhanced analytical techniques allowed for the precise determination of the number of terminal primary amino groups, which is necessary in order to determine the extent of the reactions required to partially or fully acetylate the terminal amino groups. Potentiometric titration was performed to determine the number of primary and tertiary amino groups. Theoretically, the G5 PAMAM dendrimer has 128 primary amine groups, on its surface, and 126 tertiary amine groups. These values can be determined through the use of mathematical models.[3,8,9] Potentiometric titration revealed that there were 110 primary amines present on the surface of the G5 PAMAM dendrimer carrier. Figure 3 shows the titration curves performed by direct titration with a 0.1 M HCl volumetric solution and back-titration with a 0.1 M NaOH volumetric solution. DI water (pH = 5.8) was used to prepare 10 mL of a 0.1 N NaCl solution for use as a reference titration. The result was subtracted from the gross titration. A 10 mL solution of 0.1 N NaCl was added to 100 mg of PAMAM dendrimer to shield amine group interactions. The average number of primary amino groups was calcu-

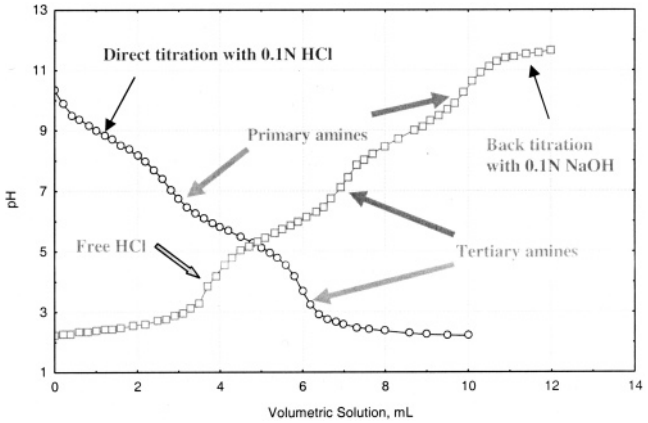

Figure 3. Potentiometric titration curves of the G5 PAMAM dendrimer.

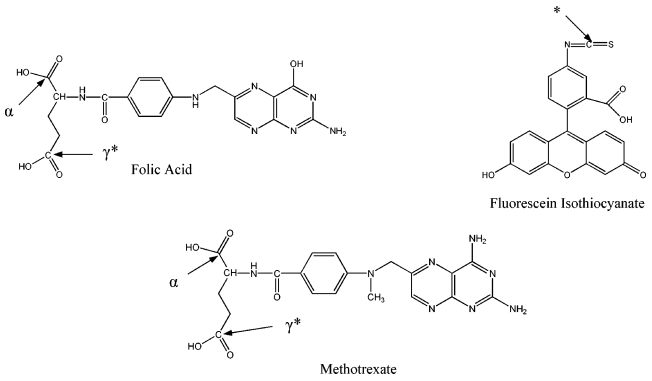

Figure 4. FITC, FA, and MTX structures with the group used for conjugation marked with an asterisk.

lated using data from back-titration performed with a 0.1 M NaOH volumetric solution.

Partial acetylation is used to neutralize a fraction of the dendrimer surface from further reaction or intermolecular interaction within the biological system, thereby, preventing nonspecific interactions from occurring during synthesis and during drug delivery. Leaving a fraction of the surface amines nonacetylated allows for the attachment of biologically important molecules. Acetylation of the remaining amino groups resulted in increased solubility of the FITC conjugate in DMSO (as the nonacetylated dendrimer and FITC conjugate was not soluble in DMSO), allowing the dendrimer to disperse more freely within the aqueous media with increased targeting specificity and giving it greater potential for use as a targeted delivery system as compared to many conventional media.[1]

The inverse relationship between the degree of acetylation and the diameter is demonstrated by a comparison of the structures of nonacetylated and 20%, 40%, 60%, 80%, and 100% acetylated G5 PAMAM dendrimer. As the degree of acetylation rises, the diameter of the dendrimer decreases, demonstrating an inverse relationship between the degree of acetylation and the diameter of the dendrimer.[23] The lower number of primary amines available for protonation (at a higher degree of acetylation, as compared to a lower degree) leads to a structure less impacted by charge−charge interactions, therefore, leading to a more compact structure. The molecular weight ($\overline{M}_n$), however, has a parallel relationship to the degree of acetylation: the molecular weight increases as the degree of acetylation rises.

A description and characterization of the reaction sequence has been reported previously.[23] Intensive dialysis, lyophilization, and repeated membrane filtration using PBS and DI water were used to yield the purified, partially acetylated G5-Ac^2(82) and G5-Ac^3(82) PAMAM dendrimers. After conjugation of FITC and FA, the dendrimer was fully acetylated again, as needed for in vitro uptake study following the reaction sequence described previously.[15] Again, intensive dialysis, lyophilization, and repeated membrane filtration were performed, yielding the fully acetylated G5-Ac^1(82)-FITC and G5-Ac^1(82)-FITC-FA.

The structures of FITC, FA, and MTX are presented in Figure 4 with the group to be attached to the dendrimer marked with an asterisk. The α- and γ-carboxyl groups are labeled on both the FA and MTX molecules. When the γ-carboxylic group on FA is used for conjugation to the dendrimer, FA retains a strong affinity toward its receptor, allowing the FA moiety of the conjugate to retain its ability to act as a targeting agent. Additionally, the γ-carboxylic group possesses a higher reactivity during carboiimide-mediated coupling to amino groups as compared to the α-carboxyl group.[1]

2. Conjugation of FITC to the Acetylated Dendrimer. A partially acetylated G5-Ac^3(82) PAMAM dendrimer was used for the conjugation of FITC. The partially acetylated dendrimer was allowed to react with FITC, and after intensive dialysis, lyophilization, and repeated membrane filtration, the G5-Ac^3(82)-FITC product was formed. The formed thiourea bond was stable during the investigation of the devices.

3. Conjugation of FA to the Acetylated Monofunctional Dendritic Device. Conjugation of FA to the partially acetylated monofunctional dendritic device was carried out via condensation between the γ-carboxyl group of FA and the primary amino groups of the dendrimer. The active ester of FA, formed by reaction with EDC in DMSO−DMF (a 1:3 solvent mixture), was added dropwise to a solution of DI water containing G5-Ac^3(82)-FITC and was vigorously stirred for 2 days to allow for the FA to conjugate to the G5-Ac^3(82)-FITC.[1] It is obvious that the α-carboxyl group may participate in the condensation reaction, but its reactivity is much lower when compared to the γ-carboxyl group. It was not our goal to detect the ratio of their reactivities. NMR was also used to confirm the number of FA molecules attached to the dendrimer. In the case that free FA is present in the sample, sharp peaks would appear in the spectrum. The broadening of the aromatic proton peaks in the G5-Ac^3(82)-FITC-FA spectrum indicates the presence of a covalent bond between the FA and the dendrimer. On the basis of the integration values of the methyl protons in the acetamide groups and the aromatic protons in the FA, the number of attached FA molecules was calculated to be 4.5. The number of FA molecules (4.8) was determined by UV spectroscopy, utilizing the concentration calibration curve of free FA.

4. Conjugation of MTX to the Acetylated Bifunctional Dendritic Device (Amide Link). A trifunctional conjugate with MTX as the drug was synthesized from G5-Ac^3(82)-FITC-FA. The similarity in structure of MTX, a commonly used anti-cancer drug,[1] to FA allows for its attachment to G5-Ac^3(82)-FITC-FA through the same condensation reaction used to attach FA to the primary amino groups. The MTX was attached through an amide bond, producing the control product for comparison with our goal product G5-Ac^3(82)-FITC-FA-OH-MTX^e (for use for in vitro study). It was expected, from the molar ratio of the reactants, that five drug molecules would be attached per dendrimer. The ^{1}H NMR spectrum of the trifunctional device was taken (not shown). The broadening of the aromatic proton peaks indicates the presence of a covalent bond between methotrexate and the dendrimer. After dialysis in DI water and repeated membrane filtration (using PBS and DI water, a minimum of five times each), UV spectrophotometry was used to detect the presence of MTX in the filtrate. Free methotrexate was not detected, further demonstrating that MTX was covalently bonded to the dendrimer and that physical loading did not occur.

Table 1. PAMAM Dendrimer Carrier and Its Mono-, Bi-, and Trifunctional Conjugates with Molecular Weights and Molecular Weight Distribution[a]

	$\overline{M_n}$, g/mol	$\overline{M_w}$, g/mol	$\overline{M_w}/\overline{M_n}$
G5	26 380	26 890	1.020
G5-Ac^2	29 830	30 710	1.030
G5-Ac^2-FA	32 380	35 470	1.095
G5-Ac^2-FA-OH	34 460	40 580	1.178
G5-Ac^2-FA-OH-MTX^e	36 730	36 960	1.006
G5-Ac^3	29 880	30 760	1.030
G5-Ac^3-FITC	32 150	32 460	1.100
G5-Ac^3-FITC-OH	34 380	34 790	1.012
G5-Ac^3-FITC-OH-MTX^e	37 350	37 800	1.012
G5-Ac^3-FITC-FA	34 710	35 050	1.010
G5-Ac^3-FITC-FA-OH	36 820	37 390	1.016
G5-Ac^3-FITC-FA-OH-MTX^e	39 550	39 870	1.008

[a] $\overline{M_n}$ = number average molecular weight, $\overline{M_w}$ = weight average molecular weight calculated from GPC data, and $\overline{M_w}/\overline{M_n}$ = molecular weight distribution.

Studies performed in our laboratory have additionally shown that the complex survives under denaturing conditions in an HPLC mobile phase (0.14% TFA, 90% water, and 10% ACN) at pH 2.2, indicating the presence of a covalent bond between methotrexate and the dendrimer.[27] On the basis of the integration values of the methyl protons in the acetamide groups and the aromatic protons in the conjugated molecules, the number of attached methotrexate molecules was calculated to be five. Fréchet and colleagues have previously synthesized a dendritic nanodevice with MTX conjugated to the dendrimer by an amide bond.[22] Here, however, methotrexate conjugation by an amide bond will serve as a control device to methotrexate conjugation by an ester bond for in vitro tests. Attachment of methotrexate via an ester bond allows for relatively easy cleavage and release of the drug into the system as compared to linkage of MTX to the dendrimer by an amide bond and was, therefore, chosen for use in our target drug for synthesis and characterization during this study.

5. Conjugation of Glycidol to the Acetylated Bifunctional Dendritic Device. The conjugation of glycidol to the acetylated bifunctional device was a necessary precursory step in order to attach MTX via an ester linkage and eliminate the remaining NH_2 groups to avoid any unwanted nonspecific targeting within the biological system. Conjugation of glycidol to the G5-Ac^3(82)-FITC-FA converted all the remaining primary amino groups to alcohol groups, producing G5-Ac^3(82)-FITC-FA-OH.

6. Conjugation of Methotrexate to the Acetylated and Glycidylated Bifunctional Dendritic Device (Ester Link). MTX conjugation via an ester linkage was tested for improved cleavage as compared to conjugation to the dendrimer via an amide linkage. The MTX was attached by use of EDC chemistry as previously described for conjugation of MTX to the acetylated bifunctional dendritic device with an amide link.[1]

7. Conjugation of MTX to the Acetylated and Glycidylated Monofunctional Dendritic Devices (Ester Link). For characterization purposes, the conjugation of MTX to glycidylated monofunctional dendritic devices containing FA or FITC produced G5-Ac^2-FA-OH-MTX^e, which will serve as a candidate device for in vitro and in vivo studies and clinical trials, and

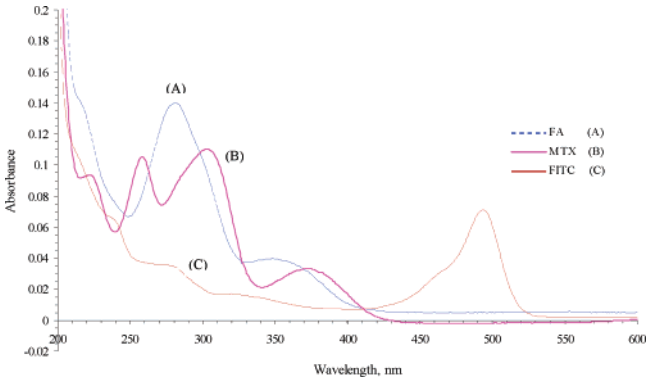

Figure 5. UV spectra of free FA, MTX, and FITC.

G5-Ac^3-FITC-OH-MTX^e, which has served as a control device in in vivo studies.[26]

The determination of the molecular weight of each conjugate structure was also necessary in order to produce a well-defined multifunctional dendritic device. A GPC instrument equipped with multiangle laser light scattering and a refraction index (RI) concentration detector was utilized for this purpose. Table 1 presents the PAMAM dendrimer carrier and its mono-, bi-, and trifunctional conjugates with molecular weights and molecular weight distribution given for each. The superscript numerals 2 and 3 (i.e., G5-Ac^2 and G5-Ac^3) indicate that these are two independent acetylation reactions. Analytical data shows very high reproducibility.

The measured molecular weight ($\overline{M_n}$) of the G5 dendrimer of 26 380 g/mol is slightly lower than the theoretical one (28 826 g/mol). GPC data for each conjugate was used in order to derive the precise number of each functional group attached to the carrier. The average number of each functional molecule can be calculated by subtracting the $\overline{M_n}$ value of the conjugate without the functional molecule in question from the $\overline{M_n}$ value of the conjugate containing the functional molecule and dividing by the molecular weight of the functional molecule.

On the basis of GPC analysis, the average number of conjugated FITC, FA, MTX, and glycidol molecules has been determined to be as follows: FITC, 5.8; FA, 5.7; MTX^e, 5−6; and OH, 28−30. The number of conjugated molecules as determined by GPC was slightly higher than assumed. We were unsure of the reasoning behind this occurrence, and further investigation is merited for the near future. These values along with values obtained through analysis of NMR and UV spectra have been utilized in combination to precisely determine the number of each conjugate molecule attached to the dendrimer.

The combined UV spectra for free FA, MTX, and FITC (Figure 5) are presented for comparison to Figure 6, the UV spectra of G5-Ac(82) and mono-, bi-, and trifunctional dendritic devices. Figure 5 presents defining peaks for FA at precisely 281 and 349 nm; for MTX at 258, 304, and 374 nm; and for FITC at 493 nm. The distinguishing peaks for FA, FITC, and MTX visible in Figure 6 are dependent on the conjugation of each molecule to the dendrimer. Characterization of each dendritic device by comparison of the UV spectra of free material and dendrimer-conjugated material was used

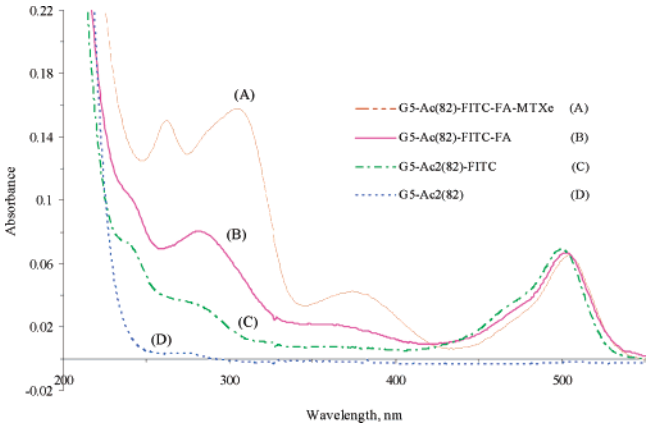

Figure 6. UV spectra of G5-Ac and mono-, bi-, and trifunctional devices.

to determine which function has been attached to the dendrimer. The theory that UV spectra possess additive properties is described in depth elsewhere.[28]

G5-Ac^2(82), the carrier dendrimer presented in Figure 6 as spectrum D, demonstrates no characteristic peaks above 300 nm. With the attachment of FITC to the dendrimer, the monofunctional dendritic device G5-Ac^3(82)-FITC, denoted as C in Figure 6, is formed. The UV peak characteristic of FITC, as demonstrated by Figure 6, is present at 500 nm, a slight shift from the peak of 493 nm for free FITC. Building upon this same principle, the attachment of FA, to form the bifunctional dendritic device G5-Ac^3(82)-FITC-FA (denoted as B in Figure 6), shifts the peak for FA to approximately 358 nm, as compared to 349 nm for free FA. The location of the other peak characteristic of free FA, 281 nm, remains unchanged in the conjugated device, and the peak for FITC in spectrum B (Figure 6) is present at 502 nm. The trifunctional device G5-Ac^3(82)-FITC-FA-MTX^e (spectrum A) also has slightly shifted peaks. Peaks for MTX appear at 262 and 304 nm; peaks representing a combination of the MTX and FA converge at 372 nm, and a peak occurs at 505 nm for FITC. UV spectroscopy permits identification of what has been attached to the dendritic carrier through comparison of the characteristic absorption peaks of each functional group and the carrier after conjugation has occurred. UV spectroscopy also allows us to determine how the wavelength at which maximum absorption occurs for each attached function is affected by its conjugation to the dendrimer.

Conclusion

Dendrimers conjugated with multiple functional molecules are being explored for use in a wide variety of biomedical applications, most prominently in the field of targeted drug delivery and imaging. We have presented a detailed synthetic study and analytical methods for the preparation and characterization of PAMAM dendrimer-based multifunctional therapeutic devices. Synthesis of these nanodevices has shown high reproducibility. Multifunctional devices have been synthesized and characterized for use in in vitro, in vivo, and control studies to be described in further detail elsewhere.[25,26]

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Supporting Information Available: ^{1}H NMR, GPC, and HPLC data for characterization of the nanodevices. This material is available free of charge via the Internet at http://pubs.acs.org.

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