Published in final edited form as:
Bioconjug Chem. 2011 April 20; 22(4): 679–689. doi:10.1021/bc100360v.

Design, Synthesis, and Biological Functionality of a Dendrimer-based Modular Drug Delivery Platform

Douglas G. Mullen[†,‡], **Daniel Q. McNerny**[‡,⊥], **Ankur Desai**[‡], **Xue-min Cheng**[‡], **Stassi C. DiMaggio**[§], **Alina Kotlyar**[‡], **Yueyang Zhong**[‡,+], **Suyang Qin**[‡], **Christopher V. Kelly**[‡,∥], **Thommey P. Thomas**[‡], **Istvan Majoros**[‡], **Bradford G. Orr**[‡,∥], **James R. Baker Jr.**[‡], and **Mark M. Banaszak Holl**[†,‡,∥,+,*]

Programs in Macromolecular Science and Engineering and Applied Physics, Departments of Chemistry and Chemical Engineering, Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan, Ann Arbor, MI 48019. Department of Chemistry, Xavier University, New Orleans, LA 70125

Abstract

A modular dendrimer-based drug delivery platform was designed to improve upon existing limitations in single dendrimer systems. Using this modular strategy, a biologically active platform containing receptor mediated targeting and fluorescence imaging modules was synthesized by coupling a folic acid (FA) conjugated dendrimer with a fluorescein isothiocyanate (FITC) conjugated dendrimer. The two different dendrimer modules were coupled via the 1,3-dipolar cycloaddition reaction ('click' chemistry) between an alkyne moiety on the surface of the first dendrimer and an azide moiety on the second dendrimer. Two simplified model systems were also synthesized to develop appropriate 'click' reaction conditions and aid in spectroscopic assignments. Conjugates were characterized by ^{1}H NMR spectroscopy and NOESY. The FA-FITC modular platform was evaluated *in vitro* with a human epithelial cancer cell line (KB) and found to specifically target the over-expressed folic acid receptor.

Introduction

The high toxicity of conventional cytotoxic anti-cancer drugs often forces these agents to be given at sub-optimal dosages and this can result in treatment failure (1). To resolve this problem, delivery platforms that can discriminate between healthy and malignant cells have been developed (1, 2). Actively targeted therapeutic delivery platforms consist of three different components: a targeting component comprised of targeting ligands with affinities for molecules expressed on cancer cells; a payload consisting of drug and/or imaging agents; and a nano-scale structure to which the targeting and payload moieties are attached. This platform targeting of anti-cancer drugs with cancer cell-specific ligands can dramatically improve a drug's therapeutic index. Conjugating multiple targeting ligands to a single

[*]Corresponding author: Mark M. Banaszak Holl, mbanasza@umich.edu, 930 N. University Avenue, Ann Arbor, MI 48109-1055. TEL: 734-763-2283, FAX: 734-936-2307.
[†]Program in Macromolecular Science and Engineering
[‡]Michigan Nanotechnology Institute for Medicine and Biological Sciences
[∥]Program in Applied Physics
[⊥]Department of Chemical Engineering
[+]Department of Chemistry, University of Michigan
[§]Department of Chemistry, Xavier University

Supporting Information **Available:** ^{1}H NMR characterization of dendrimer conjugates and a plot of Mean Fluorescence for the Un-targeted Modular Platform (**18**) control. This material is available free of charge via the Internet at http://pubs.acs.org.

platform molecule further increases the potential for specific targeting of cancer cells by allowing the possibility of multivalent interactions (3, 4).

The structural design of these types of delivery platforms is critical to the success of the delivery device. Numerous classes of targeted drug delivery platforms have been developed that potentially meet the requirements needed to combine targeting ligands, imaging agents, and drug molecules together to deliver the therapeutic payload to a desired location in the body. These include drug-target conjugates, linear polymers, lipid-based carriers (liposomes and micelles), carbon nanotubes, inorganic nanoparticles, and dendrimers. Several of these different delivery platforms are progressing towards or through clinical trials for cancer treatments with promising results (2). Each approach, however, is not without limitations and the potential for widespread application of these platforms in their present design is unclear.

Dendrimer-based platforms have a unique branching structure which results in exceptionally high degrees of monodispersity and well defined terminal groups that provide the ability to form soluble conjugates containing multiple copies of hydrophobic drug and/or targeting molecules. The compact, branched structures appear to enhance the ability of the targeting molecules to interact in a fashion conducive to multivalent binding to cell membrane receptors (3). The dendrimer's small size enables efficient diffusion across the vascular endothelium to find tumors and also allows the rapid clearance of these molecules from the blood stream. This clearance avoids potential long-term toxicities and reduces the necessity of a rapidly-degradable platform. The most widely used dendrimer in biomedical applications, poly(amidoamine) (PAMAM), is non-imunogenetic and non-toxic once the surface primary amines have been modified (5-10). There have been numerous, recent examples describing the development of dendrimer-based targeted delivery systems using a wide variety of targeting ligands including monoclonal antibodies (11-15), peptides (16), T-antigens (17-19), and folic acid (20-29).

Despite the success of these dendrimer-based platforms, this approach has several challenges associated with its implementation. First, the synthesis of dendrimers with different functional groups for targeted delivery (including targeting, drug, and imaging agents) requires a laborious chemical process that is unique for each different molecular combination. Second, the carrying capacity of a single dendrimer, although significantly better than other types of delivery platforms, is finite due to limits in surface molecule density and solubility. This becomes a problem when one attempts to conjugate multiple copies of the target, drug, and/or dye molecules to the same dendrimer. Third, significant increases in heterogeneity occur with the conjugation of each additional molecule to a single dendrimer platform due to the stochastic nature of these chemical reactions (30). This has limited the flexibility of these systems.

To address the drawbacks of the single dendrimer platforms, several groups have sought to apply modular design concepts to dendrimer systems (31-41). The common premise of this new approach is to use dendrimers or dendrons as modular units, each with multiple copies of a single functional molecule. Multi-functional platforms can be generated by combining different modules through a universal coupling mechanism. The main benefits of this design are two fold: First, segregating each functional group (drug/target/dye) to a different dendrimer module avoids the need to develop a new orthogonal coupling chemistry for each new combination of functional groups. This advantage should not be underestimated. Significant time is spent developing new orthogonal coupling strategies for desired functional combinations because many of the component drug molecules and targeting ligands (Taxol and RGD for example) are susceptible to a loss of activity due to undesired cross reactions as well as degradation by hydrolysis. The second benefit of the modular

strategy is a greater carrying capacity of the delivery platform because the different functional molecules are localized on separate dendrimer units and the water solubilizing dendrimer backbone is effectively double the mass of the single dendrimer.

In the modular dendrimer systems synthesized to-date two coupling strategies have been used to combine different dendrimer-based modules together: oligonucleotide self-assembly and the 1,3-dipolar cycloaddition between an azide and an alkyne, commonly called ‘click’ chemistry. Oligonucleotide self-assembly has been used to link both dendron and dendrimer modular units. Choi and co-workers demonstrated this strategy by using two complementary oligonucleotides to link two PAMAM dendrimers together (31, 32). DeMattie, Huang, and Tomalia used the same method to connect two un-functionalized PAMAM dendrons together (33). Characterization of these systems was accomplished by gel electrophoresis, AFM, MALDI, and UV/vis due to the small synthetic scales employed. For the dendrimer-based system, isolated dendrimer samples were not obtained, rather the samples were generated in solution. Choi et al. did demonstrate biological functionality of the modular system in a cell culture assay (32) and an *in vivo* model (34).

The use of ‘click’ chemistry to create dendritic modular systems has mainly involved dendrons. ‘Click’ chemistry is a particularly attractive coupling method because it can be performed with a wide variety of solvent conditions including aqueous environments. The stable triazole ring bridge, resulting from coupling alkyne with azide moieties, is frequently achieved at near quantitative yields and is considered to be biologically stable (42-44). Furthermore, the ‘click’ coupling chemistry is orthogonal to the coupling chemistries typically used to attach functional groups to the dendrimer. Lee and co-workers have detailed the synthesis of multi-module platforms using both un-functionalized PAMAM dendrons (35-37) as well as unfunctionalized Frechet-type dendrons (38) for each of the modules. In all of these systems, the focal point of the dendron possessed either an azide or alkyne moiety. Wu and co-workers developed a 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) based asymmetric modular dendron with 16 mannose units and 2 coumarin chromophores, and demonstrated binding in a hemagglutination assay (39). Goyal, Yoon, and Weck have also developed a poly(amine) dendrimer that possessed a single aldehyde or azide moiety on the dendrimer periphery capable of orthogonal functionalization by small molecule functional groups (40, 41). These approaches appear promising but only the bis-MPA dendron system has been demonstrated to have function as a targeted drug delivery platform. In all cases, the dendrons used were G3 or smaller. This has significant limitations for widespread therapeutic use because of the limited carrying capacity of low generation dendrons. To date, click chemistry has not been applied to a modular dendrimer system.

In this paper we report on a new modular approach based upon ‘clicking’ together generation 5 (G5) PAMAM dendrimers containing either the targeting agent folic acid (FA) (**13**) or the dye fluorescein isothiocyanate (FITC) (**14**). The molecules were characterized by ^{1}H NMR spectroscopy and two-dimensional Nuclear Overhauser Effect spectroscopy (NOESY). To the best of our knowledge, this is the first example of the successful ‘click’ chemistry conjugation of dendrimers (as opposed to dendrons). Linking together two dendrimer-based modules was achieved by first conjugating one dendrimer module to an alkyne linker **(2b**) and conjugating a second dendrimer module to an azide linker (**3c**). The ability of this modular system to specifically target folic acid receptor (FAR) expressing cells has been verified by flow cytometric documentation of the uptake of the clicked FA-FITC modules into KB cells.

This dendrimer-based modular system has several potential benefits over predecessor systems. In using ‘click’ chemistry rather than oligonucleotide linking, the modular system can be scaled up with far greater ease and at a substantially lower cost. Oligonucleotides are

typically purchased in nano-gram quantities where-as the ‘click’ linkers in this study were produced at the gram scale. Additionally, because the clicked dendrimers are covalently linked rather than joined via the hydrogen-bond base-pairing oligonucleotide bridge, the platform is less likely to become unlinked. This characteristic proves beneficial when attempting to isolate and characterize multi-module platforms. In using generation 5 dendrimers with diameters of approximately 5 nm and over 630 hydrogen bonding sites, the carrying capacity is substantially greater than the previously used dendrons which were approximately 2 nm in diameter and possess approximately 150 hydrogen bonding sites. In fact, efforts in our group to generate G2 and G3 PAMAM dendrons functionalized with folic acid, failed due to a loss of platform solubility.

Experimental Procedures

Reagents and Materials

Biomedical grade, generation 5 PAMAM (poly(amidoamine)) dendrimer was purchased from Dendritech Inc. and purified by dialysis, as previously described,(30) to remove lower molecular weight impurities including trailing generation dendrimer defect structures.

MeOH (99.8%), acetic anhydride (99.5%), triethylamine (99.5%), dimethyl sulfoxide (99.9%), fluorescein isothiocyanate (98%), dimethylformamide (99.8%), 1-[3-(dimethylamino)-propyl-3-ethylcarbodiimide HCl (EDC) (98%), acetone (ACS reagent grade ≥ 99.5%), methyl 3-(4-hydroxyphenyl)propanoate (97%), sodium azide (99.99%), 1-bromo-2-chloroethane (98%), ethyl acetate (EtOAc) (99.5%), copper sulfate pentahydrate (99%), sodium ascorbate, 18-crown-6, K_2CO_3, tetrahydrofuran (99.9%), N,N-diisopropylethylamine, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (98%), D_2O, NaCl, 1 N HCl, 2 M KOH, and volumetric solutions (0.1 M HCl and 0.1 M NaOH) for potentiometric titration were purchased from Sigma Aldrich Co. and used as received. Hexanes (HPLC grade) and 10,000 molecular weight cut-off centrifugal filters (Amicon Ultra) were from Fisher Scientific. 1× phosphate buffer saline (PBS) (Ph = 7.4) without calcium or magnesium was purchased from Invitrogen. Sephadex G-25 and Sephadex LH-20 were purchased from GE Lifesciences.

Nuclear Magnetic Resonance Spectroscopy

All ^{1}H NMR experiments were conducted using a Varian Inova 500 MHz instrument. For all dendrimer samples the delay time was 10s. No delay time was used for small molecule samples. NOESY experiments on the dendrimer samples found in Figure 1 and utilized for Table 1 were also conducted using a Varian Inova 500 MHz instrument. For these experiments the mixing time was 250 ms, the relaxation time was 1s, and the number of increments was 128 with 32 scans per increment. Temperature was controlled at 25 °C. The NOESY experiments on the small molecule model system found in Figure 1 and utilized in Table 1 were conducted using a Varian MR400 (400 MHz) instrument. For the experiments on the small molecules, the mixing time was 800 ms, the relaxation time was 1.2 s, and the number of increments was 200 with 4 scans per increment. Based on work published by Hoffman, the internal solvent reference peak for all experiments in $CDCl_3$ was set to 7.26 ppm. For experiments conducted in D_2O, the internal reference peak was set to 4.72 ppm (45).

Gel Permeation Chromatography

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). Columns employed were 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). 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 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.

Reverse Phase High Performance Liquid Chromatography

HPLC analysis was carried out on a Waters Delta 600 HPLC system equipped with a Waters 2996 photodiode array detector, a Waters 717 Plus auto sampler, and Waters Fraction collector III. The instrument was controlled by Empower 2 software. For analysis of the conjugates, a C5 silica-based RP-HPLC column (250 × 4.6 mm, 300 Å) connected to a C5 guard column (4 × 3 mm) was used. The mobile phase for elution of the conjugates was a linear gradient beginning with 90:10 (v/v) water/acetonitrile and ending with 10:90 (v/v) water/acetonitrile over 25 min at a flow rate of 1 mL/min. Trifluoroacetic acid (TFA) at 0.14 wt % concentration in water as well as in acetonitrile was used as a counter ion to make the dendrimer surfaces hydrophobic.

Cell Culture and Treatment

The uptake of the synthesized conjugates was performed using FA-receptor-expressing KB cells (ATCC #CCL-17) as we have described previously.(23) Cells were maintained in FA-free Roswell Park Memorial Institute-1640 (RPMI 1640) medium supplemented with 10% Fetal Bovine serum (FBS), 2 μM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37° C. Cells were planted into 24 wells plate at density 250,000 per well and allowed to reach ~90% confluent on the day of the experiment. They were rinsed and incubated in serum free medium with conjugates for 1 hour at 37° C in 5% CO_2. In some wells, a 20-fold excess of free folic acid or the folic acid conjugated dendrimer was added 30 minutes prior to the addition of the dendrimer platform for the blocking of folate receptors.

Flow Cytometric Analysis

The cellular fluorescence was quantified on a Beckman-Coulter EPICS-XL MCL flow cytometer, and the data were analyzed using Expo32 software (Beckman-Coulter, Miami, FL). Cells were trypsinized, rinsed and suspended in PBS containing 0.1% bovine serum albumin (PBSB). The viable cells were gated, and the mean FL1-fluorescence of 10,000 cells was quantified. Error bars are calculated using the half-peak coefficient of variation (HPCV) (46).

Synthesis

Dendrimers are identified by the core dendrimer (G5) and conjugated groups: Ac, Alkyne, Azide, FA, and FITC. In the cases where dendrimers are linked together via the triazole ring, Alkyne and Azide labels are replaced with “L.” Ac refers to the acetamide termination, alkyne to linker **2b**, azide to linker **3c**, FA to folic acid, and FITC to fluorescein isothiocyanate.

Synthesis of Partially Acetylated Dendrimer 1

Partial acetylation of G5 PAMAM dendrimer was previously described.(5, 30) The number average molecular weight (27,336 g/mol) and PDI (1.018) of the unacetylated dendrimer was determined by GPC. Potentiometric titration was conducted to determine the average

number of primary amines per dendrimer (112 ± 5). Three batches of partially acetylated dendrimer were synthesized for further modification. **1′ G5Ac$_{72\%}$:** ^{1}H NMR integration determined the degree of acetylation to be 72%. Number average molecular weight (30,722 g/mol) was computed based on the addition of mass to the dendrimer from the acetyl groups as determined by NMR. PDI (1.019) of the purified acetylated dendrimer were determined by GPC. **1″ G5Ac$_{65\%}$:** ^{1}H NMR integration determined the degree of acetylation to be 65%. Number average molecular weight (30,394 g/mol) was computed based on the addition of mass to the dendrimer from the acetyl groups as determined by NMR. PDI (1.060) of the purified acetylated dendrimer were determined by GPC. **1‴ G5Ac$_{67\%}$:** ^{1}H NMR integration determined the degree of acetylation to be 67%. Number average molecular weight (30,473 g/mol) was computed based on the addition of mass to the dendrimer from the acetyl groups as determined by NMR. **1⁗ G5Ac$_{58\%}$:** ^{1}H NMR integration determined the degree of acetylation to be 58%. Number average molecular weight (30,064 g/mol) was computed based on the addition of mass to the dendrimer from the acetyl groups as determined by NMR. **1⁗′ G5Ac$_{68.8\%}$:** ^{1}H NMR integration determined the degree of acetylation to be 69%. Number average molecular weight (30,572 g/mol) was computed based on the addition of mass to the dendrimer from the acetyl groups as determined by NMR. **1⁗″ G5Ac$_{64\%}$:** ^{1}H NMR integration determined the degree of acetylation to be 64%. Number average molecular weight (30,347 g/mol) was computed based on the addition of mass to the dendrimer from the acetyl groups as determined by NMR.

Synthesis of Alkyne Linker (3-(4-(prop-2-ynyloxy)phenyl)propanoic acid) 2

Synthesis of methyl 3-(4-(prop-2-ynyloxy)phenyl)propanoate **2a** has been previously reported (30)

Synthesis of (3-(4-(prop-2-ynyloxy)phenyl)propanoic acid) **2b** has also been previously reported (30).

Synthesis of Azide Linker (3-(4-(2-azidoethoxy)phenyl)propanoic acid) 3

To a solution of methyl 3-(4-hydroxyphenyl)propanoate (1.699 g, 9.43 mmol) in dry acetone (47.5 mL) was added anhydrous K_2CO_3 (3.909 g, 0.0283 mole) followed by 1-bromo-2-chloroethane (1.56 mL, 0.0189 mole). The resulting suspension was refluxed for 43 h with vigorous stirring. The reaction mixture was cooled to room temperature and the salt was removed by filtration followed by washing with portions of EtOAc (3 × 70 mL). The crude material was purified by silica chromatography (25:75 EtOAc:Hexane) and the solvent was removed under vacuum to give the desired product, methyl 3-(4-(2-chloroethoxy)phenyl)propanoate **3a**, as an oil (0.75 g, 33%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12 (d, *J* = 8.7, 2H), 6.84 (d, *J* = 8.7, 2H), 4.21 (t, *J* = 5.9, 2H), 3.80 (t, *J* = 5.9, 2H), 3.66 (s, 3H), 2.90 (t, *J* = 7.8, 2H), 2.60 (t, *J* = 7.8, 2H).

To a solution of methyl 3-(4-(2-chloroethoxy)phenyl)propanoate **3a** (0.75 g 3.1 mmol) in anhydrous DMF (6.1 mL) was added 18-crown-6 (3.4 mg, 0.013 mmol) and sodium azide (0.44 g, 6.8 mmol). The resulting solution was heated at 78 °C for 11 h. The reaction mixture was cooled to room temperature, diluted with ethyl acetate (50 mL), washed with a saturated $NaHCO_3$ solution (4 × 70 mL), and then dried over $MgSO_4$. The solvent was removed under vacuum to give methyl 3-(4-(2-azidoethoxy)phenyl)propanoate **3b** as a yellow oil (0.58 g, 75%) ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12 (d, *J* = 8.6, 2H), 6.85 (d, *J* = 8.6, 2H), 4.13 (t, *J* = 5.0 2H), 3.67 (s, 3H), 3.58 (t, *J* = 5.0, 2H), 2.90 (t, *J* = 7.8, 2H), 2.60 (t, *J* = 7.8, 2H).

To a solution of methyl 3-(4-(2-azidoethoxy)phenyl)propanoate **3b** (3.88 g, 0.0156 mole) in methanol (102 mL) was added potassium hydroxide (2 M, 28.3 mL, 0.0566 mole). The resulting solution was refluxed at 70 °C for 3 h. The solution was cooled to room temperature and condensed under reduced pressure. The residue was dissolved in water (30 mL) and was acidified by addition of 1N HCl to pH 1. The white cloudy solution was diluted with EtOAc. Layers were separated and the aqueous layer was extracted with EtOAc (2 × 70 mL). The combined organic extracts were washed with a saturated NaCl solution and dried over $MgSO_4$. Solvent was evaporated under vacuum to give the (3-(4-(2-azidoethoxy)phenyl)propanoic acid) **3c** as a white solid (3.44 g, 94%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.14 (d, *J* = 8.5, 2H), 6.86 (d, *J* = 8.5, 2H), 4.13 (t, *J* = 5.0 2H), 3.58 (t, *J* = 5.0, 2H), 2.91 (t, *J* = 7.7, 2H), 2.65 (t, *J* = 7.7, 2H).

Synthesis of Small Molecule Model System 4

The methyl-ester forms of the Alkyne Linker **2a** (448.0 mg, 1.80 mmol) and Azide Linker **3b** (371.5 mg, 1.70 mmol) were dissolved in a mixture of THF (1.6 mL) and water (0.4 mL). To this mixture was added copper sulfate pentahydrate (43.1 mg, 86.0μmol) and sodium ascorbate (170.9 mg, 431 μmol). The resulting reaction mixture was stirred at room temperature for 24 hrs. The solution was then diluted in EtOAc (60 mL) and water (60mL). Layers were separated and the aqueous layer was extracted twice with EtOAc solution (2 × 70 mL). The combined organic extracts were washed with a saturated $NaHCO_3$ solution (3 × 70 mL) and then with saturated NaCl solution (2 × 70 mL). The organic extracts were finally dried over $MgSO_4$. Solvent was evaporated under reduced pressure to give the desired product **4** as a white solid (0.54 g, 95%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.80 (s, 1H), 7.108 (overlapping d, *J* = 8.4, 4H), 6.91 (d, *J* = 8.6, 2H), 6.78 (d, *J* = 8.6, 2H), 5.18 (s, 2H), 4.75 (t, *J* = 5.0, 2H), 4.33 (t, *J* = 5.0, 2H), 3.66 (s, 3H), 3.657 (s, 3H), 2.890 (t, *J* = 7.8, 2H), 2.88 (t, *J* = 7.7, 2H), 2.59 (t, *J* = 7.8, 2H), 2.59 (t, *J* = 7.7, 2H).

Synthesis of G5-$Ac_{72\%}$-$Alkyne_{1.4}$ 5

A solution of partially acetylated dendrimer **1′** (54.6 mg, 1.78 μmol) was prepared with anhydrous DMSO (12.133 mL). The Alkyne Linker **2b** (0.9 mg, 4.4 μmol) was dissolved in DMSO (453 μL) and add to the dendrimer-DMSO solution. To this mixture was added N,N-Diisopropylethylamine (4.6 μL, 26 μmol) and the resulting solution was stirred for 45 minutes. Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (2.3 mg, 4.4 μmol) was dissolved in DMSO (462 μL) and added in a dropwise manner to the dendrimer/Alkyne Linker solution. The resulting solution was stirred for 24 hrs. All reaction steps were carried out in glass flasks at room temperature under nitrogen.

The reaction mixture was purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of two cycles using 1× PBS and eight cycles using DI water. All cycles were 10 minutes at 5,000 rpm. The resulting product **5** was lyophilized for three days to yield a white solid (41.5 mg, 75%). ^{1}H NMR integration determined an average of 1.4 Alkyne Linkers coupled to the dendrimer. The quantification of the number of linkers by NMR integration is described previously.(30) See Supporting Information for ^{1}H NMR spectrum of **5**.

Synthesis of G5-$Ac_{72\%}$-$Azide_{1.3}$ 6

A solution of partially acetylated dendrimer **1′** (60.5 mg, 1.97 μmol) was prepared with anhydrous DMSO (13.444 mL). The Azide Linker **3c** (1.2 mg, 4.9 μmol) was dissolved in DMSO (578 μL) and add to the dendrimer-DMSO solution. To this mixture was added N,N-Diisopropylethylamine (5.1 μL, 30 μmol) and the resulting solution was stirred for 45 minutes. Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (2.6 mg, 4.9 μmol) was dissolved in DMSO (511 μL) and added in a dropwise manner to the dendrimer/

Azide Linker solution. The resulting solution was stirred for 24 hrs. All reaction steps were carried out in glass flasks at room temperature under nitrogen.

The reaction mixture was purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of two cycles using 1× PBS and eight cycles using DI water. All cycles were 10 minutes at 5,000 rpm. The resulting product **6** was lyophilized for three days to yield a white solid (50 mg, 91%). ^{1}H NMR integration determined an average of 1.3 Alkyne Linkers coupled to the dendrimer. See Supporting Information for ^{1}H NMR spectrum of **6**.

Synthesis of Model Dendrimer System G5-Ac$_{72\%}$-L-G5Ac$_{72\%}$ 7

Partially acetylated dendrimer with an average of 1.4 Alkyne Linkers **5** (15.30 mg, 0.493 μmol) and partially acetylated dendrimer with an average of 1.3 Azide Linkers **6** (15.4 mg, 0.496 μmol) was dissolved in deuterium oxide (0.820 mL) and placed in a glass microwave reactor vessel. Sodium ascorbate (6.9 mg, 35 μmol) and copper sulfate pentahydrate (5.9 mg, 24 μmol) was added to the dendrimer solution. The resulting solution was placed in a microwave reactor for 40 seconds at 100 watts with a cut-off temperature of 100 °C. The microwave conditions were repeated for an additional 40 seconds. The cut-off temperature was then increased to 110 °C and the microwave was run at 100 watts for 2 minutes. The resulting crude product was a turbid yellow. The crude product was transferred to an NMR tube and analyzed by NOESY and ^{1}H NMR. After two days, the crude product in solution turned to a red-brown solution with a precipitate at the bottom of the NMR tube. NOESY and ^{1}H NMR experiments were repeated at this time point. The supernatant was separated from the precipitate and lyophilized to yield 4.9 mg of a brown solid. See Supporting Information for ^{1}H NMR spectrum of **7**.

Synthesis of G5-Ac$_{65\%}$-Alkyne$_{1.6}$ 8

The Alkyne Linker was conjugated to the partially acetylated dendrimer in two consecutive reactions. First, a stock solution of the Alkyne Linker **2b** (9.5 mg, 0.047 mmol) was generated with a mixture of DMF (6.198 mL) and DMSO (3.099 mL). To this mixture was added EDC (124.9 mg, 0.651 mmol). The resulting solution was stirred for 2 h at room temperature to create the active ester form of the Alkyne Linker.

A solution of partially acetylated dendrimer **1″** (58.8 mg, 1.930 μmol) was prepared with DI water (13.099 mL). The active ester form of the Alkyne Linker (5.784 mL, 0.0289 mmol) in DMF/DMSO was added in a dropwise manner (0.13 mL/min) to the dendrimer-water solution. The resulting reaction mixture was stirred for 2 days. All reaction steps were carried out in glass flasks at room temperature under nitrogen. The reaction mixture was purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of five cycles using 1× PBS and five cycles using DI water. All cycles were 30 minutes at 5,000 rpm. The resulting product **8** was lyophilized for three days to yield a white solid (55 mg, 92%). ^{1}H NMR integration determined an average of 1.6 Alkyne Linkers coupled to the dendrimer. See Supporting Information for ^{1}H NMR spectrum of **8**.

Synthesis of G5-Ac$_{65\%}$-Azide$_{2.5}$ 9

The Azide Linker was conjugated to the partially acetylated dendrimer in two consecutive reactions. First, a stock solution of the Azide Linker **3c** (7.6 mg, 0.032 mmol) was generated with a mixture of DMF (4.96 mL) and DMSO (2.48 mL). To this mixture was added EDC (86.7 mg, 0.452 mmol). The resulting solution was stirred for 1.75 h at room temperature to create the active ester form of the Azide Linker.

A solution of partially acetylated dendrimer **1‴** (58.8 mg, 1.930 μmol) was prepared with DI water (13.10 mL). The active ester form of the Azide Linker (6.66 mL, 0.0289 mmol) in DMF/DMSO was added in a dropwise manner (0.13 mL/min) to the first dendrimer-water aliquot. The resulting mixture was stirred for 2 days. All reaction steps were carried out in glass flasks at room temperature under nitrogen. The reaction mixture was purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of five cycles using 1× PBS and five cycles using DI water. All cycles were 10 minutes at 5,000 rpm. The resulting product **9** was lyophilized for three days to yield a white solid (55 mg, 89%). ^{1}H NMR integration determined an average of 2.5 Azide linkers coupled to the dendrimer. See Supporting Information for ^{1}H NMR spectrum of **9**.

Synthesis of G5-Ac$_{65\%}$-Alkyne$_{1.6}$-FA$_{1.7}$ 10

Folic acid was conjugated to the Alkyne Linker-conjugated dendrimer **8** in two consecutive reactions. First, a solution of folic acid (1.9 mg, 4.26 μmol) was generated with a mixture of DMF (1.23 mL) and DMSO (0.62 mL). To this mixture was added EDC (11.4 mg, 59.7 μmol). The resulting solution was stirred for 1 h at room temperature to create the active ester form of folic acid.

A solution of partially acetylated dendrimer with an average number of 1.6 Alkyne Linkers **8** (20.8 mg, 0.752 μmol) was prepared with DI water (4.638 mL). The active ester form of folic acid (1.85 mL, 4.26 μmol) in DMF/DMSO was added in a dropwise manner to the dendrimer-water solution. The resulting reaction mixture was stirred for 3 days. All reaction steps were carried out in glass flasks at room temperature under nitrogen. The reaction mixture was purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of five cycles using 1× PBS and four cycles using DI water. All cycles were 10 minutes at 5,000 rpm. The resulting product **10** was lyophilized for three days to yield a yellow solid (15.6 mg, 73%). ^{1}H NMR integration determined an average of 1.7 folic acid molecules coupled to the dendrimer. See Supporting Information for ^{1}H NMR spectrum of **10**.

Synthesis of G5-Ac$_{65\%}$-Azide$_{2.5}$-FITC$_{3.2}$ 11

Partially acetylated dendrimer with an average number of 2.5 Azide Linkers **9** (21.5 mg, 0.694 μmol) was dissolved in DMSO (1.5 mL). Fluorescein isothiocyanate (1.4 mg, 3.5 μmol) was dissolved in DMSO (0.54 mL) and added in a dropwise fashion to the dendrimer solution. The resulting mixture was stirred for 24 hours at room temperature. The reaction mixture was purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of six cycles using 1× PBS and six cycles using DI water. All 1× PBS cycles were 15 minutes at 5,000 rpm and all DI water cycles were 15 minutes at 5,000 rpm. HPLC analysis of the lyophilized product detected un-conjugated FITC remaining in the sample. To remove the remaining un-reacted FITC, the conjugate was purified by size exclusion chromatography using Sephadex G-25 beads in 1× PBS. The dendrimer fraction was collected and the elution buffer was exchanged with DI water using 10,000 MWCO centrifugal filtration devices (four cycles of 10 minutes at 5,000 rpm). The purified product **11** was lyophilized to yield a yellow-orange solid (10.1 mg, 46%). ^{1}H NMR integration determined an average of 3.2 FITC coupled to the dendrimer. See Supporting Information for ^{1}H NMR spectrum of **11**.

Synthesis of G5-Ac$_{65\%}$-Alkyne$_{1.6}$-FA$_{3.5}$ 12

Additional folic acid was conjugated to the partially acetylated dendrimer with an average of 1.6 Alkyne Linkers and 1.7 folic acid molecules **10** in two consecutive reactions. First, a solution of folic acid (1.1 mg, 2.4 μmol) was generated with a mixture of DMF (0.69 mL) and DMSO (0.34 mL). To this mixture was added EDC (6.3 mg, 33 μmol). The resulting

solution was stirred for 1 h at room temperature to create the active ester form of the folic acid.

A solution of partially acetylated dendrimer with an average number of 1.6 Alkyne Linkers and 1.7 folic acid molecules **10** (8.5 mg, 0.30 μmol) was prepared with DI water (1.90 mL). The active ester form of folic acid (1.03 mL, 2.4 μmol) in DMF/DMSO was added in a dropwise manner to the dendrimer-water solution. The resulting reaction mixture was stirred for 3 days. All reaction steps were carried out in glass flasks at room temperature under nitrogen. The reaction mixture was purified by size exclusion chromatography using Sephadex G-25 in 1× PBS. The dendrimer fraction was collected and the elution buffer was exchanged with DI water using 10,000 MWCO centrifugal filtration devices (four cycles of 10 minutes at 5,000 rpm). The purified product **12** was lyophilized for three days to yield a yellow solid (7.0 mg, 80%). ^{1}H NMR integration determined an average of 3.5 folic acid molecules coupled to the dendrimer. See Supporting Information for ^{1}H NMR spectrum of **12**.

Synthesis of G5-Ac_{107}-$Alkyne_{1.6}$-$FA_{3.5}$ 13

Partially acetylated dendrimer with an average number of 1.6 Alkyne Linkers and 3.5 folic acid **12** (7.0 mg, 0.22 μmol) was dissolved in anhydrous methanol (1.124 mL). Triethylamine (1.7 μL, 0.012 mmol) was added to this mixture and stirred for 30 minutes. Acetic anhydride (0.9 μL, 9.6 μmol) was added in a dropwise manner to the dendrimer solution. The reaction was carried out in a glass flask, under nitrogen, at room temperature for 24 hours. Methanol was evaporated from the resulting solution and the product was purified by size exclusion chromatography using Sephadex LH-20 in methanol. The purified dendrimer **13** was lyophilized for three days to yield a yellow solid (6.6 mg, 90%). ^{1}H NMR integration determined the degree of acetylation to be 100%. See Supporting Information for ^{1}H NMR spectrum of **13**.

Synthesis of G5-Ac_{106}-$Azide_{2.5}$-$FITC_{3.2}$ 14

Partially acetylated dendrimer with an average number of 2.5 Azide Linkers and 3.2 FITC **11** (7.5 mg, 0.23 μmol) was dissolved in anhydrous methanol (1.21 mL). Triethylamine (1.8 μL, 0.013 mmol) was added to this mixture and stirred for 30 minutes. Acetic anhydride (1.0 μL, 10.0 μmol) was added in a dropwise manner to the dendrimer solution. The reaction was carried out in a glass flask, under nitrogen, at room temperature for 24 hours. Methanol was evaporated from the resulting solution and the product was purified by size exclusion chromatography using Sephadex LH-20 in methanol. The purified dendrimer **14** was lyophilized for three days to yield a yellow solid (7.1 mg, 91%). ^{1}H NMR integration determined the degree of acetylation to be 100%. See Supporting Information for ^{1}H NMR spectrum of **14**.

Synthesis of Folic Acid Targeted Dendrimer System $FA_{3.5}$-G5-Ac_{107}-L-G5-Ac_{106}-$FITC_{3.2}$ 15

Dendrimer with an average of 1.6 Alkyne Linkers and 3.5 folic acid molecules **13** (3.1 mg, 91 nmol) and dendrimer with an average of 2.5 Azide Linkers and 3.2 FITC molecules **14** (3.0 mg, 88 nmol) were dissolved in deuterium oxide (0.65 mL) and placed in a glass microwave reactor vessel. Sodium ascorbate (1.1 mg, 4.5 μmol) and copper sulfate pentahydrate (1.1 mg, 5.4 μmol) was added to the dendrimer solution. The resulting solution was placed in a microwave reactor for 6.5 minutes at 100 watts with a cut-off temperature of 100 °C. The reaction mixture was transferred to an NMR tube and analyzed by NOESY and ^{1}H NMR spectroscopy using. Lyophilization yielded 6.7 mg of a red solid. See Supporting Information for ^{1}H NMR spectrum of **15**.

Synthesis of G5-$Ac_{67\%}$-$Alkyne_{1.3}$ 16

A solution of partially acetylated dendrimer **1‴** (176.7 mg, 5.8 μmol) was prepared with anhydrous DMSO (39.27 mL). The Alkyne Linker **2b** (2.6 mg, 13 μmol) was dissolved in DMSO (1.31 mL) and add to the dendrimer-DMSO solution. To this mixture was added N,N-Diisopropylethylamine (13.4 μL, 76.7 μmol) and the resulting solution was stirred for 45 minutes. Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (6.7 mg, 13 μmol) was dissolved in DMSO (1.331 mL) and added in a dropwise manner to the dendrimer/Alkyne Linker solution. The resulting solution was stirred for 24 hrs. All reaction steps were carried out in glass flasks at room temperature under nitrogen.

The reaction mixture was purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of two cycles using 1× PBS and eight cycles using DI water. All cycles were 10 minutes at 5,000 rpm. The resulting product **5** was lyophilized for three days to yield a white solid (116.2 mg, 65.2%). ^{1}H NMR integration determined an average of 1.3 Alkyne Linkers coupled to the dendrimer. See Supporting Information for ^{1}H NMR spectrum of **16**.

Synthesis of G5-$Ac_{110.7}$-$Alkyne_{1.3}$ 17

Partially acetylated dendrimer with an average number of 1.3 Alkyne Linkers **16** (22.6 mg, 0.737 μmol) was dissolved in anhydrous methanol (3.0 mL). Triethylamine (5.8 μL, 0.042 mmol) was added to this mixture and stirred for 30 minutes. Acetic anhydride (3.2 μL, 34 μmol) was added in a dropwise manner to the dendrimer solution. The reaction was carried out in a glass flask, under nitrogen, at room temperature for 24 hours. Methanol was evaporated from the resulting solution and the product was purified by size exclusion chromatography using Sephadex LH-20 in methanol. The purified dendrimer **17** was lyophilized for three days to yield a white solid (19.1 mg, 80.5%). ^{1}H NMR integration determined the degree of acetylation to be 100%. See Supporting Information for ^{1}H NMR spectrum of **17**.

Synthesis of Un-targeted Dendrimer System G5-$Ac_{110.7}$-L-G5-Ac_{106}-$FITC_{3.2}$ 18

Dendrimer with an average of 1.3 Alkyne Linkers **17** (1.0 mg, 31 nmol) and dendrimer with an average of 2.5 Azide Linkers and 3.2 FITC molecules **14** (1.0 mg, 29 nmol) were dissolved in deuterium oxide (0.74 mL) and placed in a glass microwave reactor vessel. Sodium ascorbate (0.36 mg, 1.8 μmol) and copper sulfate pentahydrate (0.38 mg, 1.5 μmol) was added to the dendrimer solution. The resulting solution was placed in a microwave reactor for 6.5 minutes at 100 watts with a cut-off temperature of 100 °C. The reaction mixture was transferred to an NMR tube and analyzed by NOESY and ^{1}H NMR spectroscopy using. Lyophilization yielded 2.4 mg of a red solid. See Supporting Information for ^{1}H NMR spectrum of **18**.

Results and Discussion

Synthesis and characterization of the small-molecule model system

A small molecule model system (**4**) was first synthesized to facilitate the spectroscopic assignment of triazole-related atoms resulting from successful ‘click’ reactions between dendrimer modules with an azide linker (**3c**) and dendrimer modules possessing an alkyne linker (**2b**). This model system utilized the methyl ester forms of the two linkers (**2a** and **3b**). Proton assignments were based upon ^{1}H NMR and NOESY experiments in $CDCl_3$. Figure 1, panel a, displays the cross-peaks for the triazole related protons in the clicked product (**4**) and Table 1 contains the chemical shifts for these in both the pre- and post-‘click’ reaction states. Protons c, e, and f experience the greatest change in chemical shift as

a result of the ‘click’ reaction. Also of interest is the region between 6.4 and 8.5 ppm (Figure 2, panel a and b), which shows the up-field shift for peak g from 6.85 ppm to 6.78 ppm.

Synthesis and characterization of the model dendrimer system

Dendrimers without target or dye functionalities, possessing only the ‘click’ reaction functional groups (**5** and **6**), were employed to develop ‘click’ reaction conditions (Figure 3). Because many of the proton peaks associated with the dendrimer-conjugated alkyne and azide linkers (particularly those closest to the ‘click’ reaction sites) overlap in the ^{1}H NMR spectra with other protons belonging to the PAMAM dendrimer, NOESY experiments were used to document proton chemical shifts via the resolution of cross peaks in the 2-D spectra (Figure 1, panel b). The chemical shifts for the triazole related protons in the dendrimer system both pre- and post-‘click’ reaction can be found in Table 1. The region between 6.4 and 8.5 ppm in the proton spectra for the pre- and post-‘click’ed dendrimer samples can be found in Figure 2, panel c and d. In the spectra for the pre-‘click’ reaction mixture (panel c), both sets of aromatic protons overlap at 6.90 ppm (b and g) and 7.13 ppm (a and h). In the sample post-‘click’ reaction (panel d), the aromatic protons no longer overlap. Protons a and h partially overlap at 7.09 ppm and 7.06 ppm, and protons b and g are found at 6.90 ppm and 6.74 ppm, respectively.

A comparison of the chemical shifts in Table 1 for the small molecule system and the dendrimer system reveals good correlations for both the pre- and post-reaction states. This indicates that a successful ‘click’ reaction has occurred between the azide and alkyne conjugated dendrimers. It is important to note that whereas chemical shifts for the small molecule model system are determined in $CDCl_3$, the chemical shifts for the dendrimer sample were detected in D_2O. Although the different solvents could influence the proton chemical shifts, this does not appear to be an issue for these particular molecules.

A peak for the single proton in the triazole ring is absent from both the NOESY and 1D experiments. In the small molecule system this peak is found at 7.80 ppm (Figure 2, panel b). Working with a similar system using PAMAM dendrons that contained the alkyne and azide moieties at the dendron focal point, Lee et. al. found that the triazole proton peak gradually shifted down-field from 7.77 ppm to 7.93 ppm as the generation of the clicked dendrons increased from 1 to 3 (47). If this downfield change in chemical shift also holds for the generation 5 dendrimer case, the triazole proton would be overlapped by several peaks between 7.80 ppm and 8.20 ppm associated with the dendrimer (Figure 2, panel d). The NOESY spectra did not expose any cross peaks in this region with other protons in the linkers that are in close proximity to the triazole ring. The cross-peaks associated with the triazole proton appear to be below the intensity required for NMR detection.

Synthesis and characterization of the folic acid targeted dendrimer system

Synthesis of the folic acid targeted modular dendrimer platform is outlined in Figure 4. Dendrimers with an average of 1.6 alkyne linkers (**8**) were functionalized with the targeting molecule folic acid. The formation of an amide linkage between one of the remaining primary amines on the dendrimer and one of the carboxylic acid groups in folic acid was achieved by EDC coupling chemistry as previously reported (22). This reaction conjugated an average of 1.7 folic acid molecules per dendrimer as determined by NMR (**10**). Because this average was below the optimal range for multi-valent binding (3), the reaction was repeated using the dendrimer with an average of 1.6 alkyne linkers and 1.7 folic acid (**10**). The second reaction resulted in the addition of 1.8 folic acid molecules per dendrimer bringing the final average to 3.5 folic acid molecules per dendrimer (**12**). The remaining dendrimer primary amines were then fully acetylated to avoid positive charge-based cellular interactions (**13**) (6, 9, 10). Dendrimers with 2.5 azide linkers (**9**) were functionalized with

the dye molecule FITC. Using conditions similar to previously published work (25), an average of 3.2 FITC molecules were coupled to the dendrimer via the formation of a thiourea bond between the primary amine on the dendrimer and the isothiocyanate group in FITC (**11**). This dendrimer conjugate also was fully acetylated (**14**). Reverse phase HPLC confirmed that any un-reacted FITC or folic acid molecules had been removed by purification of dendrimer **13** and **14**.

The two dendrimer modules (**13** and **14**) were coupled together using the Cu-catalyzed 1,3-dipolar cycloaddition reaction under conditions similar to those used with the model dendrimer system. NOESY experiments provided direct spectroscopic proof that the functionalized dendrimers had been clicked together. Specifically, the AA′BB′ pattern was observed to shift in a fashion identical to that observed for the two model systems previously described (Table 1 and Figure 2).

In vitro testing of the folic acid targeted dendrimer system with KB cells

Cellular uptake of the folic acid targeted dendrimer system (**15**) at four different concentrations (30 nM, 100 nM, 300 nM, and 1000 nM of **15**) was measured in KB cells that express a high cellular membrane concentration of folic acid receptor (FAR). Fluorescence uptake was quantified by Flow Cytometry. As seen in Figure 5a and 5f-blue, a dose dependent uptake was observed with saturation occurring at 100 nM. This binding affinity is consistent with previous studies on single dendrimer platforms possessing multiple FITC and multiple FA molecules (23).

A series of control experiments were performed in order to ensure that uptake of the folic acid targeted dendrimer system (**15**) was occurring via receptor-mediated endocytosis and not non-specific membrane interactions. The first set of controls measured uptake of single dendrimers possessing the azide linker and multiple FITC (**14**) at 30 nM, 100 nM, 300 nM, and 1000 nM (Figure 5b and 5f-purple). No uptake was observed for this sample above the background level. The second control sample contained a non-conjugated (un-clicked) mixture of the two dendrimers functionalized with either FITC or folic acid (**13** and **14**). Uptake of this mixture was quantified at 30 nM, 100 nM, and 300 nM (Figure 5c and 5f-teal). At all three concentrations, no florescence uptake was observed. This control eliminates the possibility that the dendrimer modules could form a non-covalently linked complex that would be internalized. A third control sample (**18**) was composed of an un-targeted dendrimer module (**17**) coupled to the FITC conjugated imaging module (**14**). The un-targeted dual module platform (**18**) was assembled under the same conditions used to form the folic acid targeted platform (**15**). Mean fluorescence uptake of the un-targeted platform can be found in the supporting information. At concentrations up to 300 nM, no uptake was observed beyond the background level. This un-targeted dendrimer platform is an essential control because it matches the molecular weight and size of the folic acid targeted dendrimer system (**15**). This control will become even more important as evaluation of this system moves to an *in vivo* model.

The final set of controls investigated active blocking of the folic acid receptor by either free folic acid (Figure 5d and 5f-green) or a folic acid-dendrimer conjugate without a fluorescent dye (**13**) (Figure 5e and 5f-orange) to prevent the specific up-take of the folic acid targeted dendrimer system. A 20 fold excess of blocking agent was employed relative to the targeted platform. For the blocking experiment using the single dendrimer-folic acid conjugate (**13**), molar equivalence was based on the folic acid content of the sample rather than the dendrimer content. Both blocking agents were evaluated at 30 nM, 100 nM, 300 nM, and 1000 nM. Complete blocking is achieved using free folic acid concentrations up to 100 nM. While the dendrimer-folic acid conjugate is not as effective at blocking as the free folic acid, approximately 75% blocking is achieved. These binding data indicate that the cellular

association of the folic acid targeted dendrimer system occurs through the folic acid receptor rather than via non-specific interactions. On a more fundamental level, the biological results prove again that the folic acid conjugate dendrimer module is covalently linked by 'click' chemistry to dendrimer module functionalized with FITC.

Design Analysis

One of the limitations of the modular platform design in its current iteration is that each module is composed of a heterogeneous distribution of dendrimer-ligand components (30, 48-50). To a first approximation, these distributions are consistent with Poissonian statistics. Not only are the modules composed of a distribution of dendrimer particles with different numbers of the biologically active ligand (folic acid, FITC), the modules are also composed of a distribution of dendrimer with different numbers of the coupling ligands (Azide and Alkyne). The implication of these distributions is that the final 'dual' module platform is most likely composed of a large number of different platforms with different numbers of modules linked together and different numbers of ligands per module. Future designs should incorporate characterization and control of these distributions in order to make a more controlled, reproducible material.

Conclusion

A delivery platform covalently linking two different PAMAM dendrimer modules through 'click' chemistry was successfully designed and synthesized. Two model systems were also generated to assist in the NMR spectroscopy characterization of the modular platform. The folic acid targeted system was evaluated for functional activity *in vitro* with KB cells and found to selectively target the cancer cell line through the high-affinity folate receptor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported with Federal funds from the National Cancer Institute, National Institutes of Health, under award 1 R01 CA119409.

References

1. Allen TM. Ligand-targeted therapeutics in anticancer therapy. Nature Reviews Cancer. 2002; 2:750–763.
2. Peer D, Karp JM, Hong S, FaroKhzad OC, Margalit R, Langer R. Nanocarriers as an emerging platform for cancer therapy. Nature Nanotechnology. 2007; 2:751–760.
3. Hong S, Leroueil PR, Majoros IJ, Orr BG, Baker JR, Holl MMB. The binding avidity of a nanoparticle-based multivalent targeted drug delivery platform. Chemistry & Biology. 2007; 14:105–113.
4. Mammen M, Choi SK, Whitesides GM. Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors. Angewandte Chemie-International Edition. 1998; 37:2755–2794.
5. Majoros IJ, Keszler B, Woehler S, Bull T, Baker JR. Acetylation of poly(amidoamine) dendrimers. Macromolecules. 2003; 36:5526–5529.
6. Hong SP, Bielinska AU, Mecke A, Keszler B, Beals JL, Shi XY, Balogh L, Orr BG, Baker JR, Holl MMB. Interaction of poly(amidoamine) dendrimers with supported lipid bilayers and cells: Hole formation and the relation to transport. Bioconjugate Chemistry. 2004; 15:774–782. [PubMed: 15264864]

7. Lee CC, MacKay JA, Frechet JMJ, Szoka FC. Designing dendrimers for biological applications. Nature Biotechnology. 2005; 23:1517–1526.
8. Svenson S, Tomalia DA. Commentary - Dendrimers in biomedical applications - reflections on the field. Advanced Drug Delivery Reviews. 2005; 57:2106–2129. [PubMed: 16305813]
9. Hong SP, Leroueil PR, Janus EK, Peters JL, Kober MM, Islam MT, Orr BG, Baker JR, Holl MMB. Interaction of polycationic polymers with supported lipid bilayers and cells: Nanoscale hole formation and enhanced membrane permeability. Bioconjugate Chemistry. 2006; 17:728–734. [PubMed: 16704211]
10. Leroueil PR, Hong SY, Mecke A, Baker JR, Orr BG, Holl MMB. Nanoparticle interaction with biological membranes: Does nanotechnology present a janus face? Accounts of Chemical Research. 2007; 40:335–342. [PubMed: 17474708]
11. Patri AK, Myc A, Beals J, Thomas TP, Bander NH, Baker JR. Synthesis and in vitro testing of J591 antibody-dendrimer conjugates for targeted prostate cancer therapy. Bioconjugate Chemistry. 2004; 15:1174–1181. [PubMed: 15546182]
12. Shukla R, Thomas TP, Peters JL, Desai AM, Kukowska-Latallo J, Patri AK, Kotlyar A, Baker JR. HER2 specific tumor targeting with dendrimer conjugated anti-HER2 mAb. Bioconjugate Chemistry. 2006; 17:1109–1115. [PubMed: 16984117]
13. Wu G, Barth RF, Yang WL, Kawabata S, Zhang LW, Green-Church K. Targeted delivery of methotrexate to epidermal growth factor receptor-positive brain tumors by means of cetuximab (IMC-C225) dendrimer bioconjugates. Molecular Cancer Therapeutics. 2006; 5:52–59. [PubMed: 16432162]
14. Wu G, Barth RF, Yang WL, Chatterjee M, Tjarks W, Ciesielski MJ, Fenstermaker RA. Site-specific conjugation of boron-containing dendrimers to anti-EGF receptor monoclonal antibody cetuximab (IMC-C225) and its evaluation as a potential delivery agent for neutron capture therapy. Bioconjugate Chemistry. 2004; 15:185–194. [PubMed: 14733599]
15. Backer MV, Gaynutdinov TI, Patel V, Bandyopadhyaya AK, Thirumamagal BTS, Tjarks W, Barth RF, Claffey K, Backer JM. Vascular endothelial growth factor selectively targets boronated dendrimers to tumor vasculature. Molecular Cancer Therapeutics. 2005; 4:1423–1429. [PubMed: 16170035]
16. Shukla R, Thomas TP, Peters J, Kotlyar A, Myc A, Baker JR. Tumor angiogenic vasculature targeting with PAMAM dendrimer-RGD conjugates. Chemical Communications. 2005:5739–5741. [PubMed: 16307130]
17. Sheng KC, Kalkanidis M, Pouniotis DS, Esparon S, Tang CK, Apostolopoulos V, Pietersz GA. Delivery of antigen using a novel mannosylated dendrimer potentiates immunogenicity in vitro and in vivo. European Journal of Immunology. 2008; 38:424–436. [PubMed: 18200633]
18. Baek MG, Roy R. Synthesis and protein binding properties of T-antigen containing GlycoPAMAM dendrimers. Bioorganic and Medicinal Chemistry. 2002; 10:11–17. [PubMed: 11738602]
19. Taite LJ, West JL. Poly(ethylene glycol)-lysine dendrimers for targeted delivery of nitric oxide. Journal of Biomaterials Science-Polymer Edition. 2006; 17:1159–1172.
20. Kono K, Liu MJ, Frechet JMJ. Design of dendritic macromolecules containing folate or methotrexate residues. Bioconjugate Chemistry. 1999; 10:1115–1121. [PubMed: 10563782]
21. Shukla S, Wu G, Chatterjee M, Yang WL, Sekido M, Diop LA, Muller R, Sudimack JJ, Lee RJ, Barth RF, Tjarks W. Synthesis and biological evaluation of folate receptor-targeted boronated PAMAM dendrimers as potential agents for neutron capture therapy. Bioconjugate Chemistry. 2003; 14:158–167. [PubMed: 12526705]
22. Majoros IJ, Myc A, Thomas T, Mehta CB, Baker JR. PAMAM dendrimer-based multifunctional conjugate for cancer therapy: Synthesis, characterization, and functionality. Biomacromolecules. 2006; 7:572–579. [PubMed: 16471932]
23. Thomas TP, Majoros IJ, Kotlyar A, Kukowska-Latallo JF, Bielinska A, Myc A, Baker JR. Targeting and inhibition of cell growth by an engineered dendritic nanodevice. Journal of Medicinal Chemistry. 2005; 48:3729–3735. [PubMed: 15916424]
24. Myc A, Douce TB, Ahuja N, Kotlyar A, Kukowska-Latallo J, Thomas TP, Baker JR. Preclinical antitumor efficacy evaluation of dendrimer-based methotrexate conjugates. Anti-Cancer Drugs. 2008; 19:143–149. [PubMed: 18176110]

25. Majoros IJ, Thomas TP, Mehta CB, Baker JR. Poly(amidoamine) dendrimer-based multifunctional engineered nanodevice for cancer therapy. Journal of Medicinal Chemistry. 2005; 48:5892–5899. [PubMed: 16161993]
26. Kukowska-Latallo JF, Candido KA, Cao ZY, Nigavekar SS, Majoros IJ, Thomas TP, Balogh LP, Khan MK, Baker JR. Nanoparticle targeting of anticancer drug improves therapeutic response in animal model of human epithelial cancer. Cancer Research. 2005; 65:5317–5324. [PubMed: 15958579]
27. Myc A, Patri AK, Baker JR. Dendrimer-based BH3 conjugate that targets human carcinoma cells. Biomacromolecules. 2007; 8:2986–2989. [PubMed: 17877400]
28. Myc A, Majoros IJ, Thomas TP, Baker JR. Dendrimer-based targeted delivery of an apoptotic sensor in cancer cells. Biomacromolecules. 2007; 8:13–18. [PubMed: 17206782]
29. Landmark KJ, DiMaggio S, Ward J, Kelly C, Vogt S, Hong S, Kotlyar A, Myc A, Thomas TP, Penner-Hahn JE, Baker JR, Holl MMB, Orr BG. Synthesis, characterization, and in vitro testing of superparamagnetic iron oxide nanoparticles targeted using folic acid-conjugated dendrimers. ACS Nano. 2008; 2:773–783. [PubMed: 19206610]
30. Mullen DG, Desai AM, Waddell JN, Cheng XM, Kelly CV, McNerny DQ, Majoros IJ, Baker JR, Sander LM, Orr BG, Banaszak Holl MM. The implications of stochastic synthesis for the conjugation of functional groups to nanoparticles. Bioconjug Chem. 2008; 19:1748–52. [PubMed: 18729391]
31. Choi YS, Mecke A, Orr BG, Holl MMB, Baker JR. DNA-directed synthesis of generation 7 and 5 PAMAM dendrimer nanoclusters. Nano Letters. 2004; 4:391–397.
32. Choi Y, Thomas T, Kotlyar A, Islam MT, Baker JR. Synthesis and functional evaluation of DNA-assembled polyamidoamine dendrimer clusters for cancer cell-specific targeting. Chemistry and Biology. 2005; 12:35–43. [PubMed: 15664513]
33. DeMattei CR, Huang BH, Tomalia DA. Designed dendrimer syntheses by self-assembly of single-site, ssDNA functionalized dendrons. Nano Letters. 2004; 4:771–777.
34. Choi, Y. Biomedical Engineering. University of Michigan; Ann Arbor, MI: 2005. p. 191
35. Lee JW, Kim JH, Kim HJ, Han SC, Kim JH, Shin WS, Jin SH. Synthesis of symmetrical and unsymmetrical PAMAM dendrimers by fusion between azide- and alkyne-functionalized PAMAM dendrons. Bioconjugate Chemistry. 2007; 18:579–584. [PubMed: 17335177]
36. Lee JW, Kim HJ, Han SC, Kim JH, Jin SH. Designing poly(amido amine) dendrimers containing core diversities by click chemistry of the propargyl focal point poly(amido amine) dendrons. Journal of Polymer Science Part a-Polymer Chemistry. 2008; 46:1083–1097.
37. Lee JW, Kim BK, Kim HJ, Han SC, Shin WS, Jin SH. Convergent synthesis of symmetrical and unsymmetrical PAMAM dendrimers. Macromolecules. 2006; 39:2418–2422.
38. Lee JW, Kim JH, Kim BK, Shin WS, Jin SH. Synthesis of Frechet type dendritic benzyl propargyl ether and Frechet type triazole dendrimer. Tetrahedron. 2006; 62:894–900.
39. Wu P, Malkoch M, Hunt JN, Vestberg R, Kaltgrad E, Finn MG, Fokin VV, Sharpless KB, Hawker CJ. Multivalent, bifunctional dendrimers prepared by click chemistry. Chemical Communications. 2005:5775–5777. [PubMed: 16307142]
40. Goyal P, Yoon K, Weck M. Multifunctionalization of dendrimers through orthogonal transformations. Chemistry-a European Journal. 2007; 13:8801–8810.
41. Yoon K, Goyal P, Weck M. Monofunctionalization of dendrimers with use of microwave-assisted 1,3-dipolar cycloadditions. Organic Letters. 2007; 9:2051–2054. [PubMed: 17472392]
42. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. A stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective “ligation” of azides and terminal alkynes. Angewandte Chemie-International Edition. 2002; 41:2596–+.
43. Wu P, Feldman AK, Nugent AK, Hawker CJ, Scheel A, Voit B, Pyun J, Frechet JMJ, Sharpless KB, Fokin VV. Efficiency and fidelity in a click-chemistry route to triazole dendrimers by the copper(I)-catalyzed ligation of azides and alkynes. Angewandte Chemie-International Edition. 2004; 43:3928–3932.
44. Wu P, Fokin VV. Catalytic azide-alkyne cycloaddition: Reactivity and applications. Aldrichimica Acta. 2007; 40:7–17.

45. Hoffman RE. Standardization of chemical shifts of TMS and solvent signals in NMR solvents. Magnetic Resonance in Chemistry. 2006; 44:606–616. [PubMed: 16534833]

46. Marie D, Brown SC. A Cytometric Exercise in Plant DNA Histograms, with 2c-Values for 70 Species. Biology of the Cell. 1993; 78:41–51. [PubMed: 8220226]

47. Lee JW, Kim JH, Kim BK, Kim JH, Shin WS, Jin SH. Convergent synthesis of PAMAM dendrimers using click chemistry of azide-functionalized PAMAM dendrons. Tetrahedron. 2006; 62:9193–9200.

48. Mullen DG, Fang M, Desai A, Baker JR, Orr BG, Holl MMB. A Quantitative Assessment of Nanoparticle-Ligand Distributions: Implications for Targeted Drug and Imaging Delivery in Dendrimer Conjugates. Acs Nano. 2010; 4:657–670. [PubMed: 20131876]

49. Mullen DG, Borgmeier EL, Fang M, McNerny DQ, Desai A, Baker JR, Orr BG, Banaszak Holl MM. Effect of mass transport in the synthesis of partially acetylated dendrimer: Implications for functional ligand-nanoparticle distributions. Macromolecules. 201010.1021/ma100663c

50. Mullen DG, Borgemeier EL, Desai A, van Dongen MA, Barash M, Cheng X, Baker JR, Banaszak Holl MM. Isolation and Characterization of Dendrimer with Precise Numbers of Functional Groups. Chemistry A European Journal. 201010.1002/chem.201001175

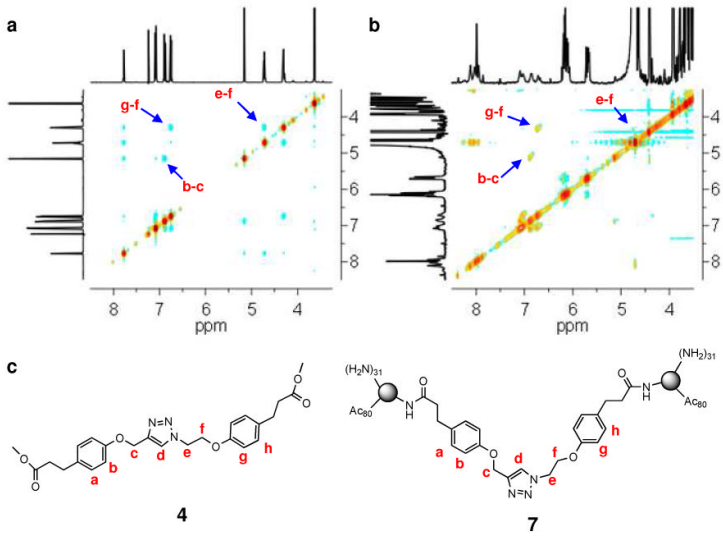

Figure 1.

a) NOESY of the small-molecule model system after the ‘click’ reaction (**4**). NOE cross-peaks between triazole related protons (b, c, e, f, and g) are labeled. **b)** NOESY of the model dendrimer system after the ‘click’ reaction (**7**). NOE cross-peaks for the triazole related protons are similarly labeled. The cross-peaks in the 2D spectra reveal proton chemical shifts for the several of the triazole related protons that are otherwise obscured by overlapping dendrimer peaks. **c)** Chemical structure and proton labels for the clicked small molecule model system (**4**) and the clicked dendrimer model system (**7**). The G5 PAMAM dendrimer is represented by a gray sphere.

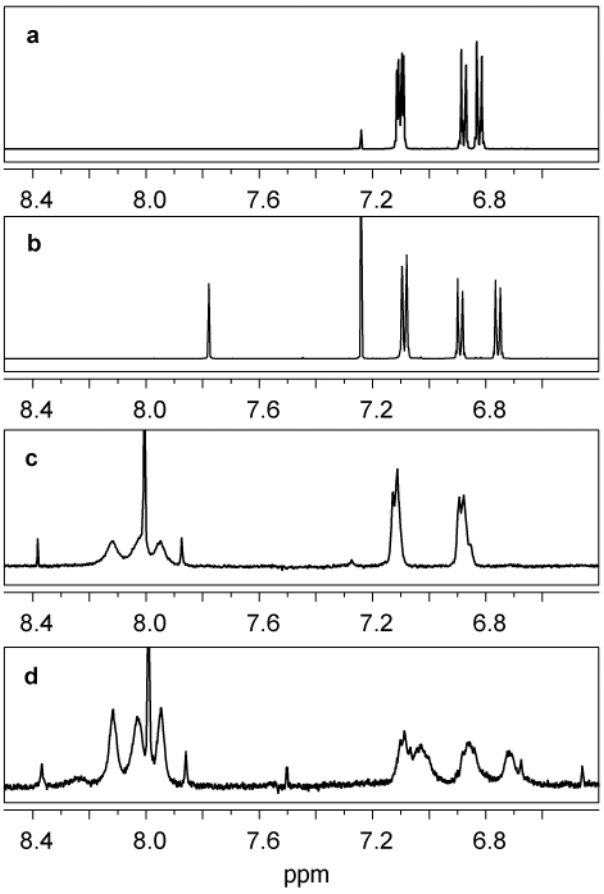

Figure 2.

Proton NMR spectra of the small-molecule model system and model dendrimer system both pre- and post-'click' reaction. An up-field shift is observed for aromatic proton g as a result of the 'click' reaction. **a)** Spectrum of the small molecule model system before the 'click' reaction (2a and 3b), taken in $CDCl_3$. Proton g has a chemical shift of 6.85 ppm. **b)** Spectrum of the small molecule model system after the 'click' reaction (4), taken in $CDCl_3$. As a result of the 'click' reaction, g has experienced an up-field change to 6.78 ppm. **c)** Spectrum of the model dendrimer system before the 'click' reaction (5 and 6), taken in D_2O. Proton g overlaps proton b at 6.90 ppm. **d)** Spectrum of the model dendrimer system after the 'click' reaction (7), taken in D_2O. Similar to the small molecule model system, proton g experiences an up-field change as a result of the 'click' reaction. In the model dendrimer system, the new chemical shift is 6.74 ppm.

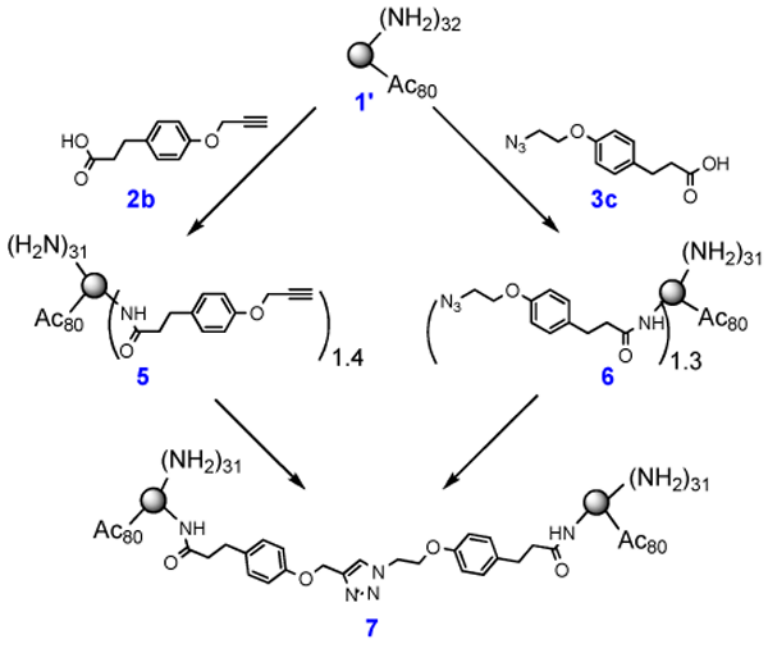

Figure 3.

Synthetic scheme for the model dendrimer system (**7**). This simplified modular platform was developed to assist with the spectroscopic characterization of the folic acid targeted dendrimer system. The G5 dendrimer, used in this study, had an average of 112 end groups as determined by GPC and potentiometric titration.

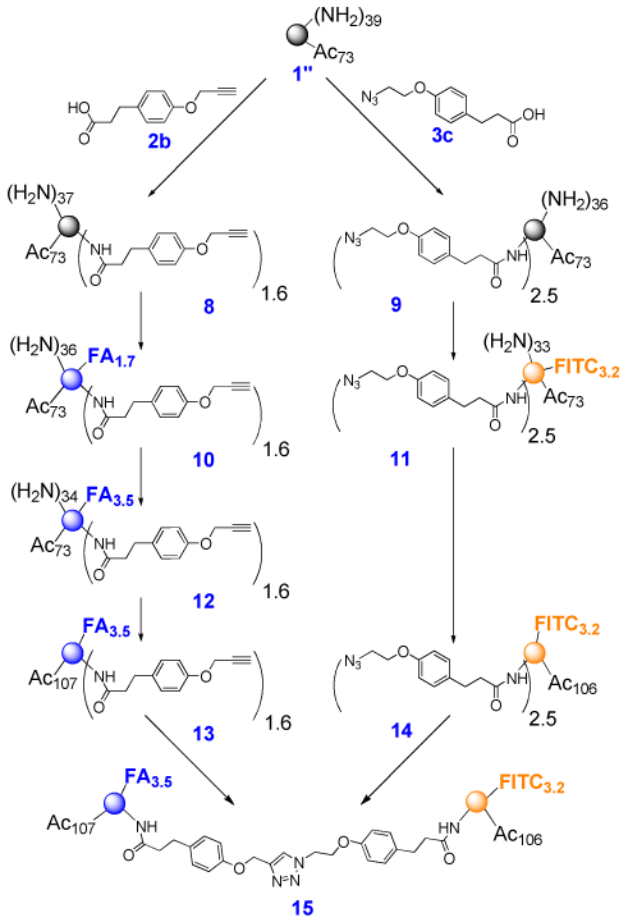

Figure 4.

Synthetic scheme for the folic acid targeted modular dendrimer-based platform (**15**). A module possessing a terminal alkyne moiety and Folic Acid (**13**) is coupled to a second module possessing a terminal azide moiety and FITC (14) via the Cu-catalyzed 1,3-dipolar cycloaddition reaction. Both modules were fully acetylated to avoid non-specific cellular interactions.

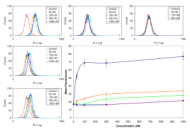

Figure 5.

Binding and uptake of the fluorescent modular targeted dendrimer platform in KB cells as measured by Flow Cytometry. **a)** Uptake $FA_{3.5}$-G5-Ac_{107}-L-G5Ac_{106}-$FITC_{3.2}$ (**15**). **b)** Uptake of G5-Ac_{106}-$Azide_{2.5}$-$FITC_{3.2}$ (**14**) is not observed for 30 nM, 100 nM, and 300 nM. Very minimal uptake of this un-targeted module is observed at 1000 nM. **c)** Similarly, no uptake is observed for an uncoupled mixture of G5-Ac_{106}-$Azide_{2.5}$-$FITC_{3.2}$ (14) and G5-Ac_{107}-$Alkyne_{1.6}$-$FA_{3.5}$ (**13**). **d)** Uptake of $FA_{3.5}$-G5-Ac_{107}-L-G5Ac_{106}-$FITC_{3.2}$ (**15**) is successfully blocked using a 20 fold excess of free folic acid. e) Uptake of $FA_{3.5}$-G5-Ac_{107}-L-G5Ac_{106}-$FITC_{3.2}$ (**15**) is also successfully blocked using a 20 fold excess (with respect to the folic acid content) of G5-Ac_{107}-$Alkyne_{1.6}$-$FA_{3.5}$ (**13**). **f**) Summary of mean fluorescence values for **a-e**. Uptake of $FA_{3.5}$-G5-Ac_{107}-L-G5Ac_{106}-$FITC_{3.2}$ (**15**) is displayed in blue. Uptake of the targeted platform (**15**) blocked by a 20 fold excess of G5-Ac_{107}-$Alkyne_{1.6}$-$FA_{3.5}$ (**13**) is shown in orange. Uptake of the targeted platform (**15**) blocked by a 20 fold excess of free folic acid can be found in green. Uptake of a mixture of G5-Ac_{106}-$Azide_{2.5}$-$FITC_{3.2}$ (14) and G5-Ac_{106}-$Alkyne_{1.6}$-$FA_{3.5}$ (**13**) can be found in teal. Finally, uptake of G5-Ac_{106}-$Azide_{2.5}$-$FITC_{3.2}$ (**14**) can be found in purple. Error bars indicate standard deviation as computed from half-peak coefficient of variation (HPCV) values.

Table 1

Good correlation is found between the small molecule model system (**2a**, **3b**, and **4**) and the dendrimer model system (**5**, **6**, and **7**) for the chemical shifts (ppm) of triazole related protons (a-h) both before and after the 'click' reaction. Chemical shifts for protons in the model dendrimer system were detected primarily via NOESY experiments.

	Compound			
	Before Reaction		After Reaction	
Proton	2a and 3b	5 and 6	4	7
a	7.14	7.13	7.11	7.09
b	6.91	6.90	6.91	6.90
c	4.67	4.69	5.19	5.15
d	n.a.	n.a.	7.80	--
e	3.58	3.61	4.75	4.76
f	4.13	4.13	4.33	4.35
g	6.85	6.90	6.78	6.74
h	7.13	7.13	7.11	7.06