ADVANCED HEALTHCARE MATERIALS

# Supporting Information

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## *N*-Acetylgalactosamine-Targeted Delivery of Dendrimer-Doxorubicin Conjugates Influences Doxorubicin Cytotoxicity and Metabolic Profile in Hepatic Cancer Cells

*Sibu P. Kuruvilla, Gopinath Tiruchinapally, Mahmoud ElAzzouny, and Mohamed E. H. ElSayed**

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## *N*-Acetylgalactosamine-targeted delivery of dendrimer-doxorubicin conjugates influences doxorubicin cytotoxicity and metabolic profile in hepatic cancer cells

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### 1. Synthesis of NAcGal$_\beta$-PEG*c*-G5-L(x)-DOX Particles:

**General Experimental Procedures:** All reactions were carried out under nitrogen with anhydrous solvents in flame-dried glassware, unless otherwise noted. All glycosylation reactions were performed in the presence of molecular sieves, which were flame-dried right before the reaction under high vacuum. Solvents were dried using a solvent purification system and used directly without further drying. Chemicals used were reagent grade as supplied except where noted. Analytical thin-layer chromatography was performed using silica gel 60 F254 glass plates. Compound spots were visualized by UV light (254 nm) and by staining with a yellow solution containing $Ce(NH_4)_2(NO_3)_6$ (0.5 g) and $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (24.0 g) in 6% $H_2SO_4$ (500 mL). Flash column chromatography was performed on silica gel 60 (230–400Mesh). NMR spectra were referenced using $Me_4Si$ (0 ppm), residual $CHCl_3$ (δ $^1$H-NMR 7.26 ppm, $^{13}$C-NMR 77.0 ppm, $CD_3OD$ (δ $^1$H-NMR 3.30 ppm, $^{13}$C-NMR 49.00 ppm, $CD_3SOCD_3$ (δ $^1$H-NMR 2.49 ppm, $^{13}$C-NMR 39.5 ppm and $D_2O$ (δ $^1$H-NMR 4.56 ppm). Peak and coupling constant assignments are based on $^1$H-NMR.

**Characterization of anomeric stereochemistry:** The stereochemistry of the newly formed glycosidic linkages in N-acetyl galactosamine derivative was determined by $J_{H1,H2}$ through $^1$H-NMR. Smaller coupling constants of $J_{H1,H2}$ (below 4 Hz) indicate α linkages and larger coupling constants $J_{H1,H2}$ (6.0 Hz or larger) indicate β linkages.

**Mass spectrometry (MS) analysis:** ESI-MS measurements were performed according to the published protocols on a Q-TOF Ultima API LC-MS instrument with Waters 2795 Separation Module (Waters Corporation, Milford, MA). All samples passed through an EagleEye HPLC $C_{18}$ column, 3 mm × 150 mm, 5 μm at a flow rate of 0.5 mL/min with a linear gradient from 10% eluent B to 26% eluent B over eight minutes with the column temperature maintained at 45 °C. All injections were performed in the full-loop injection mode using a 10 μL sample loop. Eluent A consisted of a pure aqueous solution and eluent B contained 75% acetonitrile/25% aqueous solution (v/v). The following instrument settings were common for

analyses S16 performed in both positive and negative ion modes: source temperature 120 °C, desolvation temperature 400 °C, collision energy 10 eV. When operated in negative ion mode, the mass spectrometer used the following instrument settings: capillary voltage 2.0 kV, cone voltage 35 V, extraction cone 4 V. The following instrumental parameters were used for data acquisition in positive ion mode: capillary voltage 3.5 kV, cone voltage 35 V. Sample concentrations were 1mg/mL. MALDI mass spectra were recorded on a Shimadzu Axima-CFR plus MALDI-TOF. The matrix used was 2,5-dihydroxy-benzoic acid (DHB) and Melittin from honeybee venom (M2272 from Sigma-Aldrich) as the calibration compound.

**We have reported the synthesis and analytical data for L3-DOX, L4-DOX linkers and compounds 1-8 in our previous work[18]. Below, we describe the synthesis and analytical data for compounds 9-13.**

*1.1 N-((2R,3R,4R,5R,6R)-2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-4,5-dihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-3-yl)acetamide-PEG-NH-Cis-Ac-COOH* (**9**):

Compound **8** (0.195 g, 0.074 mmol) was dissolved in MeOH (6 mL) followed by addition of $K_2CO_3$ (0.102 g, 0.74 mmol), 1 M NaOMe solution (1 mL, pH was adjusted to 9.0-9.7 by drop wise addition) and stirred for 1 h at 0 $^oC$ then for 12 h at room temperature. The reaction solution was gradually acidified by adding ice-cold 1N HCl solution while stirring the mixture at 0 °C till the pH dropped to 3.0. The reaction mixture was dialyzed (MWCO 1kDa) against deionized water for 36 hours and lyophilized to obtain compound **9** as an off-white solid (175 mg) in 94.5% yield.

$^1$H NMR (500 MHz, $CDCl_3$): δ 1.85 (s, 3H, $CH_3$, OAc), 2.02 (s, 3H, $CH_3$, OAc), 2.04 (s, 3H, $CH_3$, OAc), 2.16 (s, 3H, $CH_3$, OAc), 3.08-3.20 (m, 6H), 3.26-3.44 (m, 4H,*$CH_2$*-COOH), 3.46-3.56 (m, 8H, $H_{a,b.c.d.e.}$), 3.58-3.3.72 (m, 180H, PEG-H), 3.72-3.86 (m, 4H, $H_{f,}$ $H_{a'}$), 3.94-4.02 (m, 2H, $H_a$), 4.04-4.16 (m, 3H, $H_2$, $H_{6,6'}$), 4.32-4.38 (m, 1H, $H_3$), 4.41 (dd, 1H, *J* = 1.6 & 1.0 Hz, $H_5$), 5.31 (d, 1H, *J* = 1.6 Hz, $H_4$), 6.36 (d, 1H, *J* = 6.8 Hz, $H_1$), 6.78 (s, 1H, olefin), 7.70-7.72 (2bs, 2H, COOH). ESI-MS: $[M+H]^-$ calculated for $C_{14}H_{28}N_2O_8$-PEG-NH-cis-Ac is 2508.30, found 2507.20.

*1.2 Dendrimer coupled-4-pentynoic acid to form G5- pent-4-ynamide compound* (G5-$(alkyne)_{15}$ or **10**):

Commercially available G5-Dendrimer (0.2 g, 0.00693 mmol) and 1-pentynoic acid (13.6 mg, 0.138 mmol) were dissolved in anhydrous DMSO (7 mL) and added PyBOP (108 mg, 0.208 mmol), DIPEA (base, 0.12 mL, 0.693 mmol) and stirred at RT for 36 h. Reaction mixture was transferred in to dialysis cassette (7KDa) and dialyszed for 2 days followed by lyophilization afforded compound **10**, (0.2 g) in 96% yield.

$^1$H-NMR (500 MHz, $D_2O$): δ 2.18-2.34 (m, 240H, G5-H), 2.40-2.50 (m, 120H, G5-H), 2.56 (s, 14H, pentyne-H), 2.58-2.74 (m, 290H, 240 G5-H + 50 H from $CH_2$ of 4-pentynoic acid), 2.97(t, 10H, *J* = 6.0 Hz, pentyne-H), 3.03-3.24 (m, 240H, G5-H), 3.44 (bs, 240H).

MALDI analysis: The molecular weight of parent G5-$(NH_2)_{128}$ is 28, 826, and the molecular weight observed for G5-alkyne is 30,033, which has 1,207 daltons more than its parent dendrimer. This is attributed to alkyne units; each 4-pentynoic acid contributes 81 daltons. Therefore obtained alkyne functionality is 15 units.

*1.3 (N-((2R,3R,4R,5R,6R)-2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide-PEG-NH-Cis-Ac)$_{16.6}$-G5-(alkyne)$_{15}$* (**11**):

Compound **9** (112 mg, 0.0449 mmol, 18 eq) was dissolved in 7.5 mL of 0.1 M potassium phosphate buffer (pH 6.0) followed by addition of EDC.HCl (34 mg, 0.178 mmol, 1:4 eq with acid), catalytic amount of HOBt (4 mg) and the reaction mixture was stirred at room temperature for 30 minutes. G5-$(alkyne)_{15}$-$(NH_2)_{115}$ dendrimer **10** (75 mg, 0.00249 mmol, 1 eq) was dissolved in 5 mL of MeOH and added to the reaction mixture followed by pH adjustment to 8.0, by drop wise addition of 0.5 M NaOH solution. The reaction mixture was stirred for 36 hours at room temperature before dialyzing (MWCO 10kDa) the reaction solution against deionized water for 36 hours followed by lyophilization to obtain compound **11** as a light orange fluffy solid (140 mg) in 93% yield.

$^1$H NMR (500 MHz, $D_2O$): δ 1.82-1.88 (m, 31H, $CH_3$, NHAc), 2.15-2.36 (m, 316H, G5-H, along with other ethylene dioxide protons), 2.40-2.52 (m, 120H, G5-H, un-overlapped G5 protons), 2.54-2.76 (m, G5-H, along with other ethylene dioxide protons), 2.76 (bs, 9H, -OH), 2.82 (bs, 8H, -OH), 2.86 (bs, 27H), 2.92-3.00 (m, 42H), 3.02-3.26 (m, 361H, G5-H, along with other ethylene dioxide protons), 3.26-3.38 (m, 62H), 3.40-3.72 (m, 2795H, PEG-protons); 3.78 (bs 13H), 3.90 (bs 14H), 3.92 (bs 16.4H), 4.20 (bs 12H), 4.36 (bs 14H), 5.42 (d, 12H, *J* = 4.4 Hz), 5.78 (d, 1H, *J* = 7.4 Hz, $H_1$), 7.22 (bs, NH protons), 7.52 (bs, NH protons), 7.62 (bs, NH protons), 7.94 (bs, NH protons).

NMR analysis: We took un-overlapped G5-protons as standard G5-120 protons at 2.40-2.52 ppm, and we obtained 2795 PEG- protons at3.40-3.72 ppm. Each 2KDa PEG unit contains approximately 172 protons, and then we were able to attach 16.25 *cis*-Ac-PEG-NAcGAL units on to the G5 surface.

MALDI analysis: The molecular weight of the compound **9** is 2508, and compound **10** is 30033. The molecular weight observed for $(alkyne)_{15}$-G5-(cis-Ac-PEG-NAcGAL) is 71,922 which has 41,889 daltons more than its parent dendrimer. This is attributed to *cis*-Ac-PEG-NAcGAL units; each *cis*-Ac-PEG-NAcGAL contributes 2508.2 daltons. Therefore obtained *cis*-Ac-PEG-NAcGAL functionality is 16.6 units.

*1.4 (N-((2R,3R,4R,5R,6R)-2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide-PEG-NH-Cis-Ac)$_{16.6}$-G5-(L3-Dox)$_{11.6}$* (**12**):

First Flask: Sodium ascorbate (2 mg g, 0.002 mmol), bathophenonthroline sulfonated sodium salt (SBP, 5.5 mg, 0.002 mmol) and Cu(I) 1 mg, 0.001 mmol) was dissolved THF:$H_2O$, 1:1= 3 mL) and bubbled the nitrogen for 10 min.

Second Flask: L3-Dox-azide (3.7 mg, 0.0042 mmol) was dissolved in THF and $(N\text{-}Ac\text{-}Gal)_{16.6}$-G5-$(alkyne)_{15}$ (**11**, 0.021 g, 0.00035 mmol) in $H_2O$ and bubbled the nitrogen for 10 min. The catalyst flask was heated to 75 $^{o}$C for 3-4 min (during this time the solution becomes red in color), cool down to RT, and syringe out the catalyst solution while bubbling the nitrogen and added to L3-dox-azide flask carefully (drop wisely), flushed the nitrogen one more time and closed the flask and covered with aluminum foil and stirred for 48 h. Stirring should be slow and constant around 350 rpm. After 2 days, the reaction mixture was transferred into dialysis cassette (10KDa) and dialyzed for 2 days followed by lyphilization afforded **12**, approximately (19 mL, 1 mg/mL, 19 mg, 77% yield).

$^{1}$H NMR (500 MHz, $CD_3OD$ + 4 drops of $D_2O$): δ 0.62-0.82 (m, aliphatic protons), 0.86-1.32 (m, G5-protons), 1.52-1.62 (m, G5-protons), 1.72-2.12 (m, including NHAc protons), 2.26-2.46 (m, G5-H, along with other ethylene dioxide protons), 2.52-2.72 (m, G5-H), 3.40-3.72 (m, G5-protons, PEG-protons merged with $CD_3OD$ peak), 3.78-4.12 (m, G5-H), 6.50-8.80 (m, L3 linker and doxorubicin protons), 9.12 (bs, Doxorubicin protons).

MALDI analysis: The molecular weight of parent particle $(alkyne)_{15}$-G5-$(cis\text{-}Ac\text{-}PEG\text{-}NAcGAL)_{16.6}$ is 71,922. The molecular weight observed for $(alkyne)_{15}$-(cis-Ac-PEG-

NAcGAL)$_{16.6}$-G5-L3-DOX is 82,254 which has 10,332 daltons more than its parent dendrimer. This is attributed to L3-DOX units; each L3-DOX contributes 893.2 daltons. Therefore obtained L3-DOX functionality is 11.6 units.

*1.5 (N-((2R,3R,4R,5R,6R)-2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide-PEG-NH-Cis-Ac)$_{16.6}$-G5-(L4-Dox)$_{13.4}$* (**13**):

First Flask: Sodium ascorbate (2 mg g, 0.002 mmol), bathophenonthroline sulfonated sodium salt (SBP, 5.5 mg, 0.002 mmol) and Cu(I) 1 mg, 0.001 mmol) was dissolved THF:$H_2O$, 1:1= 3 mL) and bubbled the nitrogen for 10 min.

Second Flask: L4-Dox-azide (3.8 mg, 0.0042 mmol) was dissolved in THF and (N-Ac-Gal)$_{16.6}$-G5-(alkyne)$_{15}$ (**11**, 0.021 g, 0.00035 mmol) in $H_2O$ and bubbled the nitrogen for 10 min. The catalyst flask was heated to 75 $^{o}$C for 3-4 min (during this time the solution becomes red in color), cool down to RT, and syringe out the catalyst solution while bubbling the nitrogen and added to L3-dox-azide flask carefully (drop wisely), flushed the nitrogen one more time and closed the flask and covered with aluminum foil and stirred for 48 h. Stirring should be slow and constant around 350 rpm. After 2 days, the reaction mixture was transferred into dialysis cassette (10KDa) and dialyzed for 2 days followed by lyphilization afforded **13**, approximately (17 mL, 1.25 mg/mL, 21.25 mg) in 85% yield.

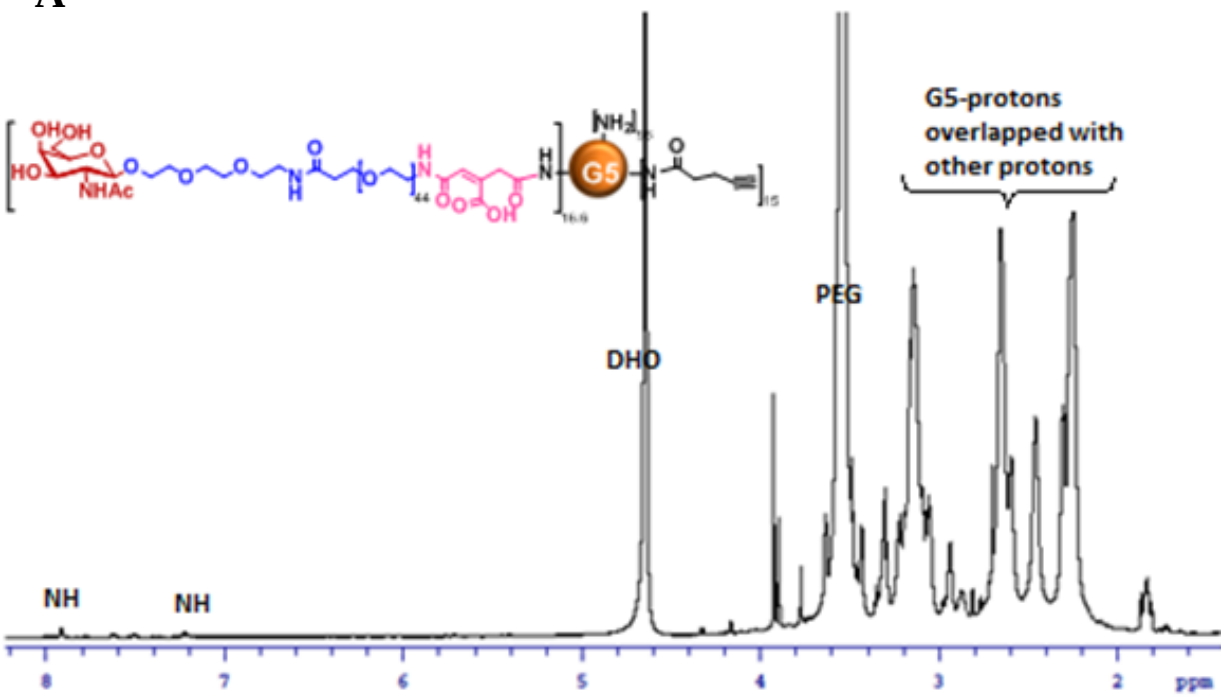
$^1$H NMR (500 MHz, $CD_3OD$ + 4 drops of $D_2O$): δ 0.68-0.88 (m, aliphatic protons), 1.02-1.52 (m, G5-protons), 1.54-1.64 (m, G5-protons), 1.80-2.12 (m, including NHAc protons), 2.14-2.50 (m, G5-H, along with other ethylene dioxide protons), 2.52-2.82 (m, G5-H), 3.40-3.92 (m, G5-protons, PEG-protons merged with $CD_3OD$ peak), 3.92-4.12 (m, G5-H), 6.60-8.50 (m, L3 linker and doxorubicin protons), 9.20 (bs, Doxorubicin protons).

MALDI analysis: The molecular weight of parent particle (alkyne)$_{15}$-G5--(cis-Ac-PEG-NAcGAL)$_{16.6}$ is 71922. The molecular weight observed for (alkyne)$_{15}$-(cis-Ac-PEG-NAcGAL)$_{16.6}$-G5-L4-DOX is 84,313 which has 12,391 daltons more than its parent dendrimer. This is attributed to L4-DOX units; each L4-DOX contributes 923.2 daltons. Therefore obtained L4-DOX functionality is 13.4 units.

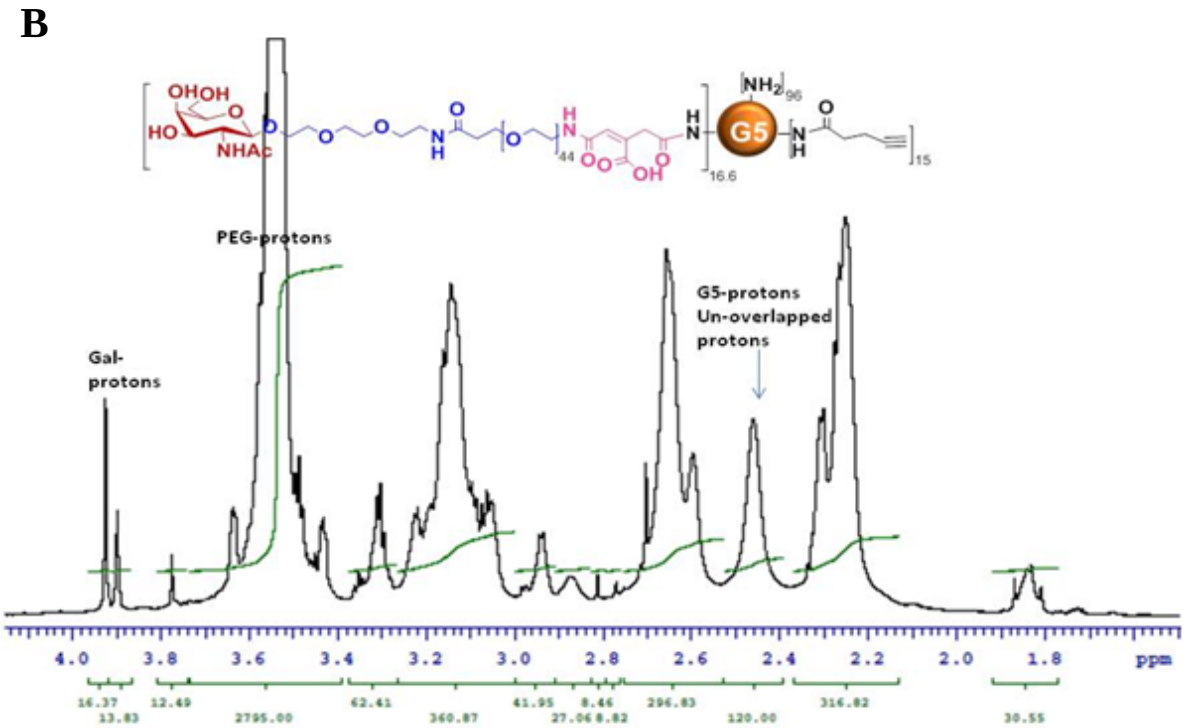
References:

1. (a) G. Tiruchinapally, Scott H. Medina, Maxim V. Chevliakov, Yasemin Y. Durmaz, Rachell N. Stender, William D. Ensminger, Donna S. Shewach, and Mohamed E.H. ElSayed, "Targeting hepatic cancer cells with PEGylated dendrimers displaying N-acetylgalactosamine and SP94 peptide ligands", Advanced Healthcare Materials, (**2013**) 2, 1337-1350. (b) S. H. Medina, Maxim V. Chevliakov, Gopinath Tiruchinapally, Yasemin Y. Durmaz, Sibu Kuruvilla, and Mohamed E.H. ElSayed, "Enzyme-activated nanoconjugates for tunable release of chemotherapeutic agents in hepatic cancer cells",Biomaterials, (**2013**) 34, 4655-4666.

**A**


G5-protons
overlapped with
other protons
PEG
DHO
NH
NH
8
7
6
5
4
3
2
ppm

**B**


PEG-protons
G5-protons
Un-overlapped
protons
Gal-
protons
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
ppm

**C**

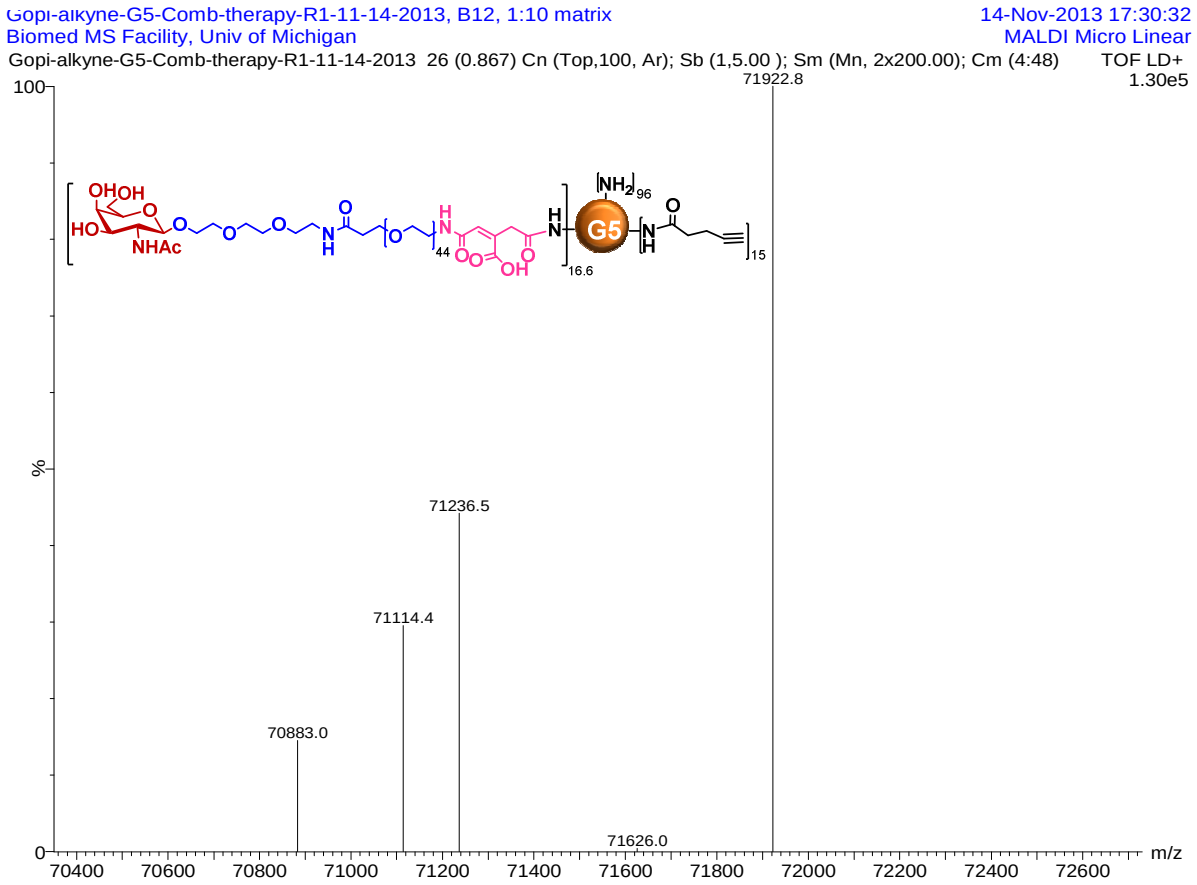



**Figure S1.**

**A:** Compound **11** $^{1}$H NMR in $D_2O$, 500 MHz

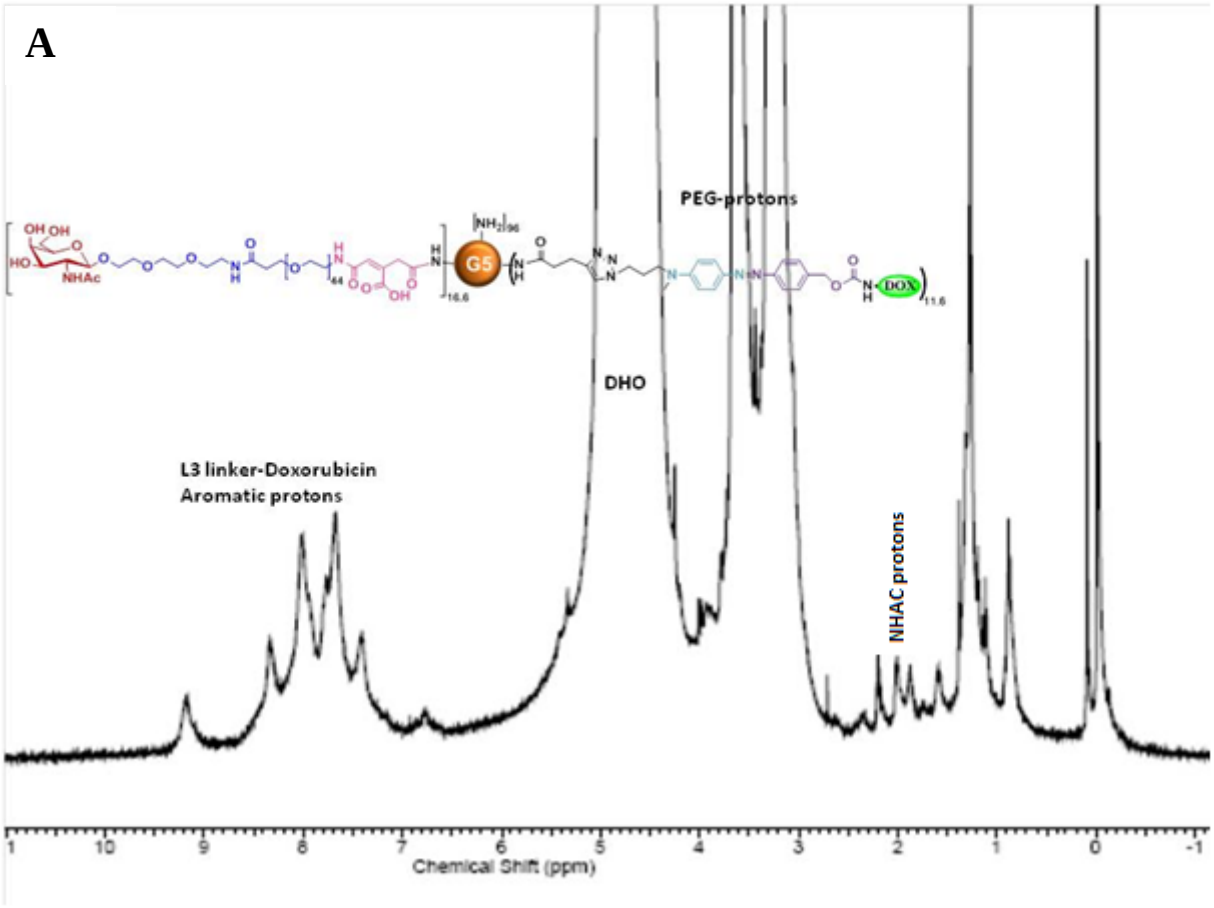
**B:** Compound **11** $^{1}$H NMR in $D_2O$ (expanded region 0.0-4.0 ppm region)

**C:** Compound **11** MALDI spectrum:

Analysis:

1. The molecular weight of parent particle G5-$(alkyne)_{15}$ is 30,033.

2. The molecular weight observed for $_m$(NAcGal$_\beta$-PEG*c*)-G5-$(alkyne)_{15}$ is 71,922 which has 41,889 daltons more than its parent dendrimer. This is attributed to NAcGal-PEG*c* units; each NAcGal-PEG*c* contributes 2508.2 daltons. Therefore the obtained NAcGal-PEG*c* functionality is 16.6 units.


A
PEG-protons
DHO
L3 linker-Doxorubicin
Aromatic protons
NHAC protons
Chemical Shift (ppm)

**B**

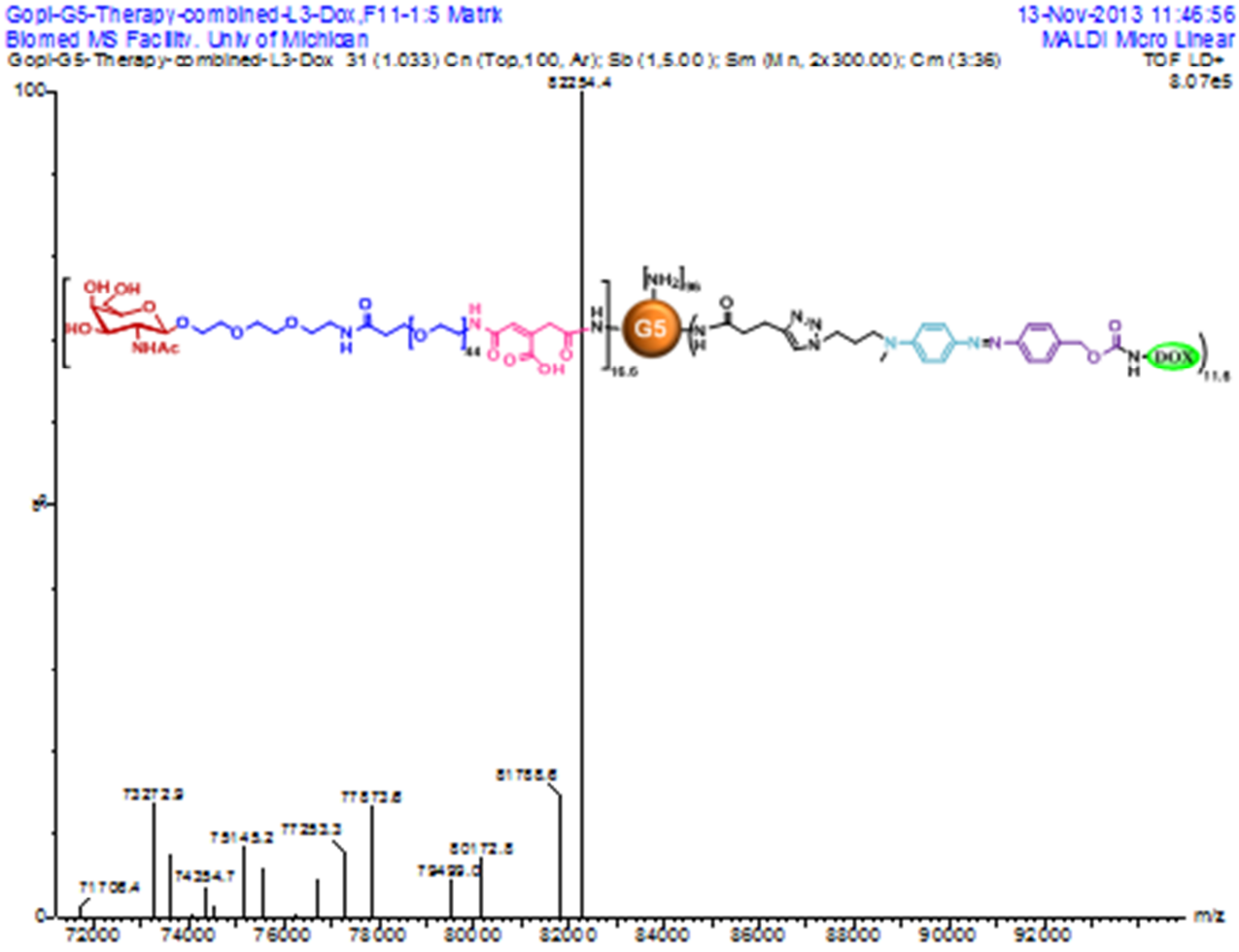



**Figure S2.**

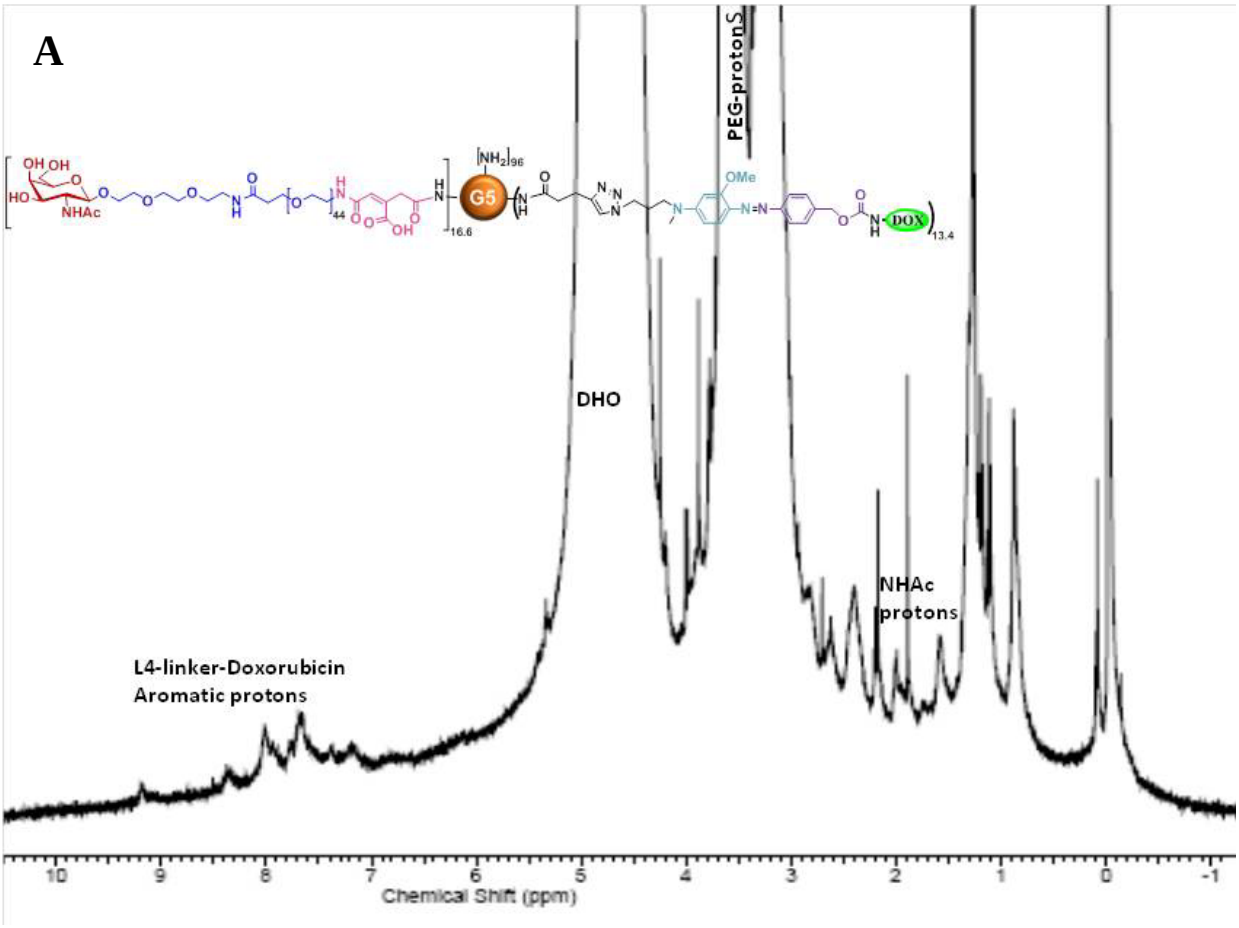
**A:** Compound **12** $^{1}$H NMR in $CD_3OD$ + 4 drops of $D_2O$, 500 MHz

**B:** Compound **12** MALDI spectrum:

Analysis:

1. The molecular weight of parent particle $_{16.6}$(NAcGal$_\beta$-PEG*c*)-G5-(alkyne)$_{15}$ is 71,922.

2. The molecular weight observed for $_{16.6}$(NAcGal$_\beta$-PEG*c*)-G5-L3-DOX is 82,254 which has 10,332 daltons more than its parent dendrimer. This is attributed to L3-DOX units; each L3-DOX contributes 893.2 daltons. Therefore the obtained L3-DOX functionality is 11.6 units.


A
PEG-protons
DHO
NHAc
protons
L4-linker-Doxorubicin
Aromatic protons
Chemical Shift (ppm)

**B**

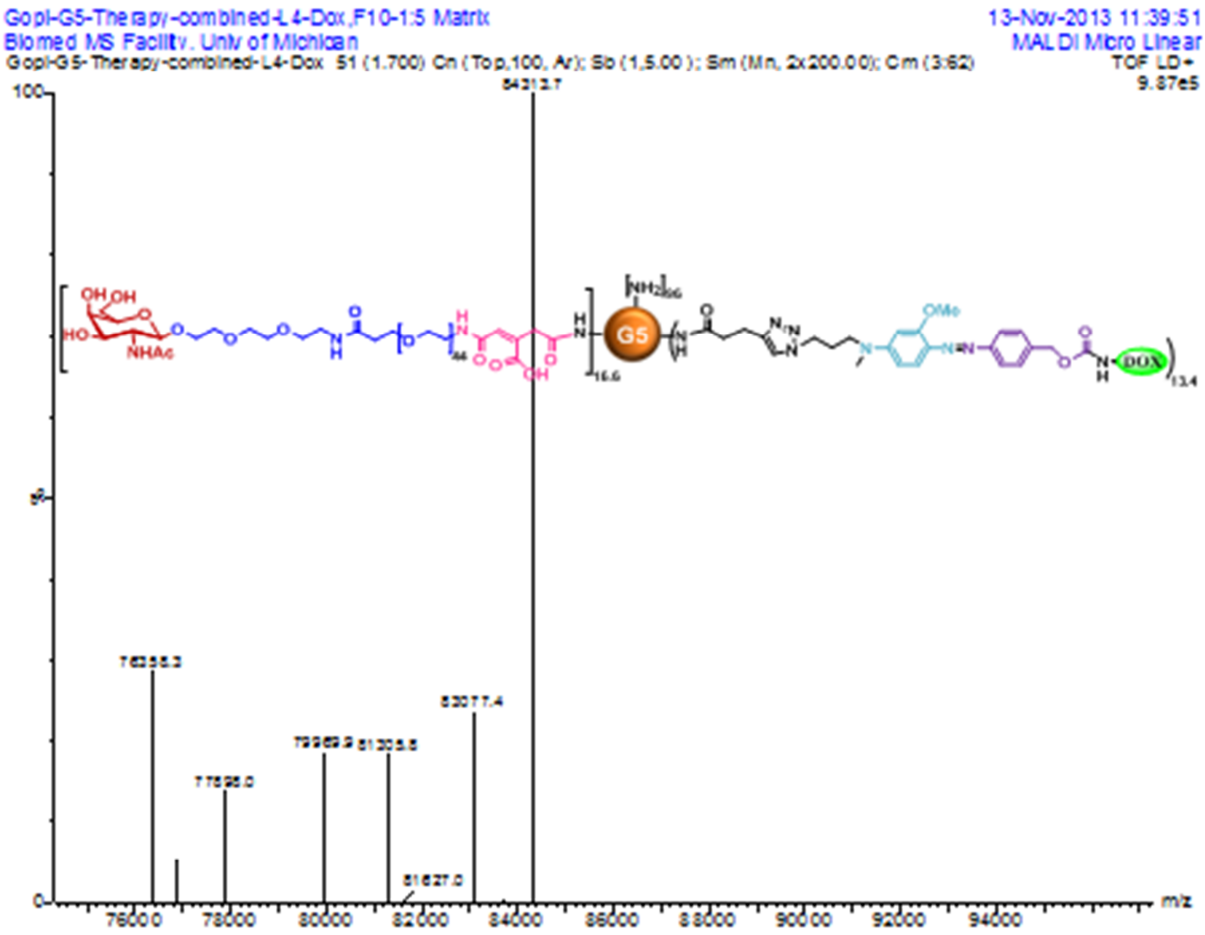



**Figure S3.**

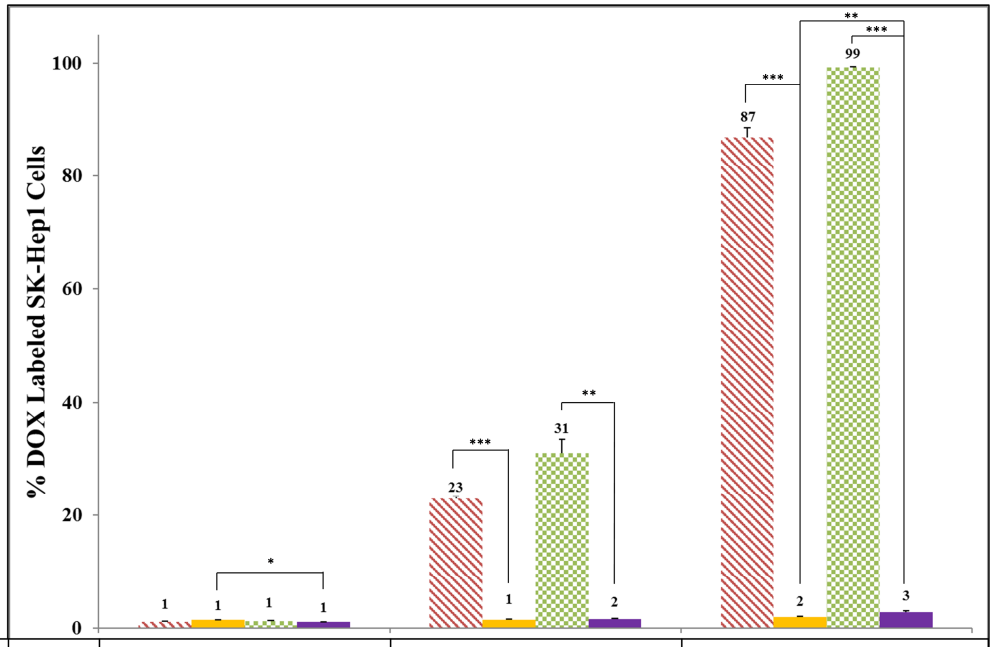
**A:** Compound **13** $^{1}$H NMR in $CD_3OD$ + 4 drops of $D_2O$, 500 MHz

**B:** Compound **13** MALDI spectrum:

Analysis:

1. The molecular weight of parent particle $_{16.6}$(NAcGal$_\beta$-PEG*c*)-G5-(alkyne)$_{15}$ is 71922.

2. The molecular weight observed for $_{16.6}$(NAcGal$_\beta$-PEG*c*)-G5-L4-DOX is 84,313 which has 12,391 daltons more than its parent dendrimer. This is attributed to L4-DOX units; each L4-DOX contributes 923.2 daltons. Therefore the obtained L4-DOX functionality is 13.4 units




| | | | | |
|---|---|---|---|---|
| DOX Concentration (nM) | for P1 | 7.0 | 349 | 2795 |
| | for P2 | 8.1 | 404 | 3229 |
| Particle Concentration (nM) | | 0.6 | 30 | 240 |
| NAcGal Concentration (nM) | | 10 | 500 | 4000 |

**Figure S4**: **Uptake of P1 and P2 particles into a control cell line, SK-Hep1**.
SK-Hep1 is known to be an ASGPR-deficient cell line, and flow cytometry results show that P1 and P2 conjugates are not internalized into these cells. Meanwhile, free DOX is internalized in SK-Hep1 cells at similar levels to HepG2 and Hep3B cells, presumably by passive diffusion. These results support that P1 and P2 internalization into HepG2 and Hep3B cells is mediated by the ASGPR. Values are presented as the mean of four replicates ± SEM. A student's t-test was used to compare the statistical significance between different treatment groups, with *P<0.05, **P<0.01, and ***P<0.001.

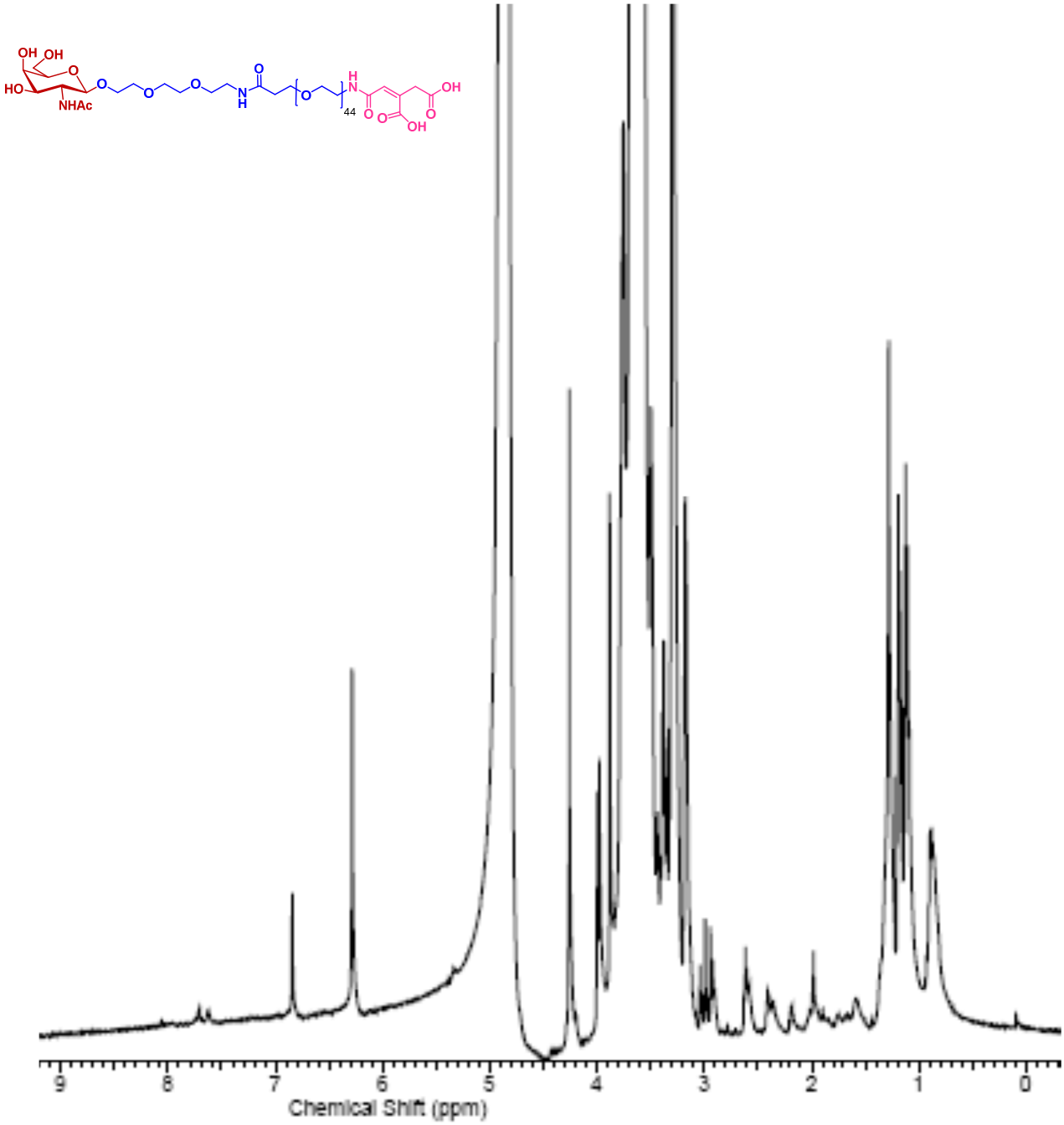
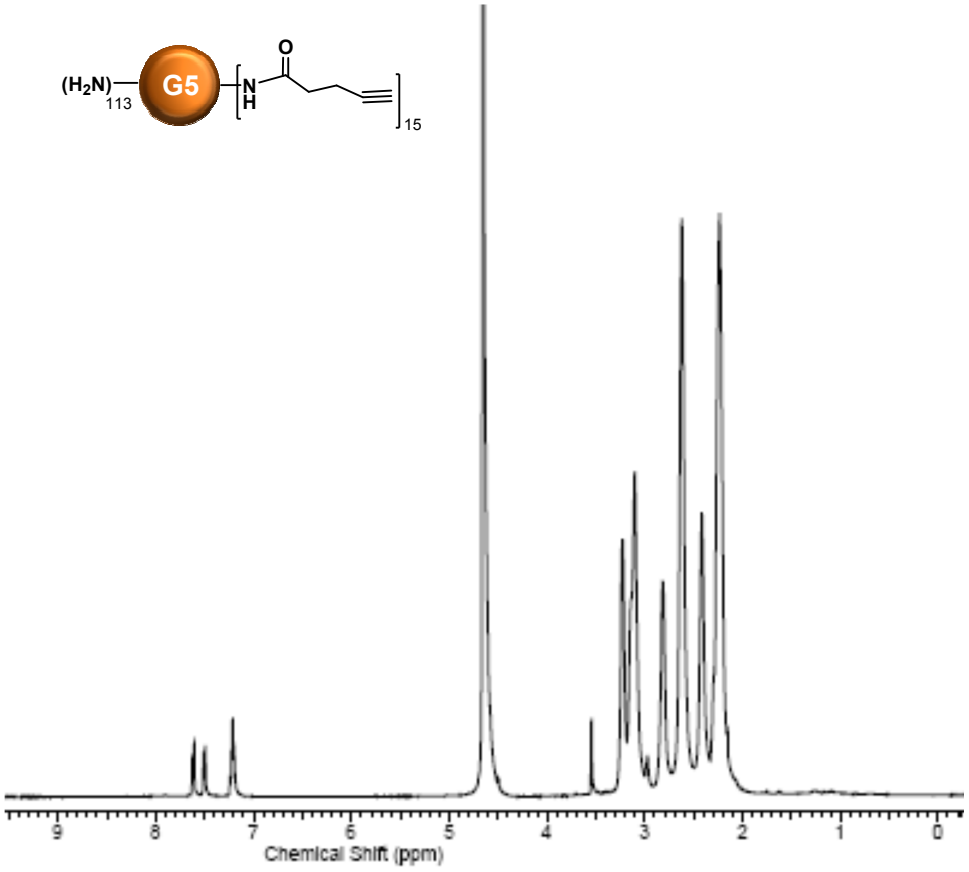
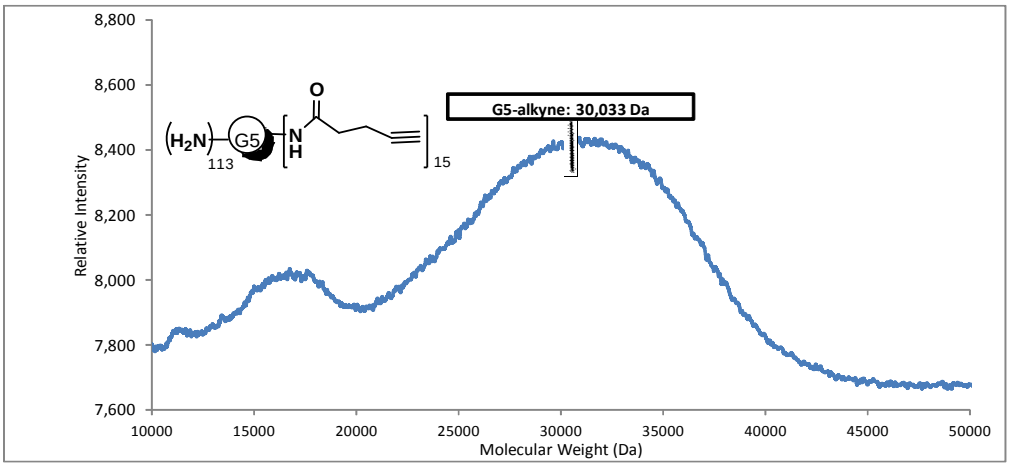



**Figure S5**. Compound **9** $^{1}$H NMR in $CD_3OD$, 500 MHz

**A**


(H2N)
113
G5
N
H
O
15
9
8
7
6
5
4
3
2
1
0
Chemical Shift (ppm)


8,800
8,600
8,400
8,200
8,000
7,800
7,600
Relative Intensity
G5-alkyne: 30,033 Da
(H2N)
113
G5
N
H
O
15
10000
15000
20000
25000
30000
35000
40000
45000
50000
Molecular Weight (Da)

**S6.**

**B**

**A:** Compound **10** $^{1}H$ NMR in $D_2O$, 500 MHz.

**B:** Compound **10** MALDI spectrum:

Analysis:

1. The molecular weight of parent G5-$(NH_2)_{128}$ is 28,826 Da.

2. The molecular weight observed for G5-alkyne is 30,033, which is 1,207 daltons more than its parent dendrimer. This is attributed to alkyne units; each 4-pentynoic acid contributes 81 daltons. Therefore the obtained alkyne functionality is 15 units.