Targeted Amplification of Delivery to Cell Surface Receptors by Dendrimer Self-Assembly

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Supporting Information

Cyscore(FITC)$_8$. The methanol from a commercial G4 cystamine core polyamidoamine dendrimer solution was removed and the solid (62.95 mg) was redissolved in 150 μL of water to a concentration of 30 mM. The pH was adjusted to 9 using concentrated NaOH aqueous solution and the dendrimer was diluted further to 15 mM with 150 μL of dimethylformamide (DMF). The dendrimer was reacted with 3.93 mg (10 equivalents) of fluorescein isothiocyanate (FITC) in 100 μL of DMF for 4 h at 40 °C. After the solvent was evaporated, the crude product was re-dissolved in 1 mL of water. The low molecular weight material was removed on a Sephadex G-25 desalting column. The column was equilibrated with 25 mL of water, the crude sample was loaded and 1.5 mL of water was added to a total volume of 2.5 mL. The product was eluted in 3.5 mL, to yield **Cyscore(FITC)$_8$** (90% recovery of dendrimer, as determined by NMR spectroscopy, using a standard). It was shown to have an average of eight fluorophores per dendrimer as determined by comparison of the integration of aromatic to aliphatic peaks using ^{1}HNMR (400 MHz, D_2O): 7.8 (broad s, 1H, Ar H), 7.4 (broad s, 1H,), 6.8-7.1 (overlapping resonances, 3H, Ar H), 6.5 (broad s, 2H, Ar H), 6.4 (broad s, 2H, Ar H) 3.6 (broad s, 15H, CH_2), 3.3-3.4 (overlapping resonances, 62H, CH_2, NH), 3.0-3.2 (overlapping resonances, 88H,CH_2, CH_2), 2.9 (s, 2H, NH_2), 2.7 (broad s, 106H, CH_2), 2.42 (broad s, 53H, CH_2), 2.3 (broad s, CH_2). The product was used without further purification.

Cyscore(FITC)$_8$ (Ald)$_{16}$ (1)

The quantity 76.7 mg of **Cyscore(FITC)$_8$** was dissolved in 150 μL of water to a concentration of 30 mM. The pH was adjusted to 7.5 with concentrated HCl aqueous solution. The solution was then diluted to 15 mM by addition of 150 μL DMF. Next, 19.2 mg of succinimidyl 4-formylbenzoate was dissolved in a minimal amount of DMF (100 μL) and then an equivalent amount of H_2O (100 μL) was added to the dissolved ester. The resultant solution was added dropwise to the **Cyscore(FITC)$_8$** solution and reacted for 4 h at 40°C. The solvent was evaporated, and the excess succinimidyl 4-formylbenzoate was removed with a Sephadex G-25 column to yield **1** (90% recovery of the dendrimer, as determined by NMR spectroscopy using a standard). The product was shown to have an average of sixteen aldehyde moieties per dendrimer, as determined by comparison of the integration between the resultant broad singlet at 9.9 ppm and the aliphatic region by ^{1}HNMR. ^{1}HNMR (400 MHz, D_2O): 9.9 (broad s, 11H, CHO), 8.2 (broad s, 2H), 7.75 (broad s, 62H, Ar H), 7.0 (broad s, 5H, Ar H), 6.4-6.6 (overlapping resonances,5H), 3.7 (broad s, 11H, CH_2), 3.4 (broad s, 308H, CH_2), 3.25-2.75 (overlapping resonances, 618H, CH_2), 2.4-2.7 (overlapping resonances, 322H, CH_2).

[CysCore(FITC)$_4$ (Ald)$_{8)}$]$_{1.5}$(HuBrE3) (2)

The HuBrE3 antibody was diluted with 1x phosphate buffered saline pH 7.4 to a concentration of 10 mg/mL. To this solution was added 4.8 mg of the water-soluble crosslinker sulfosuccinimidyl 4-N-maleimidomethyl cyclohexane-1-carboxylate (sulfo-SMCC), in 1 mL of 10 mM PBS. After 30 min of shaking at room temperature, the excess crosslinker was removed on a Sephadex G-25 column. The purified product was lyophilized to a white powder. The disulfide bond in compound **1** was reduced using

immobilized tris(2-carboxyethyl)phosphine hydrochloride (TCEP). After reduction for 1 h at room temperature, the mixture containing the immobilized TCEP and **1** was filtered using a 0.45 μm syringe filter and the beads were washed three times with water. The reduced dendrimer was lyophilized to remove the water and the resultant compound was re-dissolved in 250 μL of water and added to the derivatized antibody. The resultant antibody concentration was 40 mg/mL. After shaking for 3 h at room temperature, the excess unreacted maleimide groups on the Ab were quenched with 1.1 mg (100 molar equivalents per Ab) L-cysteine in 50 mM PBS 7.4 (freshly prepared) and the excess, unreacted dendrimer was separated from product using YM-100 spin filters to yield **2**. Construct **2** was analyzed using SDS gel electrophoresis, under reducing conditions. A 7.5 μL aliquot of **2** was added to a freshly prepared 2X reducing buffer and incubated at 95 °C for 5 min. The 15μL sample was then loaded onto a precast 12% polyacrylamide gel, with a 4% stacking gel. A fluorescent image of the gel, obtained on a Storm 840 phosphoimager, showed the presence of higher molecular weight bands, corresponding to labeled heavy and light chains of the Ab, and the absence of unconjugated dendrimer (Figure S5). Construct **2** was found to have an average of 1.2 dendrons per antibody, as calculated from comparison of absorbances at 494 nm (fluorescein-labeled dendrimer) and the absorbance at 280 nm (antibody).

HuBrE3(FITC)$_4$

Concentrated aqueous NaOH was added to 1 mL of a 10 mg/mL solution of HuBrE3 in 10 mM PBS, to a pH of 9. FITC, 0.075 mg (6 eq), in 100 μL DMF was added dropwise at room temperature and the reaction was shaken for 1 h. Excess FITC was removed by passing the solution through a G-25 desalting column. The product was shown to have an

average of four fluorophores per antibody as determined by comparing the absorbance at 494 nm (fluorescein) with the absorbance at 280 nm (antibody).

PAMAM(Rhod)$_2$(Hydr)$_5$ (3)

The G4 PAMAM dendrimer was obtained as a methanol solution. This solution (430 μL) was added to a vial, and the methanol was removed under vacuum. The resulting clear gel (71.5 mg) was dissolved in 250 μL water to a concentration of 20 mM. The pH was adjusted to 9 with concentrated NaOH. The dendrimer was reacted with 19.0 mg (10 equivalents) of rhodamine B isothiocyanate (RBITC) in 150 μL DMF for 4 h at 40 °C. After the solvent was evaporated, the crude product was re-dissolved in 1 mL of water. Unreacted RBITC was removed on a Sephadex G-25 desalting column. The product was shown to have an average of two fluorophores per dendrimer by UV spectroscopy.

The PAMAM dendrimer previously labeled with two rhodamine dyes **(**50 mg**)** was dissolved in 225 μL of water and 225 μL of DMF. To this solution was added 19.6 mg (20 equivalents) of succinimidyl 6-hydrazinonicotinate acetone hydrazone, dissolved in 100 μL of DMF. The solution was reacted for 4 h at 40 °C. The solvent was evaporated, and the excess succinimidyl 6-hydrazinonicotinate acetone hydrazone was removed on a Sephadex G-25 desalting column. To remove the protecting group, the pH of the resulting solution was adjusted to 6 with concentrated HCl. The average number of hydrazines per dendrimer was calculated as follows: 1 mg of product was dissolved in 100 μL of sodium acetate buffer pH 5.6 (NaAc), and then 50 μL of a 4.50 mM solution of 4-nitrobenzaldehyde in NaAc buffer was added. The solution was allowed to react for 1 h at

37 °C, diluted to 1 mL with NaAc buffer and the number of hydrazines per dendrimer was determined by measuring the absorbance at 354 nm.

Cys-Core(Ald)$_{16}$ (4). The methanol from a G4 Cystamine core polyamidoamine dendrimer solution was removed and the solid (62.95 mg) was re-dissolved in 150 μL of water to a concentration of 30 mM. The pH was adjusted to 7.5 using concentrated HCl and the dendrimer was diluted further to 15 mM with 150 μL of DMF. Next, 19.2 mg of succinimidyl 4-formylbenzoate was dissolved in a minimal amount of DMF (100 μL) and then an equivalent amount of H_2O (100 μL) was added to the dissolved ester. The resultant solution was added dropwise to the dendrimer and reacted for 4 h at 40 °C. The solvent was evaporated under vacuum, and the excess succinimidyl 4-formylbenzoate was removed with a Sephadex G-25 column to yield **5** (90% recovery of dendrimer, as determined by ^{1}HNMR using a standard). The product was shown to have an average of sixteen aldehyde moieties per dendrimer, as determined by comparison of the integration between the resultant broad singlet at 7.8 ppm and the aliphatic region by ^{1}HNMR. ^{1}HNMR (400 MHz, D_2O): 9.9 (broad s, 16H, CHO), 7.8 (broad s, 66H, Ar H), 3.2-3.6 (overlapping resonances, 748H, CH_2), 3.15 (broad s, 116H, CH_2), 2.75 (overlapping resonances, 272H, CH_2) 2.6 (s, 100H, NH_2).

PAMAM(Hydr)$_5$ (5). Commercial G4 dendrimer solution (430 μL) was added to a vial and the methanol was removed under high vacuum. The resulting clear gel (71.5 mg) was dissolved in 250 μL of water to a concentration of 20 mM. The pH was adjusted to 7.5 with concentrated HCl and 250 μL of DMF was added. To this solution was added 19.6 mg (20 equivalents) of succinimidyl 6-hydrazinonicotinate acetone hydrazone, dissolved in

100 μL of DMF. The solution was reacted for 4 h at 40 °C. The solvent was evaporated and the excess succinimidyl 6-hydrazinonicotinate acetone hydrazone was removed on a Sephadex G-25 desalting column. To remove the protecting group, the pH of the resulting solution was adjusted to 6 with dilute HCl to yield **6**. ^{1}HNMR: 8.4 (broad s, 7H, Ar H), 7.9 (broad s, 7H, Ar H), 6.9 (broad s, 7H, Ar H), 3.55 (broad s, 138H, CH_2), 3.5-3.35 (overlapping resonances 440H, CH_2), 3.35-3.2 (overlapping resonances, 214H, CH_2), 3.18-3.05 (overlapping resonances, 146H, CH_2), 2.9-2.6 (overlapping resonances, 314H, CH_2). The average number of hydrazines per dendrimer was confirmed using UV spectroscopy as follows: 1 mg of product was dissolved in 100 μL of sodium acetate buffer pH 5.6, and then 50 μL of a 4.50 mM solution of 4-nitrobenzaldehyde in NaAc buffer was added. The solution was allowed to react for 1 h at 37 °C and diluted to 1 mL with NaAc buffer. The number of hydrazines per dendrimer was determined by measuring the absorbance at 354 nm.

***In vitro* Experiments**

Human PANC-1 cells (ATCC, Manassas, VA) were grown on a 10 cm polystyrene plate to ~80% confluence in Minimum Essential Medium (MEM) containing 10% Fetal Bovine Serum (FBS) (v/v). The cells were trypsinized and subsequently suspended in 10 mL of MEM (10% FBS). After mixing, this cell suspension was further diluted 1:15 in MEM (10% FBS) and the cells were plated on a 96 well plate. The cells were then incubated at 37 °C and allowed to grow to ~50% confluence. The cells were washed twice with 1x PBS and fixed by incubating in -20 °C MeOH for 1 min. Following the MeOH incubation, the cells were washed twice with 1x PBS. For initial incubation, **2** or **HuBrE3-FITC** (1.0 x 10^{-7} M in 1x PBS) was added to the fixed cells and incubated for 5 min at 37 °C. The cells

were then washed three times with 1x PBS to remove unbound **2** or **HuBrE3-FITC**. The cells were then imaged on a fluorescent microscope (Leica) by examination through the fluorescein channel. Dendrimer **3**, at 1.0 x 10^{-7} M in 1x PBS, was added to the cells and the cells were incubated for 1 min at 37 °C. The cells were then washed twice with 1x PBS. Following washing, the cells were imaged with a fluorescent microscope (Leica) by examination through the Rhodamine filter.

FACS Measurements

Human PANC-1 cells were grown on a 10 cm polystyrene plate to ~80% confluence in MEM (10% FBS). The cells were trypsinized, resuspended and pelleted at 1200 rpm for 5 min using a Beckman Coulter centrifuge outfitted with a swinging-bucket rotor. The medium was removed and the cells were washed three times with 1x PBS to remove any residual medium. After the washing, the cells were re-suspended in a 1x PBS solution containing 1.0x10^{-7} M **2** or **3** and allowed to incubate for 1 h at 37 °C with gentle shaking. After 1 h incubation, the cells were pelleted as described above and washed three times with 1x PBS. The cells incubated with **3** were then resuspended in 1x PBS and submitted for flow cytometry analysis on a FACS Aria cell counter (Becton Dickinson, Franklin Lakes, NJ). The cells incubated with **2** were then resuspended in 1 mL 1x PBS and split into two 500 μL fractions. One fraction was then incubated with **4**, at 1.0x10^{-7} M in 1x PBS and allowed to incubate for 1 h at 37 °C with gentle shaking. To the other fraction, 500 μL of 1x PBS was added and the cells were incubated for 1 h at 37 °C with gentle shaking. After 1 h, the cells were centrifuged as described above and washed three times with 1x PBS. The cells were then resuspended in 1x PBS and submitted for flow cytometry analysis. To distinguish the cell population, 488 nm forward and side scatter

light was used. The fluorescence gates were set so that less than 1% of the unstained cells spilled into the positive gates. Single color controls were used in order to compensate for any fluorescent spill between fluorescein and rhodamine dyes. The data are reported as mean fluorescence intensity.

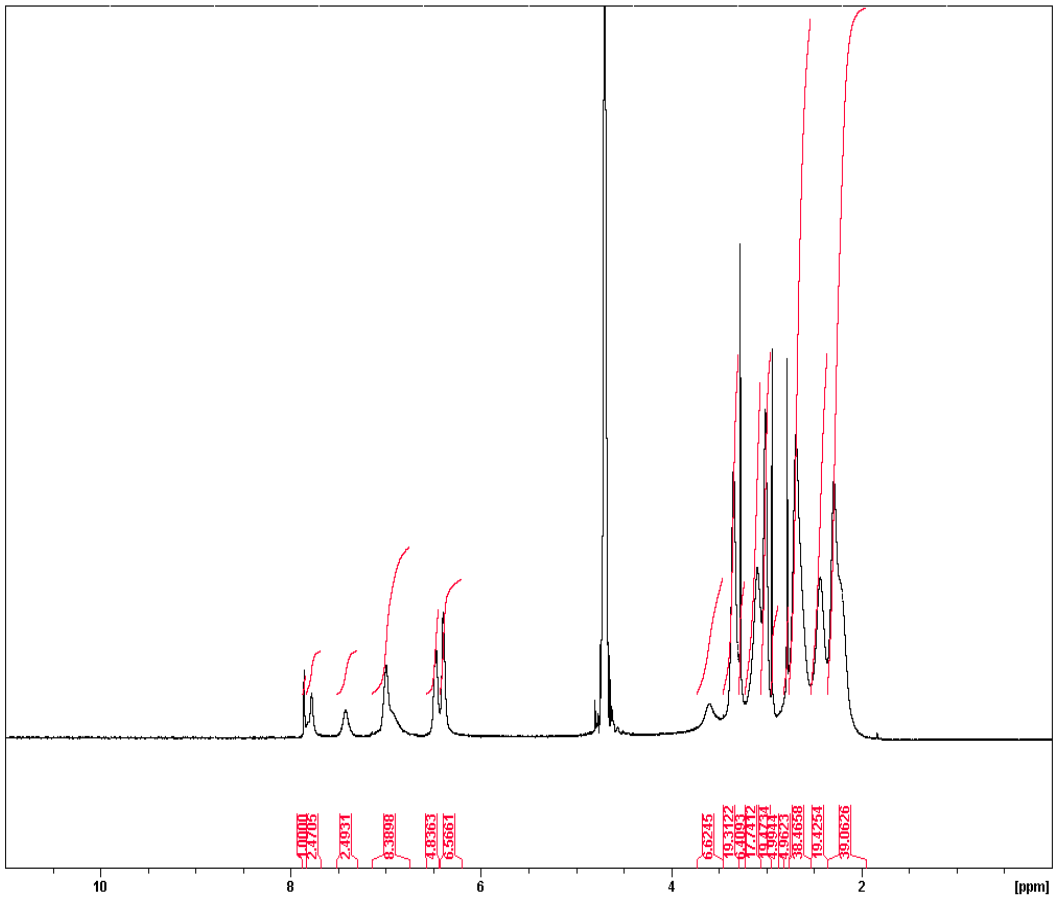

Figure S1. ^{1}HNMR (400 MHz, D_2O) showing **CysCore(FITC)$_8$**. Comparison of the aliphatic to aromatic regions of the spectra indicates an average of eight FITC molecules per dendrimer.

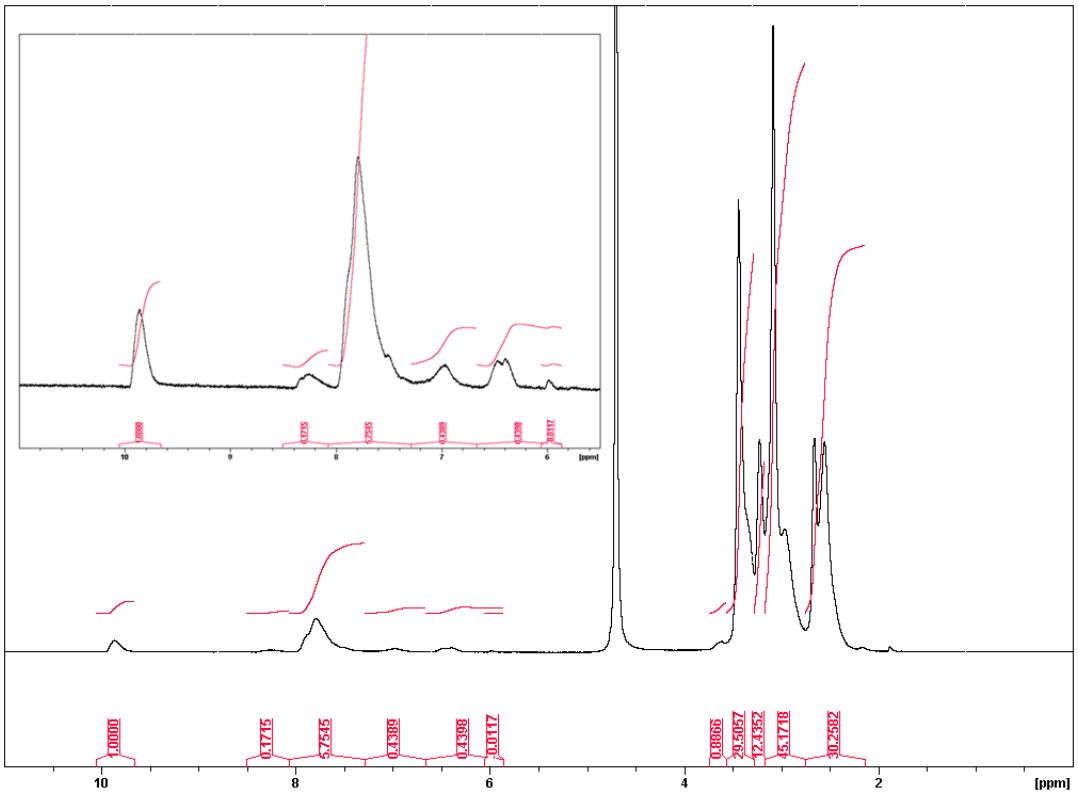

Figure S2. ^{1}HNMR (400 MHz, D_2O) showing **1**. Comparison of the peak at 9.9 ppm, aromatic peaks and aliphatic peaks indicates an average of 16 aldehyde moieties per dendrimer. The inset shows an expanded region from 11 ppm to 5.5 ppm.

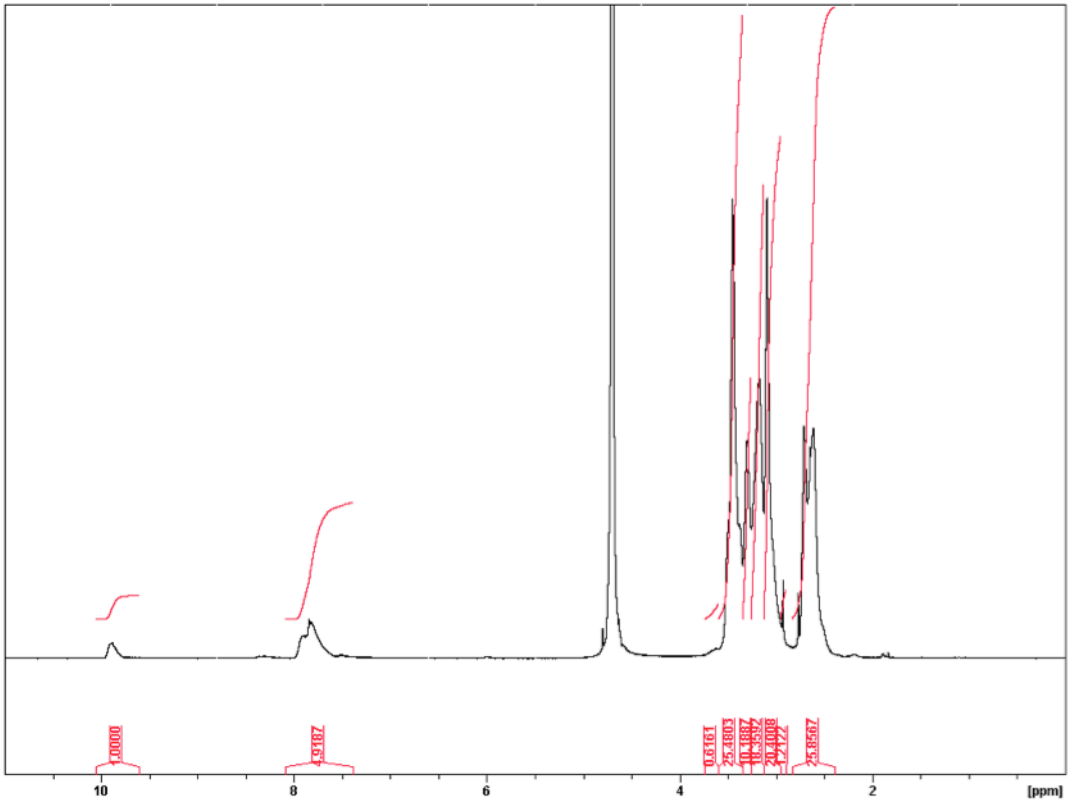

Figure S3. ^{1}HNMR (400 MHz, D_2O) showing **5**. Comparison of the integration between the peaks at 9.9 ppm, aromatic peak and aliphatic peaks indicates an average of 16 aldehyde moieties per dendrimer.

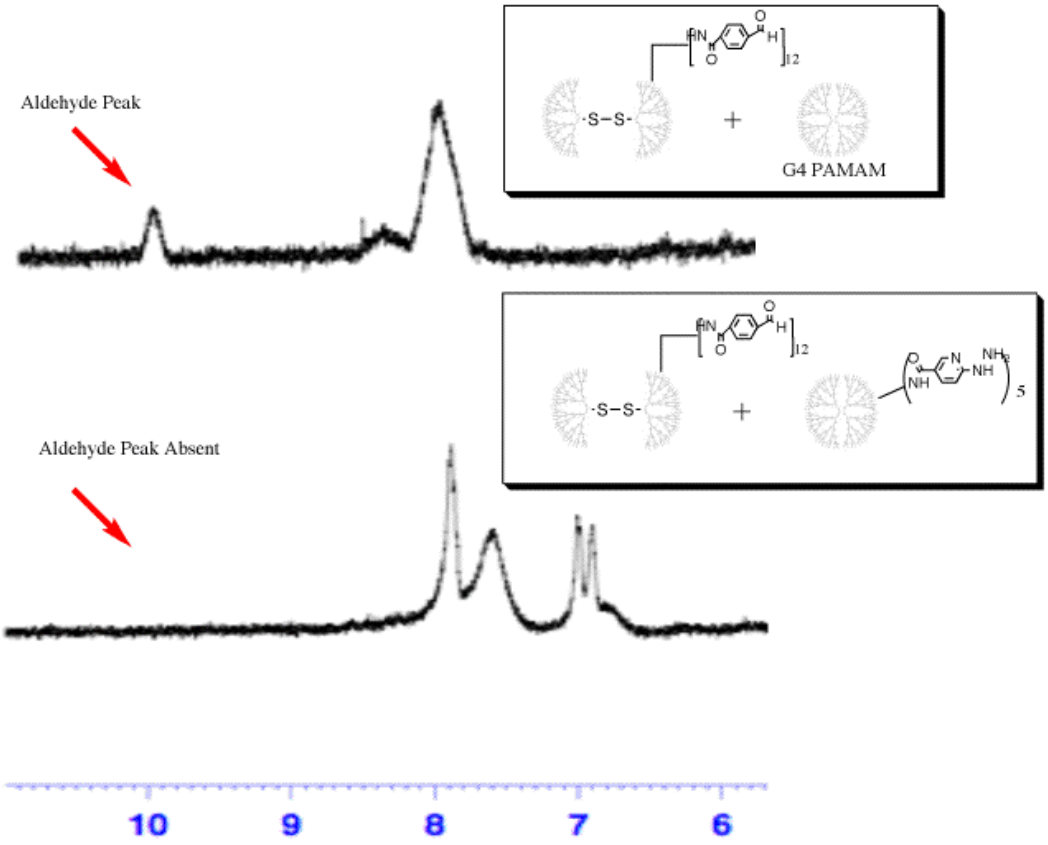

Figure S4. [1]HNMR data showing (A) **5** at 1 mM in pD 7.4 mixed with PAMAM G4, at 1 mM in pD 7.4, and allowed to react for 24 h. at 37 °C (B) **5** at 1 mM in pD 7.4 mixed with **6** at 1 mM in pD 7.4. The disappearance of the peak at 10 ppm (aldehyde) only occurs in the presence of **6**.

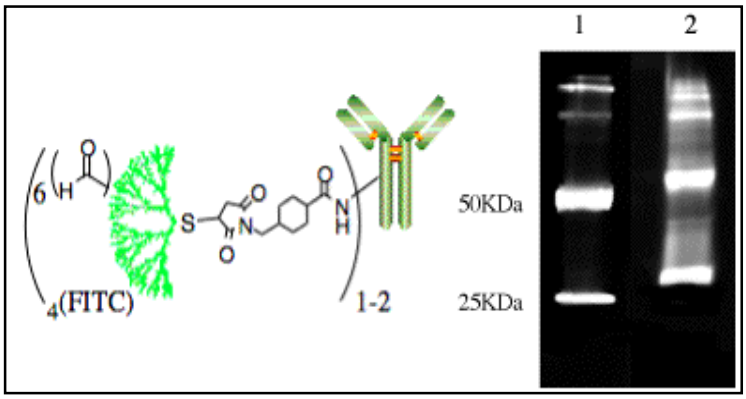

Figure S5. Fluorescent image (fluorescein channel) of a SDS reducing gel (12% polyacrylamide). Lane 1: Control showing the fluorescein-labeled antibody, **HuBrE3(FITC)$_4$**, reduced into heavy (50kDa) and light (25kDa) chains by β-mercaptoethanol. Lane 2: Compound **2**.

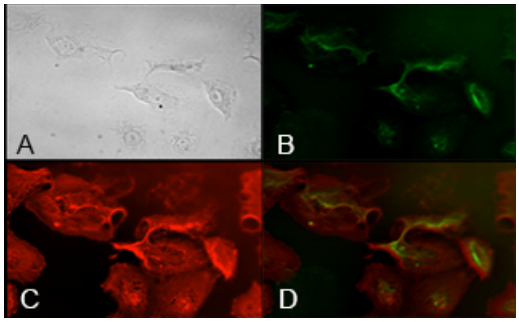

Figure S6. Fluorescence microscopy of human PANC1 cells incubated with **2**, 1.0 x 10^{-7} M, washed to remove excess, unbound dendrimer, and then incubated with **3** at 1.0 x 10^{7} M and washed. (A) Bright field image. (B) Fluorescein filtered image. (C) Rhodamine filtered image. (D) Overlay of (B) and (C), in which the intensity of the red signal was scaled down by 50% to show both color clearly.