SUPPLEMENTARY INFORMATION

Design of riboflavin-presenting PAMAM dendrimers as a new nanoplatform for cancer-targeted drug delivery

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General synthetic methods

All solvents and reagents were purchased from commercial suppliers, and used without further purification including methotrexate hydrate (TCI; purity >98.0%), and fluorescein 5(6)-isothiocyanate (Sigma; purity ~90%). Reactions were run under nitrogen atmosphere unless noted otherwise. Characterization of reaction products was routinely carried out by ^{1}H NMR spectroscopy and mass spectrometry. For NMR (^{1}H) measurement, samples were dissolved in deuterated solvent (D_2O, DMSO-d_6), and NMR spectra were acquired with a Varian nuclear magnetic resonance spectrometer at 400 MHz or 300 MHz for ^{1}H NMR spectra under standard observation conditions. Molecular weights of PAMAM G5 dendrimer and its conjugates were measured by matrix assisted laser desorption ionization-time of flight (MALDI TOF) with a Waters TOfsPec-2E spectrometer. The MALDI spectra were acquired using a matrix solution of 2,5-dihydroxybenzoic acid (10 mg/ml in 50% aqueous acetonitrile) in a linear mode with a high mass detector. The spectrometer was mass calibrated with BSA in sinapinic acid, and data was acquired and processed using Mass Lynx 3.5 software. UV-vis absorption spectra were recorded on a Perkin Elmer Lamda 20 spectrophotometer.

HPLC analysis was carried out on a Waters Acquity Peptide Mapping System equipped with a Waters photodiode array detector (referred to Ultra Performance Liquid Chromatography). For analysis the PAMAM dendrimer conjugates were run on a C4 BEH column (150 x 2.1 mm, 300 Å) connected to Waters Vanguard column. Elution of the conjugates was performed in a linear gradient beginning with 98:2 (v/v) water/acetonitrile (with trifluoroacetic acid at 0.14 wt % in each of the eluents) at a flow rate of 1 mL/min.

Size exclusion chromatography (SEC) was used to measure molecular weights and polydispersity index (PDI) of PAMAM G5 dendrimer. The GPC experiments were performed on an Alliance Waters 2695 separation module equipped with a 2487 dual wavelength UV absorbance detector (Waters Corporation), a Wyatt HELEOS Multi Angle Laser Light Scattering (MALLS) detector, and an Optilab rEX differential refractometer (Wyatt Technology Corporation). Columns employed were TosoHaas TSK-Gel Guard PHW 06762 (75 mm × 7.5 mm, 12 mm), G 2000 PW 05761 (300 mm × 7.5 mm, 10 mm), G 3000 PW 05762 (300 mm × 7.5 mm, 10 mm), and G 4000 PW (300 mm × 7.5 mm, 17 mm). 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. The weight average molecular weight, M_w, has been determined by GPC, and the number average molecular weight, M_n, was calculated with Astra 5.3.14 software (Wyatt Technology Corporation) based on the molecular weight distribution.

1. Synthesis of 5

To a solution of glutaric acid-terminated PAMAM G5 dendrimer **3** (50 mg, 1.24 μmol) in DMF (12 ml) was added and 4-dimethylaminopyridine (15.0 mg, 0.123 mmol), FITC-butane amine **3** (3.0 mg, 6.28 μmol) and a solution of riboflavin (3.75 mg, 9.96 μmol) dissolved in DMSO (3 mL). While stirring the mixture under nitrogen atmosphere, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI, 12 mg, 62.60 μmol) was added. After stirring the mixture for 48 h, the reaction mixture was concentrated *in vacuo* to ~5 mL in volume and diluted with 10 mL of phosphate-buffered saline (PBS, pH 7.2). The solution was loaded into a membrane dialysis bag (MWCO 10 kDa), and dialyzed against PBS (2 x 2L), and deionized water (3 x 2L) over 3 days. The aqueous solution was collected and lyophilized to afford PAMAM-$RF_{6.3}$-$FITC_{1.3}$ **5** as orange solid (36 mg). MALDI TOF mass spectrometry: *m/z* = 43200. UV/vis (PBS, pH 7.2): λ_{max} = 500 nm (ε = 116625 $M^{-1}cm^{-1}$ calculated on the basis of FITC), 367 nm (ε = 5353 $M^{-1}cm^{-1}$ calculated on the basis of riboflavin), 268 nm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.95 (br), 7.84 (br), 6.61 (m), 6.52 (m), 3.50 (br s), 3.01 (br s), 2.80-2.0 (br m), 1.65 (br m), 1.16 (m) ppm. Average number of riboflavin and FITC attached to the surface of PAMAM G5-glutaric acid was estimated to be 6.3 and 1.3 respectively from the analysis of UV/vis spectral data (FITC = 1.3 per dendrimer molecule) in combination with MALDI mass spectral data (number of RF per G5 molecule = [43200 (**5**) − 40200 (**3**) − 620 (mass corresponding to **3**)] ÷ 376 (MW of riboflavin) = 6.3).

2. Stepwise synthesis of conjugates 6-7

6: To a solution of glutaric acid-terminated PAMAM G5 dendrimer **3** (100 mg, 2.5 μmol) in DMF (20 mL) was added and 4-dimethylaminopyridine (9.17 mg, 75 μmol), and a solution of riboflavin (14.12 mg, 37.5 μmol) dissolved in a mixture of DMF (10 mL) and DMSO (2 mL). While stirring the mixture under nitrogen atmosphere, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI, 14.4 mg, 75 μmol) was added. After stirring the mixture for 24 h, the reaction mixture was concentrated *in vacuo* to ~5 mL in volume and diluted with 10 mL of phosphate-buffered saline (PBS, pH 7.2). The solution was loaded into a membrane dialysis bag (MWCO 10 kDa), and dialyzed against PBS (2 x 2L), and deionized water (3 x 2L) over 3 days. The aqueous solution was collected and lyophilized to afford G5-$RF_{2.5}$ **6** as orange solid (73.14 mg). MALDI TOF mass spectrometry: *m/z* = 41100. UV/vis (PBS, pH 7.2): 448 (λ_{max}), 350, 268 nm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.92 (br), 4.8-4.0 (multiple peaks), 3.65-3.50 (br s), 3.35-3.00 (br s), 2.75-2.55 (br s), 2.50-2.30 (m), 2.30-2.20 (m), 1.87 (m), 1.87-1.70 (m) ppm. Average number of riboflavin covalently attached to the surface of PAMAM G5-glutaric acid was calculated to be 2.5 from the analysis of MALDI mass spectral data: number of RF per G5 molecule = [41100 (**6**) − 40150 (**3**)] ÷ 376 (MW, riboflavin) ≈ 2.5.

7: To a solution of G5-$RF_{2.5}$ **6** (30 mg, 0.723 μmol) in DMF (10 mL) was added and 4-dimethylaminopyridine (8.83 mg, 36 μmol), and a solution of methotrexate-linker **1** (prepared freshly by treatment of its *N*-Boc protected form (7.54 mg, 11.3 μmol) with 1:1 TFA/CH_2Cl_2) dissolved in DMF (6.5 mL). While stirring the mixture under nitrogen atmosphere, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI, 13.86 mg, 72 μmol) and *N*-hydroxysuccinimide (8.32 mg, 72 μmol) was added. After stirring the mixture for 24 h, the reaction mixture was concentrated *in vacuo* to ~5 mL in volume and diluted with 10 mL of phosphate-buffered saline (PBS, pH 7.2). The solution was loaded into a membrane dialysis bag (MWCO 10 kDa), and dialyzed against PBS (2 x 2L), and deionized water (3 x 2L) over 3 days. The aqueous solution was collected and lyophilized to afford G5-$RF_{2.5}$-$MTX_{3.9}$ **7** as orange solid (20.4 mg). MALDI TOF mass spectometry: *m/z* = 43300. UV/vis (PBS, pH 7.2): 448 (λ_{max}), 368 nm. Average number of methotrexate covalently attached to the surface of PAMAM G5-$RF_{2.5}$ was calculated to be 4 from the analysis of MALDI mass spectral data (number of MTX per G5 molecule = [43300 (**7**) − 41100 (**6**)] ÷ 568 (**1**) **≈** 3.9).

3. One pot synthesis of conjugate 8

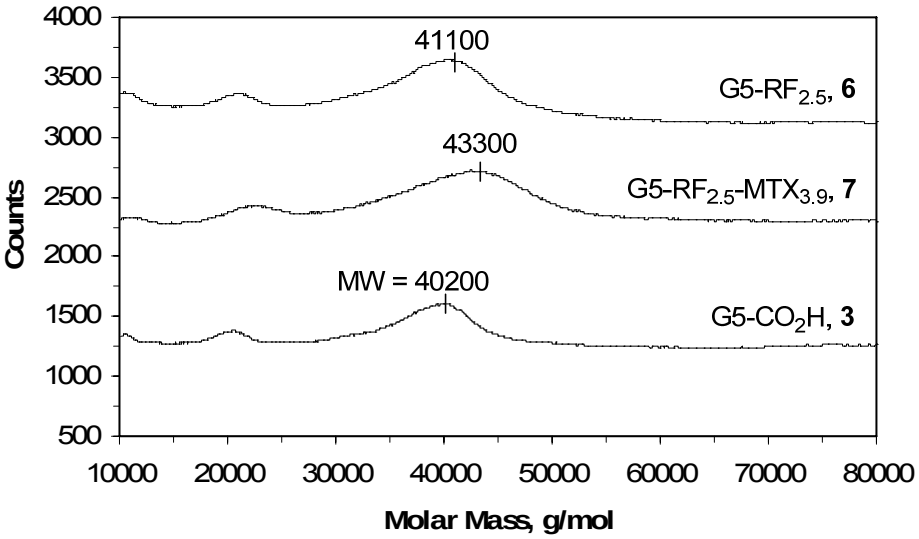
To a solution of glutaric acid-terminated PAMAM G5 dendrimer **3** (50 mg, 1.25 μmol) in DMF (10 mL) was added and 4-dimethylaminopyridine (15.3 mg, 125 μmol), triethylamine (50 μL, 359 μmol), methotrexate-linker **1** (prepared freshly by treatment of its *N*-Boc protected form (12 mg, 18.8 μmol) with 1:1 TFA/CH_2Cl_2) and a solution of riboflavin (7.1 mg, 18.8 μmol) dissolved in DMF (5 mL). While stirring the mixture under nitrogen atmosphere, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI, 24.5 mg, 125 μmol) was added. After stirring the mixture for 48 h, the reaction mixture was concentrated *in vacuo* to ~5 mL in volume and diluted with 10 mL of phosphate-buffered saline (PBS, pH 7.2). The solution was loaded into a membrane dialysis bag (MWCO 10 kDa), and dialyzed against PBS (2 x 2L), and deionized water (3 x 2L) over 3 days. The aqueous solution was collected and lyophilized to afford G5-RF_4-MTX_{10} **8** as orange solid (26.3 mg). MALDI TOF mass spectrometry: *m/z* = 47500. UV/vis (PBS, pH 7.2): 448 (λ_{max}), 368, 268 nm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.57-8.53 (m), 7.92 (br), 7.69-7.64 (m), 6.79 (m), 4.76 (br s), 4.55-4.40 (m), 4.30-3.60 (m), 3.40-3.26 (m), 3.16 (m), 2.80-2.10 (br m), 1.68 (m), 1.40-1.0 (m) ppm. Average number of riboflavin and MTX covalently attached to the surface of PAMAM G5-glutaric acid was calculated to be 4 and 10 respectively from the analysis of UV/vis spectral data (RF = 4 per a dendrimer molecule) in combination with MALDI mass spectral data (number of MTX per G5 molecule = [47500 (**8**) − 40200 (**3**) − 1504 (molar weight, riboflavin)] ÷ 568 (**1**) **≈** 10).

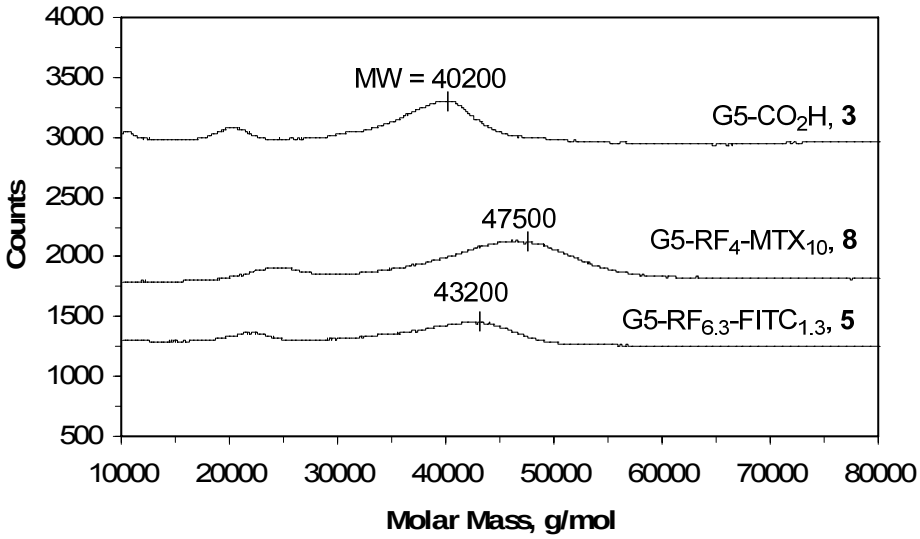
Table 1. Summary of molecular mass, yield and purity of G5 PAMAM dendrimer riboflavin nanoconjugates

Nanoconjugate G5-RF_n-MTX_n-$FITC_o$	MW (*m/z*, $gmol^{-1}$)[a]	Number[b] of RF (n), MTX (m), FITC (o)	Yield (%)[c]	Purity (%)[d]
3 G5-$(COOH)_{100}$	40200 (42730[e])	n = m = o = 0	-	≥98
5 G5-$RF_{6.3}$-$FITC_{1.3}$	43200	n = 6.3, o = 1.3	72	99
6 G5-$RF_{2.5}$	41100	n = 2.5, m = 0	73	98
7 G5-$RF_{2.5}$-$MTX_{3.9}$	43300	n = 2.5, m = 3.9	68	96
8 G5-RF_4-MTX_{10}	47200	n = 4, m = 10	53	98

[a]Molecular weight (*m/z*) refers to the mass value at or around the peak of each MALDI spectrum; [b]The number was calculated by dividing the molecular weight difference between two directly comparable conjugates by the molecular weight corresponding to riboflavin (x $gmol^{-1}$), methotrexate-linker (568.3 $gmol^{-1}$), or FITC-butane-1,4-diamine (477.1 $gmol^{-1}$); [c]based on the mass percentage of each conjugate isolated relative to the amount of a reactant dendrimer or conjugate used; [d]purity of each nanoconjugate was assessed by analytical reversed phase HPLC at a detection wavelength of 285 nm to monitor RF- and MTX-associated species; [e]M_w (weight averaged molecular weight) obtained by gel permeation chromatography (GPC), polydispersity index (PDI) = M_w/M_n = 1.046.

4. MALDI TOF mass spectra of PAMAM G5 conjugates 3, 5-8





5. UV/vis spectra of 5, 6, and 8 (PBS, pH 7.2)