SUPPORTING INFORMATION

A Lipopolysaccharide Binding Heteromultivalent Dendrimer Nanoplatform for Gram Negative Cell Targeting

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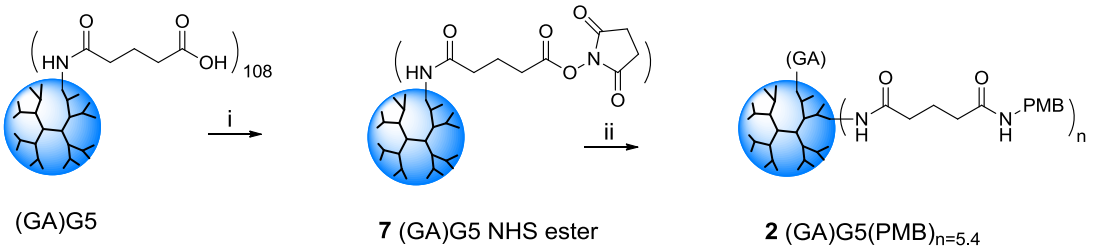
1. Synthesis of conjugate 2 (GA)G5(PMB)$_{n=5.4}$ (Scheme S1)



(GA)G5 **7** (GA)G5 NHS ester **2** (GA)G5(PMB)$_{n=5.4}$

reagents and conditions: i) EDC, NHS, DMAP, DMF, rt, 12 h; ii) polymyxin B sulfate (10 mol equiv), DIPEA, rt, 12 h.

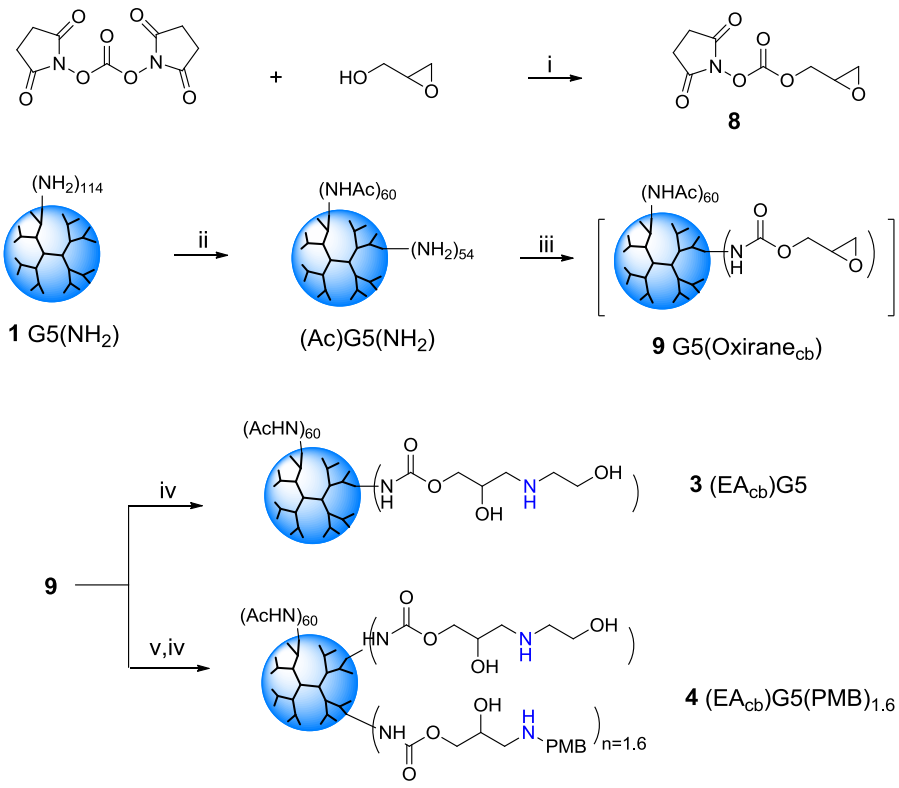
To a stirred solution of glutarate-terminated dendrimer (GA)G5[1, 2] (MW = 40,200 g mol^{-1}; 200 mg, 4.98 μmol) suspended in anhydrous DMF (15 mL) was added *N*-hydroxysuccinimide (NHS, 124 mg, 1.07 mmol), 4-dimethylaminopyridine (DMAP, 164 mg, 1.34 mmol), and then 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 257 mg, 1.34 mmol). This mixture was stirred at room temp for 12 h or longer until all dendrimer solid particles were fully solubilized. To this solution that contains an activated dendrimer (GA)G5 NHS ester (**7**) was added *N*,*N*-diisopropylethylamine (DIPEA; 0.043 mL, 0.249 mmol) and polymyxin B sulfate (PMB; 69 mg, 0.0498 mmol) as a solid while being stirred. The final mixture was stirred at room temp for 12 h and concentrated *in vacuo*. The residue was dissolved in PBS (10 mL) and loaded in a dialysis tubing (MWCO 10 kDa). The solution was dialyzed against water (4 L), PBS (4 L), and water (3 × 4 L) over 2 days. Lyphilization of the dialyzed solution afforded the conjugate **2** (GA)G5(PMB)$_n$ as a white beige solid (213 mg).

The homogeneity of conjugate **2** was analyzed by a UPLC method (Figure S2): t_r = 9.4 min, free PMB undectable, polymer purity ≥99%. MALDI-TOF mass spectrometry (*m/z*; g mol^{-1}; Figure S3): 46,400. UV–vis (PBS, pH 7.4; Figure S4): λ_{max} = 291 nm (ε = 52,600 M^{-1}cm^{-1}). ^{1}H NMR (500 MHz, DMSO-d_6; Figure S5): δ 8.8–7.8 (strong br d*; internal amide NH), 7.3–7.1 (br s, (D)-Phe), 4.6–3.8 (weak br), 3.8–2.8 (strong m), 2.8–1.9 (strong m), 1.8–1.6 (strong s; NHC(=O)CH$_3$), 1.5 (weak br s), 1.3–1.2 (br s), 1.2–0.9 (br s), 0.8 (s), 0.7–0.5 (br). *Acronyms: br (broad), s (singlet), m (multiplet). The valency (*n*) of the PMB molecule attached to the dendrimer (GA)G5(PMB)$_n$ was determined on a mean basis by two different methods. First, a NMR integration method was used in which the (D)-Phe (PMB) signal (δ 7.3–7.1 ppm) was compared to a reference group of -HNC(=O)CH$_2$C**H$_2$**CH$_2$C(=O) at δ 1.8–1.6 ppm (108 CH$_2$

residues per dendrimer), yielding n = 5.4 (±0.5). Second, the difference in MALDI-TOF spectra before and after PMB conjugation by M_r of PMB was also used to calculate the valency: n = [46,400 (**2**) − 40,200 ((GA)G5)] ÷ 1231.5 = 5.0. The UV–vis method was not applicable here since PMB has only a weak absorption at 205 nm[3] which belongs to the spectral region characterized by strong dendrimer absorption.

Three other PMB conjugates in this series (GA)G5(PMB)$_n$ (mean n = 2.2, 9.1, 13.5 determined by NMR analysis) were synthesized in a similar manner, each by varying the PMB-to-G5 molar ratio of 10 to 5, 20 or 30, respectively. (GA)G5(PMB)$_n$: n = 2.2 ± 0.2 (MW = 44,600), n = 9.1 ± 0.9 (MW = 48,400), n = 13.5 ± 0.6 (MW = 53,400).

2. Synthesis of conjugates 3–4 (EA$_{cb}$)G5(PMB)$_n$ (Scheme S2)



reagents and conditions: i) DIPEA, MeCN, rt, 12 h; ii) Ac_2O (60 mol equiv), DIPEA, MeOH, rt; iii) **8** (50 mol equiv), 12 h, rt; iv) ethanolamine (600 mol equiv), 45°C, 12 h; v) polymyxin B sulfate (5 mol equiv), NaOH (10 mol equiv), H_2O, MeOH, 45°C, 20 h

Preparation of **8**: An activated ester of glycidol was prepared following a method described elsewhere.[4] To a solution of glycidol (20.1 mg, 0.272 mmol) dissolved in acetonitrile (2 mL) was added DIPEA (52.1 μL, 0.299 mmol) and then *N,N′*-disuccinimidyl carbonate (69.6 mg, 0.272

mmol). The mixture was stirred at room temp for 12 h, and evaporated *in vacuo* to dryness. It was dissolved in MeOH (1 mL) and used immediately for next step.

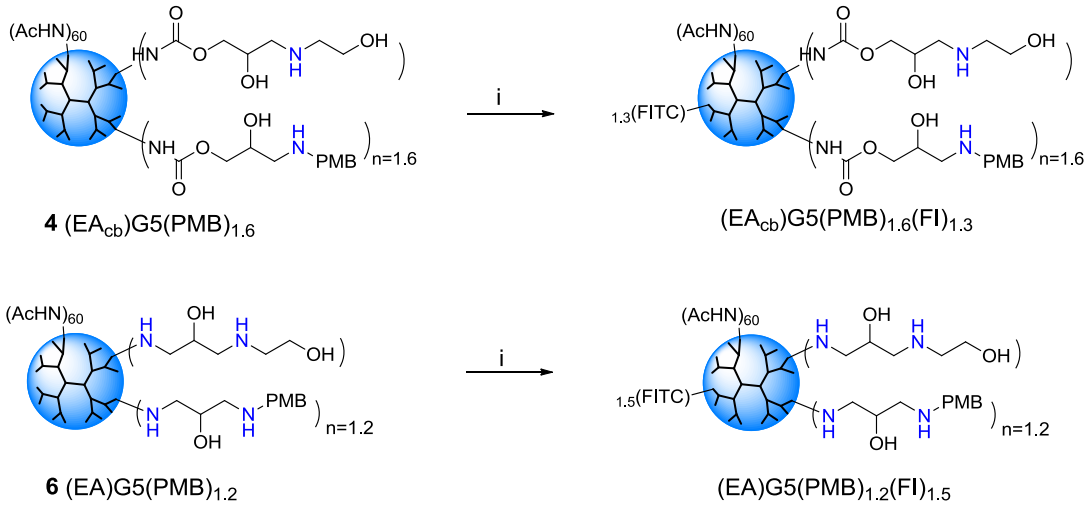
Prepwaration of **9** G5(Oxirane$_{cb}$): To a stirred solution of **1** G5(NH$_2$) (150 mg, 5.43 μmol) in methanol (20 mL) was added DIPEA (56.8 μL, 0.326 mmol) and Ac_2O (30.8 μL, 0.326 mmol) in a dropwise manner as a neat liquid. The mixture was stirred at room temp for 12 h to prepare a partially acetylated dendrimer (Ac)G5(NH$_2$) as described elsewhere.[5] To this solution was added a solution of activated glycidol carbonate **8** in methanol (1 mL) prepared above, and the reaction mixture was stirred at room temp overnight to generate a reactive conjugate **9** G5(Oxirane$_{cb}$). This solution (total 21 mL) was divided into two lots (14 mL, 7 mL each) and each was used without further treatment immediately for a next conjugation reaction.

Preparation of conjugate **3** (EA$_{cb}$)G5: To **9** (7 mL lot) was added a NaOH solution (1.0 M, 36 μL) and ethanolamine (0.132 mL, 1.09 mmol). The resulting mixture was shaken at 45^{o}C for12 h, and concentrated *in vacuo*. The residue was dissolved in water (5 mL), loaded into a membrane dialysis tubing (MWCO 10 kDa) and dialysed against water (4 L × 3) for 2 days. After lyphilization of the dialyzed solution, the conjugate **3** (EA$_{cb}$)G5 was obtained as white beige solid (43 mg).The homogeneity of conjugate **3** was analyzed by a HPLC method (Figure S2): t_r = 7.9 min, polymer purity ≥97%. GPC (Figure S1): M_n = 34,700 gmol^{-1}, PDI = 1.23. MALDI-TOF mass spectrometry (*m/z*; g mol^{-1}; Figure S3): 31,900. UV–vis (PBS, pH 7.4; Figure S4): λ_{max} = 281 nm (ε = 8,136 M^{-1}cm^{-1}). ^{1}H NMR (500 MHz, DMSO-d_6; Figure S6): 8.1–7.8 (strong m), 4.1–3.6 (weak m), 3.5–3.0 (strong m), 2.8–2.4 (strong m), 2.3–2.1 (strong m), 1.9 (m), 1.8 (strong s), 1.25 (weak m), 1.15 (weak m) ppm.

Preparation of conjugate **4** (EA$_{cb}$)G5(PMB)$_n$: To **9** (21 mL lot) was added a solution of polymyxin B sulfate (50 mg, 36.2 μmol) dissolved in 1.0 mL of water, and followed by the addition of a NaOH solution (1 M, 0.181 mL). The mixture was shaken at 45^{o}C for 20 h, and ethanolamine (0.132 mL, 1.09 mmol) was added. The final mixture was shaken at 45^{o}C for 12 h and concentrated *in vacuo*. The residue was dissolved in water (10 mL) and dialysed (MWCO 10 kDa; 4 L of water × 3) for 2 days. After lyphilization of the dialyzed solution, the conjugate **4** (EA$_{cb}$)G5(PMB)$_n$ was obtained as white beige solid (111 mg).The homogeneity of conjugate **4** was analyzed by a HPLC method (Figure S2): t_r = 7.9 min, free PMB undetectable, polymer purity ≥97%. GPC (Figure S1): M_n = 36,300 gmol^{-1}, PDI = 1.18. MALDI-TOF mass

spectrometry (*m/z*; g mol^{-1}; Figure S3): 32,100. UV–vis (PBS, pH 7.4; Figure S4): λ_{max} = 282 nm (ε = 8,280 M^{-1}cm^{-1}). ^{1}H NMR (500 MHz, DMSO-d_6; Figure S6): 8.2–7.8 (strong m), 7.3–7.1 (br; D-Phe), 4.0–3.7 (weak m), 3.6–3.0 (stong m), 2.9–2.6 (m), 2.5–2.4 (m), 2.3–2.1 (m), 1.9 (m), 1.8 (strong s), 1.5 (br), 1.3–1.0 (br m) ppm. The valency (*n*) of the PMB molecule attached was determined by a NMR integration method in which the (D)-Phe (PMB) signal (δ 7.3–7.1 ppm) was compared to a reference group of NHAc at δ 1.8 ppm (60 Ac residues per dendrimer), yielding *n* = 1.6 (±0.1). The PMB valency (*n*) calculated by M_n values between conjugate **3** and **4** is slightly lower: *n* = [36,340 − 34,660] ÷ 1231.5 = 1.4.

3. Synthesis of FITC-labeled G5 dendrimer conjugates 4, 6 G5(PMB)$_n$(FI) (Scheme S3)



reagents and conditions: i) FITC, Et_3N, DMF, 45°C

A representative procedure: To a stirred solution of **4** (20 mg, 0.62 μmol) or **6** (20 mg, 0.66 μmol), each dissolved in DMF (5 mL), was added triethylamine (50 μL, 0.356 mmol) and then fluorescein 5(6)-isothiocyanate (1 mg, 2.56 μmol). The mixture was stirred at room temp for 5 min, and placed in a water bath for incubation at 45°C for 24 h. The mixture was concentrated with a rotary evaporator to a thin layer of oily residue, and the residue was dissolved in water (5 mL). The solution was transferred to a membrane dialysis tubing (MWCO 10 kDa), and dialyzed against water (2 × 2L) over 24 h. Each solution was lyophilized to afford a dry solid. It was dissolved in 2 mL of PBS, and further purified by centrifugal membrane filtration using Amicon membrane tube (MWCO 10 kDa) at 4800 rpm for 15 min. This centrifugal filtration was

repeated with PBs for three additional times, and with water for two times. After filtration, each solution was collected and lyophilized, affording the FITC-labeled dendrimer as an orange fluffy solid (12 mg for **4**; 11 mg for **6**).

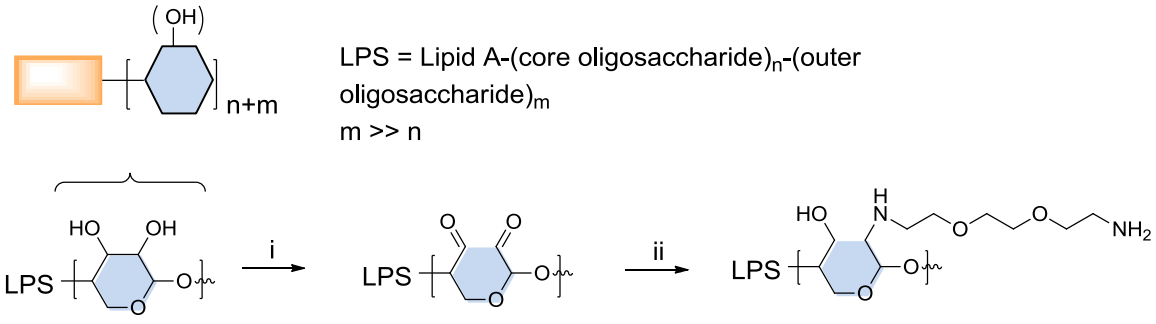
4 $(FI)_{1.3}$: HPLC: t_r = 7.4 min; polymer purity ≥95%. MALDI-TOF mass spectrometry (*m/z*; g mol^{-1}): 32,400. UV–vis (PBS, pH 7.4; Figure S4): λ_{max} = 491 nm (FITC; ε = 100,200 $M^{-1}cm^{-1}$).

5 $(FI)_{0.58}$: HPLC: t_r = 7.3 min; polymer purity ≥95%. MALDI-TOF mass spectrometry (*m/z*; g mol^{-1}): 30,500. UV–vis (PBS, pH 7.4; Figure S4): λ_{max} = 491 nm (FITC; ε = 44,240 $M^{-1}cm^{-1}$).

6 $(FI)_{1.5}$: HPLC: t_r = 7.3 min; polymer purity ≥95%. MALDI-TOF mass spectrometry (*m/z*; g mol^{-1}): 30,500. UV–vis (PBS, pH 7.4; Figure S4): λ_{max} = 491 nm (FITC; ε = 112,450 $M^{-1}cm^{-1}$).

4. Surface Plasmon Resonance (SPR) Spectroscopy

Preparation of amine-derivatized LPS (Scheme S4). Step i): To a solution of LPS (MW > 10 kDa; 5 mg ≈ 0.5 μmol) dissolved in water (1 mL) was added an aqueous solution of $NaIO_4$ (20 mM, 0.5 mL; 10 μmol). The mixture was stirred at room temp for 30 min, and unreacted $NaIO_4$ was consumed by adding excess 1,2-propanediol (0.10 mL, 1.37 mmol) in the mixture. After shaking for 10 min, the solution was transferred to an untracentrifugal filtration unit (Amicon; MWCO 10 kDa), and centrifuged (4500 rpm, 15 min). The filtrate collected in the outer tube was removed, and water (1 mL) was added to the residue in the inner tube. After mixing it, the solution was filtered again. The residual solution (~0.1 mL) in the inner tube was saved and used immediately for next step below.



reagents and conditions: i) $NaIO_4$, water, rt, 30 min; ii) 2,2'-(ethylenedioxy)bis(ethylamine), PBS 7.4; then $NaBH_4$, 5 min

Step ii): A solution of 2,2'-(ethylenedioxy)bis(ethylamine) (0.05 mL, 0.34 μmol) dissolved in 1 mL of PBS buffer (pH 7.4) was added to the $NaIO_4$-tretated LPS solution above. The mixture was shaken for 5 min prior to the addition of $NaBH_4$ (10 mg). The final mixture was shaken for 5 min, and filtered using an untracentrifugal filtration unit (Amicon; MWCO 10 kDa) at 4500 rpm for 15 min. The residue in the inner tube was dissolved in PBS (pH 9), and filtered again. Finally, the residue was reconstituted in 1 mL of PBS (pH 9.0; ≈5 mg/mL). This solution was used for LPS immobilization to a CM5 sensor chip.

LPS immobilization. A CM5 sensor chip immobilized with LPS molecules was prepared by an amide coupling method[6-8] in which the amine-terminated LPS was covalently attached to the chip surface coated with carboxymethylated dextran. First, a flow cell 1 was activated by injection of an EDC/NHS solution (70 μL; 1:1 mixture of 0.4 M EDC and 0.1 M NHS, each in water). This flow cell was immediately treated by injection of the amine-terminated LPS (35 μL, 5 mg/mL, pH 9) and excess ethanolamine for reaction with unreacted activated ester on the surface. Second, a flow cell 2 (reference cell) was treated in the same manner as above but witout injection of the LPS solution. Such treatment led to a net increase in response units, ΔRU = RU (Fc_1) – RU (Fc_2) of 230. This value is relatively lower than those from protein immobilization,[8-10] and attributable to similarity in refractive indices between water (n = 1.333) and a sugar solution (5%, n = 1.340) (cf, proteins, n = 1.6).

5. Release kinetics of ciprofloxacin complexed with PMB-conjugated dendrimers

Preparation of a ciprofloxacin alone control: To a 1 mL of water was added an aqueous solution of ciprofloxacin (10.5 μL; 6.04×10^{-3} M). This mixture served as a reference (ciprofloxacin alone).

Preparation of ciprofloxacin-dendrimer complexes: To a solution of **2** (GA)G5$(PMB)_{5.4}$ dissolved in water (1 mL; 6.47×10^{-5} M) was added an aqueous ciprofloxacin solution (10.5 μL; 6.04×10^{-3} M). The mixture was left at room temp for 15 min prior to transfer into a dialysis tube. Each of other complexes was prepared in the same manner except by replacing **2** with **4** (GA)G5$(PMB)_{5.4}$ (6.23×10^{-5} M) or **6** (GA)G5$(PMB)_{5.4}$ (6.60×10^{-5} M).

Figure S1. Gel permeation chromatography (GPC) traces of PAMAM G5 dendrimer conjugates **2**–**6**.

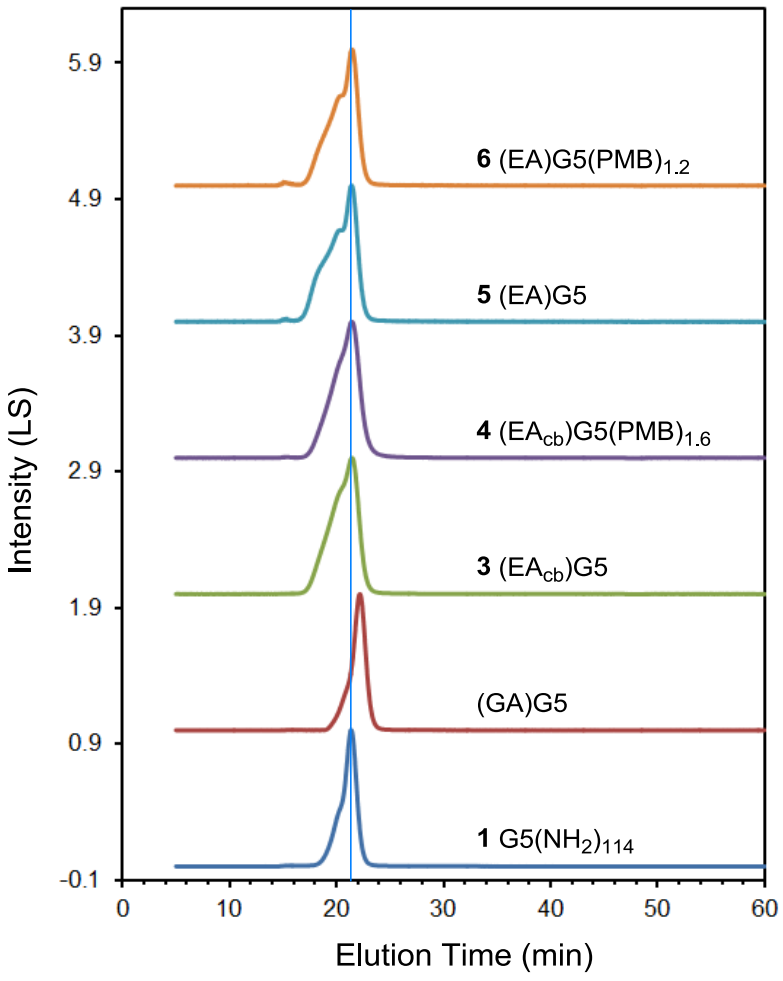


Figure S12. (A–C) HPLC traces of **1**–**6** PAMAM G5 dendrimers. Polymyxin B (PMB) = a mixture of polymyxin B_1 and polymyxin B_2

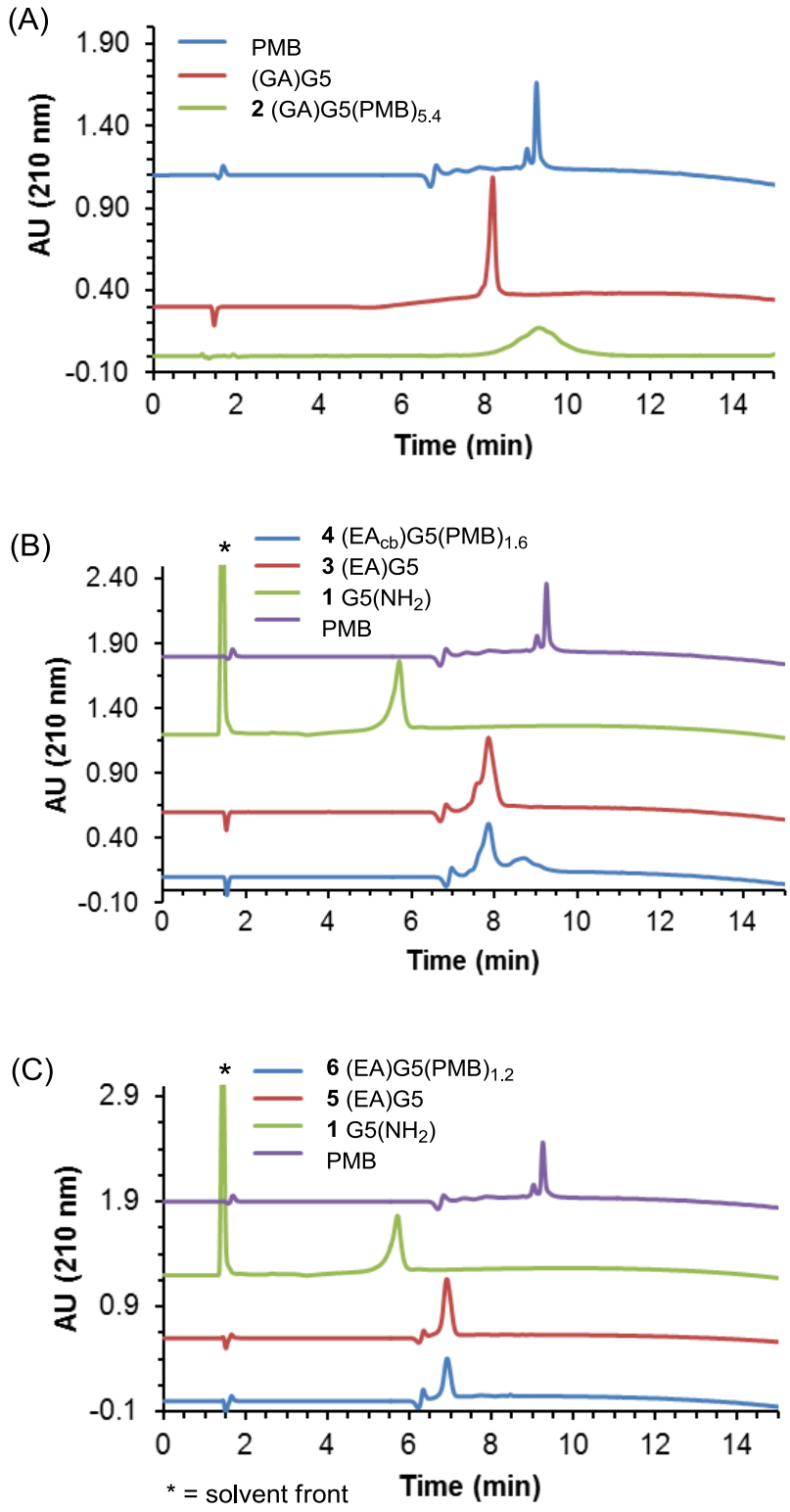


Figure S3. MALDI-TOF MS data of PAMAM G5 dendrimer conjugates **2**–**6**. (A) (GA)G5 and **2** (GA)G5(PMB)$_{5.4}$; (B) **1** G5(NH$_2$)$_{114}$, **3** (EA$_{cb}$)G5, **4** (EA$_{cb}$)G5(PMB)$_{1.6}$; (C) **1** G5(NH$_2$)$_{114}$, **5** (EA)G5, **6** (EA)G5(PMB)$_{1.2}$. The number noted on each spectrum refers to a M_r value at the peak.

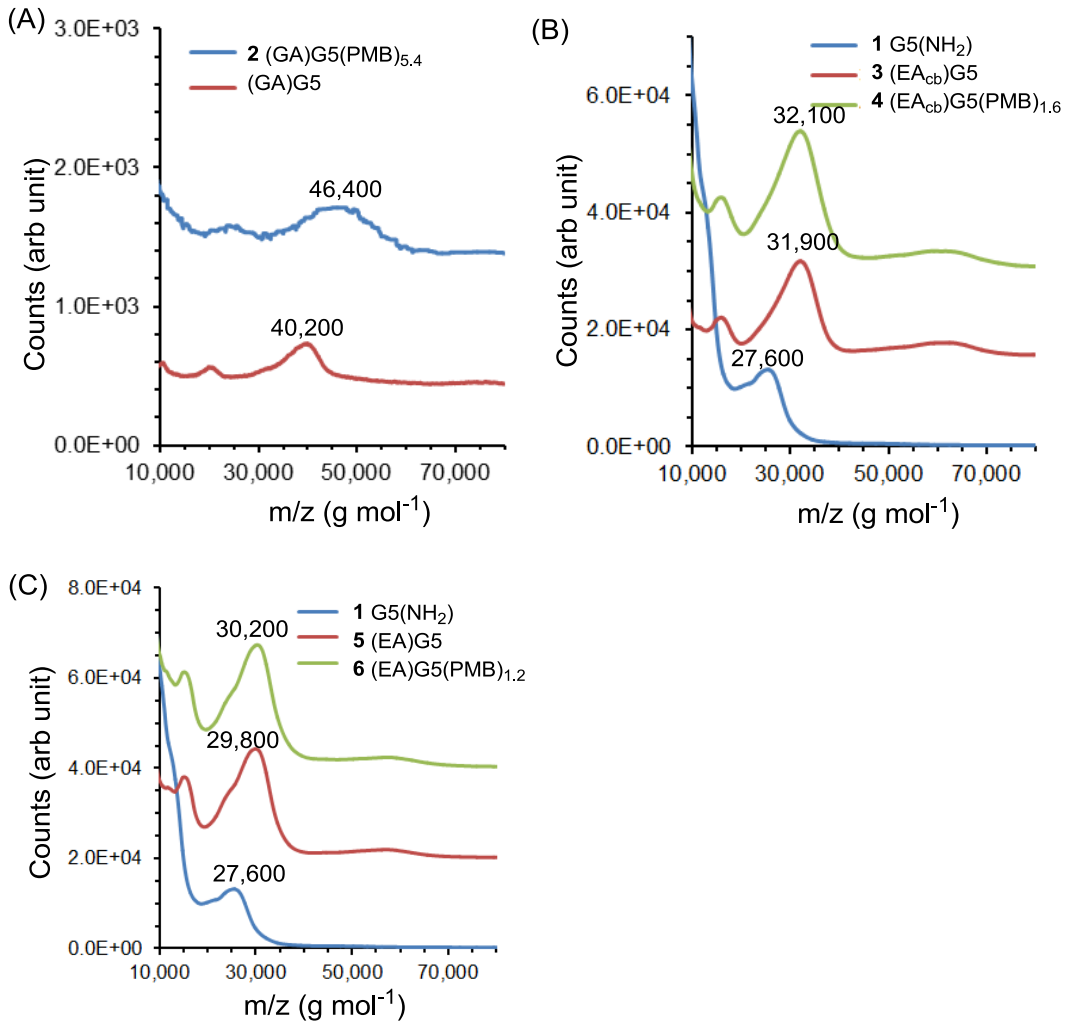


Figure S4. UV–vis absorption spectra of **2**–**6** conjugates, each measured in PBS (pH 7.4).

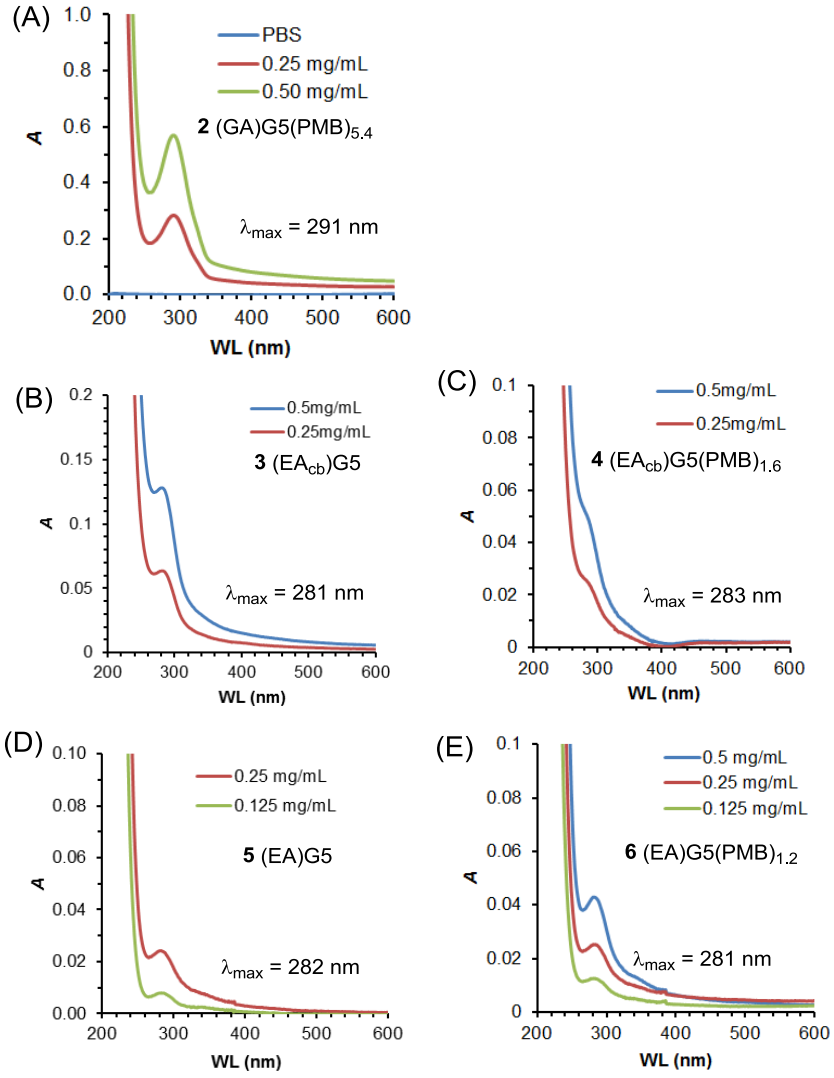


Figure S5. ^{1}H NMR (500 MHz) spectra of (GA)G5 (A; D_2O), PMB (B; D_2O) and **2** (GA)G5(PMB)$_{n=5.4}$ (C; DMSO-d_6).

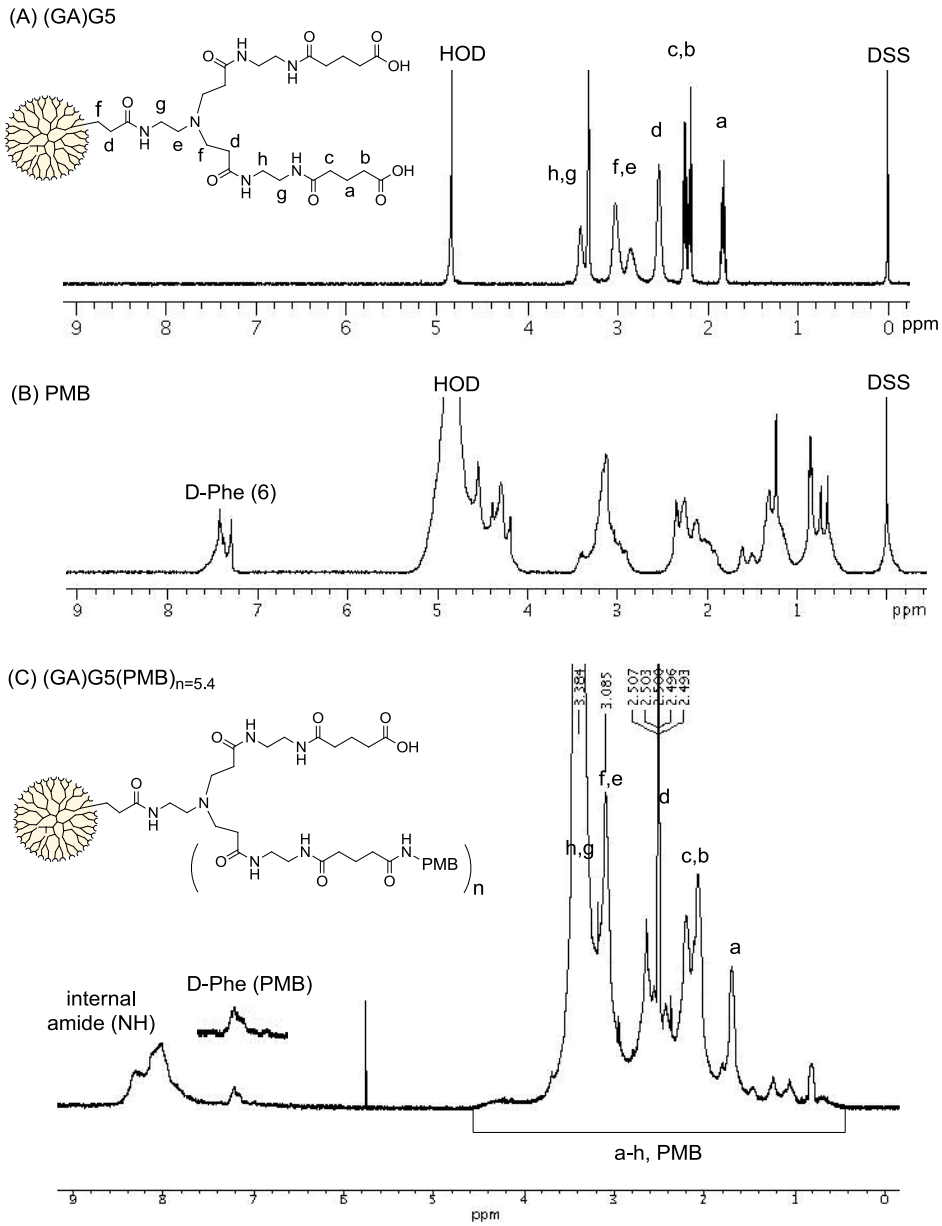
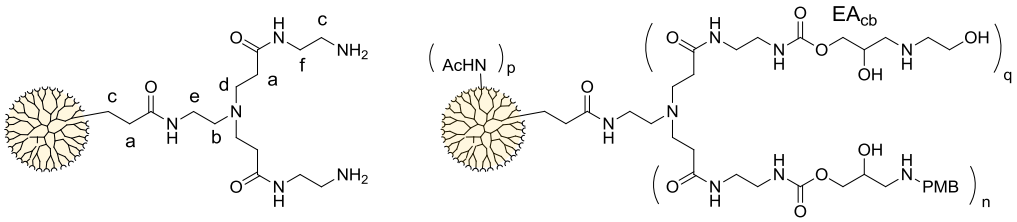


Figure S6. ^{1}H NMR spectra (500 MHz, D_2O) of **1** G5$(NH_2)_{114}$ (A), **3** (EA_{cb})G5 (B) and **4** (EA_{cb})G5$(PMB)_{n=1.6}$ (C).



1 G5(NH_2) **3**, **4** (EA_{cb})G5$(PMB)_n$: (p +q + n) = 114

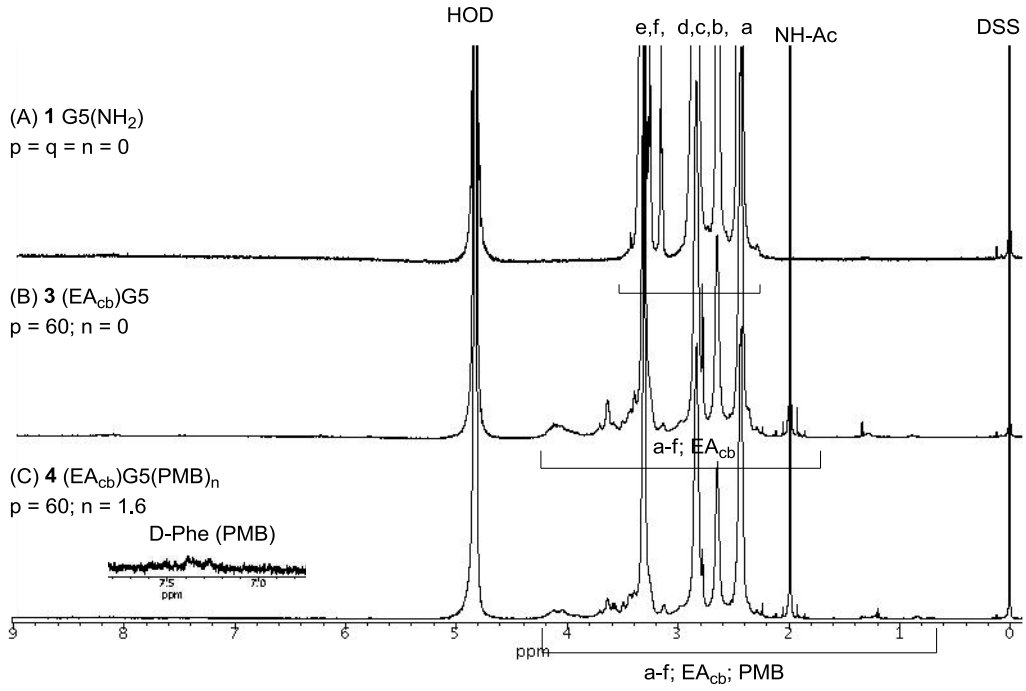
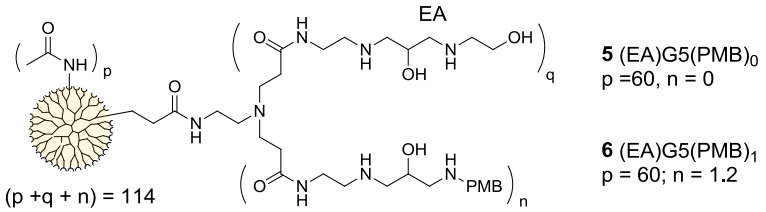


Figure S7. ^{1}H NMR (500 MHz; DMSO-d_6) spectra of **1** G5$(NH_2)_{114}$ (top), **5** (EA)G5 (middle) and **6** (EA)G5$(PMB)_{n=1.2}$ (bottom).



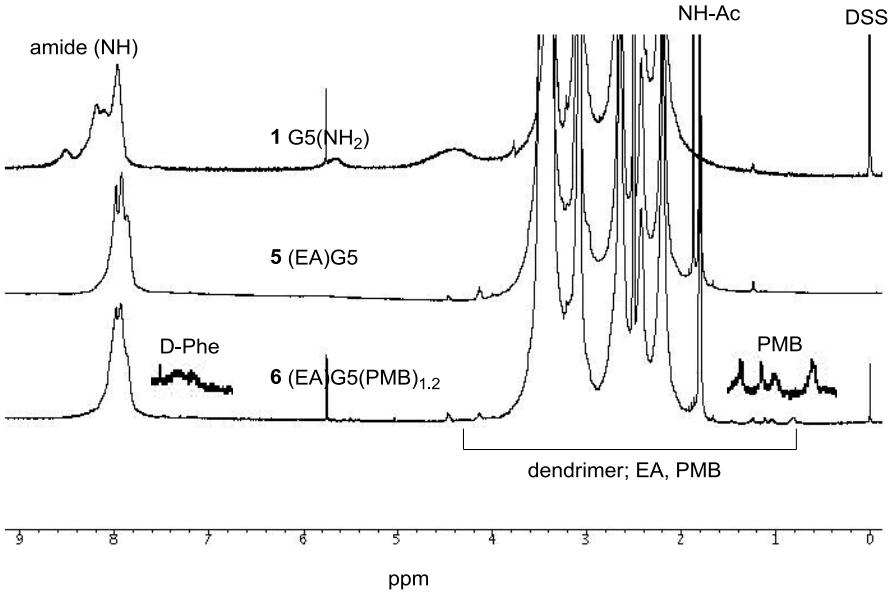


Figure S8. SPR sensorgrams of PMB (A) and dendrimer conjugates **2**–**4** G5(PMB)$_n$ (B–D) to a CM5 sensor chip immobilized with LPS. Each corrected sensorgram ΔRU (Fc_1 – Fc_2) is acquired from substraction of flow cell 1 (Fc_1; LPS immobilized) by flow cell 2 (Fc_2; reference witout LPS immobilized). The Scatchard plot (Figure A, right) is made from PMB binding sensorgrams (left). Experimenal (A–D; solid lines); simulated global fits (D; dotted lines).

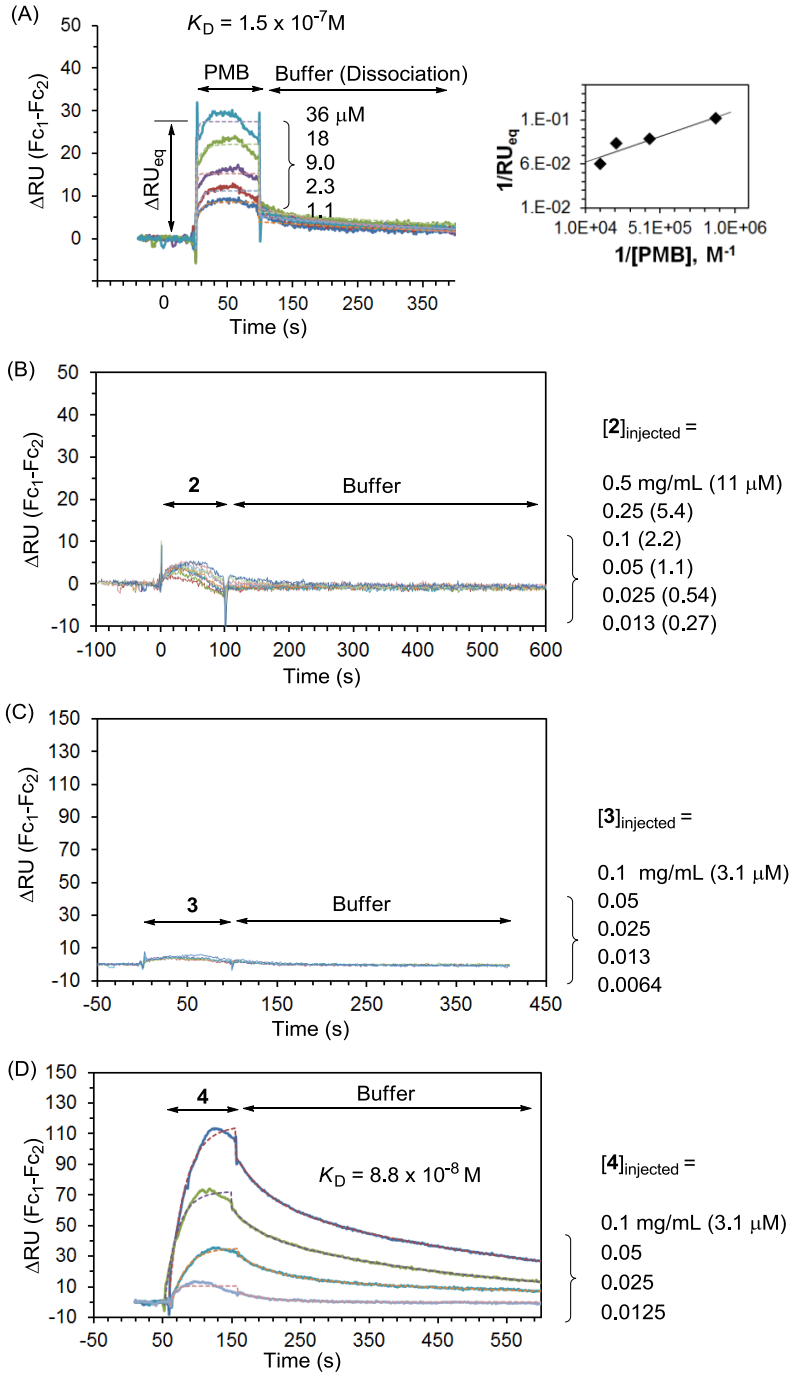


Figure S9. Dose-dependent sensorgrams of LPS (A) and its premade mixture with conjugate **5** (EA)G5 (B) prior to injection onto a CM5 sensor chip immobilized with LPS. Each mixture in (B) was prepared at a fixed concentration of **5** with the variation of LPS concentration as indicated.

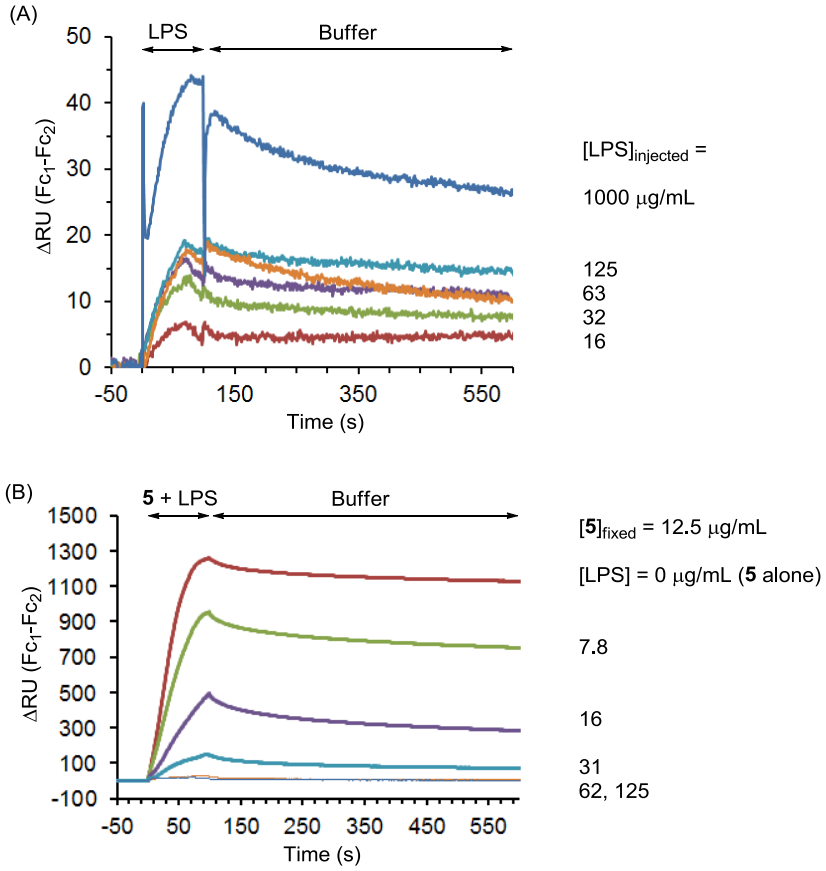


Figure S10. (A) Poissonian analysis of dendrimer distribution[11, 12] simulated for G5(PMB)$_n$ **2**, **4**, **6**, each having the mean valency of PMB at n_{mean} = 5.4, 1.6 or 1.2, respectively. (B) Sum of populations (%) of multivalent species ($n \geq 2$) distributed in each G5(PMB)$_n$ conjugate.

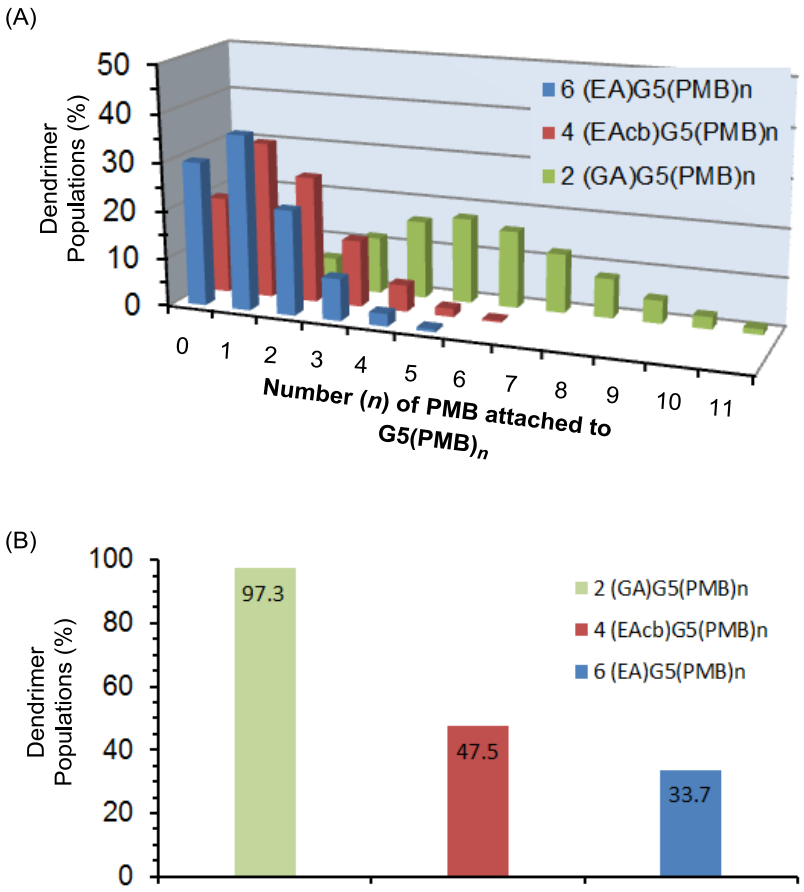


Figure S11. Effect of PMB valency (n) on the *in vitro* antibacterial activity of an amide-conjugated series (GA)G5(PMB)$_n$ (n = 2.2, 9.1, 13.5) against *E. coli*. Relative cell viability is plotted as a function of concentrations on the basis of dendrimer ([Dendrimer]; A) or PMB ([PMB] = n × [Dendrimer]; B).

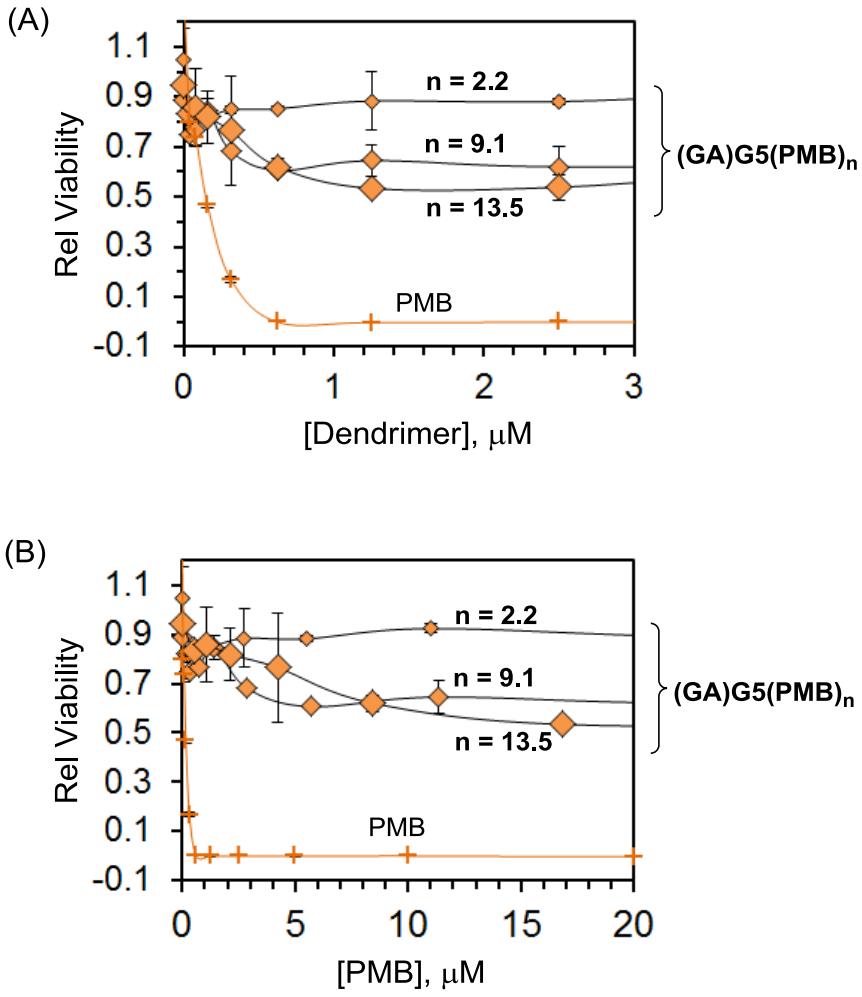


Figure S12. (A) A LCMS/MS calibration plot of ciprofloxacin. (B) Time-dependent cumulative concentrations of ciprofloxacin released from its complex made with **2** (GA)G5(PMB)$_{5.4}$, **4** (EA$_{cb}$)G5(PMB)$_{1.6}$ or **6** (EA)G5(PMB)$_{1.2}$. Each complex was prepared in a 1:1 molar ratio by adding ciprofloxacin to dendrimer (= 6.2–6.6 × 10^{-5} M), and drug release kineics was investigated by using a dialysis method as described in the supplementary method section (page S11).

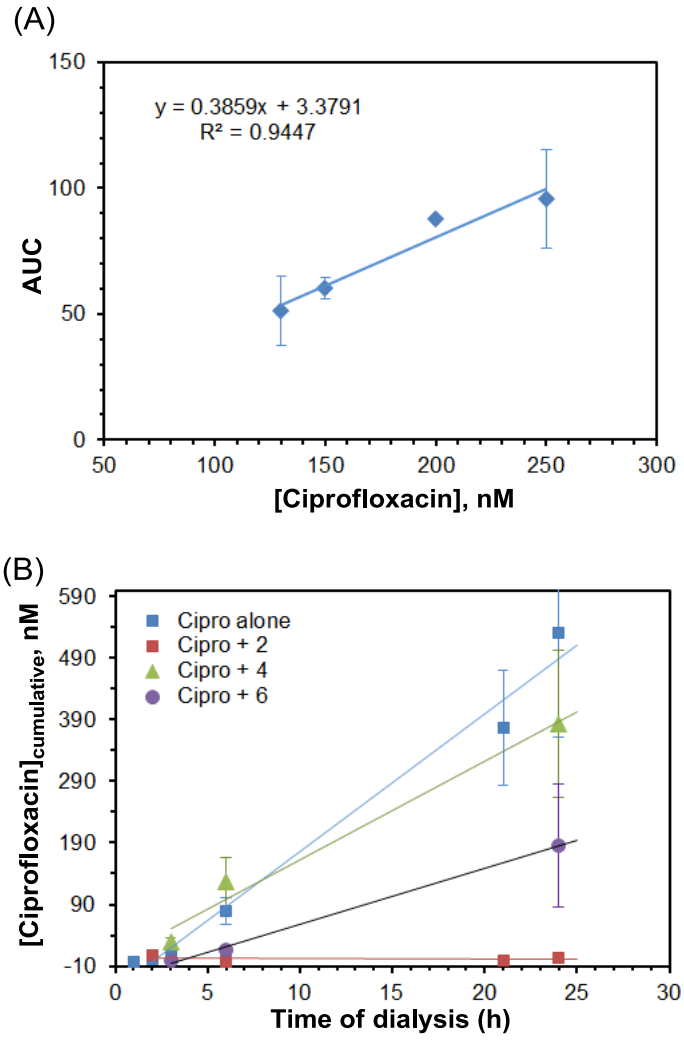


Table S1. Macromolecular properties and PMB mean valency (*n*) of G5 dendrimer conjugates G5(PMB)$_n$ **1**–**6**.

Number	G5(PMB)$_n$	M_r[a]	M_n[b] (PDI[c])	Valency (*n*, mean)
1	G5(NH$_2$)$_{n = 114}$	27,600	27,100 (1.09)	0
2	(GA)G5(PMB)$_n$	46,400	nd[d]	5.4±0.51;[e] 5.0[f]
3	(EA$_{cb}$)G5	31,900	34,700 (1.23)	0
4	(EA$_{cb}$)G5(PMB)$_n$	32,300	36,300 (1.18)	1.6±0.10;[e] 1.4[f]
5	(EA)G5	29,800	27,900 (1.33)	0
6	(EA)G5(PMB)$_n$	30,300	27,500 (1.26)	1.2±0.33[e]

[a]Measured by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF; g mol^{-1}) mass spectrometry

[b]Number-averaged molecular weight by gel permeation chromatography (GPC; g mol^{-1})

[c]Polydispersity index (PDI) = $M_w \div M_n$

[d]Not determined due to insufficient solubility in the GPC eluent (≤3 mg/mL; 0.1 M citric acid, pH 2.7)

[e]NMR integration (mean±SD)

[f]MW difference

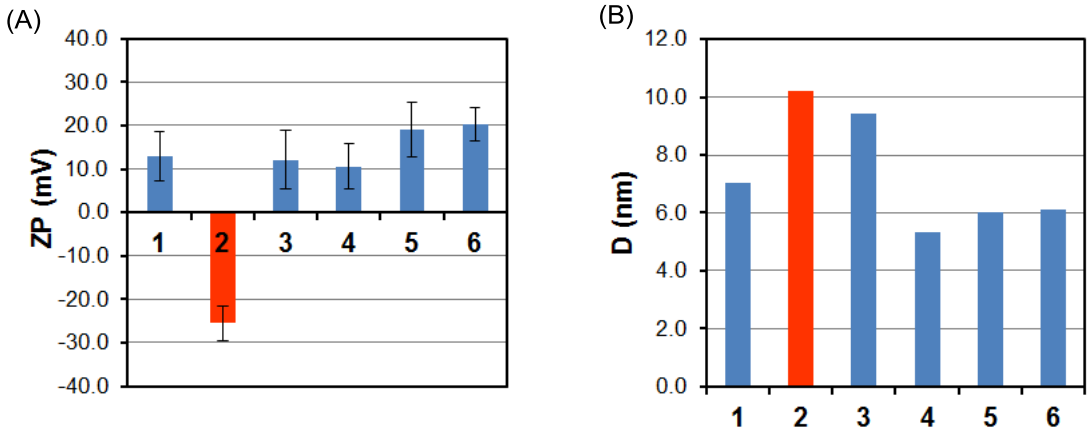
Table S2. Hydrodynamic diameter (D, nm) and zeta potential (ZP, mV) values of G5 dendrimers G5(PMB)$_n$ **1**–**6**.[a] Plots below: ZP (A); Diameter (B)

Number	G5(PMB)$_n$	ZP, mV	D, nm[b]	PDI[c]
1	G5(NH$_2$)$_{114}$	12.9 (±5.61)	7.05	0.421
2	(GA)G5(PMB)$_{5.4}$	−25.5 (±3.91)	10.2	0.76
3	(EA$_{cb}$)G5	12.1 (±6.75)	9.41	0.904
4	(EA$_{cb}$)G5(PMB)$_{1.6}$	10.5 (±5.19)	5.34	0.633
5	(EA)G5	19.1 (±6.27)	6.04	0.548
6	(EA)G5(PMB)$_{1.2}$	20.3 (±3.92)	6.12	0.61

[a] Measured in HEPES pH 7 ([dendrimer] = 0.05 mg/mL)

[b] Number-weighted diameter determined by dynamic light scattering (DLS) method

[c] Polydispersity index (PDI) determined by DLS method



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