

Supporting information

Carboxy-Terminal Dendrimers with Phenylalanine for pH-Sensitive Delivery System into Immune Cells Including T Cells

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Experimental procedures

Synthesis of carboxy-terminal dendrimers with hydrophobic amino acids

PAMAM-Suc-Phe, PAMAM-CHex-Phe, and PAMAM-Phe-Suc were synthesized in accordance with our previous reports.^{5,6} PAMAM-Suc-Leu and PAMAM-CHex-Leu were synthesized in the same procedure as PAMAM-Suc-Phe and PAMAM-CHex-Phe by replacing Phe with Leu. First, PAMAM-Suc and PAMAM-Chex were synthesized in accordance with our previous reports.⁵ Then, 80 eq. of L-leucine benzyl ester monotosylate (Leu-OBzl·Tos), 74 eq. of 1-[bis(dimethylamino)methylumyl]-1H-benzotriazole-3-oxide hexafluorophosphate (HBTU), and 76 eq. of triethylamine (TEA) to the terminal group of the dendrimer were added to the dendrimer solution in DMSO (5 mL) and stirred at room temperature. After the 4 days stirring, 1 mL of distilled water was added. The dendrimer was purified by dialysis (molecular weight cut-of (MWCO): 1000) in methanol. PAMAM-Suc-Leu-OBzl and PAMAM-Chex-Leu-OBzl were obtained following the evaporation and the lyophilization. The yields for PAMAM-Suc-Leu-OBzl and PAMAM-Chex-Leu-

OBzl were 15% and 41%, respectively. Then, these dendrimers were dissolved in methanol (4 mL). Then, 4 M NaOH methanol solution (500 μ L) was added. After stirring for 2 h at 4 °C, the dendrimer was dialyzed (MWCO: 1000) in distilled water. PAMAM-Suc-Leu was obtained following the lyophilization. The yields of PAMAM-Suc-Leu and PAMAM-CHex-Leu were ~100% and 40%, respectively.

PAMAM-Phe-CHex was synthesized in the same procedure as PAMAM-Phe-Suc by replacing Suc with CHex.⁶ PAMAM-Phe, synthesized in accordance with our previous report,⁴ was dissolved in 125 mM NaHCO₃ aqueous solution (3 mL). An excess of cyclohexanedicarboxylic anhydride (approximately 100 eq.) was added to the dendrimer solution. The solution pH was adjusted to approximately 9 using 4 M aqueous NaOH and stirred overnight at room temperature. The dendrimer was purified by dialysis (MWCO: 1000) in distilled water, and PAMAM-Phe-Chex was obtained after the lyophilization. The yield was 28%.

Labeling of green fluorescent dye to dendrimers

These carboxyl-terminal dendrimers were labelled with fluorescein isothiocyanate (FITC) in accordance with our previous report.¹⁴ Briefly, dendrimers were modified with several molecules of *N*-(*tert*-butoxycarbonyl)-1,2-diaminoethane (Boc-ethylenediamine). After the deprotection of the Boc group, FITC was reacted to the dendrimers. The bound number of FITC to each dendrimer was estimated as 3-9 from the absorbance at 495 nm of the FITC-conjugated dendrimers.

Characterization

The ¹H NMR spectra were recorded in methanol-*d*₆ or D₂O including NaOH using JEOL ECS-400 and ECX-400 spectrometers.

Solution turbidity

The aqueous solutions of various dendrimers were prepared at different pHs, in accordance with our previous reports.^{5,6} The temperature-dependent transmittance measurement was performed using a Jasco Model V-630 UV/Vis spectrophotometer equipped with ETC-717 (Jasco Inc., Tokyo, Japan) in accordance with our previous reports.^{5,6} The transmittance at 37°C in different pH solutions was picked up. The results of PAMAM-Suc-Phe, PAMAM-CHex-Phe, and PAMAM-Phe-Suc were referred to from our previous reports.^{5,6}

Investigation of interaction between dendrimers and liposome

Chloroform solution of hydrogenated soy phosphatidyl choline (HSPC) was evaporated and dried in

vacuum to make a thin lipid film. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (0.1 M, pH 7.4) was added to the HSPC thin film. The ultrasonication was irradiated for 2 min. 25 μ L of FITC-labeled dendrimer solution (100 μ M in dye) was incubated with 475 μ L of liposome solution (1.6 mg/ml) at 37 $^{\circ}$ C for 3 h. The liposomes were then precipitated by centrifugation at 37 $^{\circ}$ C at 11,000 rpm for 15 min. After the washing with the HEPES buffer, the supernatant was removed after the centrifugation. The liposomes were collapsed by adding 0.5 mL of chloroform/methanol solution (1/1, v/v), followed by the stirring at 37 $^{\circ}$ C for 10 min. Fluorescence intensity of the dendrimer was measured with methanol at a total volume of 2 mL by using FP-6200 spectrofluorometer (JASCO), which was obtained by subtracting the fluorescence intensity of the liposome alone.

Table S1. Fluorescence intensity of dendrimers adsorbed to liposomes

	Liposome-bound dendrimer	Total dendrimer	Adsorption (%)
PAMAM-CHex-Phe	1.54	86.95	2
PAMAM-Phe-CHex	18.67	33.79	55

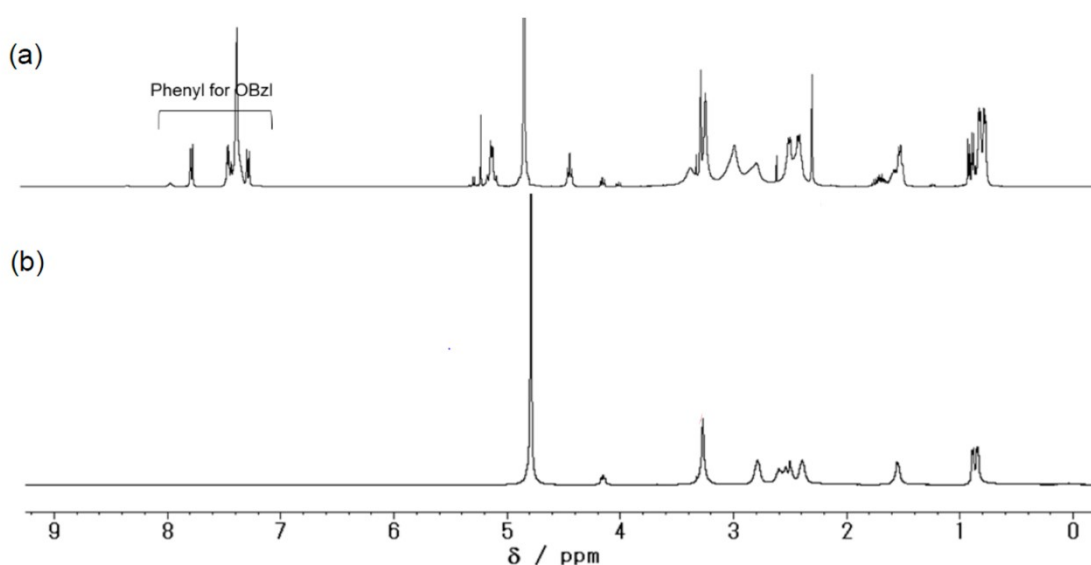


Figure S1. 1 H NMR spectra of PAMAM-Suc-Leu-OBzl in methanol- d_4 (a) and PAMAM-Suc-Leu in D_2O containing NaOD (b).

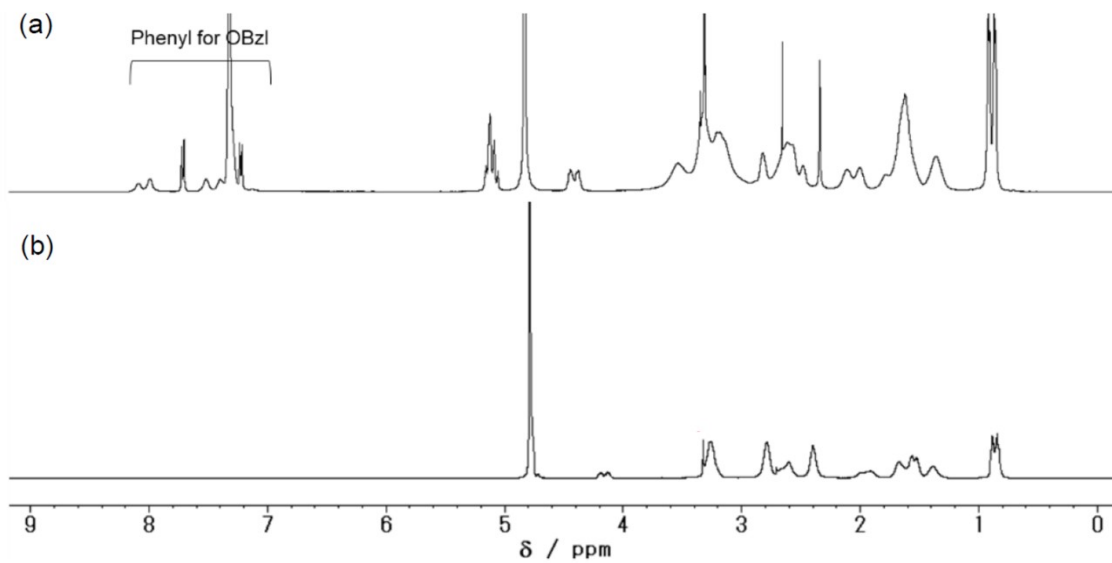


Figure S2. ^1H NMR spectra of PAMAM-CHex-Leu-OBzl in methanol- d_4 (a) and PAMAM-CHex-Leu in D_2O containing NaOD (b).

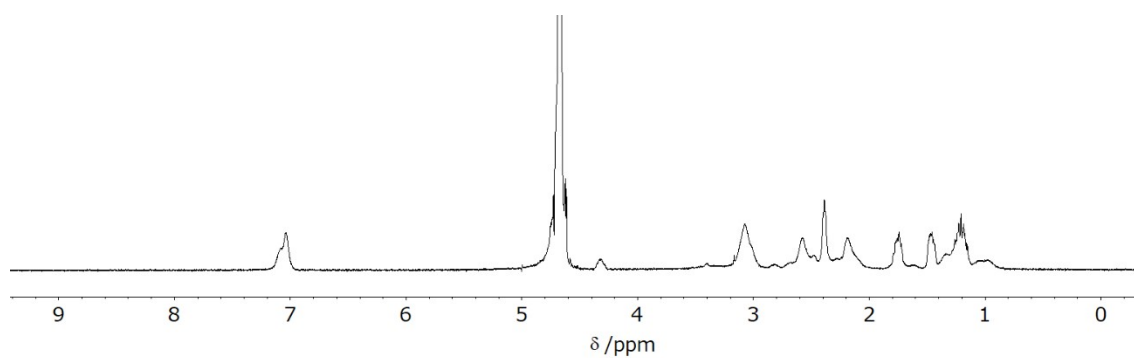


Figure S3. ^1H NMR spectra of PAMAM-Phe-CHex in D_2O containing NaOD.

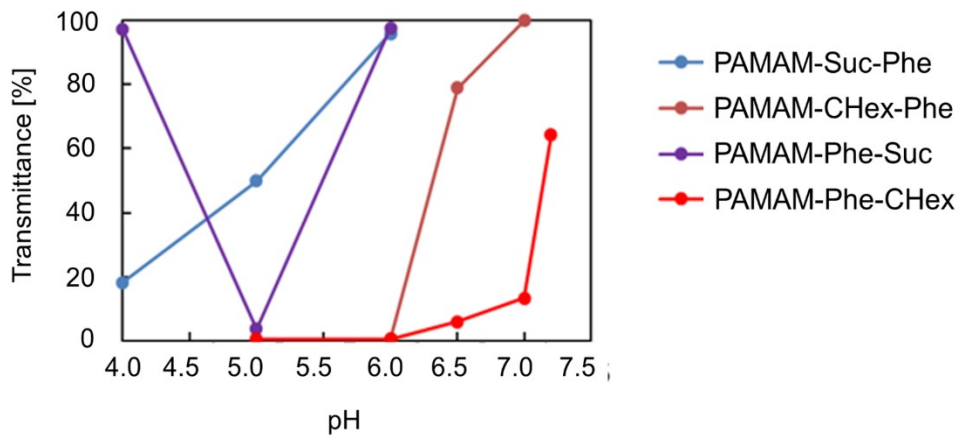


Figure S4. pH-dependent transmittance of various dendrimers (1 mg/ml) at 37°C.

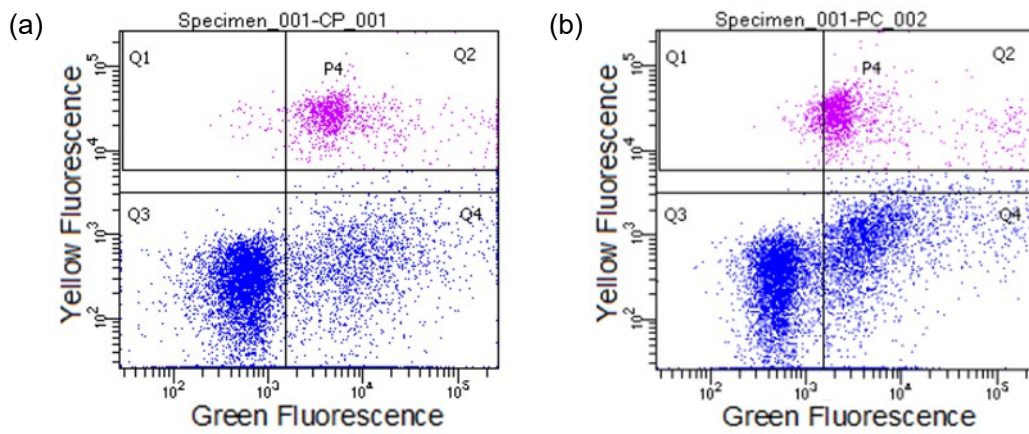


Figure S5. Cell distribution of splenocytes stained with CD3-PE, which were associated with PAMAM-CHex-Phe (a) and PAMAM-Phe-CHex (b) at 37°C, in FACS analysis.

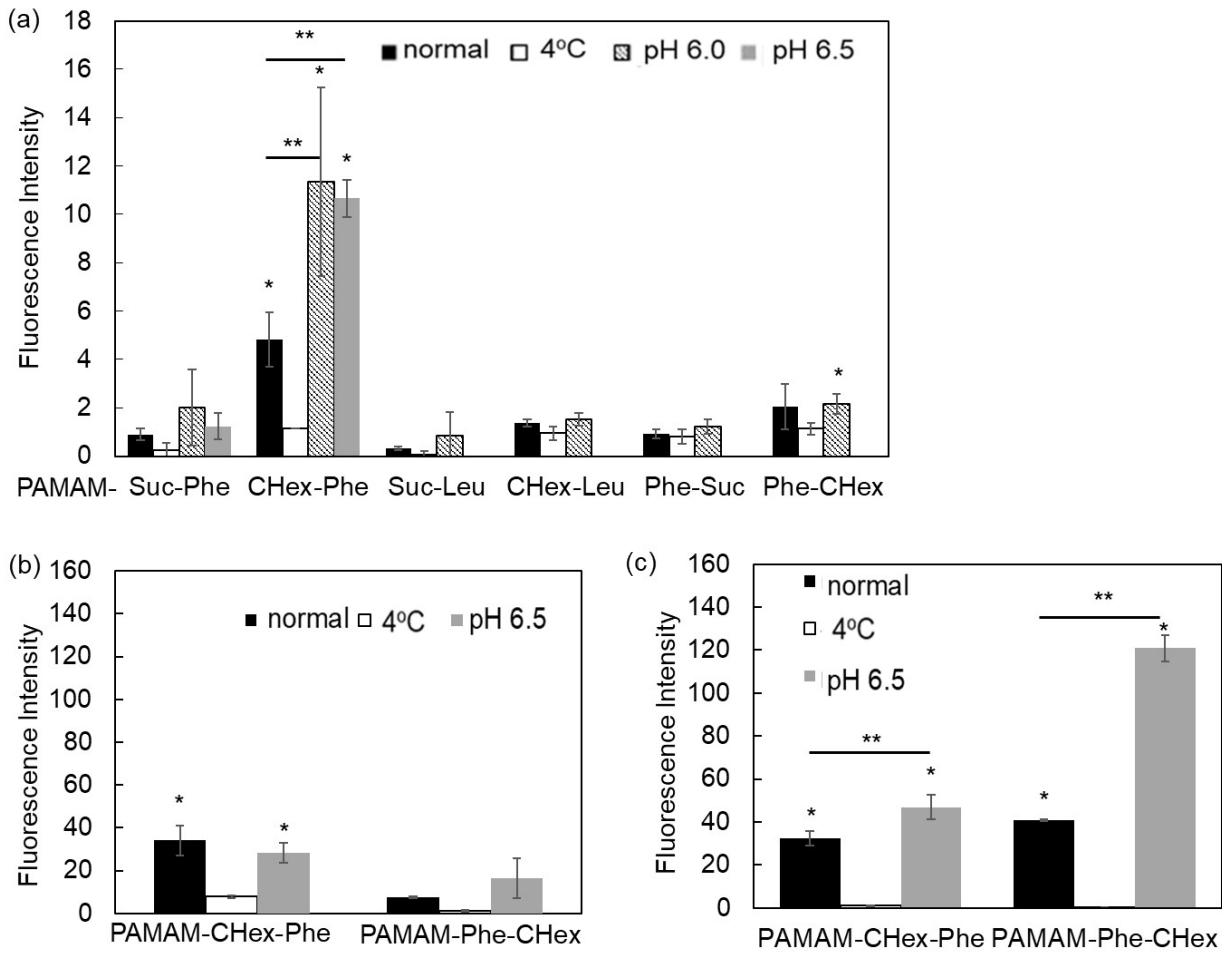


Figure S6. Mean fluorescence intensity of each dendrimer associated with Jurkat cells (a), RAW264 cells (b), and 4T1 cells (c) under the different conditions. *Higher and $p < 0.05$ vs PAMAM-CHex-Phe at 4°C. **Higher and $p < 0.05$ vs normal condition of each dendrimer and higher.

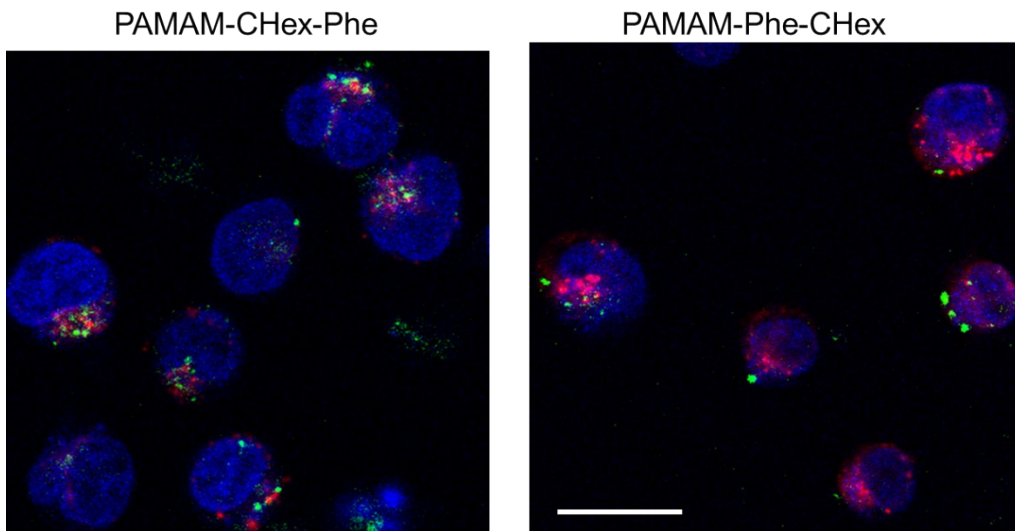


Figure S7. Expanded images of the merge panels in Figure 5.

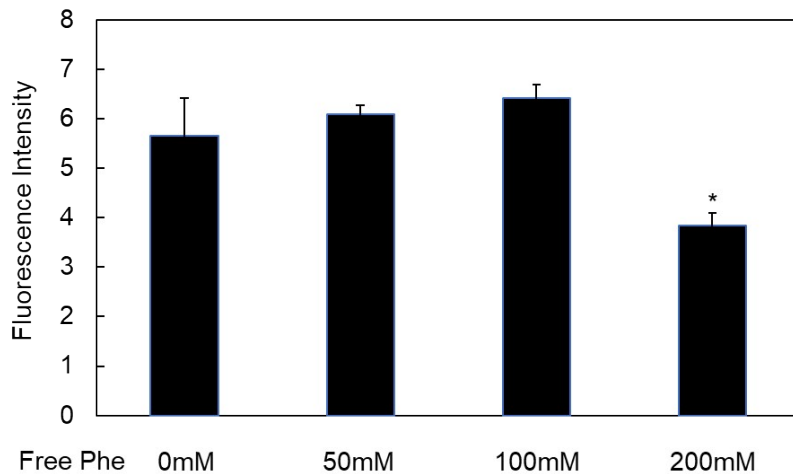


Figure S8. Association of PAMAM-CHex-Phe with Jurkat cells in the presence of free Phe at different concentrations. * $p < 0.05$ vs 0 mM.

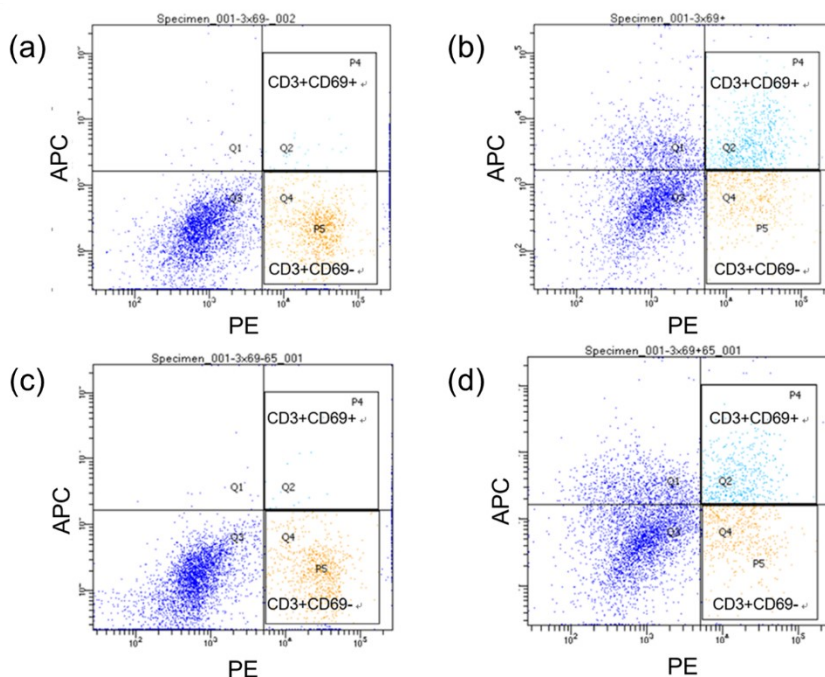


Figure S9. Cell distribution of splenocytes stained with CD3-PE and CD69-APC under the different conditions before (a,c) and after (b,d) the Con. A treatment in normal pH (a,b) and pH 6.5 (c,d) in FACS analysis.