Supporting information

pH-Switchable LCST/UCST-type Thermosensitive Behaviors of Phenylalanine-modified Zwitterionic Dendrimers

Mamiko Tamaki[1], Chie Kojima[1]*

[1]Department of Applied Chemistry, Graduate School of Engineering, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan

Experimental Section

Synthesis

PAMAM-Suc-Phe was synthesized using the PAMAM dendrimer of G4 with an ethylenediamine core, in accordance with our previous report.[1] PAMAM-Phe-Suc was synthesized, as follows. PAMAM-Phe of G4 was synthesized in accordance with our previous report.[2] Then, PAMAM-Phe (121.4 mg, 4.08 μmol) was dissolved in 125 mM $NaHCO_3$ aqueous solution (3 mL). An excess of succinic anhydride (Suc) of approximately 100 eq was added to the dendrimer solution, followed by overnight stirring at room temperature. The reaction solution was adjusted to approximately pH 10. The carboxy-terminal dendrimers were purified by dialysis (molecular weight cut-off [MWCO]: 1000) in distilled water, and white solids were obtained after the lyophilization, yielding 67 mg (55 %). PAMAMG3-Phe-Suc and PAMAMG5-Phe-Suc were also synthesized by the same method except the starting materials. Yields of

PAMAMG3-Phe-Suc and PAMAMG5-Phe-Suc were 46 mg (57 %) and 47 mg (48 %), respectively. PAMAM-Ile-Suc was synthesized using the same method as PAMAM-Phe-Suc, except for the starting material: PAMAM-Ile. The yield was 39 mg (77 %).

PAMAM-Phe-SO_3Na was synthesized, in accordance with the previous report.[3] 1.3-propane sultone (142.3 mg, 1.17 mmol) was dissolved in acetonitrile (2.8 mL), and PAMAM-Phe (92.2 mg, 3.09 μmol) was dissolved in 125 mM $NaHCO_3$ aqueous solution (2.8 mL). These two solutions were mixed and stirred at room temperature under a nitrogen atmosphere. The reaction solution was adjusted to approximately pH 9. After five days, an aqueous HCl solution was added to decrease the pH to 2, and then the pH was increased to 8 by adding an aqueous NaOH solution. The dendrimer was dialyzed with distilled water, dialyzed with 125 mM $NaHCO_3$ aqueous solution, purified once more by dialyzed with distilled water, and lyophilized. The yield was 61 mg (62 %). PAMAMG3-Phe-SO_3Na and PAMAMG5-Phe-SO_3Na were also synthesized by the same method except the starting materials. Yields of PAMAMG3-Phe-SO_3Na and PAMAMG5-Phe-SO_3Na were 78 mg (88 %) and 82 mg (97 %), respectively.

PAMAM-SO_3Na was synthesized using the same method as PAMAM-Phe-SO_3Na, except for the starting material: the amino-terminal PAMAM dendrimer. The yield was 45 mg (74 %). QPAMAM-Phe-Suc and QPAMAM-Phe-SO_3Na were synthesized in accordance with the previous report.[4] PAMAM-Phe-Suc (34.0 mg, 1.13 μmol) was mixed with methyl iodide (43 μL, 0.69 mmol) in DMF (3 mL), and the mixture was stirred at 37 °C for 25 h. The dialysis was performed using a 2 M NaCl aqueous solution followed by distilled water. White solids were obtained by the lyophilization. The same synthesis was performed by using PAMAM-Phe- SO_3Na (17.8 mg, 0.56 μmol) and 31 μL (0.50 mmol) of methyl iodide. Yields of QPAMAM-Phe-Suc and QPAMAM-

Phe-SO_3Na were 29 mg (77 %) and 5 mg (26 %), respectively.

PAMAM-Suc-AMBA was synthesized, as follows. PAMAM-Suc was synthesized in accordance with our previous report;[1] PAMAM-Suc (43.2 mg, 1.96 μmol) was dispersed in dimethyl sulphoxide (DMSO) (3 mL) and stirred for 1h. Then, methyl-4-(aminomethyl) benzoate hydrochloride (AMBA-OMe-HCl, 56.9 mg, 0.282 mmol), 1-[bis(dimethylamino)methyliumyl]-1*H*-benzotriazole-3-oxide hexafluorophosphate (HBTU, 0.11 g, 0.30 mmol), and triethylamine (TEA, 41 μL, 0.29 mmol) were added to the dendrimer solution, followed by overnight stirring at room temperature. Subsequently, 1 mL of distilled water was added to quench the reaction. The dendrimer was purified by dialysis (MWCO: 1000) in methanol. PAMAM-Suc-AMBA-OMe was obtained by the evaporation of methanol and the subsequent lyophilization. The yield was 44 mg (72 %). PAMAM-Suc-AMBA-OMe (14.2 mg, 0.46 μmol) was then dissolved in methanol (2 mL), and 4 M NaOH of methanol solution (500 μL) was added. After stirring at 4 °C for 2 h, these dendrimers were dialyzed (MWCO: 1000) in distilled water. White solids were obtained by freeze-drying, producing a yield of 13 mg (110 %).

Obtained dendrimers were characterized by ^{1}H NMR spectra (Figure S1), except PAMAM-Suc-AMBA-OMe and PAMAM-Suc-AMBA. PAMAM-Phe-Suc: ^{1}H NMR (D_2O containing NaOD, 400 MHz): δ 2.17-2.20 (m, Suc and $NCH_2C\underline{H_2}CONHCH_2CH_2N$), 2.38 (br, $NCH_2CH_2CONHCH_2C\underline{H_2}N$), 2.55 (br, $NC\underline{H_2}CH_2CONHCH_2CH_2N$), 2.75-3.07 (br, $NCH_2CH_2CONHC\underline{H_2}CH_2N$, $NCH_2CH_2CONHCH_2C\underline{H_2}N$ and H_β-Phe), 4.29 (br, H_α-Phe), 7.05-7.13 (m, phenyl). PAMAM-Phe-Ile: ^{1}H NMR (D_2O containing NaOD, 400 MHz): δ 0.71 -0.75 (br H_α-Ile), 1.03 and 1.28 (br $CHCHCH_3C\underline{H_2}CH_3$-Ile), 1.69 ($CHC\underline{H}CH_3CH_2CH_3$-Ile), 2.25-2.36 (m, $NCH_2C\underline{H_2}CONHCH_2CH_2N$ and Suc), 2.46 (br, $NCH_2CH_2CONHCH_2C\underline{H_2}N$), 2.65 (br,

$NC\underline{H_2}CH_2CONHCH_2CH_2N$), 3.93 (br, $NCH_2CH_2CONHC\underline{H_2}CH_2N$ and $NCH_2CH_2CONHCH_2C\underline{H_2}N$). PAMAM-Phe-$SO_3Na$: ^1H NMR (D_2O containing NaOD, 400 MHz): δ 1.72 (br, $NHCH_2C\underline{H_2}CH_2SO_3Na$), 2.16-2.24 (m, $NCH_2C\underline{H_2}CONHCH_2CH_2N$ and $NHCH_2CH_2C\underline{H_2}SO_3Na$), 2.44 (br, $NCH_2CH_2CONHCH_2C\underline{H_2}N$), 2.65-2.72 (m, $NC\underline{H_2}CH_2CONHCH_2CH_2N$), 2.84 (br, $NH\underline{CH_2}CH_2CH_2SO_3Na$), 2.96 (br, H_β-Phe), 3.13 (br, $NCH_2CH_2CONHC\underline{H_2}CH_2N$, $NCH_2CH_2CONHCH_2C\underline{H_2}N$) 3.28 (br, H_α-Phe) 7.08-7.19 (m, phenyl). PAMAM-SO_3Na: ^1H NMR (D_2O containing NaOD, 400MHz): δ 1.75 (br, $NHCH_2C\underline{H_2}CH_2SO_3Na$), 2.27 (br, $NCH_2C\underline{H_2}CONHCH_2CH_2N$), 2.48-2.77 (br, $NCH_2CH_2CONHCH_2C\underline{H_2}N$, $NC\underline{H_2}CH_2CONHCH_2CH_2N$, $NH\underline{CH_2}CH_2CH_2SO_3Na$ and $NHCH_2CH_2C\underline{H_2}SO_3Na$), 3.15 (br, $NCH_2CH_2CONHC\underline{H_2}CH_2N$). QPAMAM-Phe-Suc: ^1H NMR (D_2O, 400MHz): δ 2.24-2.40 (m, Suc), 2.67 (m, $N^+CH_2C\underline{H_2}CONHCH_2CH_2N$ and H_β-Phe), 2.98 (br, N^+CH_3), 3.12 (br, $N^+CH_2CH_2CONHC\underline{H_2}CH_2N$, $N^+CH_2CH_2CONHCH_2C\underline{H_2}N$), 3.33 ($N^+CH_2CH_2CONHCH_2C\underline{H_2}N$), 3.50 (br, $N^+C\underline{H_2}CH_2CONHCH_2CH_2N$), 4.36 (br, H_α-Phe), 7.13-7.22 (m, phenyl). QPAMAM-Phe-SO_3Na: ^1H NMR (D_2O, 400 MHz): δ 1.79 (br, $N^+CH_2C\underline{H_2}CH_2SO_3Na$), 2.21-2.31 ($N^+CH_2CH_2C\underline{H_2}SO_3Na$), 2.63-2.72 (br, $N^+CH_2C\underline{H_2}CONHCH_2CH_2N$), 2.78 (br, N^+CH_3), 2.88 (H_β-Phe), 2.98 (br, $N^+CH_2CH_2CONHC\underline{H_2}CH_2N$ and $N^+CH_2CH_2CONHCH_2C\underline{H_2}N$), 3.20-3.24 (br, $N^+\underline{CH_2}CH_2CH_2SO_3Na$), 3.35 (br, $N^+CH_2CH_2CONHCH_2C\underline{H_2}N$), 3.57 (br, $N^+C\underline{H_2}CH_2CONHCH_2CH_2N$), 4.00 (br, H_α-Phe), 7.17-7.27 (m, phenyl).

Since PAMAM-Suc-AMBA-OMe and PAMAM-Suc-AMBA were insoluble in any solvents, the binding of Suc and AMBA to the dendrimer was confirmed by the Fourier-transform infrared (FT-IR) spectra (Figure S2). IR of PAMAM-Suc-AMBA: 1118 cm^{-1} (1,4-substituted aromatics) and 1725 cm^{-1} (aromatic CH stretching). IR of PAMAM-Suc-

AMBA-OMe: 850 cm^{-1} (methyl group), 1118 cm^{-1} (1,4-substituted aromatics) and 1725 cm^{-1} (aromatic CH stretching).

Characterization

^1H NMR spectra were obtained by using ^1H-NMR (400 MHz) (JEOL Ltd., Tokyo, Japan). The infrared (IR) spectra were recorded using a JASCO FTIR-4600 spectrometer (Jasco Inc., Tokyo, Japan).

Microscopic observation was performed by using an inverted fluorescence microscope (ECLIPSE Ti-U) (Nikon Corp., Tokyo, Japan) equipped with a glass heater unit for cell culture (C-140A) (BLAST Inc., Kanagawa, Japan). The dendrimer solutions (1 mg / mL, ~100 μL) were prepared on a slide glass and observed at room temperatures of 40°C and 60°C.

The ζ-potential was measured by ELSZ-DN2 (Otsuka Electronics, Osaka, Japan) at 25 ^oC. Sample solutions (1 mg/mL) were prepared using 0.01 M HCl (pH 2) and 0.01 M NaOH (pH 12) containing 10 mM NaCl. Sample solutions at different pH were prepared by mixing these acidic and basic sample solutions.

Temperature-dependent transmittance measurement

The dendrimer-containing buffer solutions (dendrimer 1 mg/mL, buffer 20 mM) were prepared. Phosphate solutions (NaH_2PO_4 and Na_2HPO_4) and acetate solutions (CH_3COONa and CH_3COOH) were mixed to adjust to above pH 6 and pH 4-6, respectively. Glycine-HCl buffer was used to adjust to pH 3 and lower. The dendrimer-containing buffer solutions containing 150 mM NaCl were also prepared. In comparison of the dendrimer generations, the dendrimer-containing buffer solutions (32 μM) were

prepared. The temperature-dependent transmittance at 500 nm was measured using a Jasco Model V-630 UV/Vis spectrophotometer equipped with ETC-717 (Jasco Inc., Tokyo, Japan). The heating rate was 1.0 ^{o}C/min.

Potentiometric pH titration

5 mL of dendrimer aqueous solutions (0.5 mg / mL) were prepared by using 0.01 M HCl. 0.01 M NaOH of aqueous solution was dropped into this solution, and the pH was monitored to analyze the pKa using a pH titrator system (AT-710) (Kyoto Electronics Industry, Kyoto, Japan). The solution temperature was controlled at 20°C and 50°C using a thermostatic chamber NCB-500 (Tokyo Rikakikai Co, Ltd., Tokyo, Japan).

Separation of rose bengal (RB) from aqueous solutions

RB (175 μmol) and PAMAM-Phe-SO_3Na (32 μmol) were mixed in 1 mL of 20 mM phosphate buffer (pH 6.5). The mixture was incubated at 4 ^{o}C or 60 ^{o}C for 30 min. Before and after centrifugation (15000 g, 5 min) at 4 ^{o}C or 40 ^{o}C, the absorption spectra of the mixture (RB 4 μM) were measured by the UV/Vis spectrophotometer. The same experiment was performed using PAMAM-SO_3Na instead of PAMAM-Phe-SO_3Na. The residual RB (%) was calculated from the ratio of the absorbance of RB before and after centrifugation.

[References]

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(3) H.-T. Chen, M. F. Neerman, A. R. Parrish, E. E. Simanek, *J. Am. Chem. Soc.*, 2004, **126**, 10044-10048.

(4) J. H. Lee, Y. B. Lim, J. S. Choi, Y. Lee, T. I. Kim, H. J. Kim, J. K. Yoon, K. Kim, J. S. Park, *Bioconjugate Chem.*, 2003, **146**, 1214-1221.

Table S1. Numbers of terminal group and bound compounds to different-generation dendrimers synthesized in the present study.

dendrimer	terminal group	bound	
		Phe	Suc/SO_3Na
PAMAMG3-Phe-Suc	32	32*	31
PAMAMG5-Phe-Suc	128	128*	120
PAMAMG3-Phe-SO_3Na	32	28*	30
PAMAMG5-Phe-SO_3Na	128	112*	121

*Estimated from the ^1H NMR spectrum of PAMAMG3/G5-(Boc-Phe).

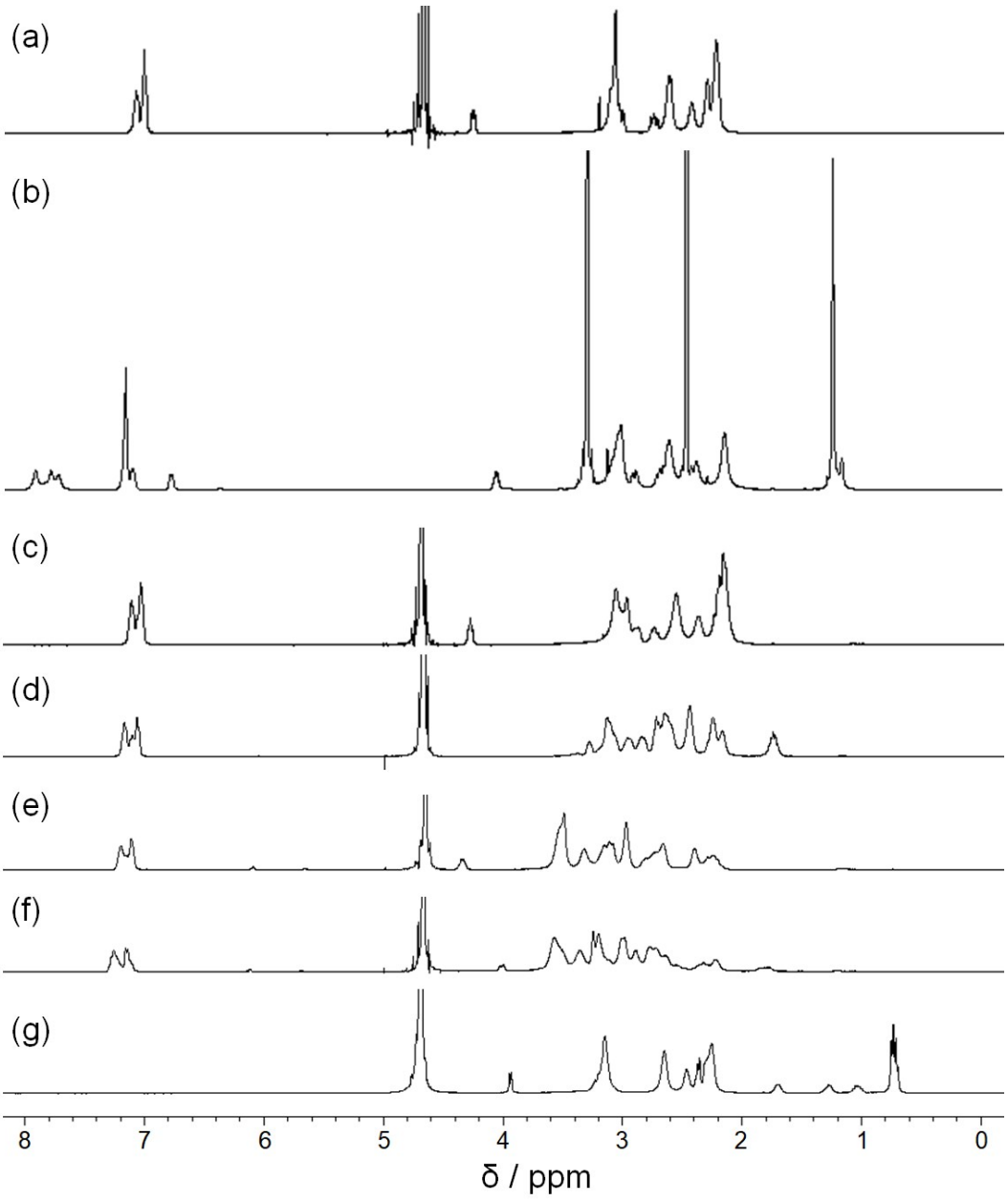

Figure S1. ^{1}H NMR spectra of (a) PAMAM-Suc-Phe, (b) PAMAM-(Boc-Phe), (c) PAMAM-Phe-Suc, (d) PAMAM-Phe-SO_3Na, (e) QPAMAM-Phe-Suc, (f) QPAMAM-Phe-SO_3Na and (g) PAMAM-Ile-Suc in D_2O with (a-c,f) and without NaOD (d,e) except PAMAM-(Boc-Phe) (b, DMSO-d6). The result of PAMAM-Suc-Phe was referred to our previous report.[1]

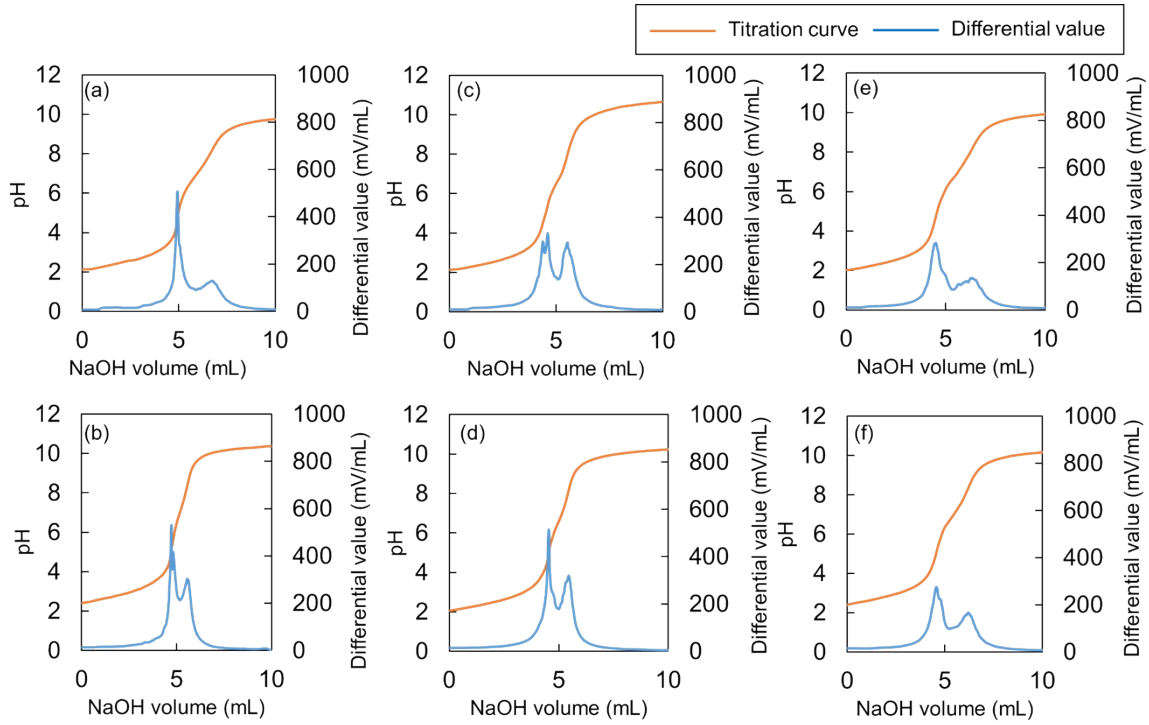

Figure S2. pH titration profiles of PAMAM-Suc-Phe (a and b), PAMAM-Phe-Suc (c and d) and PAMAM-Phe-SO_3Na (e and f) at 20°C (a, c, and e) and 50°C (b, d, and f).

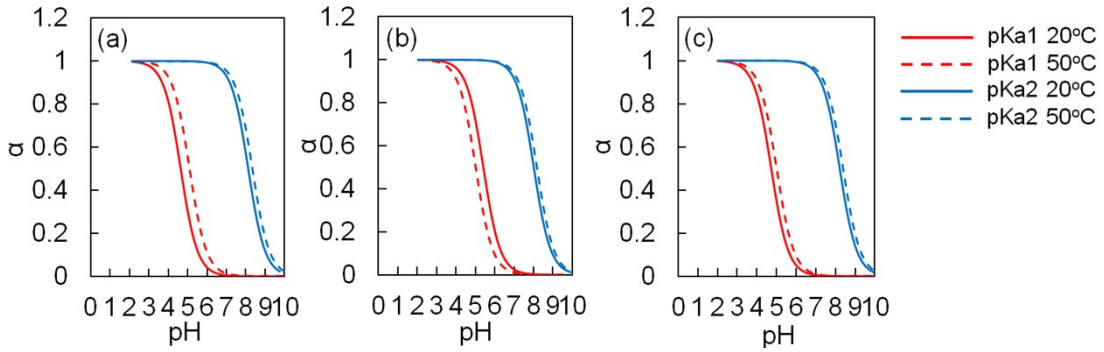

Figure S3. The degree of protonation (α) of PAMAM-Suc-Phe (a), PAMAM-Phe-Suc (b) and PAMAM-Phe-SO_3Na (c) as a function of pH. Red line: pKa1, blue line: pKa2; solid line (20 °C), and dotted line (50 °C).

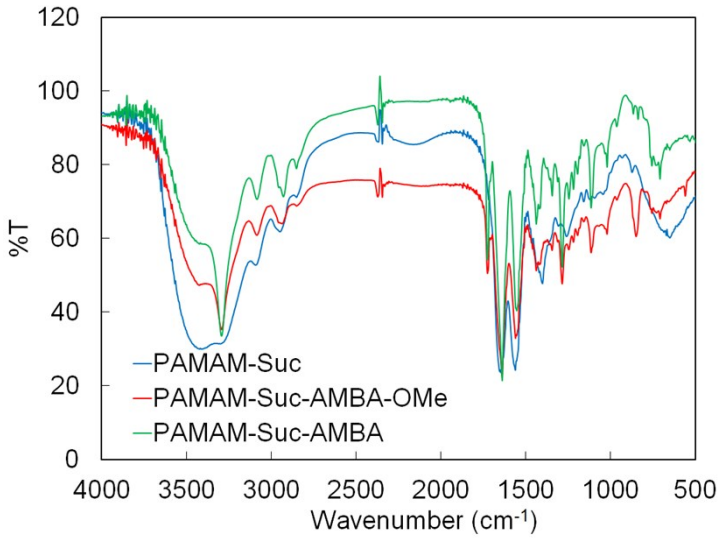

Figure S4. FT-IR spectra of PAMAM-Suc, PAMAM-Suc-AMBA-OMe and PAMAM-Suc-AMBA.

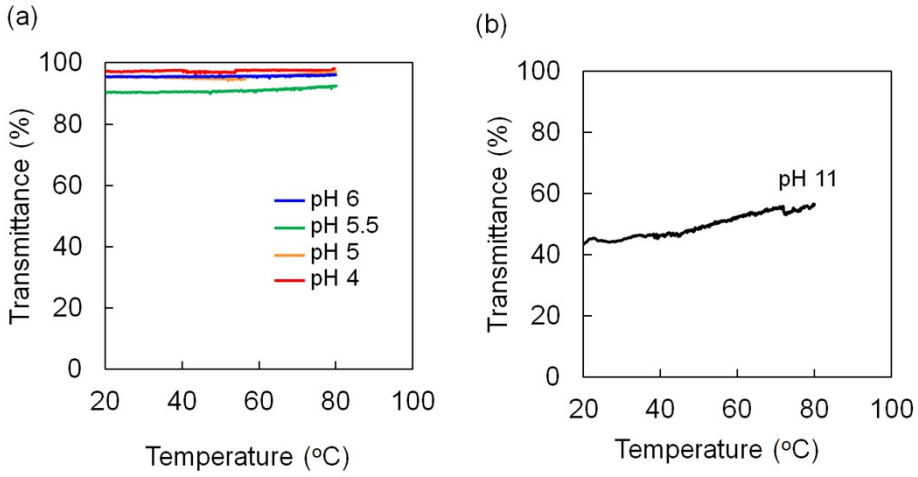

Figure S5. Temperature-dependent transmittance curves of (a) PAMAM-Ile-Suc and (b) PAMAM-Suc-AMBA at various pHs.

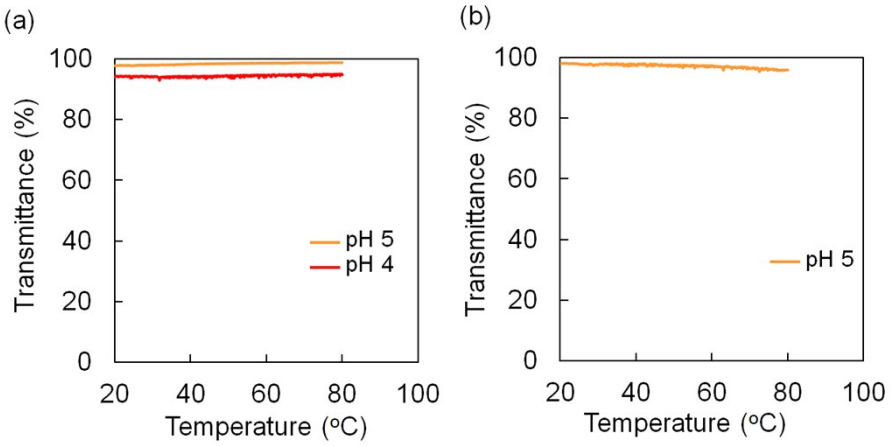

Figure S6. Temperature-dependent transmittance curves of (a) QPAMAM-Phe-Suc and (b) QPAMAM-Phe-SO_3Na at various pHs

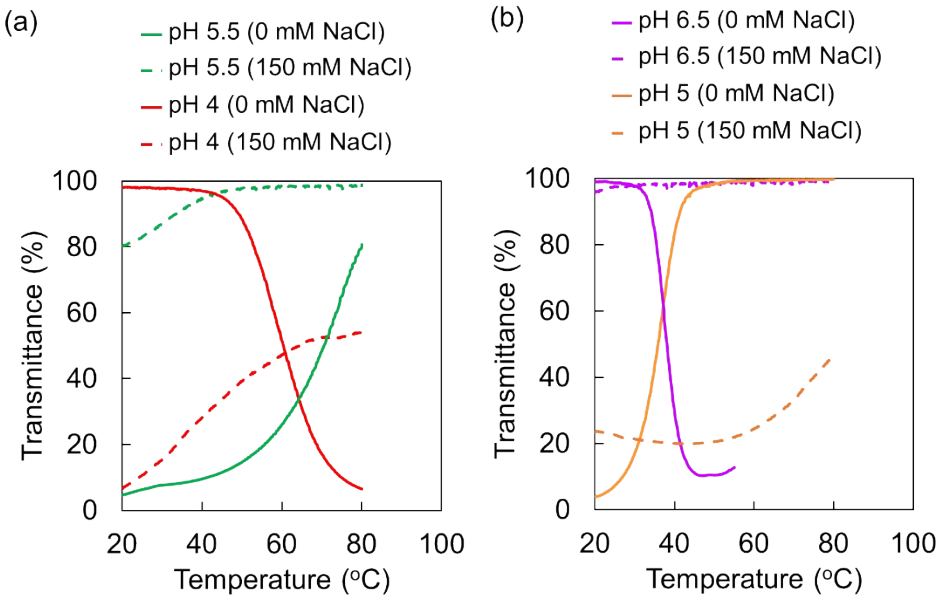

Figure S7. Temperature-dependent transmittance curves of (a) PAMAM-Phe-Suc and (b) PAMAM-Phe-SO_3Na in absence (solid lines) and presence (dotted lines) of 150 mM NaCl.

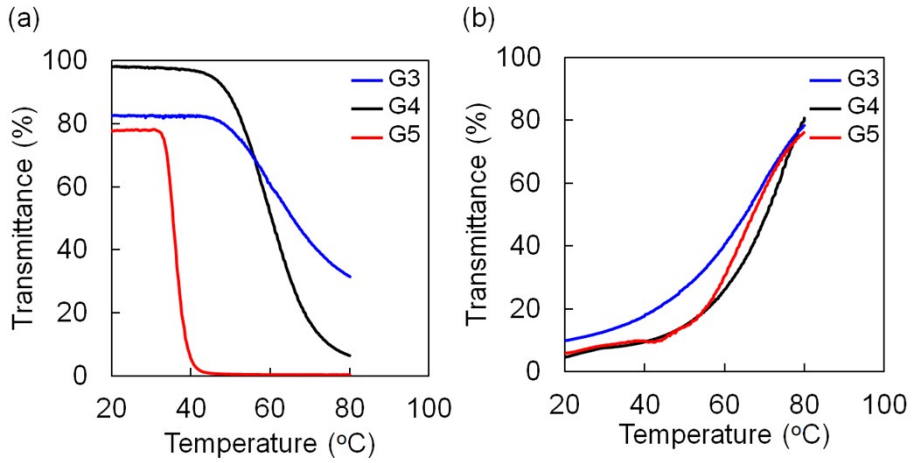

Figure S8. Temperature-dependent transmittance curves of PAMAM-Phe-Suc with different generations at (a) pH 4 (LCST) and (b) pH 5.5 (UCST).

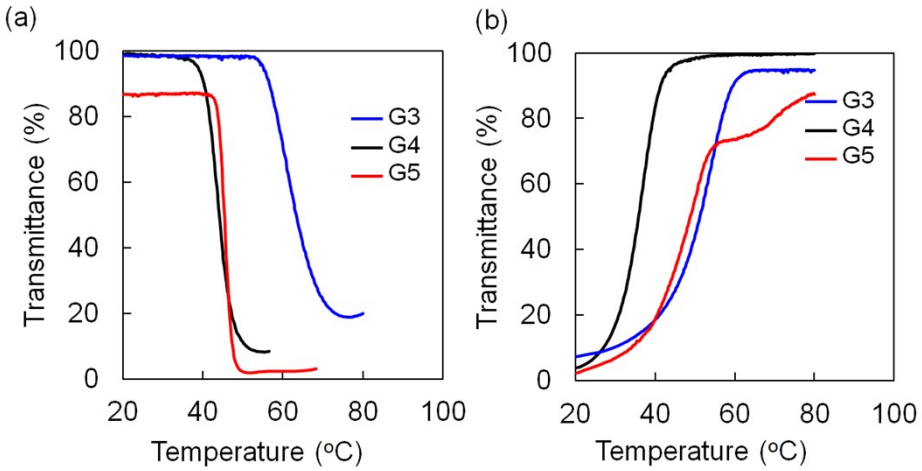

Figure S9. Temperature-dependent transmittance curves of PAMAM-Phe-SO_3Na with different generations at (a) pH 5 (LCST) and (b) pH 6.5 (UCST).

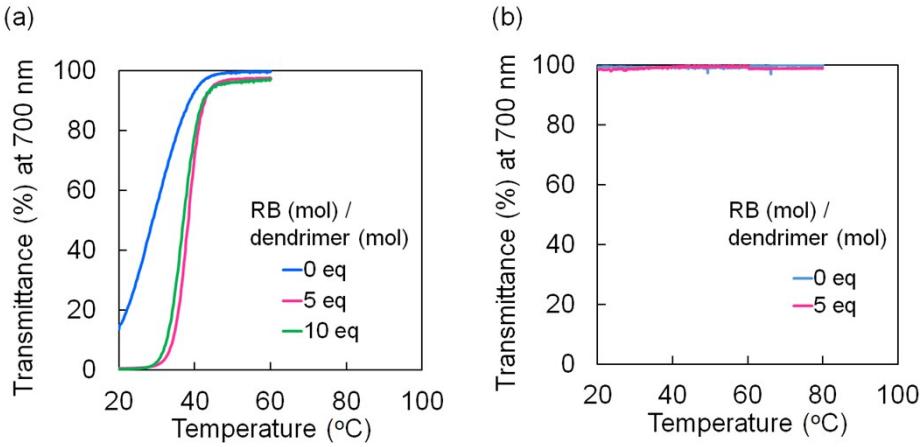

Figure S10. Temperature sensitivity of 1mg/mL of (a) PAMAM-Phe-SO_3Na and (b) PAMAM-SO_3Na at pH 6.5 in absence and presence of RB at different ratios.