Supporting Information

Enzymatically Degradable Star Polypeptides with Tunable UCST Transitions in Solution and within Layer-by-Layer Films

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1. Materials

N-δ-Cbz-L-ornithine(L-ornithine(Z)) was purchased from Chem-Impex international, Inc. Triphosgene, potassium cyanate (KOCN), imidazole, trifluoracetic acid (TFA), tetrahydrofuran (THF), diethyl ether, and dialysis tubing were purchased from Alfa Aesar chemicals. Generation four polyamidoamine dendrimer (G4-PAMAM), hexane, ethylenediamine (EDA), dimethyl formamide (DMF), hydrobromic acid solution (33 wt.% in acetic acid), branched polyethylenimine (BPEI, 750 kDa), tannic acid, sodium phosphate monobasic dihydrate, sodium phosphate dibasic dihydrate were purchased from Sigma-Aldrich. Trypsin 0.25wt% solution (with 1 mM ethylenediaminetetraacetic acid) was purchased from GenDEPOT. Solvents were purchased in anhydrous grade and used without further purification.

2. Polymer synthesis

Each star polypeptide consists of a G4-PAMAM core and a ureido modified poly(L-ornithine-*co*-L-citrulline) corona. A typical polymer synthesis involved (a) synthesis of N-carboxyanhydride (NCA) of L-ornithine(Z), (b) ring opening polymerization of the L-ornithine(Z)-NCA, initiated by the G4-PAMAM's peripheral primary amino groups, (c) deprotection of polypeptides' carboxybenzyl protecting group, and (d) ureido modification of deprotected polypeptides. Each step is described in detail below.

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2.1 Synthesis of N-carboxyanhydride (NCA) of L-ornithine(Z)

The monomer was prepared as previously described with some modifications.[1] In brief, L-ornithine(Z) (1.00g, 3.76 mmol) and triphosgene (0.50 g, 1.68 mmol) were mixed with THF (20 mL) at 23 °C in a round bottom flask. The slurry was stirred under reflux at 50 °C in an oil bath. The slurry dissolved in one hour and the reaction flask was cooled to room temperature. The resultant solution was dropped into hexane. The crude product was washed by hexane three times, then dissolved in THF, precipitated in hexane again, and finally dried to constant weight to yield white crystalline power (0.92 g, 84% yield). The monomer synthesis is shown in Scheme S1.

2.2 Synthesis of *star*-poly-L-ornithine

Star polypeptides were synthesized utilizing the primary amine groups of the G4-PAMAM *via* ring-opening polymerization of NCAs, followed by deprotection of carboxybenzyl group (Scheme 1). In a *star*-poly-L-ornithine(Z) (SPO(Z)) synthesis with NCA/[NH_2] ratio of 55, the monomer L-ornithine(Z)-NCA (0.51 g, 1.74 mmol) was dissolved in 5 mL anhydride DMF, followed by G4-PAMAM (6.83 mg, 9.89×10^{-3} mmol) as 0.39 wt.% solution in DMF. The reaction flask was purged by argon gas and subjected to three freeze-pump cycles for oxygen removal. Then, the reaction was carried out under continuous stirring for 48 hours at 23 °C. The crude product was dialyzed against DI water for 24 hours at room temperature (23^{o}C). The polypeptide was further purified by re-precipitation from DMF to diethyl ether and freeze-dried to its constant weight. Gel permeation chromatography (GPC) analysis of SPO(Z) polymers is shown in Figure S1. For removing carboxybenzyl group, the polymer (0.37 g) was first dissolved in TFA (3.5 mL) in ice bath. Then, excess amount of hydrobromic acid (33 wt.% in acetic acid) was added dropwise to the polymer solution upon stirring. The reaction was carried out for 12 hours under stirring at room temperature and the polymer was again precipitated in diethyl ether. The deprotected polypeptide, *star*-poly(L-ornithine) (SPO), was further washed with diethyl ether and dried to constant weight. The complete removal of carboxybenzyl group was confirmed by the disappearance of ^{1}H-NMR signals at 7.29 ppm and 5.03 ppm[1] (Figure S2).

2.3 Ureido modification of star polypeptides

The primary amine groups from deprotected SPO were partially converted into ureido groups by reacting with KOCN in 1M imidazole buffer solution. Specifically, KOCN was

dissolved in 1M imidazole buffer solution with a concentration of 3 wt.%. To carry out the modification, SPO was added to reaction flasks, followed by various amounts of KOCN solution with respect to the molar ratio of amino groups from SPO. For example, SPO (43 mg, obtained from NCA/[NH_2] of 55) was dissolved in KOCN solution (533 mg). After all reactants were completely dissolved, the solution was degassed by three freeze-pump-thaw cycles under argon protection. Then, the reaction was carried out at 50 °C for 13 hours. At the end of the reaction, the clear polypeptide solution was dialyzed against cold DI water for 24 hours and the UCST transitions could be observed during dialysis. The UCST star polypeptide, *star*-poly(L-ornithine-*co*-L-citrulline) (SPOC), was then recovered through freeze-drying. The degree of ureido modification was calculated by comparing peak integration from ^{1}H-NMR spectrum (with ureido group at 3.2 ppm, and amino group at 2.7 ppm)[2], in this example it was calculated as 96% (Figure S3).

2.4 Synthesis ethylenediamine-*core*-poly(L-ornithine-*co*-L-citrulline)

The linear UCST polypeptide, using ethylenediamine (EDA) as the initiator, poly(L-ornithine-*co*-L-citrulline) (LPOC with EDA core) was prepared using a similar technique described previously in 2.2 and 2.3. During the polymerization, the G4-PAMAM dendrimer was replaced by EDA with a degree of polymerization of 50 on its two ends (with M_n = 24.8 kg/mol and PDI = 1.07, measured *via* GPC), the polymerization was followed by deprotection and ureido modification, resulting in a UCST linear polypeptide with a ureido modification degree of 96 (LPOC).

3. Polymer characterization

The chemical structures of SPO and SPOC were characterized using ^{1}H-NMR (Mercury 300, 300 Hz). The ^{1}H NMR spectra were recorded in D_2O solutions (containing 0.1wt% NaOD) at 50^{o}C. GPC measurements of SPO(Z) were conducted at 30^{o}C *via* an Agilent 1260 system equipped with a Phenogel 5 μm column. DMF was used as an eluent with a flow rate of 0.2 mL/min, and the system was calibrated by linear poly(ethylene oxide) standard samples (Table S1).

4. FTIR spectroscopy

FTIR spectroscopy measurements (Bruker, Tensor II) were used to investigate the conformations present in SPOCs. Freeze-dried SPOC powders were used in attenuated total reflectance (ATR) mode with a high-pressure clamp attachment. The spectra were recorded from 4000 to 600 cm^{-1}, with a 4 cm^{-1} resolution.

5. Dynamic light scattering (DLS)

For studying polypeptide solution behavior, DLS analysis was performed at a scattering angle of 90°, using a homebuilt DLS instrument equipped with a 532 nm Whisper Mini laser and a Luma 40 temperature-controlled cuvette holder (Quantum Northwest, temperature controller). During measurements, photon count was recorded using a fiber-optic adapter for an 8 mm photomultiplier tube module (Edmund Optics) and two Hamamatsu photon counters (H10682-210). Samples were prepared as 2 mg/mL SPOC in 150 mM NaCl solution and the photon count was recorded as the number average from a 120 second measurement. The temperature was held constant for 20 minutes before measuring at each degree from 50°C to UCST of the testing sample in 1-degree steps.

6. Ultraviolet-visible spectroscopy (UV-Vis)

Transmittance measurements of 2 mg/mL SPOC 150 mM NaCl solutions were performed on a Shimadzu 2600 UV-VIS at 670 nm wavelength. The temperature of cuvettes (10 mm pathlength with 1.0 mL volume) was controlled by a Julabo CORIO CD Heating Immersion Circulator. The cuvette was equilibrated for 10 minutes before each measurement from 10 to 30°C in 1-degree steps (Fig. S6).

7. Atomic force microscopy (AFM) and transmission electron microscopy (TEM)

The morphology of the UCST SPOC was acquired using AFM (Bruker-Dimension Icon) and TEM (JEOL JEM-2010 at 100 kV). For AFM, the specimen was prepared as monolayer of SPOC on a silicon wafer *via* spin coating at 1000 RPM from diluted SPOC distilled water solution at room temperature (above UCST). The imaging was carried out using a silicon cantilever with normal stiffness of K_n = 7.4 N/m and resonance frequency of 160 kHz. For TEM imaging, the sample was prepared by casting a drop of diluted polypeptide solution (0.1 wt%) on a carbon-coated copper gird (CF400-Cu-UL 400 mesh). Excess solution was then removed by a filter paper, and the samples were dried at room temperature for at least 12 h before imaging.

8. Degradation studies on star polypeptide

To study biodegradability, 2 mg/mL solutions of SPOC were prepared by dissolving the polymer in distilled water or solution containing 0.25% trypsin. The samples were incubated at 37^{o}C and the content remaining inside the solution was measured *via* DLS, also at 37^{o}C.

9. Layer-by-layer (LbL) assembly of SPOC/tannic acid (TA) film

The star polypeptide, SPOC, was deposited on a silicon wafer *via* LbL deposition. A cleaned silicon wafer was primed with a precursor layer of BPEI by exposing the wafer to 0.2 mg/mL BPEI (750 kDa) in 10 mM phosphate buffered solution (pH 5) for 30 minutes. The primed wafer was then alternatively exposed to 0.2 mg/mL TA in 10 mM phosphate buffered solution (pH 5) and 0.2 mg/mL SPOC in 10 mM phosphate buffered solution (pH 5) for 10 minutes with a one-minute rinse of 10 mM phosphate buffered solution between each layer until reaching 6.5 bilayers with TA on the top of the film.

The thickness of the LbL-assembled film was measured by spectroscopic ellipsometry (J.A. Woollman Co. M-2000). In dry film measurements, the thickness was measured at an incidence angle of 45˚, 55˚, 65˚, and 75˚ at ambient temperature. For wet film measurements, the nominal angle of incidence was 75˚. A temperature-controlled liquid cell was attached to the ellipsometer to measure the thickness of the LbL assembly film in 150 mM NaCl aqueous solution at temperatures from 50 ˚C to 25 ˚C with 5 minutes holding time for each degree in one-degree steps. A two-layer model was used to fit the recorded ellipsometry data from dry film measurements with a native oxide layer on the silicon substrate and a polymer layer treated as a Cauchy material. An additional liquid layer was added as transparent Cauchy medium for wet film measurements.

Supporting Information Tables

Table S1. Polymers used in this study

Polymers	NCA/[NH_2] ratio	Elution time (min)	Apparent M_n of linear counterpart (kg/mol)	$\frac{\rho_{star}}{\rho_{linear^*}}$	Modified polymers	% of ureido modification**
$SPO(Z)_{41}$	41	36.92	59.4	24.5	$SPOC_{41}$-96	96
$SPO(Z)_{55}$	55	36.21	67.7	28.5	$SPOC_{55}$-93	93
					$SPOC_{55}$-96	96
					$SPOC_{55}$-99	99
$SPO(Z)_{75}$	75	34.27	85.8	30.5	$SPOC_{75}$-96	96
$SPO(Z)_{100}$	100	33.43	102.3	34	$SPOC_{100}$-96	96

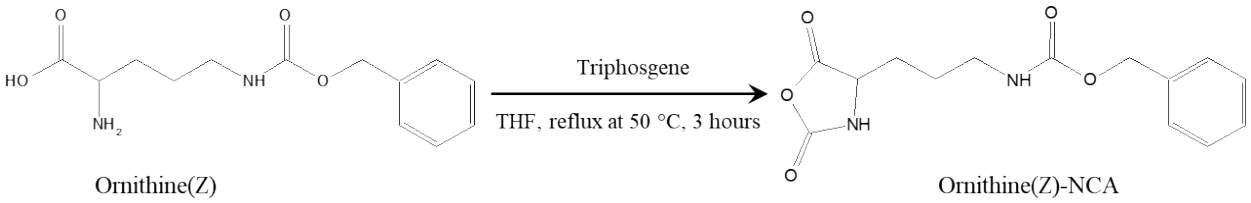
* The ratio of average density of polymer segments within star and linear polymers $\frac{\rho_{star}}{\rho_{linear}}$ was calculated as:

$$\frac{\rho_{star}}{\rho_{linear}} = \frac{Theoretical\ M_n}{Apparent\ M_n} \times \frac{\langle R_g \rangle_{linear}^3}{\langle R_g \rangle_{star}^3}$$

where and theoretical M_n was estimated from NCA/[NH_2] ratio, the apparent M_n of a hypothetical linear counterpart of a star polymer with equal hydrodynamic size. The radii of gyration of linear and star polymers were related through the condition of equality of their hydrodynamic sizes $\langle R_h \rangle = 0.79\langle R_g \rangle_{linear} = 1.29\langle R_g \rangle_{star}$.

** The uredio modification degree was calculated by comparing integrated intensities of peaks *d* and *h* from ^{1}H NMR in Figure S3

Supporting Information Schemes, and Figures



Scheme S1. Synthesis of L-ornithine(Z)-NCA monomer.

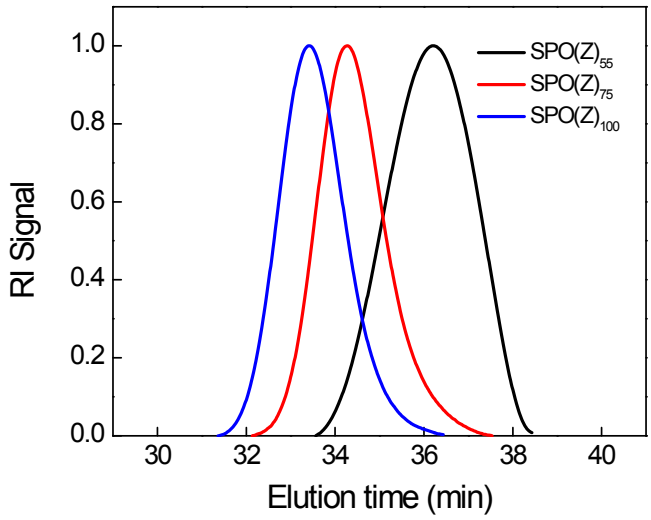


Figure S1. Size exclusion chromatography data of $SPO(Z)_{55}$, $SPO(Z)_{75}$, and $SPO(Z)_{100}$.

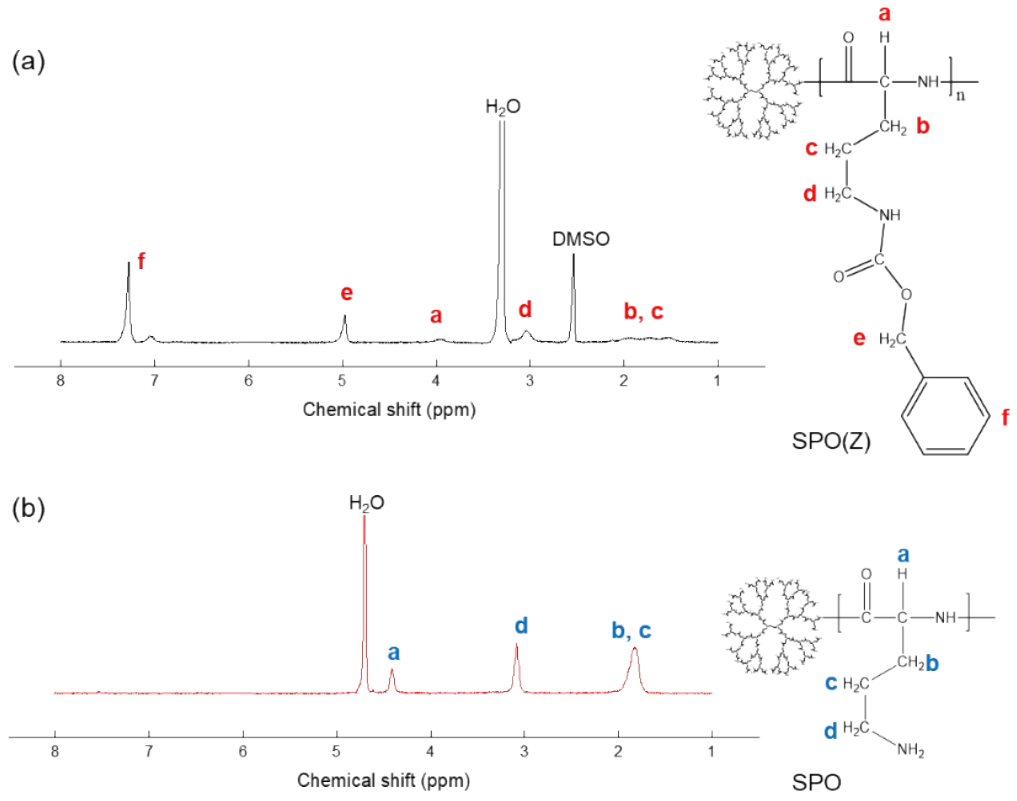


Figure S2. ^{1}H NMR spectra and chemical structures of (a) SPO(Z)$_{55}$ and (b) SPO$_{55}$.

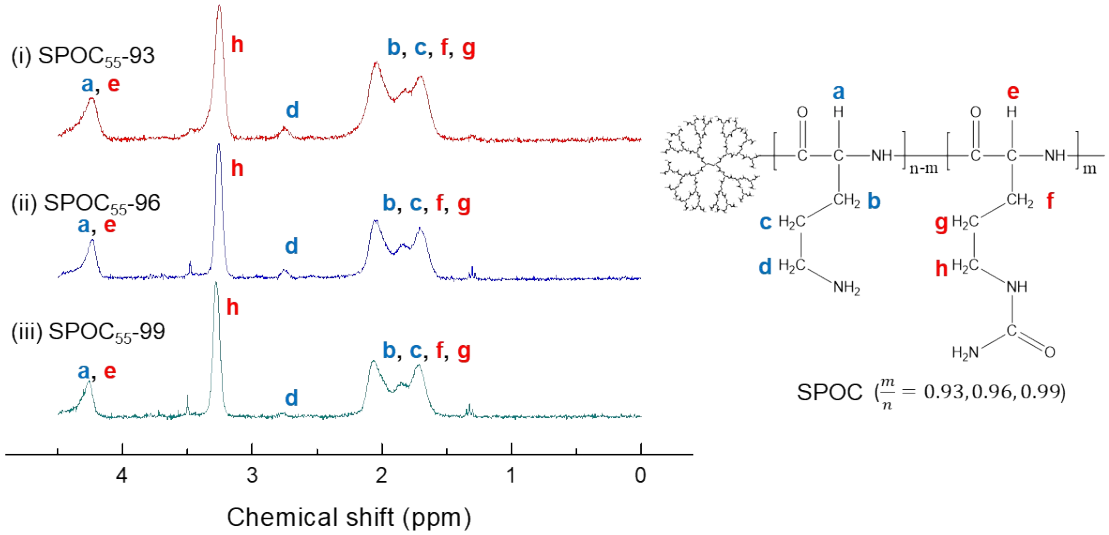


Figure S3. ^{1}H NMR spectra and chemical structure of SPOC$_{55}$ with different ureido modification degrees calculated *via* integrated intensities of peaks *d* and *h*.

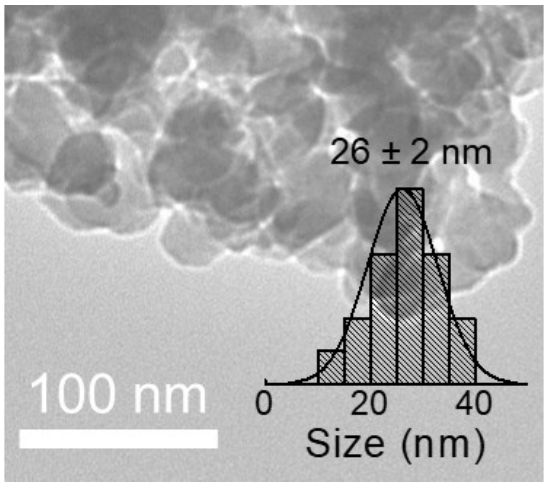


Figure S4. TEM image of SPOC$_{55}$-96 dried at room temperature (above UCST) from a drop of a diluted polypeptide solution (0.1 wt%) on a carbon-coated copper gird (CF400-Cu-UL 400 mesh).

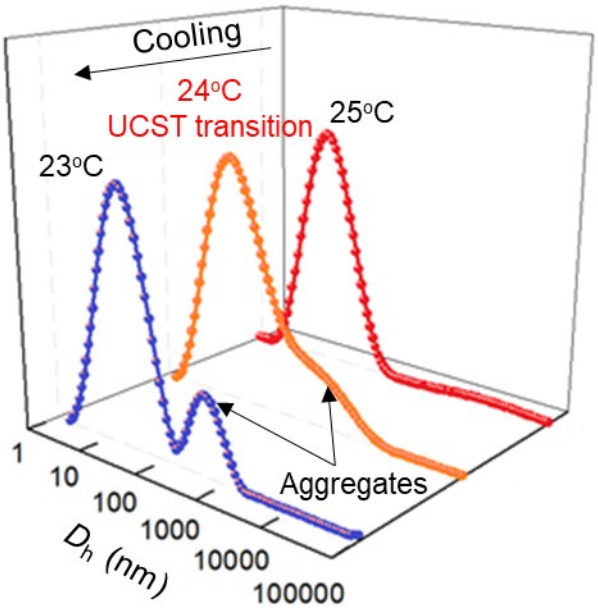


Figure S5. DLS curves showing distribution of hydrodynamic diameters in $SPOC_{55}$-96 solutions at several temperatures close to the UCST.

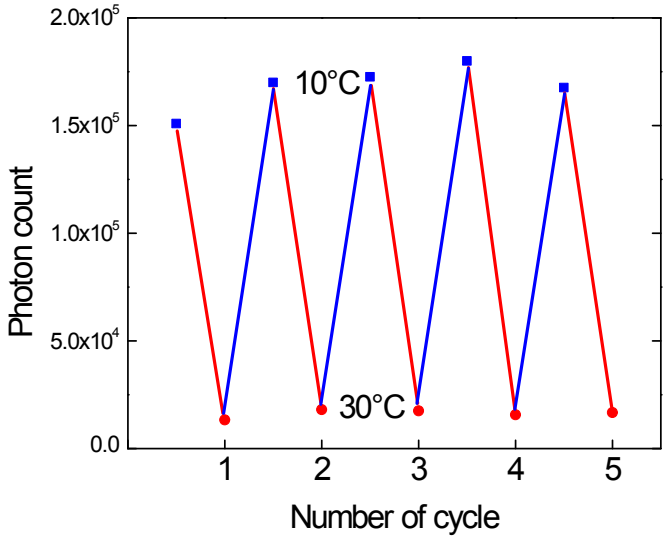


Figure S6. Reversibility of UCST transitions in 2 mg/mL $SPOC_{55}$-96 solutions in 150 mM NaCl at pH 7.

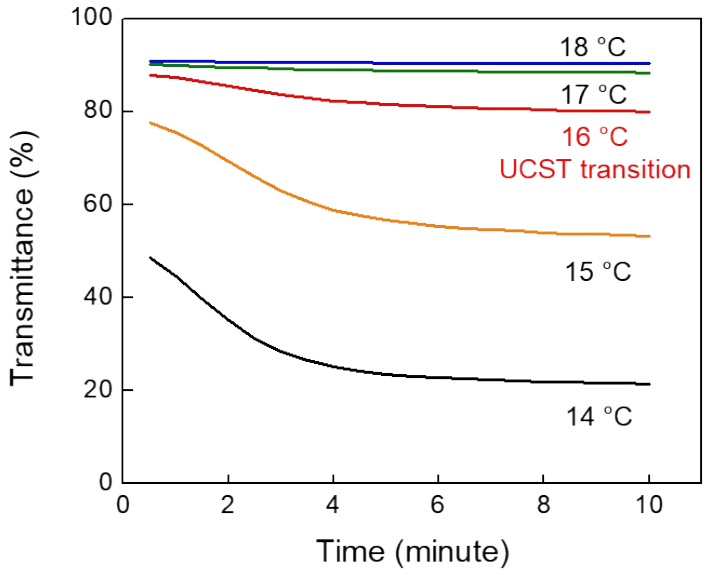


Figure S7. Transmittance of 2 mg/mL $SPOC_{55}$-96 solutions in 150 mM aqueous NaCl at pH 7 measured at 670 nm as a function of time.

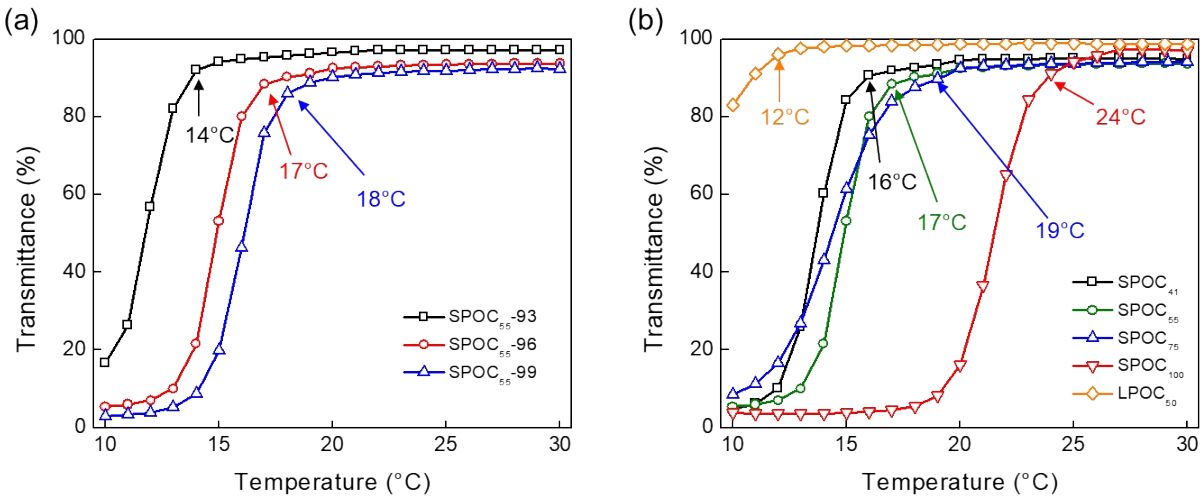


Figure S8. UCST transitions determined by UV-Vis transmittance changes at 670 nm for (a) $SPOC_{55}$ series with varied degrees of ureido modification and (b) LPOC and SPOCs acquired with different NCA/[NH_2] ratios. All aqueous solutions contained 2 mg/mL of polymers and 150 mM NaCl and were at pH 7.

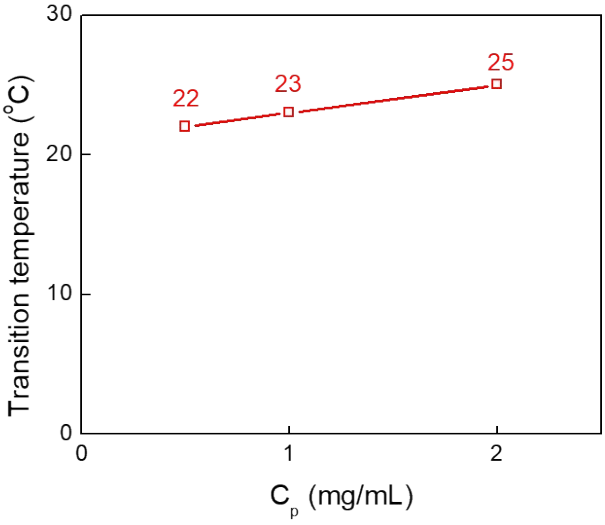


Figure S9. Concentration dependence of UCST transition temperature of $SPOC_{55}$-99 in 150 mM NaCl aqueous solution at pH 7.

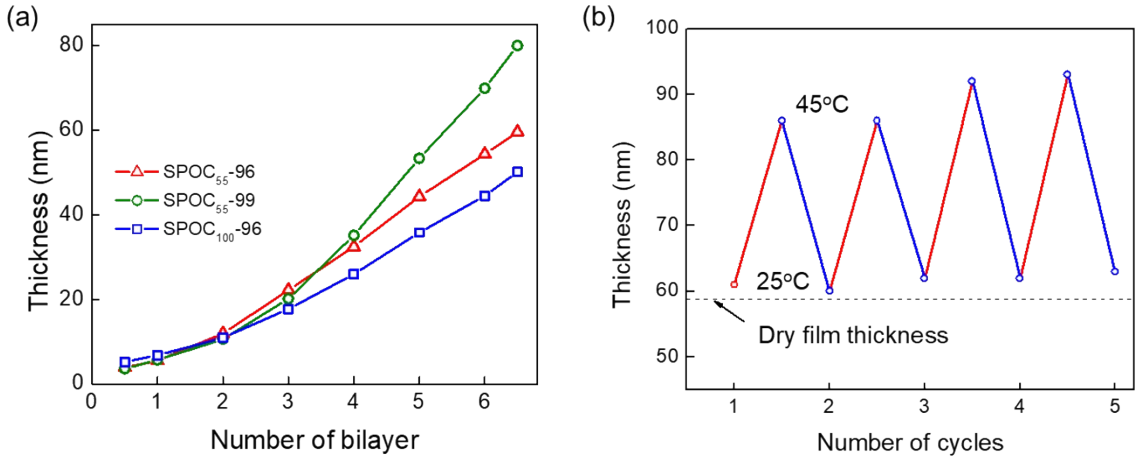


Figure S10. (a) The growth of the thicknesses of LbL films (assembled with $SPOC_{55}$-96/TA, $SPOC_{55}$-99/TA, and $SPOC_{100}$-96/TA) with number of bilayer with dry films and (b) reversible UCST swelling transitions of a 6.5-bilayer $SPOC_{55}$-96/TA assembly (TA on top) in 150 mM NaCl aqueous solution at pH 7 as measured by spectroscopic ellipsometry.

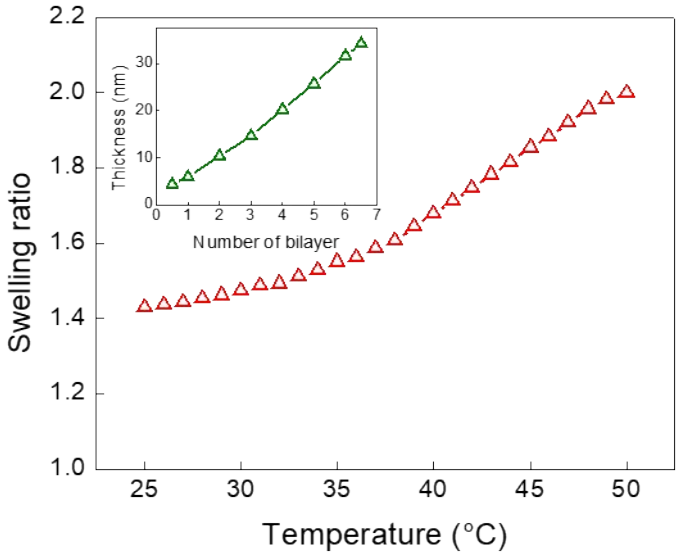


Figure S11. Temperature dependence of swelling ratio of 6.5-bilayer LPOC/TA film deposited on a silicon substrate and exposed to 150 mM NaCl solutions at pH 7 as measured by *in situ* spectroscopic ellipsometry and (inset) the thicknesses of LbL films versus number of bilayers as measured for dry films with spectroscopic ellipsometry.

References

1. Palanisamy, A.; Albright, V.; Sukhishvili, S. A., Upper Critical Solution Temperature Layer-by-Layer Films of Polyamino acid-Based Micelles with Rapid, On-Demand Release Capability. *Chem. Mat.* **2017,** *29* (21), 9084-9094.
2. Shimada, N.; Ino, H.; Maie, K.; Nakayama, M.; Kano, A.; Maruyama, A., Ureido-Derivatized Polymers Based on Both Poly(allylurea) and Poly(L-citrulline) Exhibit UCST-Type Phase Transition Behavior under Physiologically Relevant Conditions. *Biomacromolecules* **2011,** *12* (10), 3418-3422.