Supporting Information

Living unimodal growth of polyion complex vesicles via 2D supramolecular polymerization

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EXPERIMENTAL SECTION

Materials. Block-aniomer PEG-poly(*a,b*-aspartic acid) (PEG-P(Asp); M_n of PEG = 2,000, DP of P(Asp) = 75), homo-catiomer poly([5-aminopentyl]-*a,b*-aspartamide) (Homo-P(Asp-AP); DP of P(Asp-AP) = 82), and Cy3-labelled PEG-P(Asp) were prepared as previously reported.[13] 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Wako Pure Chemical Industries (Osaka, Japan). 3-Hydroxypicolinic acid (HPA) was purchased from Sigma Aldrich (St. Louis, MO). Cy3 mono-reactive dye pack was purchased from GE Healthcare (Tokyo, Japan).

Preparation of Nano-PICsomes.[13] PEG-P(Asp) and Homo-P(Asp-AP) solutions were prepared separately in 10 mM phosphate buffer (PB, 0 mM NaCl, pH 7.4). These solutions were filtered through a 0.22-μm membrane filter to remove particulates. The PEG-P(Asp) solution was mixed with Homo-P(Asp-AP) solution in a 1:1 ratio of $-COO^-$ and NH_3^+, followed by vortex mixing to form the Nano-PICsomes. Cy3-labelled Nano-PICsomes were also prepared via the same procedure, using a mixture of PEG-P(Asp) and Cy3-labelled PEG-P(Asp) in a ratio of 4:1.

Preparation of cross-linked Nano-PICsomes.[11] EDC (10 mg) was dissolved in 10 mM PB (1 mL). Nano-PICsome solution (960 μL) was added to 670 μL of the EDC solution. After 12 h, the mixed solution was subjected to size-exclusion chromatography (SEC). For cryo-transmission electron microscopy (TEM) analysis, the EDC-crosslinked mixed solutions were centrifugally concentrated at 14,000 *g* to approximately 10 mg/mL.

Cryogenic phase contrast transmission electron microscopy (Cryo-TEM). TEM samples were prepared on micro-grids and vitrified by rapid immersion into liquid ethane near its freezing point. Vitrification was performed on an EM CPC cryo-station (Leica Microsystems, Vienna, Austria). The vitrified specimen was transferred to a JEM 2011 transmission electron microscope (JEOL, Tokyo, Japan) for imaging using a cryo-holder (Model 630, Gatan, Munich, Germany). The microscope was equipped with a LaB_6 electron gun, a transfer doublet lens,[15] a Zernike phase plate,[16,17] and a 1K×1K CCD camera (F114, Tietz, Germany). Detailed settings of the Zernike phase plate is shown in Supporting Information. The temperature of the sample was maintained below -170 °C throughout the analysis.

Size Exclusion Chromatography (SEC). SEC was carried out in pH 7.4 10 mM PB (150 mM NaCl) solution using a high performance liquid chromatography (HPLC) system (JASCO, Japan) equipped with a Superdex200™ 10/300 GL column, ultraviolet (UV), refractive index

(RI), and fluorescent detectors. Columns were eluted with pH 7.4 10 mM PB (150 mM NaCl) at a flow rate of 0.5 mg/mL at a temperature of 35 °C.

Restart of growth and analysis of transfer of small PICs to Nano-PICsomes. Nano-PICsomes and small PICs were separated using a polyethersulfone ultrafiltration membrane (MWCO 300,000) at 4 °C for two weeks. Subsequently, the concentration of each solution was adjusted to that of the original solution. Then, isolated small PICs were added to the PICsome solution. The resulting mixture was subjected to dynamic light scattering (DLS) or SEC after crosslinking.

Fluorescence cross-correlation spectroscopy (FCCS). FCCS was carried out with a combination system consisting of an LSM510 and a ConfoCor2 (Zeiss, Jena, Germany). FITC was excited by a 488 nm Ar-ion laser and Cy5 was excited by a 633 nm He–Ne laser through a water immersion objective (C-Apochromat, 40×, 1.2 NA; Zeiss, Jena, Germany).

Detailed information of dynamic light scattering (DLS) analysis. The size of the polyion complexes was evaluated by DLS at specific temperatures using a Zetasizer Nano-ZS instrument (Malvern Instruments, Malvern, UK) equipped with a He-Ne ion laser (l = 633 nm). A scattering angle of 173° was used for all measurements. The autocorrelation function, g(t), was determined using the cumulant method,[3,4] where

$$g(t) = \exp[-\bar{\Gamma}t + (m_2/2)t^2 - (m_3/3!)t^3 + \ldots.] \qquad (1)$$

yields an average characteristic line width, $\bar{\Gamma}$. The z-averaged diffusion coefficient was obtained from the $\bar{\Gamma}$ based on the following equations:

$$\bar{\Gamma} = Dq^2 \qquad (2)$$

$$q = (4\pi n/\lambda)\sin(\theta/2) \qquad (3)$$

where q is the magnitude of the scattering vector, n is the refractive index of the solvent, and θ is the detection angle. The polydispersity index (PDI = $m_2/\bar{\Gamma}^2$) was derived from Equation 1.

Settings of Zernike phase plate for Cryo-TEM. The Zernike phase plate consisted of a vacuum-evaporated amorphous carbon film of 28 nm thickness that corresponded to a $-\pi/2$

phase shift for 200 kV electrons.[5] The hole in the centre of the film was 0.4–0.8 mm in diameter. The phase plate was mounted on a special heated aperture holder thermostatted between 200–280 °C at all times. This temperature control was necessary to prevent adsorption and build-up of contamination on the surface of the phase plate.[6]

Detailed information of matrix-assisted laser desorption/Ionization time-of-flight mass spectrometry (MALDI-TOF MS). Mass analyses were performed on a MALDI-TOF mass spectrometer (Bruker REFLEX III) operating at an acceleration voltage of 23 kV in reflection mode. Ions were generated by laser desorption at 337 nm (N_2 laser, 3-ns pulse width, 10^6–10^7 W/cm^2). For each spectrum, approximately 400 transients were accumulated and all spectra were recorded in reflection mode. Data evaluation was performed with the Bruker XMASS program, using reflection spectra only to achieve better signal-to-noise ratios. 3-Hydroxypicolinic acid (HPA) was selected as the matrix. To prepare the matrix, a solution of HPA in 1:2 water:acetonitrile with 0.1% trifluoroacetic acid was mixed with the sample solution. An aliquot of the mixture was placed on the target plate and applied to the probe.

Detailed information of size exclusion chromatography-multiangle laser light scattering (SEC-MALLS) analysis. SEC-MALLS is a combination technique that allows samples to be first separated by SEC and then analysed by MALLS to determine the distribution of absolute molecular weight. Here, the column was connected to an HPLC system equipped sequentially with a MALLS detector (DAWN-EOS, Wyatt Technology Co., Santa Barbara, CA) and a RI detector. The MALLS system employed a laser source at 653 nm with 18 photodiodes placed at different angles.

The molecular weights of the samples were analysed using Equation (1):

$$(\mathrm{LS}) = (dn/dc)^2 \, C \, Mw \, K_{LS} \qquad (1)$$

where (LS) is the intensity of the MALLS signal, dn/dc is the refractive index increment, C is the concentration of the sample, and K_{LS} is a constant of the MALLS detector. Before SEC-MALLS analysis, K_{LS} was calibrated with toluene (6.578×10^{-6}) and a value of 0.159 was assigned to dn/dc as previously reported. Equation (2) was then used to convert the measured intensity of the RI detector to the sample concentration:

$$(\mathrm{RI}) = (dn/dc) \, C \, K_{RI} \qquad (2)$$

where K_{RI} is a constant of the RI detector (determined using a pullulan solution; dn/dc = 0.142 in water). This value of C was finally substituted into Equation (1) to obtain M_w.

References

1. Harada, A.; Kataoka, K. *Macromolecules* **1995**, *28*, 5294-5299.

2. Harada, A.; Kataoka, K. *Macromolecules* **2003**, *36*, 4995-5001.

3. Danev, R.; Nagayama, K. *Ultramicroscopy* **2001**, *88*, 243-252.

4. Hosokawa, F.; Danev, R.; Arai, Y.; Nagayama, K. *J. Electr. Microsc.* **2005**, *54*, 317-324.

Table S1. Nano-PICsome growth during FCCS measurements as determined by DLS.

	Size (nm)	PDI
Just after preparation	102	0.015
1 day	262	0.072
3 day	284	0.056

Table S2. Peak area and percentage of peak (i) and (ii) after peak separation of uPIC.

	Area	%
Peak i	2867	17
Peak ii	13598	83

Table S3. Peak areas corresponding to initial amounts of Nano-PICsomes and uPICs as determined by SEC. Results are plotted in Fig. S4.

Polymer Concentration (mg/mL)	Peak Area (μV·min)		
	Nano-PICsomes	uPICs	Total
1.0	1140027	1052332	2192359
0.5	907600	78922	986522
0.1	187030	9844	196874

Table S4. Nano-PICsome growth as a function of time as determined by DLS.

Time (t, h)	Diameter (d(t), nm)	Diameter^2 ($\{d(t)\}^2$, nm^2)	$\{d(t+1)\}^2 - \{d(t)\}^2$	Amount of uPICs still left in the solution
0	95.4	9101.16	1099.84	A_0 = 70000
0.05	101	10201	1035	A_0-1099.84 = 68900.16
0.1	106	11236	645	A_0-1099.84-1035 = 67865.16
0.15	109	11881	888	A_0 (70000)-1099.84-1035-645 = 67220.16
…	…	…	…	…
1	140	19600	281	59501.16
1.05	141	19881	283	59220.16
1.1	142	20164	572	58937.16
1.15	144	20736	873	58365.16
…	…	…	…	…
36.0	278	77284	557	1817.16
36.05	280	77841	559	1260.16
36.1	281	78400	561	701.16
42	281	78961	-	140.16

*A_0: Initial amount of uPICs. This value was calculated by the following equation:

$$\{d(42)\}^2 - \{d(0)\}^2 = 78961 - 9101.16 \approx 70000$$

Table S5. Peak areas corresponding to Nano-PICsomes and uPICs as a function of time as determined by SEC. Results are plotted in Fig. 5a.

Time (h)	Peak area (μV·sec)		
	Nano-PICsomes	uPICs	Total
0	185693 (52.3 %)	169361 (47.7 %)	355054 (100%)
1	215904 (59.1 %)	149416 (40.9 %)	365320 (100%)
6	259060 (72.5 %)	118264 (27.5 %)	377324 (100%)
12	276178 (77.0 %)	88494 (23.0 %)	364672 (100%)
24	292533 (81.0 %)	68618 (19.0 %)	361151 (100%)
48	289398 (83.2 %)	58577 (16.8 %)	347975 (100%)
72	287787 (85.0 %)	50785 (15.0 %)	338572 (100%)

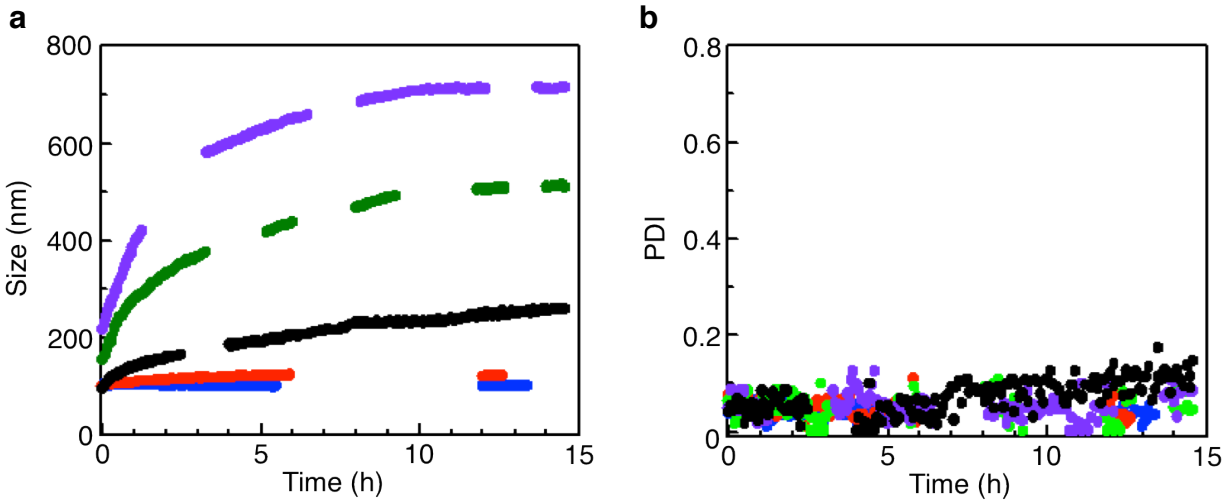


Figure. S1. Time dependent growth of Nano-PICsomes. a-b, Time dependence of size and PDI of Nano-PICsomes (purple: 3 mg/mL, green: 2 mg/mL, black: 1 mg/mL, red: 0.5 mg/mL, blue: 0.1 mg/mL).

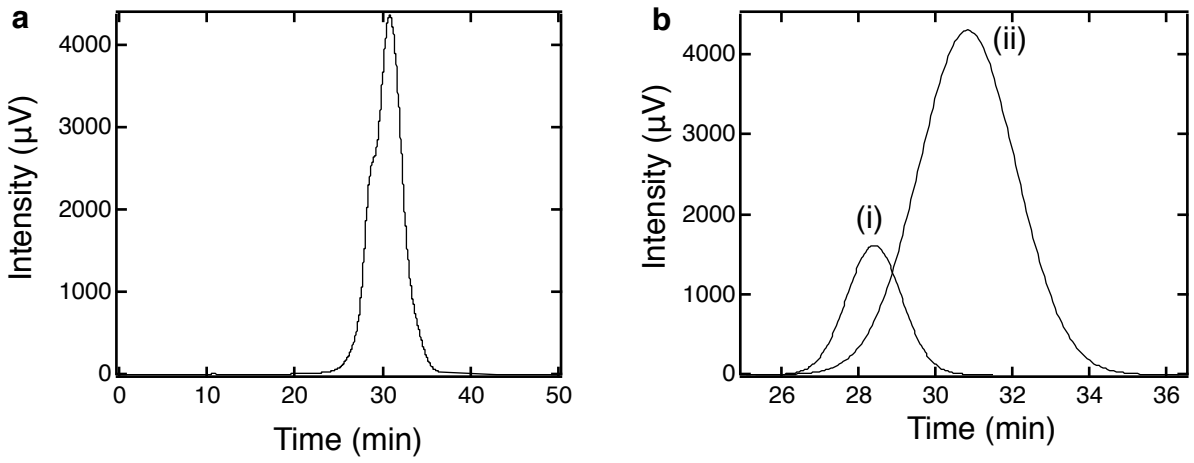
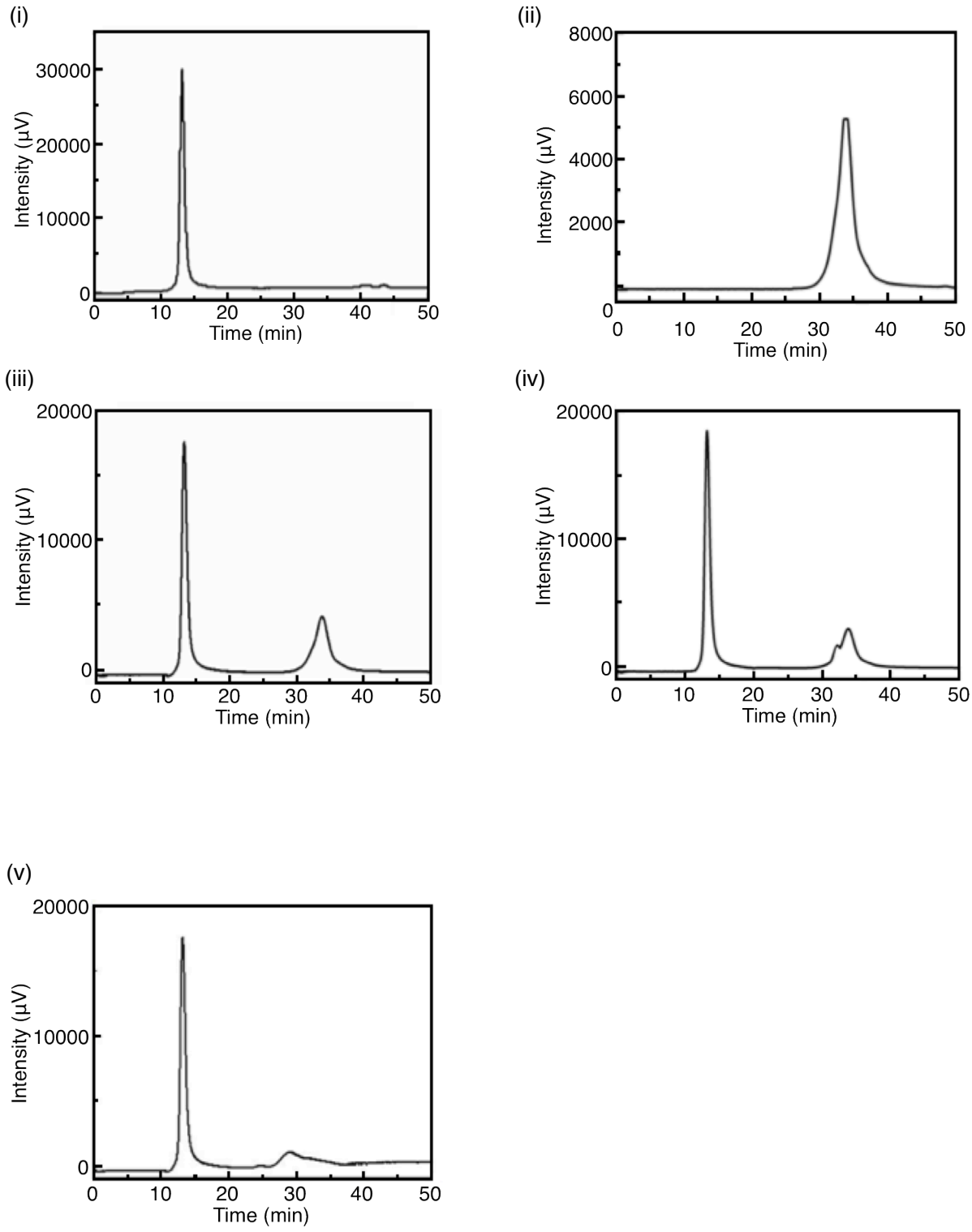


Figure S2. Peak separation of SEC profiles of uPICs using IGOR software. **a**, SEC profiles of uPICs before peak separation. **b**, Profiles of uPICs after peak separation. Peak separation was fitted as a Gaussian model.

Figure S3.

a


(i)
Intensity (μV)
Time (min)
(ii)
Intensity (μV)
Time (min)
(iii)
Intensity (μV)
Time (min)
(iv)
Intensity (μV)
Time (min)
(v)
Intensity (μV)
Time (min)

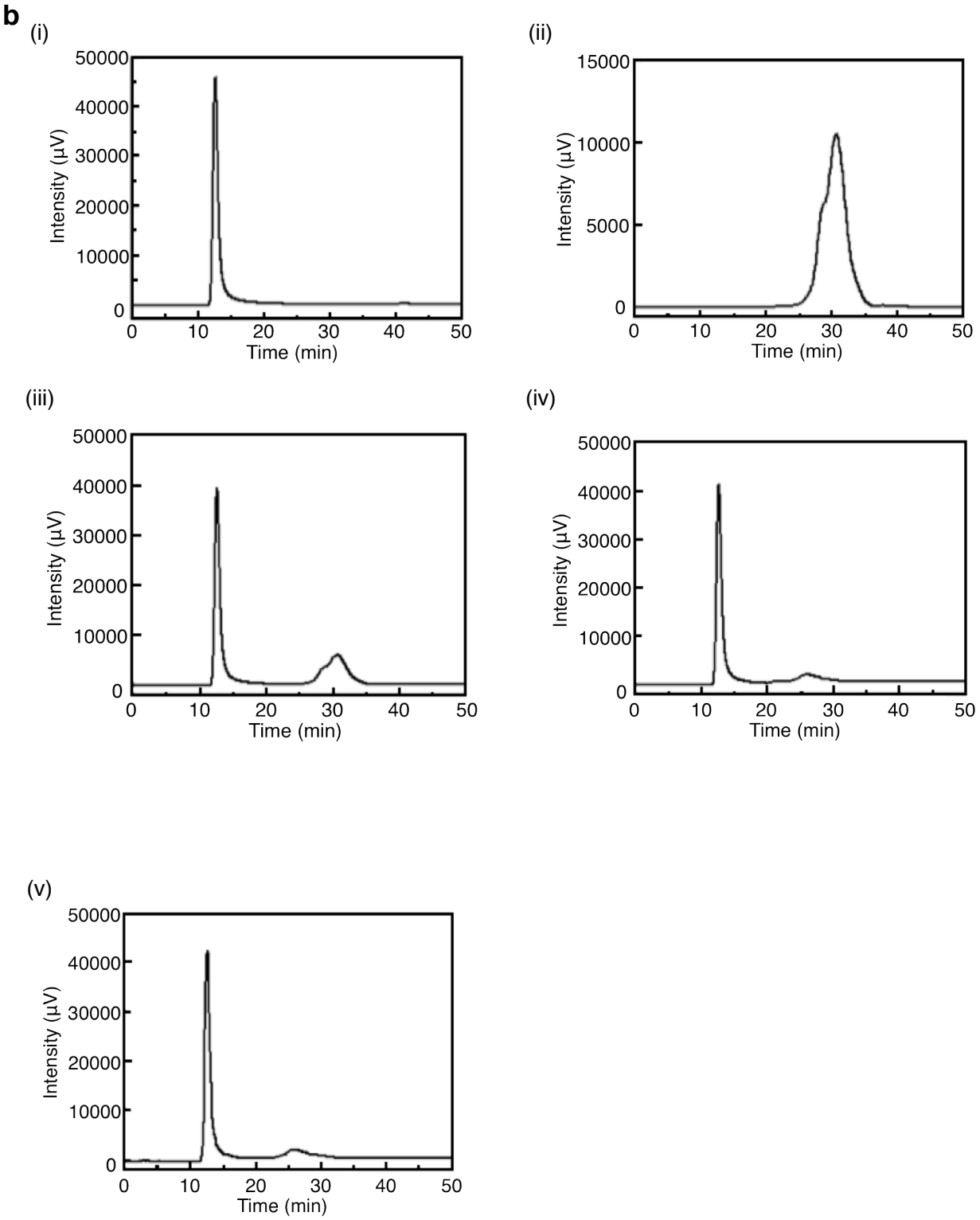


Figure S3. Analysis of transfer of small PICs to Nano-PICsomes. a-b, Time-dependent composition changes of PICs were recorded using a UV detector (220 nm), corresponding to Fig. 3a (a), and Fig. 3b (b). SEC traces of isolated unlabelled Nano-PICsomes (i) and isolated small Cy3-labelled PICs (ii). The mixture of isolated unlabelled Nano-PICsomes and Cy3-labelled small PICs was analysed at 0 h (iii), 1 h (iv), and 24 h (v) after crosslinking by EDC .

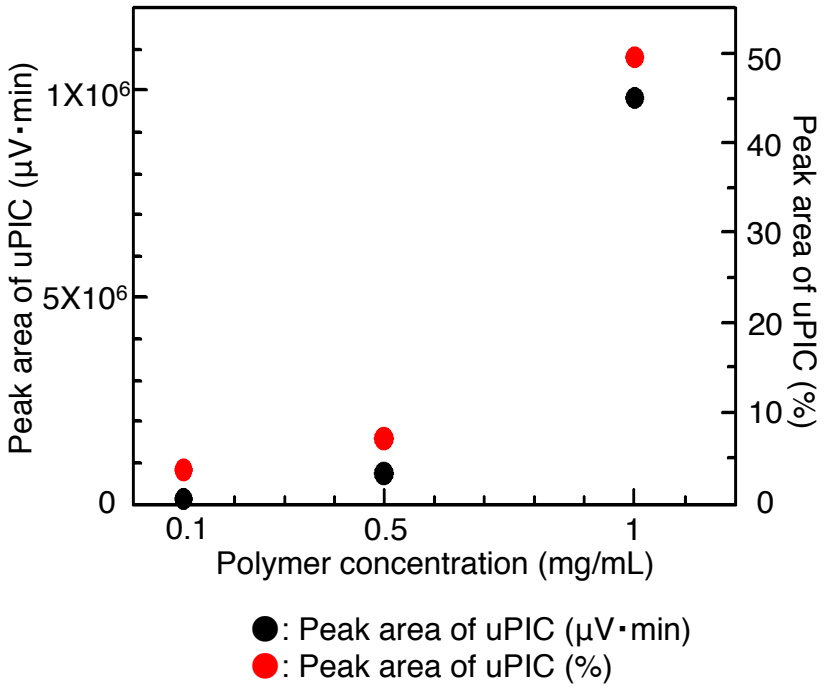


Figure S4. The initial amounts of uPICs at lower polymer concentrations. The PIC compositions were analysed just after formation of PICs using a fluorescent detector (Ex/Em = 520/550 nm), corresponding to Table S3.

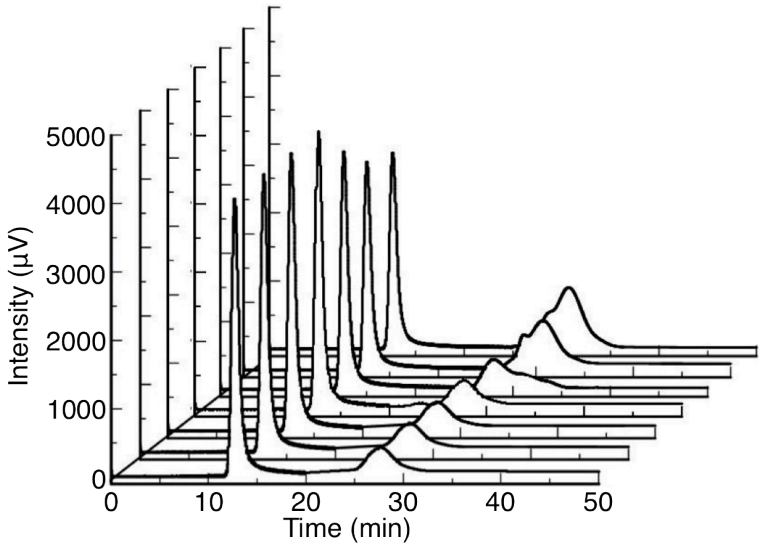


Figure S5. SEC profiles of the PIC solution at various time points (0, 1, 6, 12, 24, 48 and 72 h).

Figure S6.

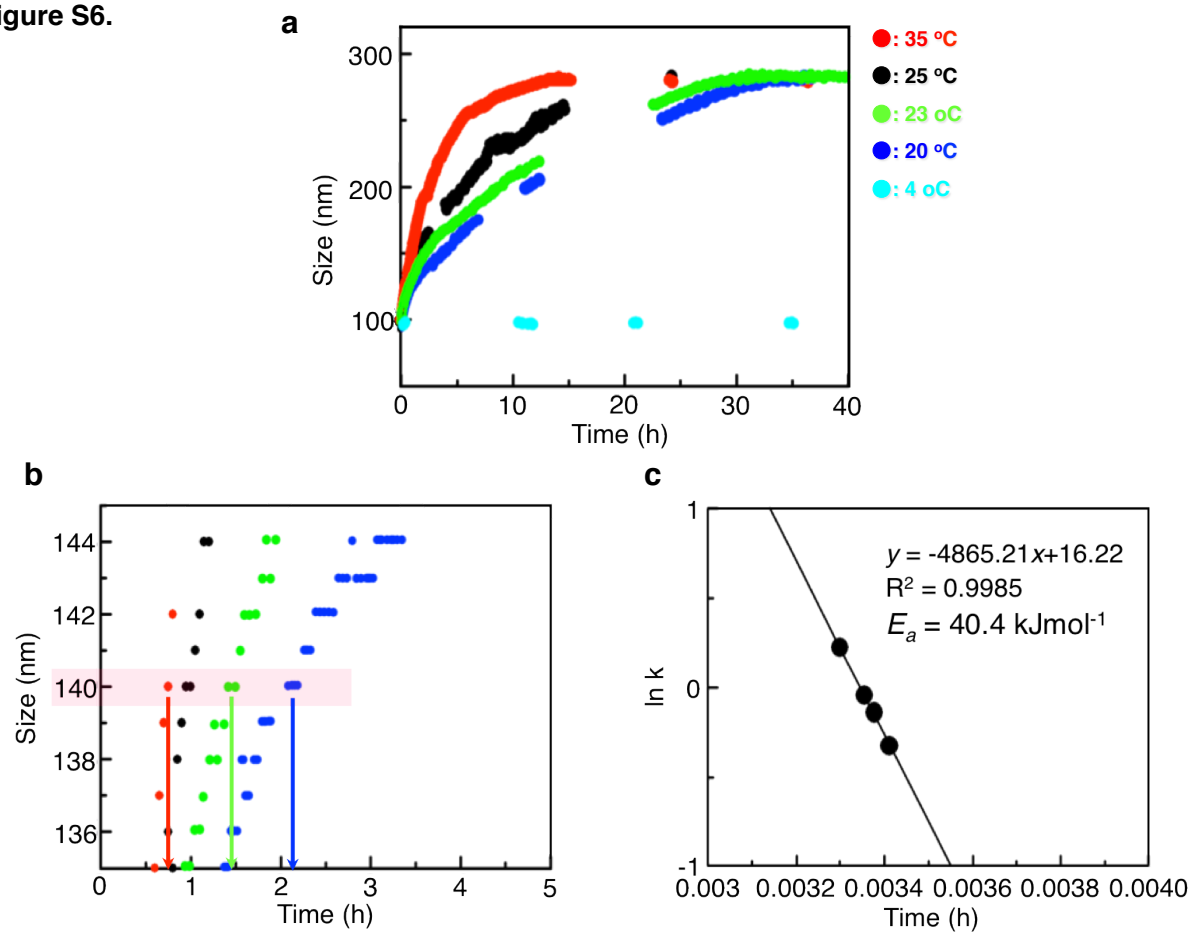


Figure S6. Growth of Nano-PICsomes at various temperatures. a, Time dependency of Nano-PICsome size at various temperatures, as determined by DLS measurements. **b,** Initial growth curves used for calculation of reaction rates at various temperatures. **c,** The Arrhenius plot for the growth of Nano-PICsomes. Activation energy (*Ea*) was calculated using the Arrhenius equation.

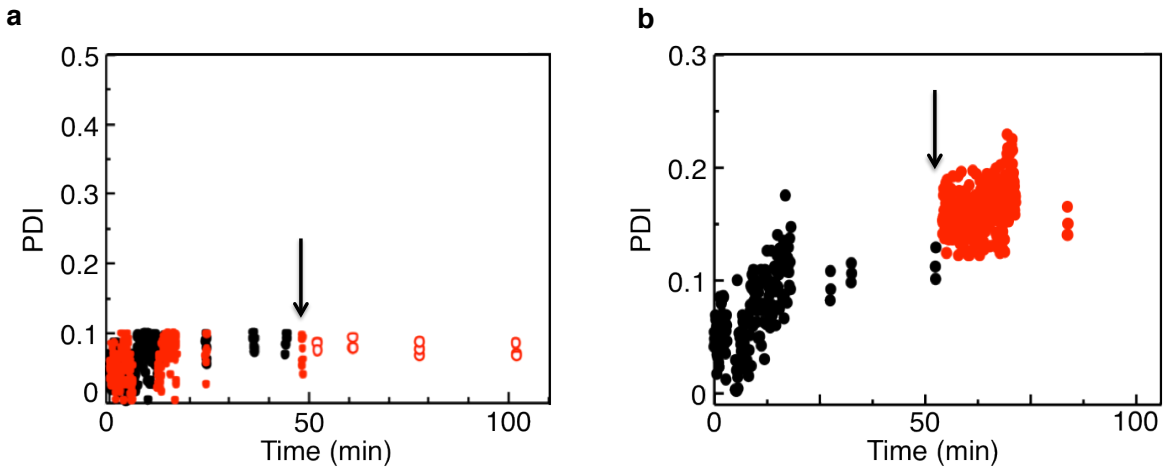


Figure S7. The effect of uPICs on PICsome growth. Time-dependency of polydispersity index (PDI) of Nano-PICsomes before (black dots) and after removal of uPICs (red dots) followed by the addition of uPICs (red circles). The total polymer concentration was 1 mg/mL. **b**, Time-dependency of the PDIs of Nano-PICsomes before (black dots) and after addition of additional uPICs (red dots). Initial total polymer concentration was 1 mg/mL. PDI of Nano-PICsomes were determined by DLS analysis. Time points at which uPICs were added are marked with arrows.

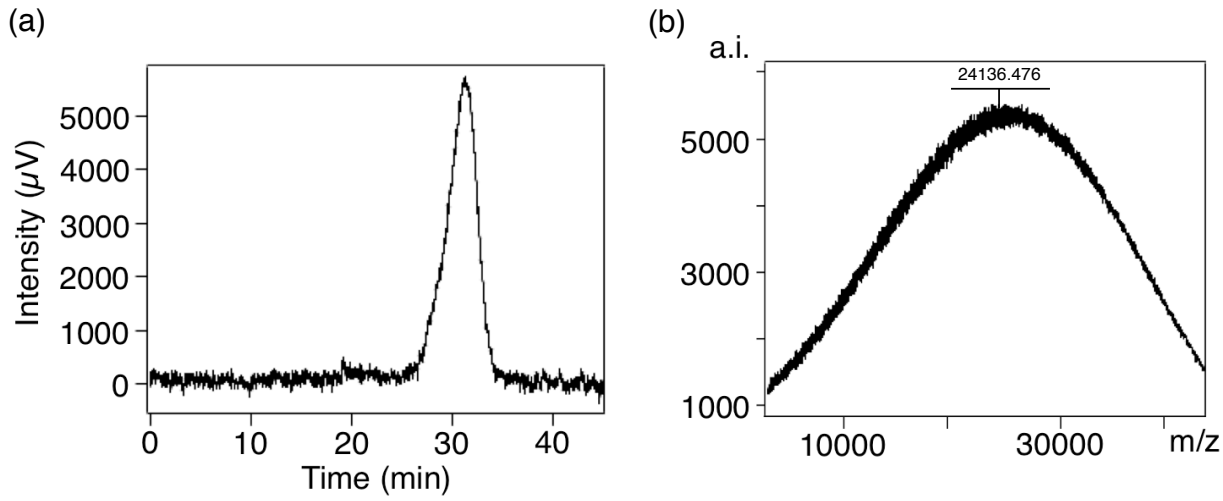


Figure S8. (a) SEC traces of Cy3-PICs were recorded using a fluorescent detector (Ex/Em = 520/550 nm). (b) Molecular weight distribution of Cy3-PICs determined by MALDI TOF mass spectrometry. PICs, obtained at the polymer concentration of 1 mg/mL, were diluted to 0.05 mg/mL, followed by vortex mixing before measurements.

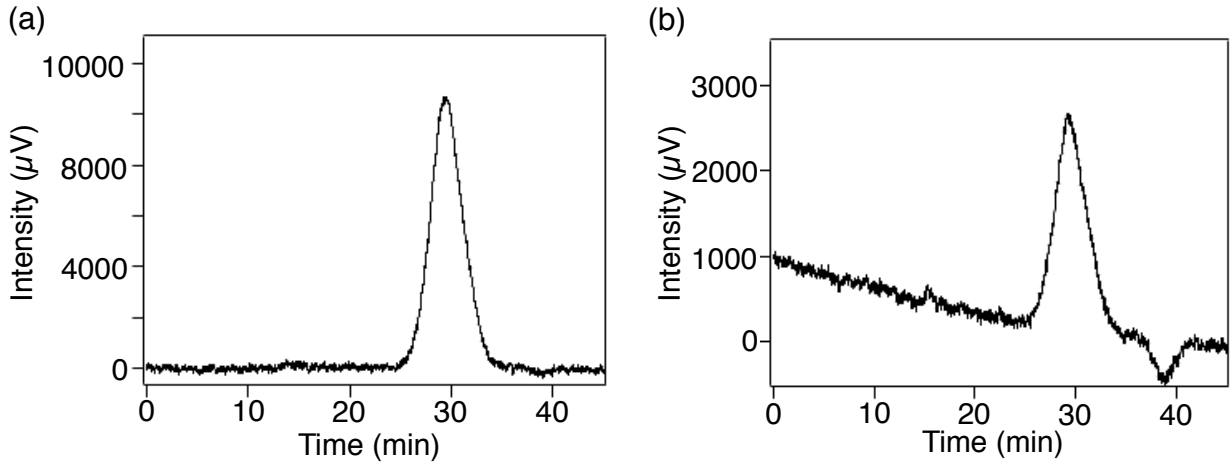


Figure S9. SEC traces of Cy3-PICs obtained at the polymer concentration of 0.05 mg/mL were recorded using a fluorescent detector (Ex/Em = 520/550 nm): (a) Just after preparation, and (b) 48 h after preparation of PICs.

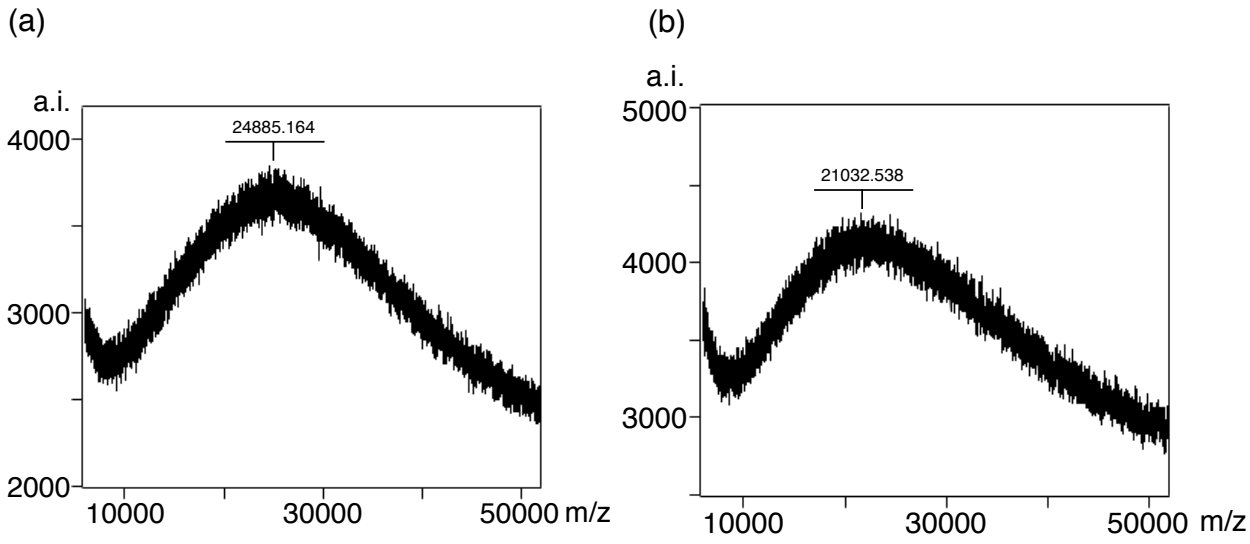


Figure S10. Molecular weight distribution of Cy3-PICs determined by MALDI TOF mass spectrometry: (a) Just after preparation, and (b) 48 h after preparation. PICs were obtained at the polymer concentration of 0.05 mg/mL.

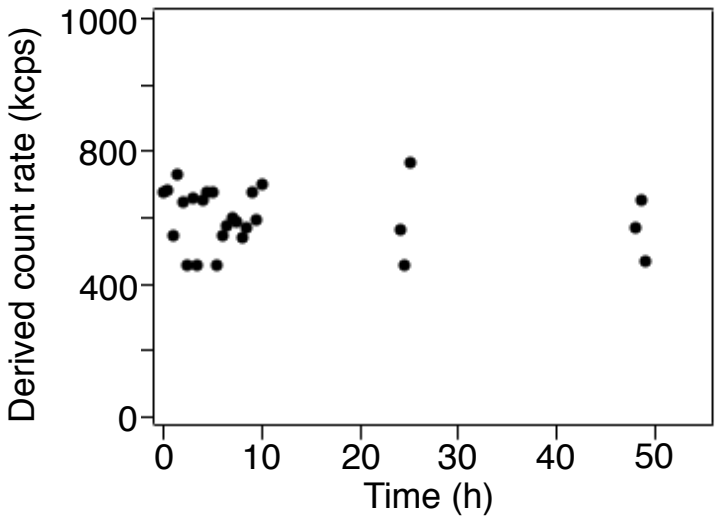


Figure S11. Time-dependence of derived count rates of PICs obtained by dynamic light scattering measurement. PICs were obtained at the polymer concentration of 0.05 mg/mL.