Supporting Information

Facile preparation of hexagonal nanosheets via polyion complex formation from α-helical polypeptides and polyphosphate-based molecules

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Experimental

1. Materials

N-Epsilon-trifluoroacetyl-L-lysine-*N*-carboxy anhydride (Lys(TFA)-NCA) and α-methoxy-ω-amino poly(ethylene glycol) (MeO-PEG-NH_2, M_n = 2,424) were purchased from Chuo Kaseihin Co., Inc. (Tokyo, Japan) and NOF Co., Ltd. (Tokyo, Japan), respectively. MeO-PEG-NH_2 was further purified by ion-exchange chromatography using CM Sephadex C-50 (Sigma; St. Louis, MO, USA). Benzene, *N*-dimethylformamide (DMF), *n*-hexane, ethyl acetate, tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), and methanol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cyanine3 (Cy3) and *N*-hydroxysuccinimide (NHS) ester were purchased from Lumiprobe Corp. (MD, USA). Deuterium oxide (D_2O) was purchased from Cambridge Isotope Laboratories Inc. (MA, USA). Adenosine 5'-triphosphate disodium salt (99%, Sigma-Aldrich; St. Louis, MO, USA), adenosine 5'-diphosphate disodium salt, (>98%, Tokyo Chemical Industry, Tokyo, Japan), adenosine 5'-monophosphate disodium salt (99.6%, Oriental Yeast Co., Ltd., Tokyo, Japan), 5-phospho-D-ribose 1-diphosphate sodium salt (phosphoribosyl pyrophosphate, PRPP, 75%, Cayman Chemical, Michigan, USA), sodium polyphosphate (triphosphate) (Wako Pure Chemical Industries Ltd., Osaka, Japan), guanosine 5'-triphosphate sodium salt (Sigma-Aldrich; St. Louis, MO, USA), and glutaraldehyde (GA, Electron Microscopy Sciences, Hatfield, UK) were also sourced commercially, as indicated. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein labelled isothiocyanate (FITC) was purchased from Invitrogen, Thermo Fischer Scientific Co., (CA, USA). Copper grids (150 mesh) coated with a thin film of Formvar and reinforced with a carbon coating were purchased from JEOL (Tokyo, Japan).

2. Measurements

2.1 ^{1}H nuclear magnetic resonance (NMR) spectroscopy

The ^{1}H NMR spectra of PEG-*b*-PLL was measured and recorded using a JNM-ECZ400S JEOL spectrometer (Tokyo, Japan) at a frequency of 400 MHz in D_2O at 25 °C.

2.2 Size exclusion chromatography (SEC)

The SEC measurements of PEG-*b*-PLL were carried out using a high-performance liquid chromatography system (JASCO International Co., Ltd., Tokyo, Japan) equipped with a Superdex 200-10/300 GL column (Cytiva, Massachusetts, USA) and a RI-4030 refractive index detector (JASCO International Co., Ltd., Tokyo, Japan) with an aqueous phosphate buffer (PB) solution (100 mM, pH 7.4) containing 500 mM NaCl as the eluent at a flow rate of 0.5 mL/min at room temperature.

2.3 Dynamic light scattering (DLS) and ζ-potential measurements

A Zetasizer Pro from Malvern PANalytical Ltd. (Malvern, UK) equipped with an ion He-Ne laser (λ = 633 nm) at measurement angles of 173 and 13° was used to analyse the size distributions and ζ-potentials of the polyion complexes (PICs) at room temperature. The hydrodynamic diameters and polydispersity indices (PDIs) of the particles were calculated using the cumulant method (*vide infra*). The derived count rate data obtained from the DLS measurements were used as the scattered light intensities.

In the DLS measurements, the autocorrelation function, $g(\tau)$, was analysed using the cumulant method[1,2] in which

$$g(\tau) = \exp[-\bar{\Gamma}\tau + (\mu_2/2)\tau^2 - (\mu_3/3!)\tau^3 + \ldots.] \quad (1)$$

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

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

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

where q is the magnitude of the scattering vector, n is the refractive index of solvent, and θ is the detection angle. In addition, the polydispersity index (PDI = $\mu_2/\bar{\Gamma}^2$) was derived from Equation 1.

2.4 Fluorescence microscopy

Fluorescence microscopy observations were performed using a BZ-X800 microscope (Keyence Corporation, Osaka, Japan) equipped with a light-emitting diode (3.7 W), a metal halide lamp (80 W), a dichroic mirror block TRITC (Model OP-87764, Keyence, Excitation λ: 545/25 nm, Emission λ:605/70), and a 100× oil immersion objective lens (Plan Apo λ, Nikon, Tokyo, Japan). The size distribution of the ATP-based hexagonal nanosheets (N = 500) was further analysed using ImageJ 1.53k software.[3] The size of the hexagon was defined as the side-to-side length.

2.5 Transmission electron microscopy (TEM)

TEM observations were performed on a JEM-1400 JEOL microscope (Tokyo, Japan) operating at 120 kV to clarify the morphologies of the PIC particles. Prior to carrying out the observations, all PIC samples

were crosslinked using 0.1% GA to fix their structures. An aliquot (2 μL) of the PIC sample was placed on a 150-mesh copper grid and hydrophilised using an HDT-400 JEOL hydrophilic treatment device (Tokyo, Japan). After natural drying for 3 min, a drop of uranyl acetate solution (2% w/v) was added for staining, and the sample was allowed to dry for another 3 min. Any excess solution was removed carefully using filter paper, and the grid was air-dried at room temperature.

2.6 Circular dichroism (CD) spectroscopy measurements

The CD spectra of the PEG-*b*-PLL and PICs particles were measured using a JASCO J-820 spectropolarimeter (Tokyo, Japan) at room temperature using a 50 mM PB solution (pH 7.4). A S10-SQ-1 quartz cell (GL Sciences Inc., Tokyo, Japan) with a path length of 2 mm and a maximum volume of 0.4 mL was used to carry out the measurements.

2.7 Atomic force microscopy (AFM)

The thicknesses of the ATP–PEG-*b*-PLL hexagonal nanosheets were evaluated in the peak force tapping mode using a BioScope Resolve microscope (Bulker) in a 50 mM PB solution (pH 7.4) at room temperature, in addition to a Biolever mini BL-AC40TS-C2 micro cantilever (k = 0.09 N/m, f = 110 kHz) at a peak force frequency of 1 kHz and peak force set point of 150 pN. Prior to carrying out the measurements, the sample was fixed using a 0.1% GA solution, purified as described in Section 4.1, and adhered to a clean surface of mica overnight in a 50 mM PB solution (pH 7.4). A minimum of three points were randomly selected and scanned to measure the thickness of each hexagonal nanosheet (N = 9).

3. Polymer synthesis and characterisation

3.1 Synthesis of polyethylene glycol-*b*-poly-L-lysine (PEG-*b*-PLL)

PEG-*b*-PLL was obtained by the deprotection of PEG-Lys(TFA), which was prepared from the ring-opening polymerisation of Lys(TFA)-NCA initiated by MeO-PEG-NH_2. More specifically, this deprotection was carried out using a 1.0 M solution of NaOH.[4,5] The degree of polymerisation (DP) of the lysine segment was determined to be 70 from the peak intensity ratio of the protons of the poly-L-lysine side chains to the methylene protons in the PEG (OCH_2CH_2; δ 3.71; 212H) and PLys (-CH-CH_2-(CH_2)$_2$-CH_2-; δ 4.3; 70H, δ 1.4; 128H, δ 1.7; 272H and δ 3.0; 140H) segments, respectively (Figure S1a). PEG-*b*-PLL with a DP of 70 PLL segments was used for the purpose of this study. The polymer unimodal distribution was further confirmed by SEC (Figure S1b), and the polydispersity (M_w/M_n) was calculated to be 1.28.

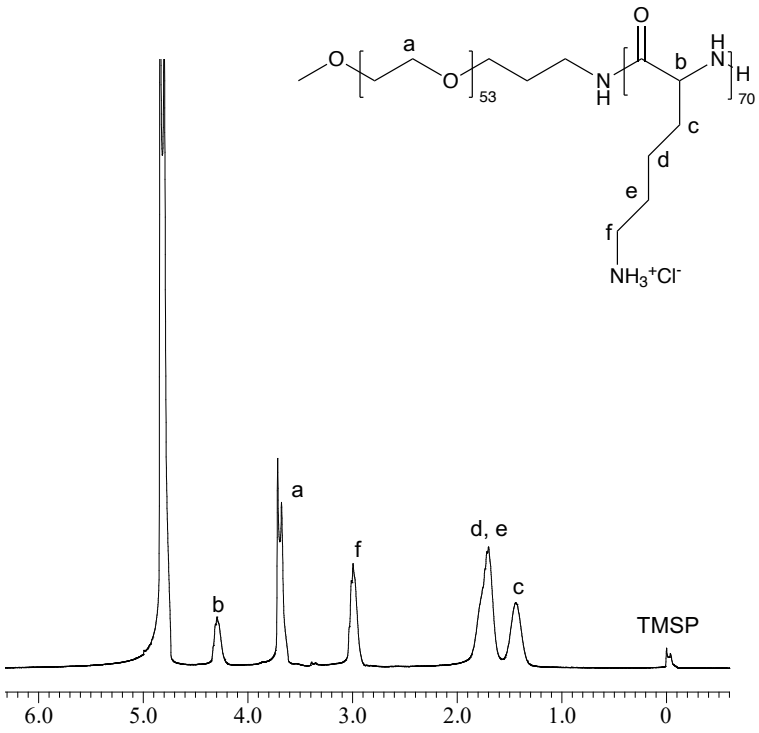

Fig. S1a. A ^{1}H NMR spectrum of PEG-*b*-PLL D_2O at 24 °C.

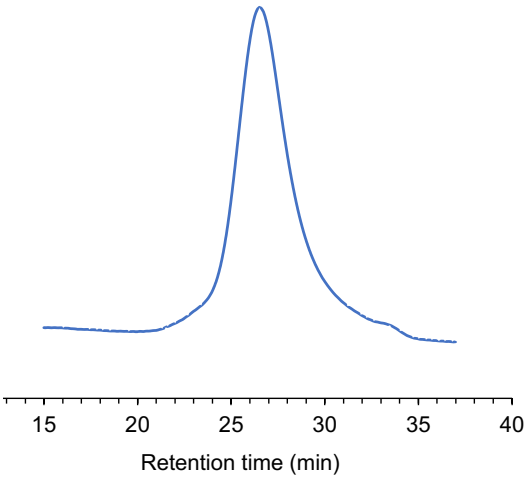

Fig. S1b. SEC chromatogram of PEG-*b*-PLL.

3.2 Synthesis of Cy3-labelled PEG-*b*-PLL (Cy3-PEG-PLL)

For fluorescence labelling, PEG-*b*-PLL was treated with Cy3-NHS ester (1 eq. per PLL chain) in a 0.1 M Na_2CO_3 solution (pH-adjusted to 9.0 using a 1 M HCl solution) at room temperature, and the reaction was allowed to proceed overnight. The resulting polymer was then subjected to dialysis against ultrapure water for 2 d using a Spectra/Por cellulose dialysis membrane (molecular weight cut-off (MWCO) = 3.5 kDa) from Spectrum Laboratories (California, USA). The final solution containing Cy3-PEG-*b*-PLL was lyophilised to obtain the powdered form. The labelling ratio was determined to be 16%.

4. PIC preparation

4.1 General protocol

PEG-*b*-PLL, ATP, ADP, AMP, PRPP, STP, and GTP were separately dissolved in a 50 mM PB solution (pH 7.4) to give a final concentration of 1.0 mg/mL. The PIC particles were prepared by mixing PEG-*b*-PLL with either ATP, ADP, AMP, PRPP, STP, or GTP at a fixed cation/anion ratio (C/A) of 1:1 at 4 °C. In addition, Cy3-labelled PIC nanosheets were prepared using Cy3-PEG-*b*-PLL. The PICs were further characterised using DLS, fluorescence microscopy, TEM, and AFM (using an ATP-based complex), immediately after preparation. For the TEM observations, all PIC particles were crosslinked with GA. More specifically, the as-prepared PIC sample was treated with a GA solution (0.1% v/v GA in 50 mM PB, pH 7.4, 0.1 eq. relative to the total number of amino residues in PLL), which was added to the PIC solution and gently mixed by pipetting. After 1 h of incubation at 4 °C, the resulting materials were purified by centrifugation at 3000G and 4 °C for 10 min. The supernatant was then removed by pipetting, and the sedimented PIC particles were suspended in ultrapure water.

4.2 BSA loading

BSA labelled with FITC (BSA-FITC) was dissolved in a 50 mM PB solution (pH 7.4) to give a final concentration of 1.0 mg/mL. The mixture of ATP and BSA-FITC at the volume ratio of 1:1 was prepared and used for the PIC formation by mixing with PEG-*b*-PLL. The final concentration of BSA-FITC after the complexation was 0.3 mg/mL.

4.3 Salt addition to the PEG-*b*-PLL–STP complex

PEG-*b*-PLL and STP were dissolved separately in a 50 mM PB solution (pH 7.4, containing 200 mM NaCl) to give a final concentration of 1.0 mg/mL. The PEG-*b*-PLL-STP complex was prepared by mixing each solution at a C/A ratio of 1:1.

4.4 Urea addition to the PEG-*b*-PLL–GTP complex

A GTP solution (1.0 mg/mL) was prepared using 50 mM PB (pH 7.4, containing 1 M urea) as the solute. The prepared solution was then mixed with a 50 mM PB solution of PEG-*b*-PLL (1.0 mg/mL). The final urea concentration in the mixture was 0.67 M.

5. Supporting Tables

Table S1. DLS results for the PEG-*b*-PLL complexed with ATP

Sample	Z-Average (nm)	Polydispersity Index	Attenuator	Derived Mean Count Rate (kcps)
PEG-*b*-PLL–ATP	1100	0.28	6	43200

Table S2. DLS results for the PEG-*b*-PLL complexed with homo $PAsp_{106}$

Sample	Z-Average (nm)	Polydispersity Index	Attenuator	Derived Mean Count Rate (kcps)
PEG-*b*-PLL–homo $PAsp_{106}$	77.5	0.014	6	48400

Table S3. DLS measurements for the PEG-b-PLL – ATP specimens before and after the addition of 1 M urea

Sample	Z-Average (nm)	Polydispersity Index	Attenuator	Derived Mean Count Rate (kcps)
PEG-*b*-PLL–ATP	1300	0.20	6	45400
PEG-*b*-PLL–ATP–1 M urea	110	0.34	10	1190

Table S4. DLS measurements for the PEG-*b*-PLL complexed with AMP or ADP

Sample	Z-Average (nm)	Polydispersity Index	Attenuator	Derived Mean Count Rate (kcps)
PEG-*b*-PLL–AMP	260	0.60	10	690
PEG-*b*-PLL–ADP	310	0.57	10	740

Table S5. DLS measurements for the PEG-*b*-PLL complexed with STP, PRPP, or GTP

Sample	Z-Average (nm)	Polydispersity Index	Attenuator	Derived Mean Count Rate (kcps)
PEG-*b*-PLL–STP	1200	0.28	7	29900
PEG-*b*-PLL–PRPP	1400	~1	6	54200
PEG-*b*-PLL–GTP	320	0.25	6	83400

6. Supporting Figures

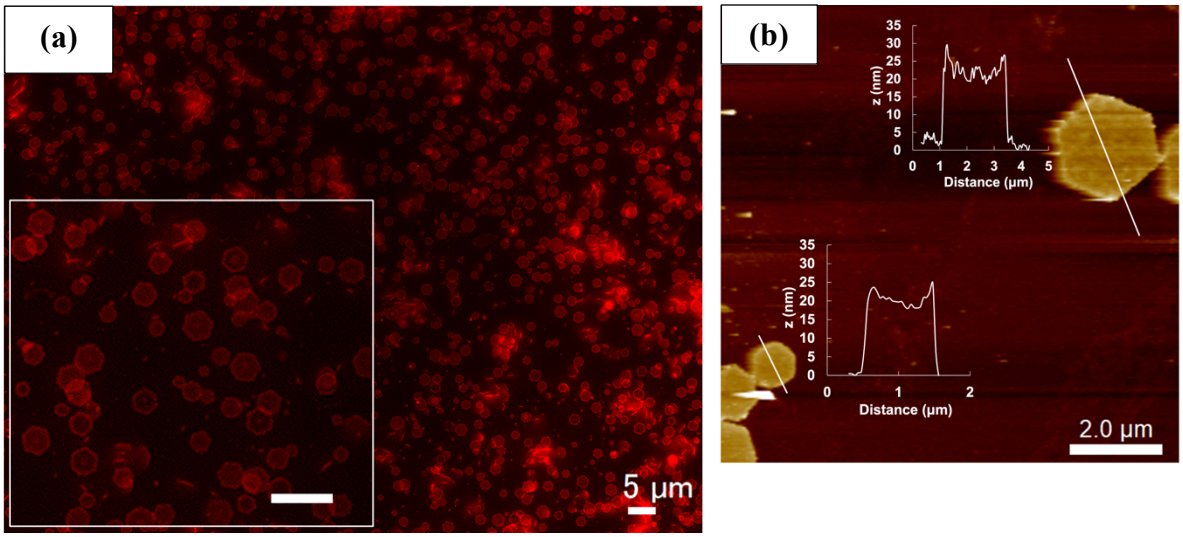

Fig. S2. (a) Fluorescent micrographs of the hexagon nanosheets prepared using Cy3-labelled PEG-*b*-PLL and ATP, and (b) an AFM image of hexagon nanosheets with selected height profile.

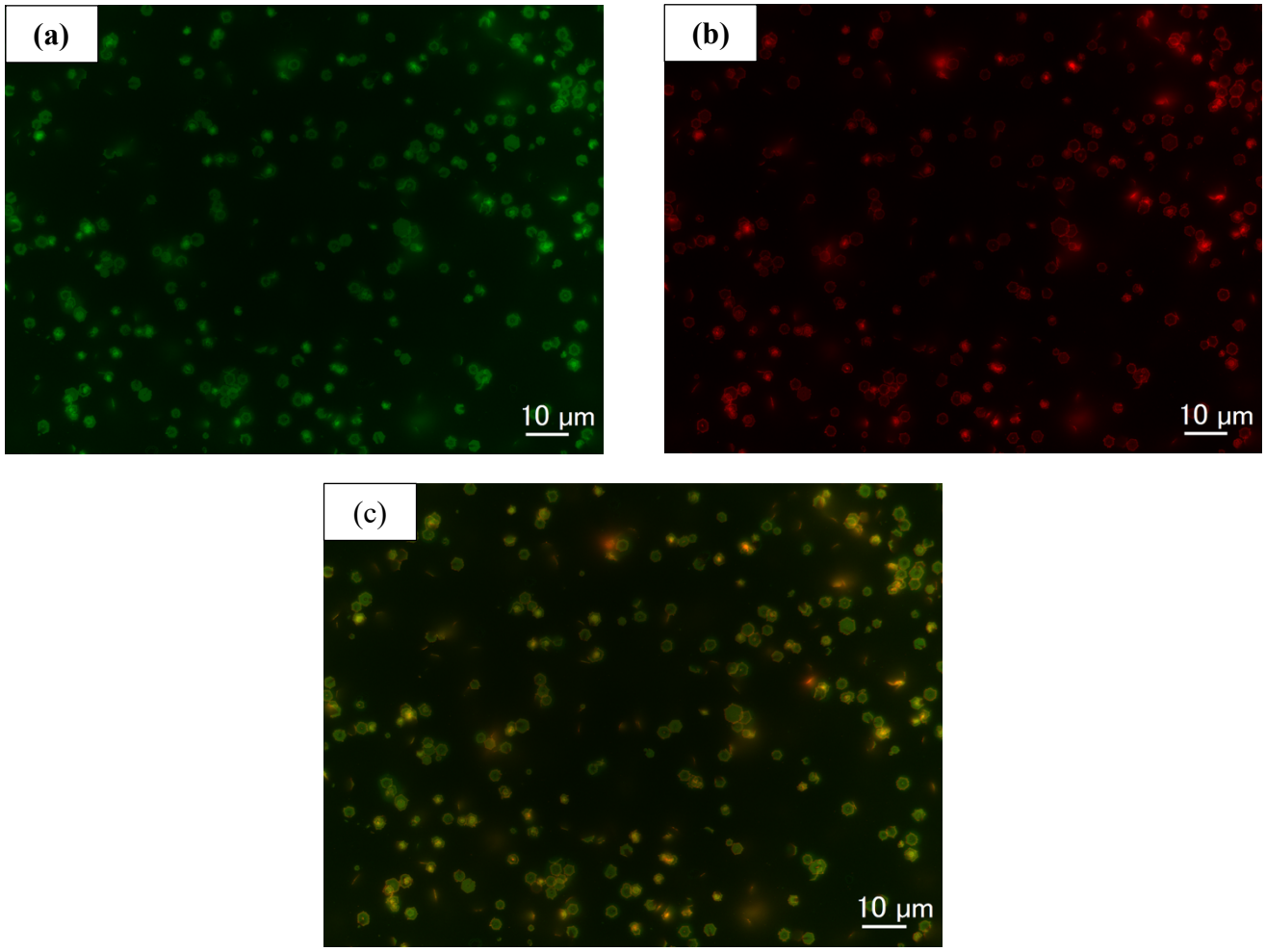

Fig. S3. Fluorescent images of the hexagon nanosheets prepared using Cy3-labelled PEG-b-PLL in the presence of 1 mg/mL FITC-labelled BSA solution (50 mM PB buffer, pH 7.4, 25 °C): (a) an FITC-channel, (b) a Cy3-channel and (c) a merged image

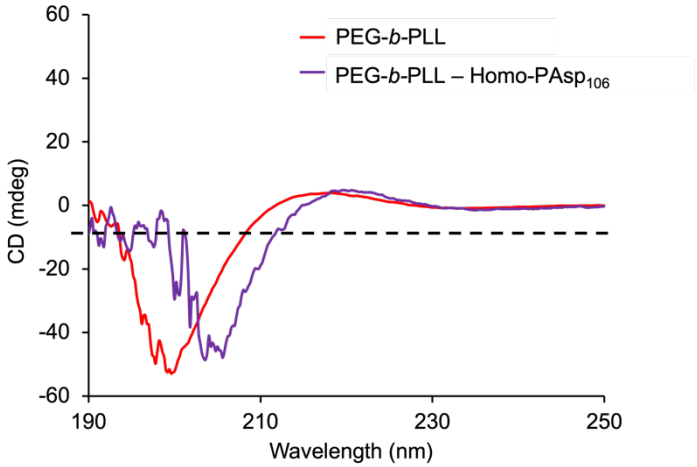

Fig. S4a. CD spectrum of the PIC prepared from PEG-*b*-PLL and homo $PAsp_{106}$.

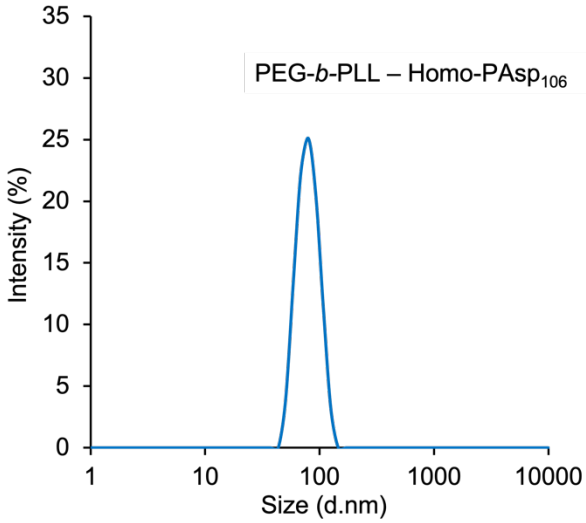

Fig. S4b. Size distribution of the PIC particles prepared from PEG-*b*-PLL and homo $PAsp_{106}$.

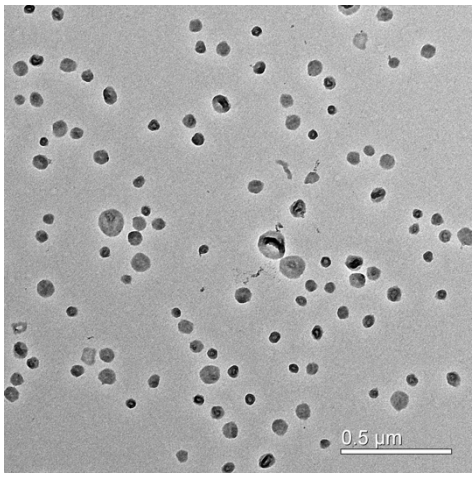

Fig. S4c. TEM image of the PIC particles prepared from PEG-*b*-PLL and homo $PAsp_{106}$.

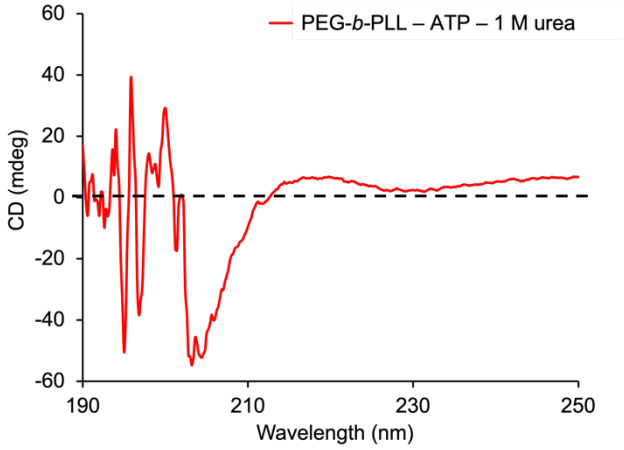

Fig. S5a. CD spectrum of the PIC prepared from PEG-*b*-PLL and ATP in the presence of urea.

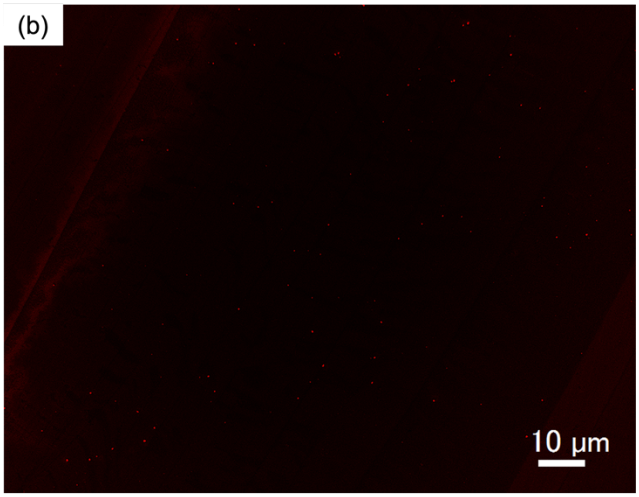

Fig. S5b. Fluorescence micrograph of the PIC prepared from PEG-*b*-PLL and ATP in the presence of urea.

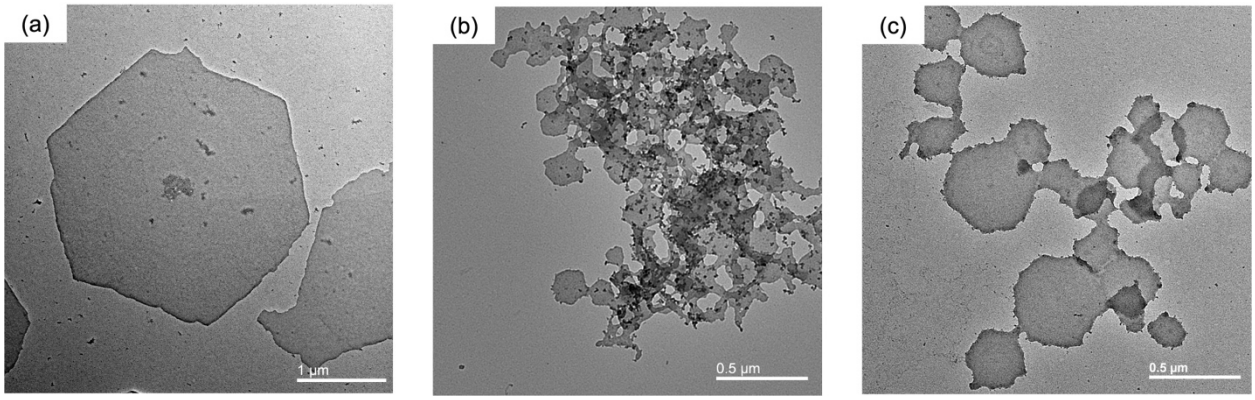

Fig. S6. TEM images of nanosheets formed from (a) PEG-*b*-PLL – PRPP, (b) PEG-*b*-PLL – STP, (c) PEG-*b*-PLL – GTP.

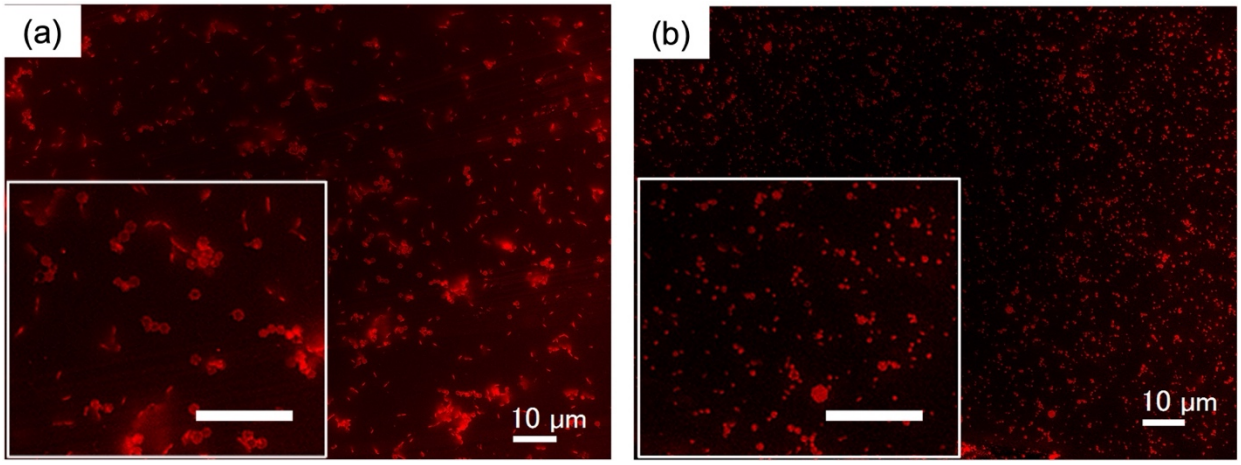

Fig. S7. Fluorescence images of the PIC hexagonal nanosheets prepared in the presence of 200 mM NaCl or 1 M urea. The PICs prepared from Cy3-labelled PEG-*b*-PLL and STP in the presence of 200 mM NaCl (a), and from PEG-*b*-PLL and GTP in the presence of 1 M urea (b).

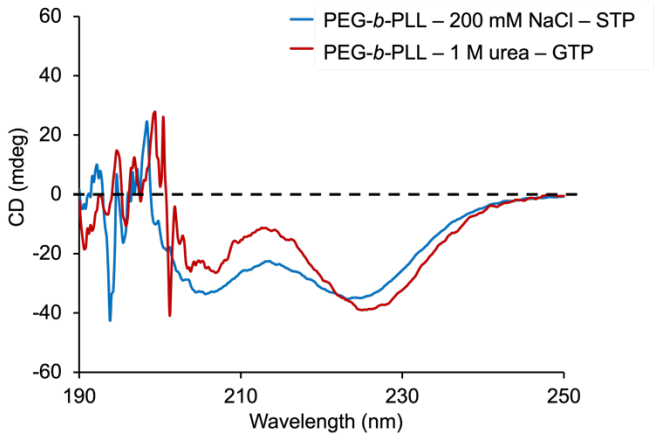

Fig. S8. CD spectra of the PICs fabricated from PEG-*b*-PLL and STP or GTP after the addition of 200 mM NaCl and 1 M urea, respectively.

7. References

1. A. Harada and K. Kataoka, *Macromolecules*, 1995, **28**, 5294.
2. A. Harada and K. Kataoka, *Macromolecules*, 2003, **36**, 4995.
3. C. A. Schneider, W. S. Rasband and K. W. Eliceiri, *Nat. Methods*, 2012, **9**, 671.
4. O. F. Mutaf, A. Kishimura, Y. Mochida, A. Kim and K. Kataoka, *Macromol. Rapid Commun.*, 2015, **36**, 1958.
5. A. Ahmad, T. Nii, T. Mori, Y. Katayama, M. Toyofuku and A. Kishimura, *Macromol. Rapid Commun.*, 2022, 2200316.