Supporting Information

Semipermeable Polymer Vesicle (PICsome) Self-Assembled in Aqueous Medium from a Pair of Oppositely Charged Block Copolymers: Physiologically Stable Micro-/Nano-containers of Water-soluble Macromolecules

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Materials.

β-Benzyl-L-aspartate *N*-carboxy-anhydride (BLA-NCA) and α-methoxy-ω-amino poly(ethylene glycol) (MeO-PEG-NH_2) (M_n=2,000, M_w/M_n=1.05) were obtained from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). 1,2-Diaminoethane (DAE) and1,5-Diaminopentane (DAP) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and distilled over CaH_2 under reduced pressure. *N*,*N*-dimethylformamide (DMF) and dichloromethane (CH_2Cl_2) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and distilled by a general method before use.

Fluorescein isothiocyanate-labeled dextran (FITC-Dex: M_n=40,000), tetramethylrhodamine isothiocyanate (TRITC: MW=443.5), tetramethylrhodamine isothiocyanate labeled-dextran (TRITC-Dex: M_n=70,000), and fetal bovine serum (FBS) were purchased from Sigma (St. Louis, MO).

Instruments.

^{1}H-NMR spectra were measured in d_6-DMSO or D_2O at 80 °C on a JNM-AL 300 (JEOL, Japan) at 300 MHz. Number-average molecular weight (M_n) and molecular weight distribution (M_w/M_n) were determined using a gel permeation chromatography (GPC) system (TOSOH HLC-8220) equipped with two TSK gel columns ($G4000H_{HR}$ and $G3000H_{HR}$) and an internal reflective index (RI) detector. Columns were eluted with DMF containing 10 mM LiCl at a flow rate of 0.8 ml/min at 40 °C. Dark-field microscopy (DFM) was performed using an Olympus model BX51 equipped with a 100× Oil-immersed objective (UPlanApo, Olympus, Japan) and a digital camera (VB-7000, Keyence, Japan) at ambient temperature. Flow particle image analysis was performed for particles whose diameters were more than 0.8 μm using a Sysmex FPIA-3000 (Sysmex, Japan), which consists of a thin capillary as a flow channel (similar to flow cytometer used for cell analysis) equipped with a CCD detector.[1] Morphological parameters are calculated from the captured images through image processor. Fluorescence observation and emission spectra measurements in the specific region of the PICsome were performed using a confocal laser scanning microscope (CLSM) (LSM510 META, Carl Zeiss, Germany) with a 63×

objective (C-Apochromat, Carl Zeiss, Germany) at excitation wavelengths of both 488 nm (Ar laser) and 543 nm (He-Ne laser) in parallel.[2] The CLSM is equipped with a grating as a dispersive element and a 32-channel PMT array to collect photons across the visible spectrum.

Experiments

1. Synthesis

1-1 Synthesis of Poly(ethylene glycol)-Poly(β-benzyl-L-aspartate) (PEG-PBLA$_{100}$ and PEG-PBLA$_{17}$)

PEG-PBLA$_{100}$ block copolymer was prepared by the ring-opening polymerization of BLA-NCA initiated by the terminal primary amino group of MeO-PEG-NH_2. MeO-PEG-NH_2 was dissolved in benzene (5 mg/mL), followed by freeze-drying to obtain the sample used for block copolymer synthesis. MeO-PEG-NH_2 (250 mg; 0.125 mmol) was dissolved in a mixed solvent of DMF (1 mL) and CH_2Cl_2 (4 mL). After BLA-NCA (2.95 mg; 12.5 mmol) was dissolved in a mixed solvent of DMF (5 mL) and CH_2Cl_2 (50 mL), this solution was added to the solution of MeO-PEG-NH_2. The reaction mixture was stirred for 40 h at 35 °C under a dry argon atmosphere. The polymerization was stopped when the characteristic bands corresponding to BLA-NCA (1850, 1760, and 915 cm^{-1}) disappeared from the IR spectrum. The resulting solution was precipitated into diethyl ether (500 mL). The crude precipitate was washed twice with diethyl ether to obtain 1.5 g of a white powder. The GPC chromatogram of the prepared PEG-PBLA was unimodal, and the M_n and the M_w/M_n were determined to be 11,000 and 1.17, respectively, using the calibration curve for PEG. From ^{1}H-NMR measurement, the degree of polymerization (DP) of the BLA units was calculated to be 100, comparing to the peak intensity ratio of the methylene protons of PEG (δ3.52) and the benzyl protons of the BLA unit (δ7.27). ^{1}H-NMR (d_6-DMSO): δ 2.59-2.89 (186H, CHCH_2CO), δ3.25 (3H, CH_3OCH_2CH_2), δ3.52 (180H, OCH_2CH_2), δ4.62 (70H, CO*CH*NH), δ5.01 (190H, COOCH_2Ph), δ7.27 (498H, COOCH_2*Ph*), δ7.92 (40H, COCH*NH*).

In a manner similar to that for PEG-PBLA$_{100}$, PEG-PBLA$_{17}$ (900 mg) was prepared from MeO-PEG-NH$_2$ (500 mg) and BLA-NCA (1.2 g). The M_n and the M_w/M_n were determined to be 3500 and 1.18, respectively by GPC measurement. From ^{1}H-NMR measurement, the DP of the BLA units was calculated to be 17. ^{1}H-NMR (d_6-DMSO): δ 2.59-2.89 (32H, CH*CH$_2$*CO), δ3.25 (3H, *CH$_3$*OCH$_2$CH$_2$), δ3.52 (180H, O*CH$_2$CH$_2$*), δ4.62 (13H, CO*CH*NH), δ5.01 (34H, COO*CH$_2$*Ph), δ7.27 (85H, COOCH$_2$*Ph*), δ7.92 (16H, COCH*NH*).

1-2 Synthesis of Poly(ethylene glycol)-Poly(α,β-aspartic acid) (PEG-P(Asp)$_{100}$ and PEG-P(Asp)$_{17}$) as Anionic Block Copolymer

PEG-P(Asp) was prepared by deprotection of PEG-PBLA. PEG-PBLA$_{100}$ (300 mg) was lyophilized from benzene solution and dissolved in CH$_3$CN (5 mL). 1 M NaOH (5 mL) was then added and stirred for 10 h at r.t.. After 10 h, the solution was dialyzed against water using a Spectrapor dialysis membrane (MWCO 8000). PEG-P(Asp)$_{100}$ (134 mg) was obtained as the sodium salt after the lyophilization. ^{1}H-NMR measurements showed a disappearance of the signals corresponding to the benzyl group and all the signals were assigned as PEG-P(Asp) (**Figure S1**). From ^{1}H-NMR measurement, the DP of the P(Asp) segment was calculated to be 101, comparing the peak intensity ratio between the methylene protons of PEG (**b**) and the methylene protons of the α,β-P(Asp) segment (**e1, e2**). Quantitative deprotection was confirmed without any chain length cleavage. ^{1}H-NMR (D$_2$O): δ2.76 (202H, **e1** and **e2**), δ3.39 (3H, **a**), δ3.70 (180H, **b**), δ4.47 (76H, **d2**), δ4.67 (27H, **d1**).

In a manner similar to that for PEG-P(Asp)$_{100}$, PEG-P(Asp)$_{17}$ (184 mg) was obtained from the deprotection of PEG-P(PBLA)$_{17}$ (300 mg). From ^{1}H-NMR measurement, the DP of the P(Asp) unit was calculated to be 17. ^{1}H-NMR (D$_2$O): δ2.76 (34H, **e1** and **e2**), δ3.39 (3H, **a**), δ3.70 (180H, **b**), δ4.47 (13H, **d2**), δ4.67 (4H, **d1**).

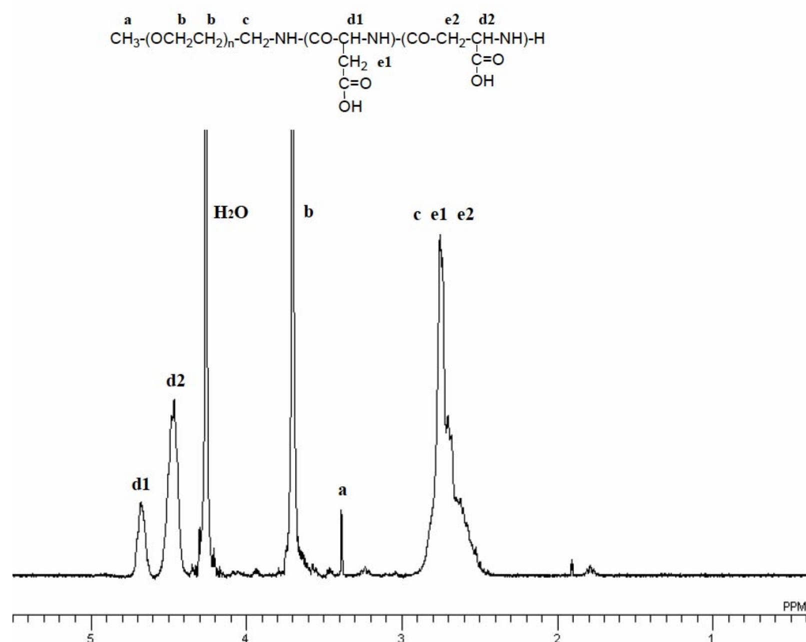

Figure S1. ^{1}H-NMR spectrum of PEG-P(Asp)$_{100}$ (solvent: D_2O, temperature: 80 °C).

1-3 Synthesis of Poly(ethylene glycol)-Poly([5-aminopentyl]-*α,β*-aspartamide) (PEG-P(Asp-AP)$_{100}$ and PEG-P(Asp-AP)$_{17}$) as Cationic Block Copolymer

PEG-PBLA$_{100}$ (200 mg) was lyophilized from benzene solution and dissolved in DMF (8 mL). DAP (3.5 mL; 30eq relative to the benzyl groups of PBLA) was then added and stirred for 24 h at 40 °C under a dry argon atmosphere. After 24 h, 16 mL of 10% acetic acid was added and stirred for 5 h at 0 °C, and the resulting solution was dialyzed against 0.01 N HCl and then water using a Spectrapor dialysis membrane (MWCO 8000). PEG-P(Asp-AP)$_{100}$ (140 mg) was obtained as the hydrochloride salt after the lyophilization. From the ^{1}H-NMR measurement (**Figure S2**), the DP of the P(Asp-AP) unit, comparing the peak intensity ratio between the methylene protons of PEG (**b**) and the methyne protons of the side chain of P(Asp-AP) segment (**e1 and e2**) was calculated to be 100, indicating the aminolysis was proceeded quantitatively. ^{1}H-NMR (D_2O): δ1.38 (196H, **h1** and **h2**), δ1.54 (200H, **i1** and **i2**), δ1.69

(190H, **g1** and **g2**), δ2.78 (200H, **e1** and **e2**), δ3.02 (180H, **f1** and **f2**), δ3.20 (202H, **j1** and **j2**), δ3.38 (3H, **a**), δ3.70 (180H, **b**), δ4.66 (102H, **d1** and **d2**).

In a manner similar to that of PEG-P(Asp-AP)$_{100}$, PEG-P(Asp-AP)$_{17}$ (110 mg) was obtained from the aminolysis of PEG-PBLA$_{17}$ (200 mg). From ^{1}H-NMR measurement, the DP of the P(Asp-AP) unit was calculated to be 17 based. ^{1}H-NMR (D_2O): δ1.38 (34H, **h1** and **h2**), δ1.54 (34H, **i1** and **i2**), δ1.69 (34H, **g1** and **g2**), δ2.78 (34H, **e1** and **e2**), δ3.02 (34H, **f1** and **f2**), δ3.20 (34H, **j1** and **j2**), δ3.38 (3H, **a**), δ3.70 (180H, **b**), δ4.66 (17H, **d1** and **d2**).

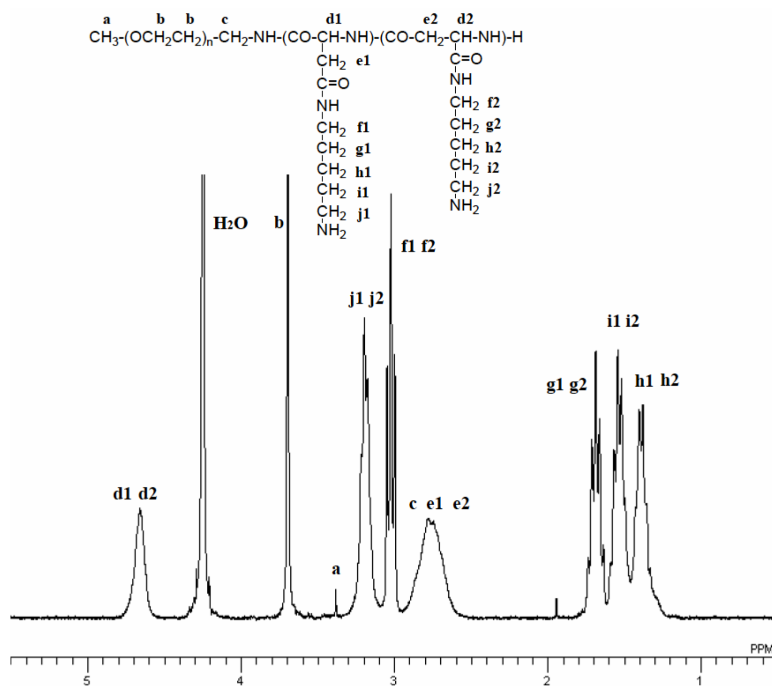

Figure S2. ^{1}H-NMR spectrum of PEG-P(Asp-AP)$_{100}$ (solvent: D_2O, temperature: 80 °C).

1-4 Synthesis of Poly(ethylene glycol)-Poly([2-aminoethyl]-α,β-aspartamide) (PEG-P(Asp-AE)$_{100}$ and PEG-P(Asp-AE)$_{17}$)

PEG-PBLA$_{100}$ (200 mg) was lyophilized from benzene solution and dissolved in DMF (8 mL). DAE (3 mL; 50eq relative to the benzyl groups of PBLA) was added and stirred for 24 h at 40 °C under a dry argon atmosphere. After 24 h, 16 mL of 10% acetic acid was added and stirred for 5 h at 0 °C, and

the resulting solution was dialyzed against 0.01 N HCl and then water using a Spectrapor dialysis membrane (MWCO 8000). PEG-P(Asp-AE)$_{100}$ (140 mg) was obtained as the hydrochloride salt after the lyophilization. From the ^{1}H-NMR measurement (**Figure S3**), the DP of the P(Asp-AE) unit, comparing the peak intensity ratio between the methylene protons of PEG (**b**) and the methyne protons of the side chain of P(Asp-AE) segment (**e1 and e2**), was calculated to be 104, indicating the aminolysis was proceeded quantitatively. ^{1}H-NMR (D_2O): δ1.38 (196H, **h1** and **h2**), δ1.54 (200H, **i1** and **i2**), δ1.69 (190H, **g1** and **g2**), δ2.78 (200H, **e1** and **e2**), δ3.02 (180H, **f1** and **f2**), δ3.20 (202H, **j1** and **j2**), δ3.38 (3H, **a**), δ3.70 (180H, **b**), δ4.66 (102H, **d1** and **d2**).

In a manner similar to that of PEG-P(Asp-AE)$_{100}$, PEG-P(Asp-AE)$_{17}$ (90 mg) was obtained from the aminolysis of PEG-PBLA$_{17}$ (200 mg). From ^{1}H-NMR measurement, the DP of the P(Asp-AP) unit was calculated to be 17. ^{1}H-NMR (D_2O): δ2.81 (34H, **e1** and **e2**), δ3.20 (34H, **f1** and **f2**), δ3.38 (3H, **a**), δ3.54 (34H, **g1** and **g2**), δ3.70 (180H, **b**), δ4.69 (17H, **d1** and **d2**).

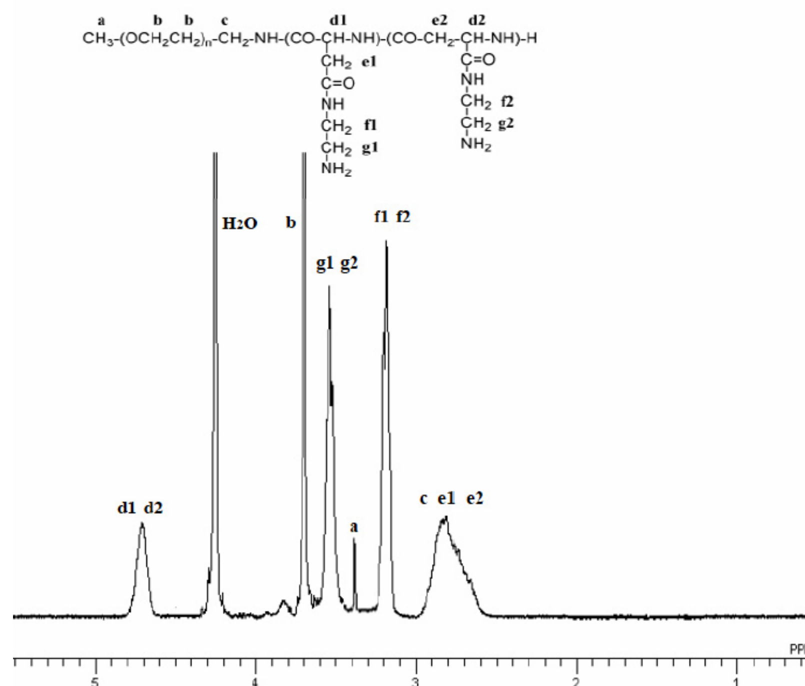

Figure S3. ^{1}H-NMR spectrum of PEG-P(Asp-AE)$_{100}$ (solvent: D_2O, temperature: 80 °C).

2. Preparation of PICsomes

PEG-P(Asp) and PEG-P(Asp-AE) or PEG-P(Asp-AP) were separately dissolved in 10 mM Tris-HCl buffer (pH 7.4) with 150 mM NaCl. These solutions were purified by filtration through a 0.22-μm membrane filter to remove dust. PEG-P(Asp) solution and PEG-P(Asp-AE) or PEG-P(Asp-AP) solution were mixed in an equal ratio of $-COO^-$ and $-NH_3^+$ units and then subjecting to sonication for 20 minutes (10 minutes ×2). Sample solutions were incubated at ambient temperature overnight.

3. Preparation of PICsomes Encapsulating FITC-Dex

PEG-P(Asp-AP)$_{100}$ solution (1 mg/mL) and PEG-P(Asp)$_{100}$ solution (1 mg/mL) containing FITC-Dex (1 mg/mL) were mixed and subjected to sonication. Sample solution was incubated at ambient temperature overnight. The final concentration of FITC-Dex was adjusted to be 380 μg/mL (10 μM).

Results

1. Flow Particle Image Analysis for PICsomes Encapsulating FITC-Dex

Flow particle image analysis was performed on a Sysmex FPIA-3000. The histogram was prepared for the particles with a diameter range of more than 0.8 μm. Circularity of particle is calculated from the circumference of the circle of equivalent area divided by the actual perimeter of the particle. The formula for the circularity is defined by equation 1

$$\text{Circularity} = 2(\pi A_P)^{1/2}/P_P \qquad (1)$$

where A_P is the area of the particle, and P_P is the perimeter of the particle. The circularity converges to unity with the image approaching to perfect circle.

1 ml of the solution of the PICsome encapsulating FITC-Dex prepared in **Experiments 3** was analyzed by this method, and the size histogram was obtained as shown in **Figure S4**. Then, the

circularity of the particles was evaluated based on equation 1, and the result was shown in **Figure S5** as a circularity histogram. Most of the fraction has the circularity near to unity, indicating the essentially spherical architecture of the obtained particles.

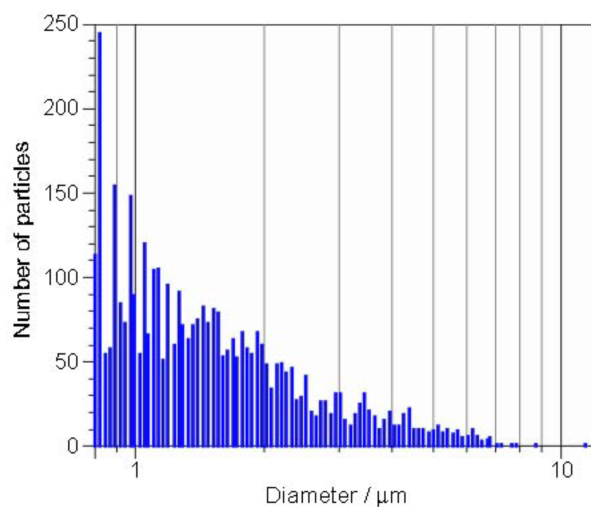

Figure S4. A size histogram of the particles prepared from PEG-P(Asp)$_{100}$/ PEG-P(Asp-AE)$_{100}$ system, which encapsulate FITC-Dex.

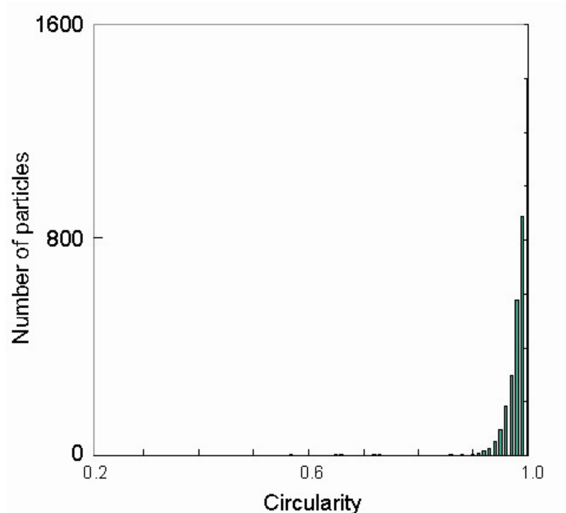

Figure S5. A circularity histogram of the particles prepared from PEG-P(Asp)$_{100}$/ PEG-P(Asp- AE)$_{100}$ system, which encapsulate FITC-Dex.

2. DFM observation for PEG-P(Asp)$_{100}$/PEG-P(Asp-AE)$_{100}$ system

DFM observation for the PEG-P(Asp)$_{100}$/PEG-P(Asp-AE)$_{100}$ system, which has shorter alkyl chain length of the cationic side chain than the system of the PEG-P(Asp)$_{100}$/PEG-P(Asp-AP)$_{100}$, showed that particles consisted of a large amount of dot-scatterings, which may be attributed to the micelle and a small amount of ring-scatterings with around 800 nm size (**Figure S6**). Obviously, these particles were smaller than that of the PEG-P(Asp)$_{100}$/PEG-P(Asp-AP)$_{100}$ system, which mainly consisted of large ring-like scatterings.

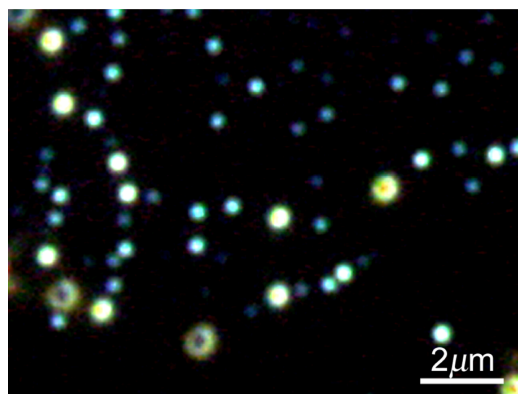

Figure S6. A DFM image of PICsomes prepared from PEG-P(Asp)$_{100}$/PEG-P(Asp-AE)$_{100}$ system.

3. Stability of PICsomes in the Presence of Serum

The stability of PICsomes in the presence of the serum was examined. 10 μL of fetal bovine serum (FBS) was added to 90 μL of the solution of PICsome prepared from PEG-P(Asp)$_{100}$/PEG-P(Asp-AP)$_{100}$ system containing 150 mM NaCl, the formation of the PICsome even after 40 h at 37 °C in 10 % FBS solution was confirmed by DFM observation as shown **Figure S7**.

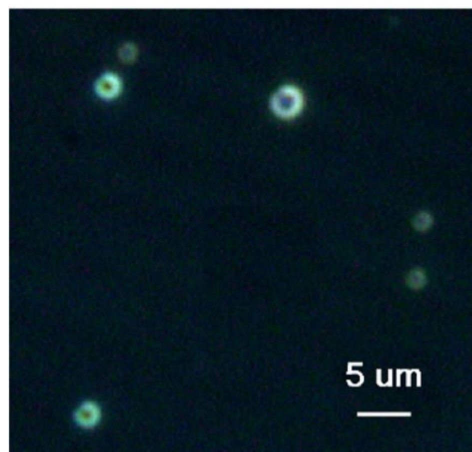

Figure S7. A DFM image of PICsomes prepared from PEG-P(Asp)$_{100}$/PEG-P(Asp-AP)$_{100}$ system in the presence of 10 % FBS. The solution was incubated for 40 h at 37 °C.

4. Fluorescence Observation and Emission Spectra of the PICsome by CLSM

Fluorescence observation and emission spectra measurements of the PICsome were performed using the CLSM. When the region of interest (ROI) was specified in the microscopic image, the mean intensity of all pixels within the ROI was obtained as a spectrum. As for the references, emission spectra of FITC-Dex, TRITC-Dex, and TRITC, which were dissolved in 10 mM Tris-HCl buffer (pH 7.4) with 150 mM NaCl, were taken under the microscopy using micro chamber (**Figure S8**). After the acquisition of the emission spectrum of the ROI inside the PICsome encapsulating FITC-Dex (**Figure 2c (dotted line)**), TRITC-Dex or TRITC was added to the outer solution to spectrophotometrically examine the permeability through the PICsome membrane under the microscopy as described in the main text of the article.

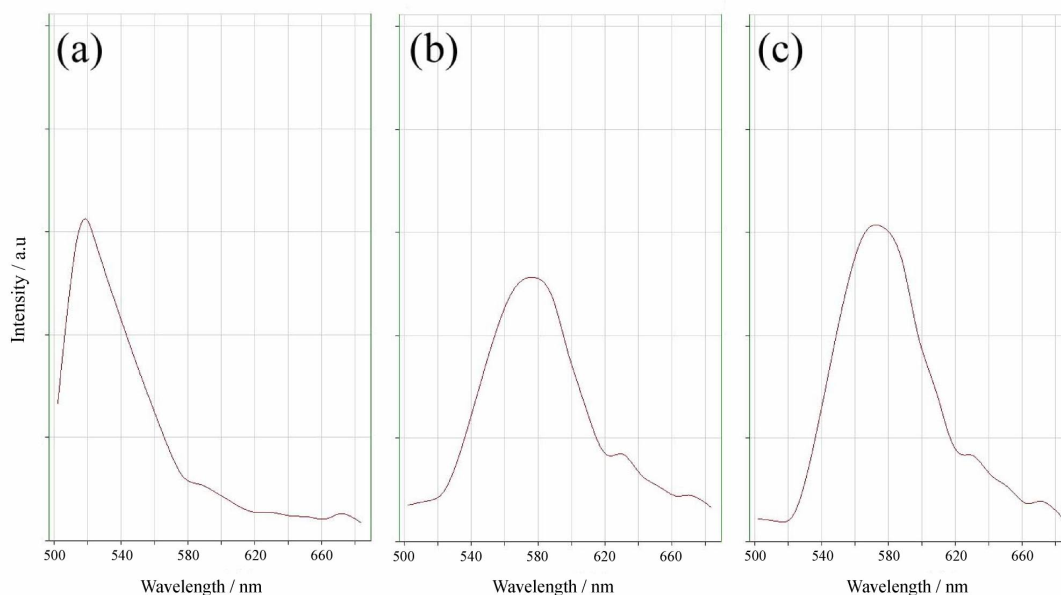

Figure S8. Emission spectra of the reference fluorophores. (a) FITC-Dex (380 μg/ml), (b) TRITC (10 μg/ml), (c) TRITC-Dex (500 μg/ml). The fluorophores were excited with 488 and 543 nm coincidentally.

References

(1) See information at Malvern Instruments Ltd.

(http://www.malvern.co.uk/LabEng/products/sysmex_fpia3000/sysmex_fpia3000.htm)

(2) Dickinson, D. E.; Tille, S.; Lansford, R.; Frase, S. E. *BioTechniques* **2001**, *31*, 1273-1278.