Supporting Information

for

Bilayer Domain Formation of Thermoresponsive Amphiphilic Block Copolymers in Hybrid Liposomes for Synthetic Molecular Channels

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Materials

Maltopentaose-*block*-poly(propylene oxide)$_{2.5K}$ (see S1 for NMR spectrum), Maltopentaose-*block*-poly(propylene oxide)$_{1.5K}$ (Fig. S1 and S2) and rhodamine functionalized maltopentaose-*block*-poly(propylene oxide) were synthesized according to a previously reported procedure.[S1] 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; purity > 99%) 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (rhodamine-DOPE; purity > 99%), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) ammonium salt (NBD-DOPE; purity > 99%), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC; purity > 99%) and 1,2-dimyristoyl-d_{54}-*sn*-glycero-3-phosphocholine (DMPC-d_{54}; purity > 99%) were purchased from Sigma-Aldrich Co. LLC (Sigma-Aldrich) and used as received. Deuterium oxide (D_2O; purity > 99.9%) and methanol (MeOH; purity > 99.8%) were purchased from FUJIFILM Wako Pure Chemical Corporation (Wako) and used as received. FITC-PEG550 was purchased from Nanocs and used as received.

Methods

Preparation of micrometer-sized hybrid vesicle solutions

A glass tube was charged with 50 μL of a mixture solution of the rhodamine-functionalized polymer or DOPC (or DMPC) containing 1 mol% of NBD-DOPE in MeOH. The solvent was removed under a flow of nitrogen gas to obtain a thin film, which was further dried for 12 h under reduced pressure. The resulting film was hydrated with distilled water (500 μL) at room temperature and allowed to stand for at least 12 h at 30 ºC. The final concentration of the amphiphiles is 0.1 mM.

Preparation of the polymer/lipid hybrid vesicle solutions

A glass tube was charged with 50 μL of a mixture solution of the rhodamine-functionalized polymer or DOPC (or DMPC) containing 1 mol% of NBD-DOPE in MeOH. The solvent was removed under

a flow of argon gas to obtain a thin film. The resulting film was hydrated with distilled water (500 μL) and stirred using a vortex mixer for 30 s. The resulting solution was passed through an Avanti Mini-Extruder apparatus. The solution was passed 21 times through a 0.1 μm polycarbonate membrane.

Dynamic Light Scattering (DLS)

DLS measurements were carried out using a Zetasizer Nano ZS instrument (Malvern Instruments, U.K.) operating at a wavelength of 632.8 nm and a detection angle of 173° or an ELSZ-2000S instrument (Otsuka Electronics Co., Ltd., Japan) operating at a wavelength of 665.2 nm and a detection angle of 165°. The obtained data were collected at 25 °C and analyzed using the cumulant method.

Confocal laser scanning microscopy (CLSM)

Micrometer-sized vesicle were prepared as described above. A solution of micrometer-sized vesicles (100 μL) was poured into a glass-bottom dish (diameter: 35 mm, Asahi Techno Glass co., Ltd., Japan) or an 8 wells chamber side (μ-Slide 8 well, ibidi GmbH, Germany). Images of the micrometer-sized vesicles were taken using a confocal laser scanning microscope (LSM780; Carl Zeiss or FV1000-D; Olympus) at a magnification of 40× with an excitation wavelength of 488 nm for NBD and 561 nm for rhodamine.

Transmission Electron Microscopy (TEM)

A solution of the block polymers in water (5 μL; 1 mg mL^{-1}) was placed on a copper grid coated with a formvar film (PVF-C10 STEM Cu100P, Okenshoji Co., Ltd. Japan). Excess sample solution was removed using filter paper. A solution of 1 wt% phosphotungstic acid (5 μL; pH = 7.0) was added as the staining agent and then removed prior to drying the sample in a desiccator. The grid was placed in a JEM-2100 (JEOL Ltd., Japan) electron microscope, which was operated at 100 kV.

Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Cryo-TEM measurements were carried out on a JEM-2100Plus (JEOL, Japan), operated at 200 kV. The samples were vitrified in a controlled environment vitrification system with relative humidity close to 80% at 23 or 30 ºC. Three micro-litter of the vesicle solutions (5 mg mL^{-1}) was deposited on holey carbon support film grids (Quantifoil R2/1, EM-Japan Co., Ltd.) and excess fluid was gently removed by blotting. The grids were then directly plunged into liquid ethane. Grids were stored in liquid nitrogen until being transferred to the electron microscope.

Small Angle X-ray Scattering (SAXS)

SAXS measurements were performed at the BL40B2 beamline of SPring-8 (Japan). A 25.4 × 28.9 cm^2 photon-counting detector (PILATUS3 S 2M) was placed 2.1 m from the sample. The wavelength (λ) of the incident X-rays was 1.0 Å. The X-ray transmittance values of the sample and of water were measured using ion chambers located in front of and behind the sample. The polymer solutions or their blank solvents were placed in a quartz capillary (diameter: 2 mm; Hilgenberg GmbH). The sample temperature was controlled using a Peltier thermostat cell holder system (TS-62, Instec, Inc., USA) and the samples were incubated at the target temperature for 10 min before the measurements. SAXS images were collected with an exposure time of 180 s. The resulting 2D SAXS images were converted into one-dimensional intensity versus q profiles by circular averaging using the FIT2D software package. The background intensity of the capillary filled with distilled water was subtracted. The scattering curves from the polymer solutions were fitted with a form factor using a bilayer vesicle model as follows.

$$I(q) = \frac{4\pi}{q^4}\left[(\rho_c - \rho_h)\sin\left(q\frac{t_c}{2}\right) + (\rho_h - \rho_s)\sin\left(q\frac{2t_h+t_c}{2}\right)\right]^2 \quad \text{(Eq. S1)}$$

where, ρ_c, ρ_s, ρ_h, t_h, and t_c are the scattering length densities of the hydrophobic core, solvent, and hydrophilic parts and the thicknesses of the hydrophilic part and hydrophobic core, respectively. q was

defined as $q = (4\pi/\lambda)\sin(\theta/2)$ (λ and θ being the wavelength and scattering angle, respectively). A Gaussian distribution of the bilayer hydrophobic thicknesses was used to take the polydispersity into account.

Small angle neutron scattering (SANS)

SANS experiments were performed using SANS-U spectrometer at the Institute for Solid State Physics (ISSP), the University of Tokyo.[S2] SANS measurements were carried out in a velocity-selector mode with a neutron wavelength of 7 Å ($\Delta\lambda/\lambda$ = 10%). The sample to detector distances were wet to 8, 2, and 1 m, respectively. The raw data was normalized to the absolute scattering values using an empty beam transmission measurement. The vesicle solutions in D_2O or D_2O/H_2O = 86 : 14 or solvents were deposited in quartz cells (sample thickness: 2 mm) and incubated at 30 °C. An absolute intensity was calibrated by using a Lupolene standard. Total counts of the scattering detected by the two-dimensional detector were utilized as scattering intensity $I(t)$ after the electronic background and scattering from an empty cell and that from solvents were subtracted. The scattering curves from the vesicle solutions were fitted with a form factor using a core shell disk model below:

$$I(q) = \frac{1}{2}\int_0^{\pi}\{(\rho_{core} - \rho_{shell})V_cF_c(q) + (\rho_{shell} - \rho_{solvent})V_sF_s(q)\}^2 \, sin\beta d\beta \qquad \text{(eq. S2)}$$

where, ρ_{core}, ρ_{shell}, $\rho_{solvent}$, V_c, V_s, and β are the electron densities of the hydrophobic core, hydrophilic shell, and solvent, the volume of the core and overall particle and scattering vector q for averaging over the orientations, respectively.

In the case of core-shell disc,

$$F_c(q) = \frac{\sin\left(\frac{qL}{2}cos\beta\right)}{\frac{qL}{2}cos\beta} \times \frac{J_1(qRsin\beta)}{qRsin\beta} \qquad \text{(eq. S3)}$$

$$F_s(q) = \frac{\sin\left(q(\frac{L}{2}+t)cos\beta\right)}{(\frac{L}{2}+t)cos\beta} \times \frac{J_1(q(R+t)sin\beta)}{q(R+t)sin\beta} \qquad \text{(eq. S4)}$$

where, $J_1(x)$, β, L, R, and t are the first-order Bessel function of x, the angle between the disc normal, the thickness of the hydrophobic core, the radius of the particle, and the thickness of the

hydrophilic shell, respectively. A Gaussian distribution of the radius of the hydrophobic core was used to take the polydispersity into account.

Permeability test

The giant hybrid vesicle solutions ([DOPC] = 0.09 mM, [polymer] = 0.01 mM) or giant DOPC liposome solution ([DOPC] = 0.1 mM) were prepared according to the aforementioned procedure. 100 μL of the solutions were poured into a glass-based dish (ϕ = 35 mm, AGC techno glass) and 50 μL of FITC-PEG550 solution (0.1 mM in water) was added. Images of the mixture solutions were taken using a confocal laser scanning microscope (LSM780; Carl Zeiss or FV1000-D; Olympus) at a magnification of 40× with an excitation wavelength of 488 nm for FITC and 561 nm for rhodamine at various time intervals.

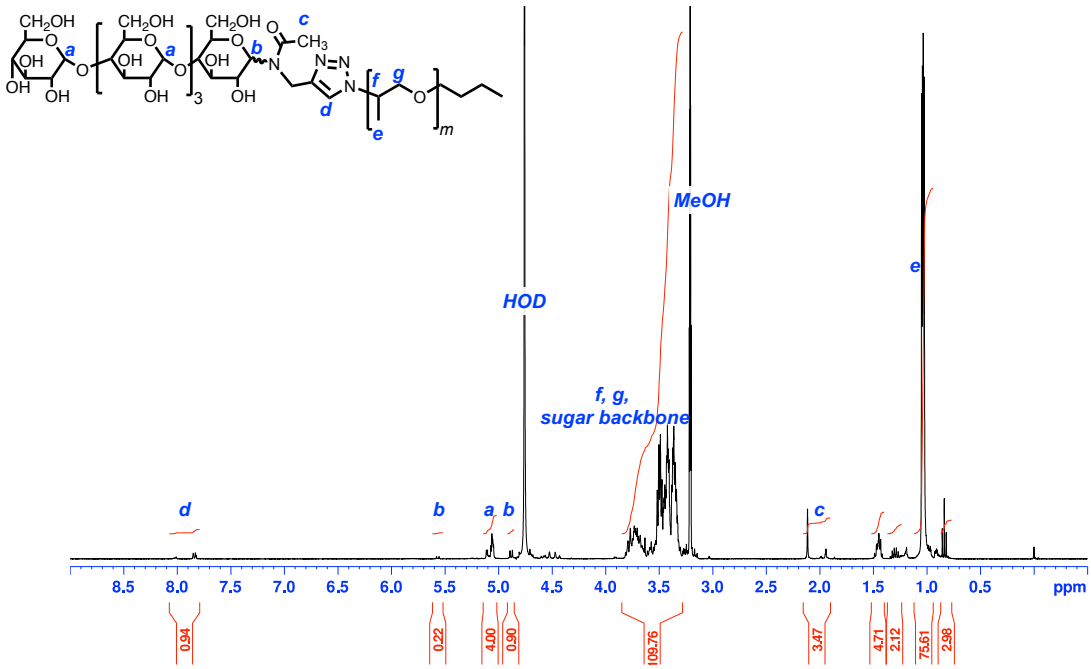


Fig. S1 ^{1}H NMR spectrum of maltopentaose-*b*-PPO$_{1.5K}$ in Methanol-d_4.

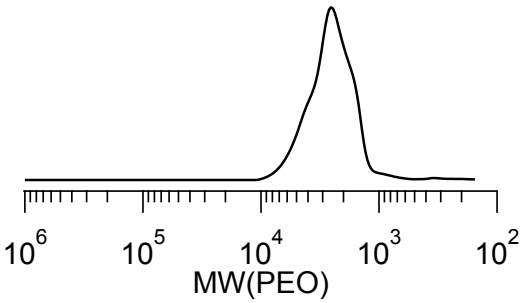


Fig. S2 GPC chromatogram of peracetylated maltopentaose-*b*-PPO$_{1.5K}$

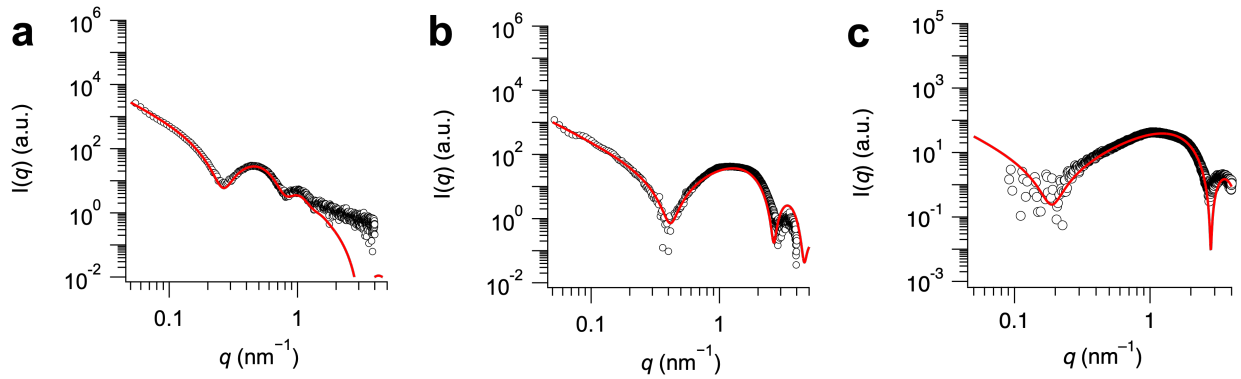


Fig. S3 SAXS profiles of (a) maltopentaose-*b*-$PPO_{2.5K}$, (b) DMPC liposome, and (c) DOPC liposomes in H_2O (open circles): [polymer] = 2.5 mg mL^{-1}, [phospholipid] = 3.6 mg mL^{-1}. The solid lines show the theoretical curves obtained using the cross-sectional bilayer model.

Table S1 Fitting parameters of the polymer vesicles and phospholipid vesicles obtained from SAXS analysis

Parameters	maltopentaose-*b*-$PPO_{2.5K}$	DMPC	DOPC
Electron density of the hydrophilic region (e nm^{-3})	367	261	261
Electron density of the hydrophobic region (e nm^{-3})	333	460	460
Electron density of the solvent (e nm^{-3})	334	334	334
Thickness of the hydrophilic region (nm)	2.0	0.95	1.10
Thickness of the hydrophobic region (nm)	9.4	2.55	2.25
Standard deviation of the hydrophobic thickness	1.7	0.12	0.03

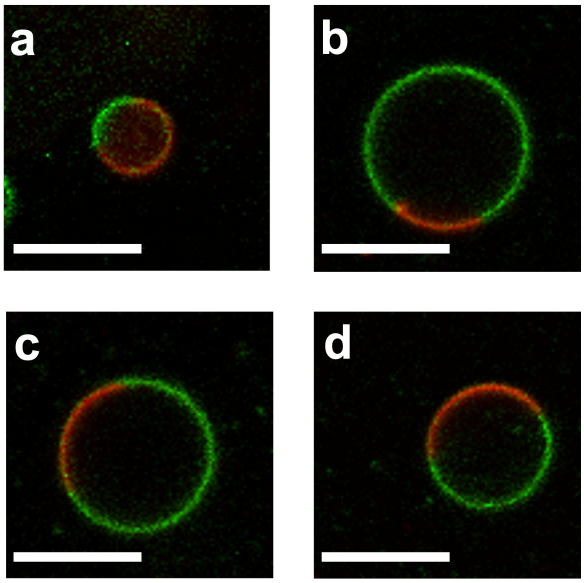


Fig. S4 CLSM images of the polymer/lipid hybrid vesicles labeled with NBD-DOPC (green channel) and rhodamine-polymer (red channel) with the molar ratio of maltopentaose-*b*-$PPO_{2.5K}$ and DOPC = 1 : 9 after incubation for a) 1 h, b) 24 h, c) 48 h, and d) 72 h. Scale bars = 10 μm.

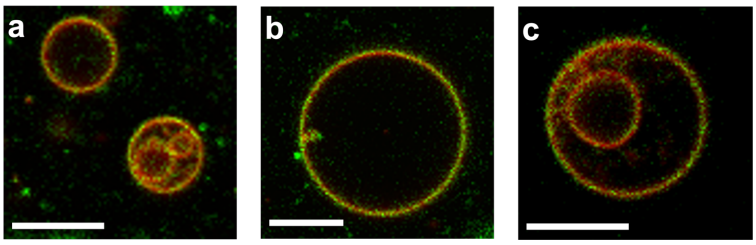


Fig. S5 CLSM images of the polymer/lipid hybrid vesicles labeled with NBD-DOPE (green channel) and rhodamine-polymer (red channel) with the different molar ratios of maltopentaose-*b*-$PPO_{2.5K}$ and DOPC. a) [polymer] : [DOPC] = 1 : 9, b) = 3 : 7, c) = 5 : 5. Scale bars = 10 μm.

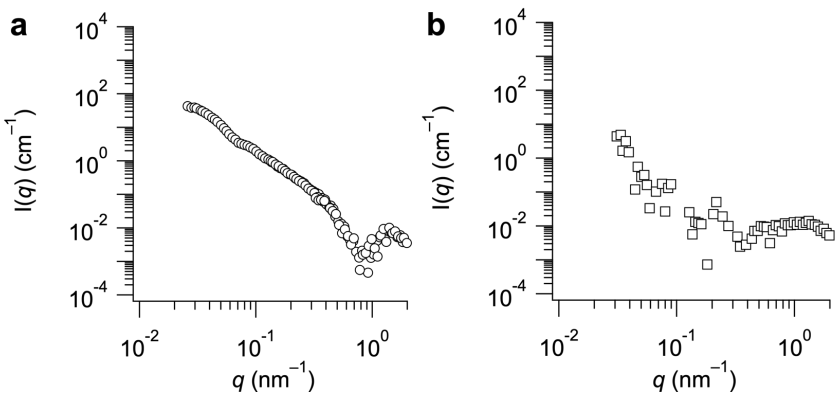


Fig. S6 a) SANS profile of DMPC-d_{54} liposome at 30 ºC in D_2O (open circles) and b) in D_2O : H_2O = 86 : 14 (open squares) Concentrations; [DMPC-d_{54}] = 7.2 mg mL^{-1}.

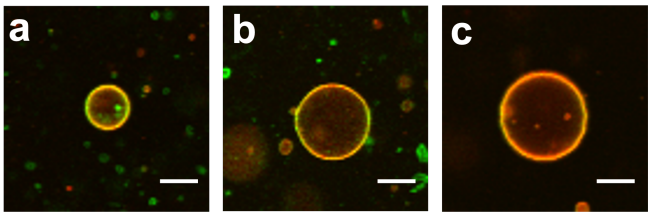


Fig. S7 CLSM images of maltopentaose-*b*-$PPO_{2.5K}$/DMPC hybrid vesicles labeled with NBD-DOPE (green channel) and rhodamine-functionalized polymer (red channel) with the different molar ratios of maltopentaose-*b*-$PPO_{2.5K}$ and DMPC. a) [polymer] : [DOPC] = 1 : 9, b) = 5 : 5, c) = 9 : 1. Scale bars = 10 μm.

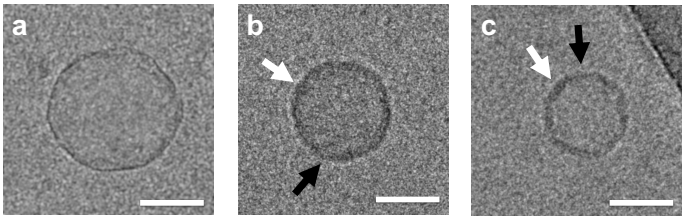
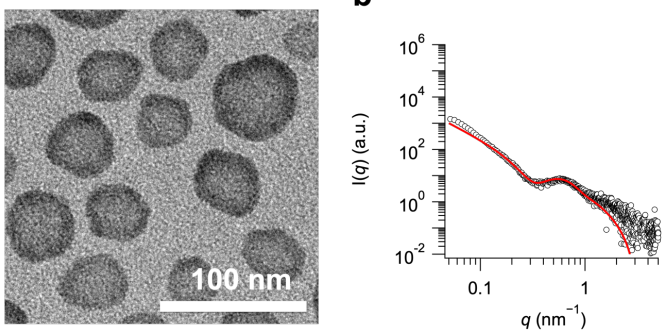


Fig. S8 Cryo-TEM images of a) DMPC liposome, b) the polymer/lipid hybrid vesicles with the molar ratio of maltopentaose-*b*-$PPO_{2.5K}$ and DMPC = 1 : 9, c) the polymer/lipid hybrid vesicles with the molar ratio of maltopentaose-*b*- $PPO_{2.5K}$ and DMPC = 2 : 8. White arrows indicate thick membranes corresponding to polymer bilayer membranes, and black arrows indicate thin membranes corresponding to DMPC bilayer membranes. Scale bars = 50 nm.



c

Parameters	
Electron density of the hydrophilic region (e nm^{-3})	360
Electron density of the hydrophobic region (e nm^{-3})	330
Electron density of the solvent (e nm^{-3})	334
Thickness of the hydrophilic region (nm)	2.0
Thickness of the hydrophobic region (nm)	6.0
Standard deviation of the hydrophobic thickness	2.2

Fig. S9 (a) A TEM image of self-assembled particle in maltopentaose-*b*-$PPO_{1.5K}$ solution using positive staining with phosphotungstic acid. Scale bar = 100 nm (b) SAXS profiles of maltopentaose-*b*-$PPO_{1.5K}$ solution (open circles) and the theoretical curve obtained from the cross-sectional bilayer model (red line). (c) fitting parameters of the polymer vesicles obtained from SAXS analysis

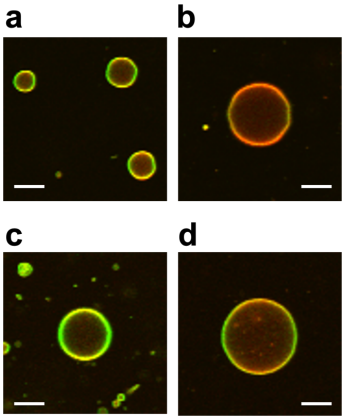
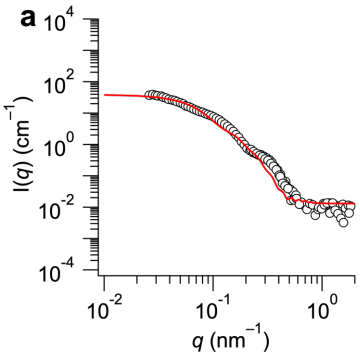


Fig. S10 CLSM images of maltopentaose-*b*-$PPO_{1.5K}$/phospholipid hybrid vesicles labeled with NBD-DOPE (green channel) and rhodamine-functionalized polymer (red channel) with different molar ratios of maltopentaose-*b*-$PPO_{1.5K}$ and DOPC: a) [polymer] : [DOPC] = 1 : 9 and b) = 5 : 5 and maltopentaose-*b*-$PPO_{1.5K}$ and DMPC: c) [polymer] : [DMPC] = 1 : 9 and d) = 5 : 5. Scale bars = 10 μm.



b

Parameters	
SLD of the hydrophilic region (cm^{-2})	1.00×10^{10}
SLD of the hydrophobic region (cm^{-2})	4.00×10^{10}
SLD of the solvent (cm^{-2})	5.35×10^{10}
Radius of the disk (nm)	35
Thickness of the hydrophobic region (nm)	5.0
Overall thickness of the disk (nm)	8.0
Standard deviation of the thickness of the disk	0.5

Fig. S11 (a) SANS profile of the malotopentaose-*b*-$PPO_{1.5K}$/DMPC-d_{54} hybrid vesicles at 30 ºC in D_2O : H_2O = 86 : 14 (open circles) and theoretical curves obtained from the core-shell disk model (red line). b) structural parameters of the polymer assemblies in the hybrid vesicles.

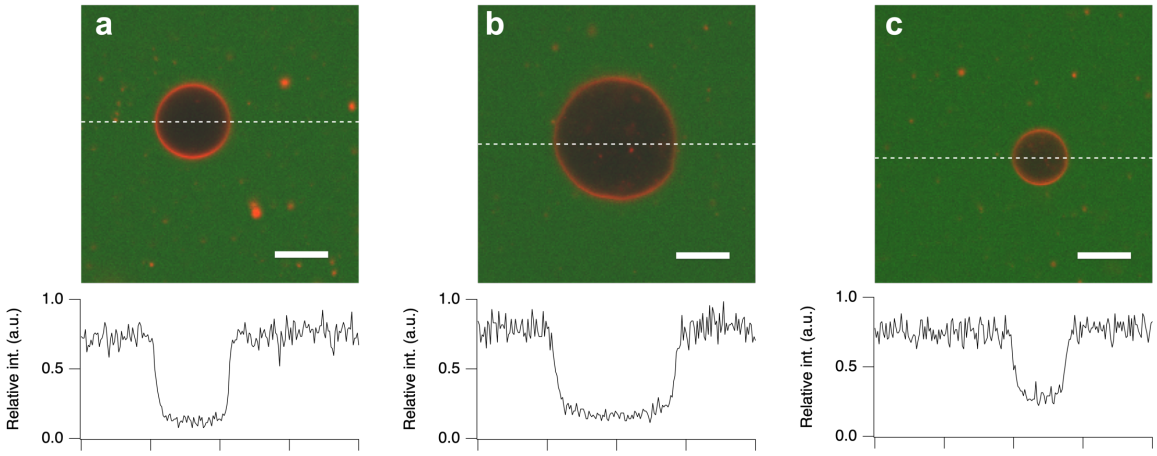


Fig. S12 Representative CLSM images of DOPC liposomes labeled with rhodamine-DOPE (red channel) after a) 5 min, b) 30 min, c) 60 min. of the addition of FITC-PEG550 (green channel); scale bars = 10 μm.

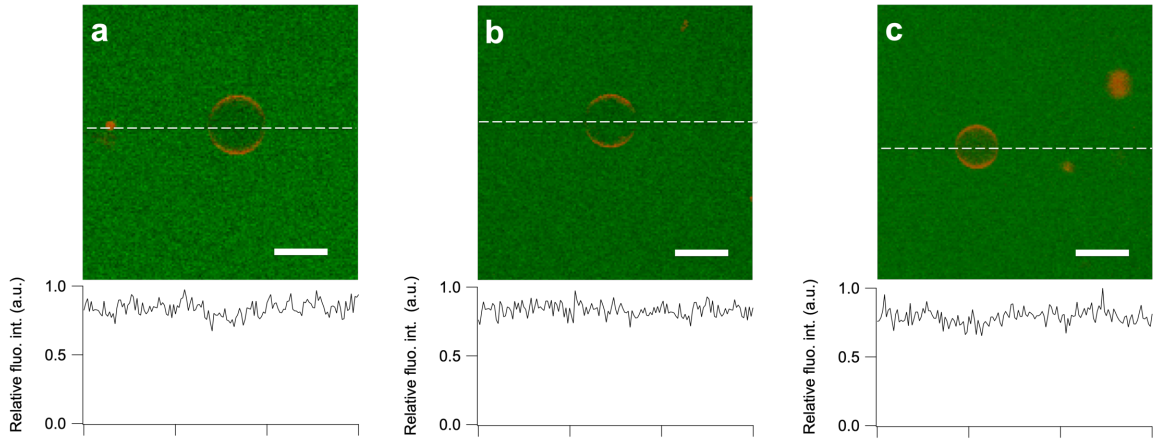


Fig. S13 Representative CLSM images of the malotopentaose-*b*-$PPO_{1.5K}$/DOPC hybrid vesicles labeled with rhodamine-DOPE (red channel) after a) 5 min, b) 30 min, c) 60 min. of the addition of FITC-PEG550 (green channel); scale bars = 10 μm.

Additional references

(1) T. Nishimura, Y. Sasaki and K. Akiyoshi, *Adv. Mater.*, 2017, **29**, 1702406.

(2) H. Iwase, H. Endo, M. Katagiri and M. Shibayama, *J. Appl. Crystallogr.*, 2011, **44**, 558-568.