Spontaneously Formed Semipermeable Organic–inorganic Hybrid Vesicle Permitting Molecular Weight Selective Transmembrane Passage

Kazuma Yasuhara*, Takahiro Kawataki, Shizuyo Okuda, Shohei Oshima and Jun-ichi Kikuchi*

Graduate School of Materials Science, Nara Institute of Science and Technology (NAIST)

8916-5 Takayama, Ikoma Nara, 6300192, Japan

Electronic Supporting Information

Contents **Page**

Experimental

Materials

The organoalkoxysilane lipid *N*, *N*-dihexadecyl-N^{α}-(6-((3-triethoxysilylpropyl)dimethylammonio) hexanoyl)alaninamide bromide (**1**) was synthesized in five steps according to the literature procedure (details in Reference 10b of the main text). Fluorescein isothiocyanate (FITC), α-chymotripsin, tripsin inhibitor from bovine pancreas were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Methoxyl poly(ethyleneglycol) amine (mPEG-NH_2, MW = 550, 1,000, 2,000, and 5,000 g mol^{-1}), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl), ammonium salt (Rh-PE) were purchased from Nanocs Inc. (Boston, MA, USA), and Avanti Polar Lipids, Inc. (Alabaster, AL, USA), respectively. All other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used without further purification.

Synthesis of FITC-PEG

FITC-labeled poly(ethyleneglycol) (FITC-PEG) was synthesized according to the literature procedure with slight modification (Reference 13 of the main text). FITC (93.6 mg, 0.24 mmol) and an appropriate weight of mPEG-NH_2 (0.08 mmol) were dissolved in a chloroform/methanol (3:2, v/v) mixture, and the solution was stirred overnight at room temperature. The solvent was then removed *in vacuo* to afford a dark yellow solid, which was purified by gel-permeation chromatography using Sephadex™ LH-20 (GE Healthcare Bio-sciences AB, Uppsala, Sweden) with methanol as eluent to remove any unreacted FITC. The obtained FITC-PEGs were characterized by nuclear magnetic resonance (NMR), absorption spectroscopy, and dynamic light scattering (DLS) analysis (see Table S1). To estimate the percentage of FITC modification, the absorbance at 490 nm was measured in an aqueous solution at pH = 9 (ε = 70,000 M^{-1} cm^{-1}).

Vesicle preparation

An appropriate amount of **1** was dissolved in chloroform. The solvent was then evaporated under nitrogen gas flow and any residual trace solvent was completely removed *in vacuo* for 3 h to give a thin film on the wall of a round-bottom flask. Hydration of the lipid film was performed at 40 °C with 0.5 mL of 10 mM HEPES buffer ([NaCl] = 50 mM, pH = 7.0) containing marker molecules (FITC-PEG, acridine orange or PEG), followed by five freeze-and-thaw cycles at −196 and 50 °C. The vesicle dispersion thus obtained was extruded 15 times through a LiposoFast miniextruder (Avestin) equipped with stacked polycarbonate membrane filters (200 nm pore) in order to obtain large unilamellar vesicles (LUVs). The LUVs formed with lipid **1** were incubated at room temperature (25 °C) for 24 h to complete the formation of the siloxane network on the surface of the vesicles. Untrapped marker in the bulk phase was removed by gel filtration using hydrophilic polyacrylamide gel beads (Bio-Gel P-100, Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 4 °C to prevent the spontaneous leakage of entrapped markers.

Cryo-TEM observation

The specimen for cryogenic TEM (cryo-TEM) was prepared by rapid freezing of a vesicular dispersion. A 200 mesh copper microgrid was used and pretreated with a glow-discharger (HDT-400, JEOL) to obtain a hydrophilic surface. An aliquot (3 μL) of a vesicular sample was placed on the mesh and immediately plunged into liquid propane using a specimen

preparation machine (EM CPC, Leica). The temperature of the specimen was maintained below −140 °C during the observation using a cryo-transfer holder (Model 626.DH, Gatan). Microscopic observations were carried out using a transmission electron microscope (JEM-3100FEF, JEOL) at an acceleration voltage of 300 kV in zero-loss imaging mode.

DLS measurements

The hydrodynamic diameter of the vesicles was measured using a dynamic light scattering spectrometer equipped with a 633 nm He–Ne laser (Zetasizer Nano-ZS, Malvern). The size distribution of the vesicles in the dispersion was obtained by analyzing the time course of the scattered light intensity at an angle of 173° from the incident light using the Cumulant method. The sample temperature was maintained at 25 °C using a thermostat temperature controller.

Fluorescence measurements

All fluorescence measurements were carried out using a Hitachi F-4500 spectrofluorometer. The fluorescence intensity of the FITC-PEG or acridine orange was recorded at 520 nm or 530 nm, respectively, with excitation at 490 nm. To initiate the release, an aliquot of the vesicular suspension (0.01 μL) chilled at 4 °C was injected into 0.99 mL of 10 mM HEPES buffer ([NaCl] = 50 mM, pH = 7.0) at 25 °C in a sample cuvette. All measurements were carried out at 25 °C.

The leakage of entrapped fluorescent marker was monitored on the basis of the increase in the fluorescence intensity due to its dilution in the outer phase. To self-quench the fluorescence of FITC-PEG, concentrations of the entrapped FITC-PEGs were set to 3 mM (a typical concentration dependence for a FITC-PEG is shown in Fig. S1). Also, acridine orange was entrapped in the hybrid vesicle at the concentration of 4.5 mM. To obtain the fluorescence intensity corresponding to complete leakage (F_{100}), 5 μL of Triton X-100 (200 mM) was added to the cuvette. The percentage of leakage was defined by the following equation:

$$\text{Leakage (\%)} = 100\{(F_t - F_0) / (F_{100} - F_0)\},$$

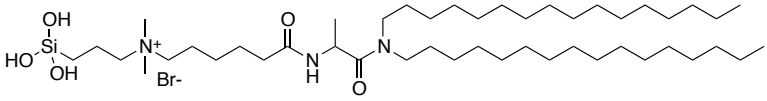
where F_t and F_0 are the fluorescence intensities of the vesicular sample at measurement time t and just after preparation, respectively.

MALDI-TOF mass spectroscopy

The molecular weight threshold for membrane permeation was determined by MALDI-TOF mass spectroscopy in combination with an ultrafiltration technique. Organic–inorganic hybrid vesicles were prepared by entrapping PEG (average MW = 1,500 g mol^{-1}). The obtained suspension of vesicles was then subjected to incubation at 25 °C for 24 h to complete the leakage of the permeable PEG fraction. The leaked fraction of PEG was separated by ultrafiltration using an Amicon Ultra-0.5 mL (molecular weight cut off = 100,000 g mol^{-1}) centrifugal filter unit (Millipore, Cork, Ireland). The separated fraction was then directly subjected to MALDI-TOF mass measurement using a Bruker Daltonics Autoflex II spectrometer in linear mode with positive ionization and α-cyano-4-hydroxycinnamic acid as the matrix.

The crosslinked product of lipid **1** formed through a sol-gel reaction was also evaluated by MALDI-TOF mass spectroscopy. An organic–inorganic hybrid vesicle was prepared with lipid **1** in water as described above. The vesicular suspension was incubated at room temperature overnight to complete the sol-gel reaction. The obtained vesicle was then

dissolved in an acetonitrile/water (1:1, v/v) mixture with *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile as the matrix, and the mass numbers were externally calibrated using PEG as the standard. The linear mode with positive ionization was used for the measurement. Six separated mass spectra with different ranges were independently taken to cover the m/z range from 600 to 12,000 g mol^{-1}, as shown in Fig. S6. A series of peaks corresponding to the oligomers of hydrolyzed lipid **1** (designated as M in Table S2, the chemical structure is shown below) were observed. Because broad peaks were observed in the high molecular weight region due to the formation of various isotropic species and differently dehydrated species, the centroid peaks were regarded as representative species, as summarized in Table S2. Thus, it is to be noted that the produced oligomeric species of lipid **1** are not limited to the plausible assignments listed in Table S2.



M

Chemical Formula: $C_{46}H_{96}BrN_3O_5Si$
Exact Mass: 877.63, Molecular Weight: 879.26

Preparation of cell-sized organic–inorganic hybrid vesicles and CLSM observation

Cell-sized vesicles were prepared via the gentle hydration of a lipid film. A chloroform solution of **1** containing 0.1 mol% of Rh-PE was dried under vacuum for 3 h to obtain the lipid thin film. The lipid film was gently hydrated with 50 mM sucrose solution at 25 °C for 12 h. The obtained suspension of cell-sized vesicles was subjected to the addition of FITC-PEG (MW = 820 or 5,300 g mol^{-1}) followed by incubation at 25 °C for one hour to complete the penetration of FITC-PEG to the inner aqueous phase of the vesicle. The final concentration of **1** was set to 500 μM. A microscopic image was acquired using a Carl Zeiss LSM710 confocal laser scanning microscope equipped with a 63x water immersion objective lens. FITC-PEG and Rh-PE were excited by the irradiation of the laser beam at 488 and 561 nm, respectively. The temperature of the sample was maintained at 25 °C during the observation using a thermostated chamber.

Kinetic measurement of α -chymotrypsin activity

α-chymotrypsin entrapping vesicles were prepared by the same procedure described above. Untrapped enzyme in the bulk phase was removed by gel-permeation chromatography using Sephacryl™ S-300 HR (GE Healthcare Bio-sciences AB, Uppsala, Sweden). Enzymatic activity of α-chymotrypsin was evaluated by means of the hydrolysis of benzoyl-*L*-tyrosine *p*-nitroanilide (Bz-Tyr-pNA). The time course of the hydrolysis was monitored by measuring the increase in absorbance at 410 nm as a function of time to quantify produced *p*-nitroaniline (ε = 8,800 M^{-1} cm^{-1}). First, the suspension of the α-chymotrypsin entrapping vesicles or stock solution of α-chymotrypsin was diluted in the buffer (Tris-HCl 50 mM, pH=7.4) in a quartz cuvette. Tripsin inhibitor from bovine pancreas (BPTI) was mixed in advance if needed for the inhibition of enzymatic activity. The reaction was initiated by the addition of the substrate (Bz-Tyr-pNA). Final concentrations of α-chymotrypsin, Bz-Tyr-pNA, and BPTI were set to 75 nM, 100 μM, and 5μM, respectively. The temperature of the reaction mixture was maintained at 25 °C during a measurement using a thermostated cuvette holder.

Vesicle dissolution test

Dissolution of the organic-inorganic hybrid vesicle was evaluated by measuring the turbidity of the samples. 10 μL of the vesicular stock ([**1**] = 10 mM) was diluted with 990 μL of methanol / water mixture. Obtained solution was incubated for 1 hour at room temperature to reach equilibrium. The scattering light intensity was measured at 400 nm and 25 °C using a Hitachi F-4500 spectrofluorometer. The scattering light intensity of each sample was normalized against that of the aqueous solution to obtain relative intensity. Dissolution of the vesicle was also confirmed by DLS measurement.

Additional Tables

Table S1. Characterization of the FITC-functionalized PEGs

MW / g mol^{-1}	$Abs_{490\ nm}$ / -	C_{FITC} *1 / µM	C_{PEG} / µM	Modification*2 / %	D_{hy} *3/ nm	PDI *4/ -
5300	0.54	7.7	10	77	8.99	0.31
2300	0.45	6.4	10	64	3.75	0.36
1200	0.44	6.3	10	63	2.53	0.47
820	0.47	6.7	10	67	1.82	0.44

*1 Calculated concentration of FITC
*2 Percentage of PEG functionalized with an FITC group
*3 Mean hydrodynamic diameter of PEG in water determined by DLS
*4 Polydispersity index of the hydrodynamic diameter

Table S2. Characterization of the crosslinked organic–inorganic hybrid lipid using MALDI-TOF mass

Degree of Oligomerization	*n* (-H_2O)*1	m/z (calcd.) *2	m/z (obsd.) *3	Assignment
1	0	798.71	798.76	$[M-Br]^+$
2	1	1660.33	1660.36	$[2M-H_2O-Br]^+$
3	2	2521.95	2522.19	$[3M-2H_2O-Br]^+$
4	4	3364.56	3365.97	$[4M-4H_2O-Br]^+$
5	5	4226.18	4225.95	$[5M-5H_2O-Br]^+$
6	7	5069.79	5068.43	$[6M-7H_2O-Br]^+$
7	8	5930.41	5928.80	$[7M-8H_2O-Br]^+$
8	10	6774.02	6770.21	$[8M-10H_2O-Br]^+$
9	12	7617.63	7619.64	$[9M-12H_2O-Br]^+$
10	13	8478.25	8474.48	$[10M-13H_2O-Br]^+$
11	15	9321.86	9319.74	$[11M-15H_2O-Br]^+$
12	17	10164.47	10160.28	$[12M-17H_2O-Br]^+$

*1 Number of intermolecularly eliminated water molecules through condensation reaction
*2 Calculated m/z values for the most abundant isotropic species
*3 Observed m/z values determined as a centroid of peaks

Additional Figures

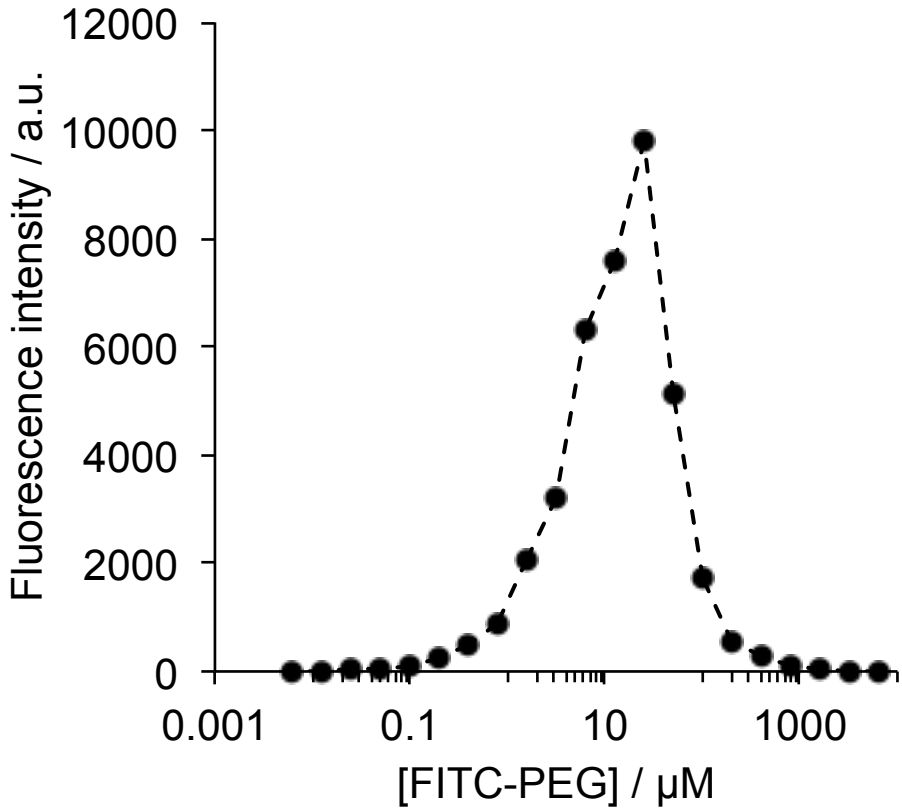


Fig. S1 Concentration dependent fluorescence of FITC-PEG (MW = 2,300 g^{-1}). λ_{ex}= 490 nm and λ_{em} = 520 nm at pH 7 and 25 °C.

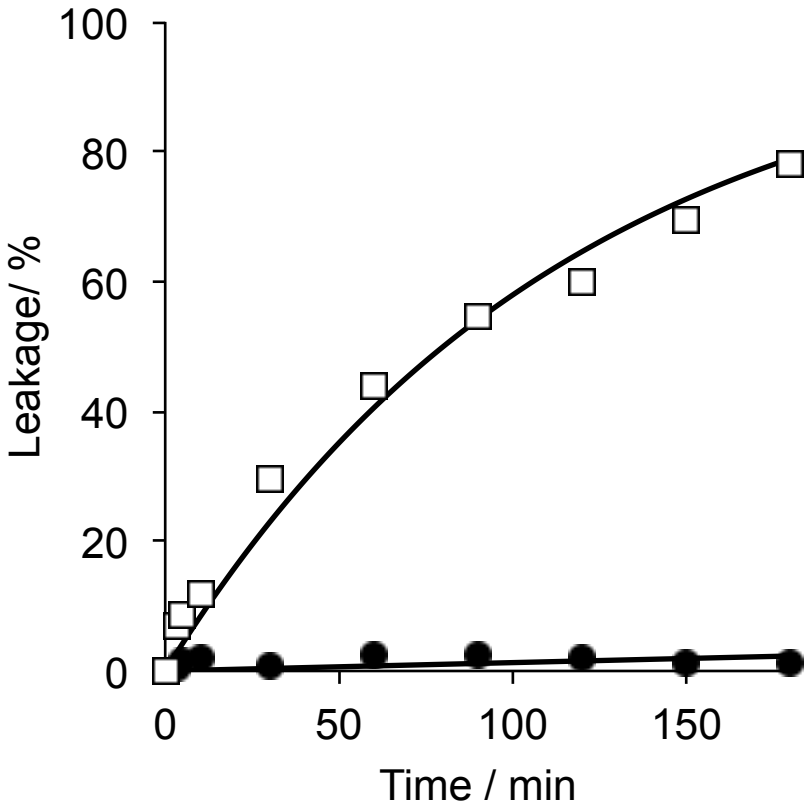


Fig. S2 Temperature-dependent leakage of FITC-PEG (MW=820 g mol^{-1}) from the hybrid vesicle. Filled circles and open squares correspond to 4 and 25 °C, respectively. [**1**] = 33 μM, [FITC-PEG] = 3 mM.

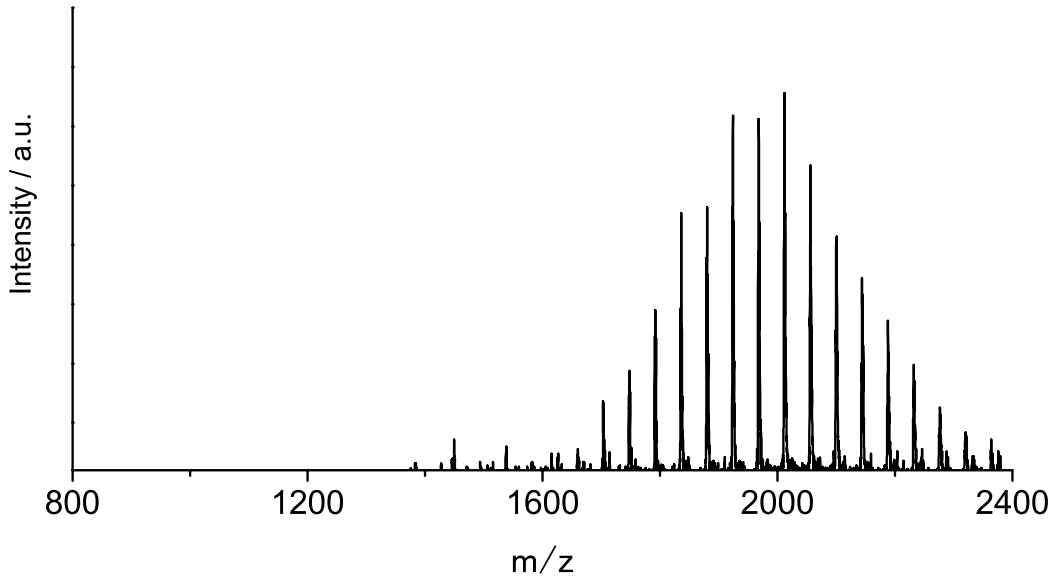


Fig. S3 MALDI-TOF mass spectrum of PEG retained in the hybrid vesicle after the complete leakage of low molecular weight fraction.

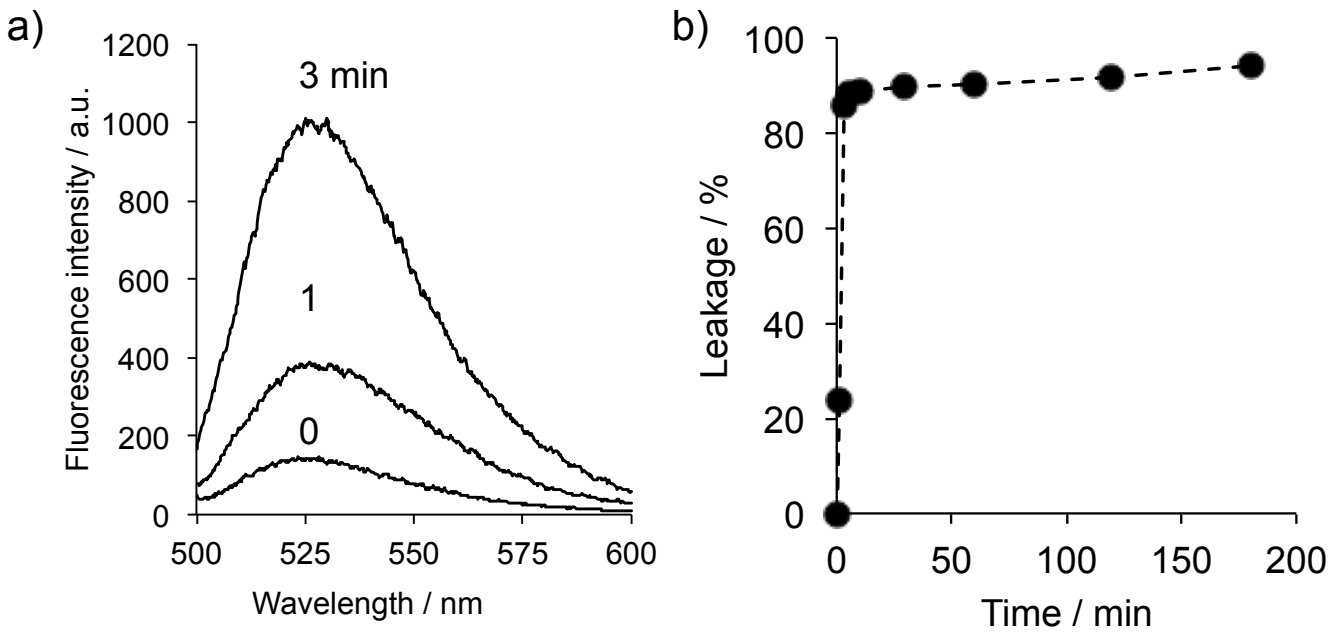


Fig. S4 (a) Fluorescence spectrum of acridine orange recorded at 0, 1 and 3 min and (b) time course of calculated leakage fraction. λ_{ex}= 490 nm, λ_{em}= 530 nm, [**1**] = 33 μM, [acridine orange] = 4.5 mM at 25 °C.

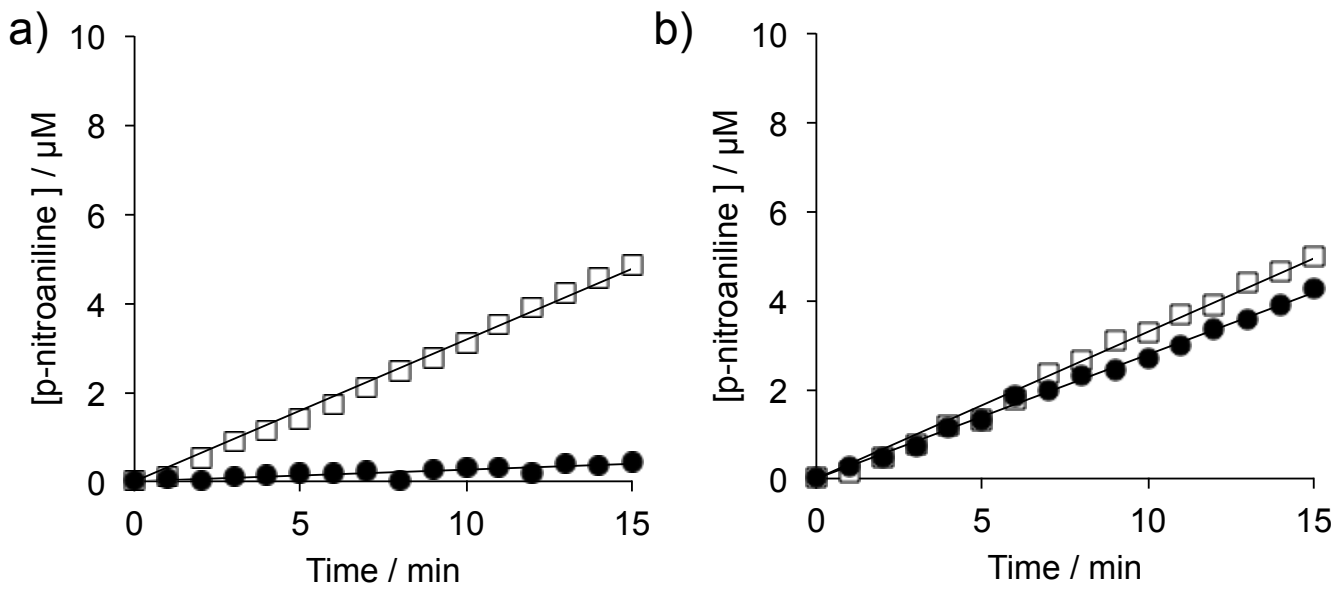


Fig. S5 Hydrolysis of Bz-Tyr-*p*NA by α-chymotrypsin (a) in an aqueous solution and (b) encapsulated within the hybrid vesicles. Filled circles and open squares correspond to the presence and the absence of BPTI as an inhibitor (5 μM), respectively. [α-chymotripsin] = 75 nM, [Bz-Tyr-*p*NA] = 100 μM at pH 7.4 and 25 °C.

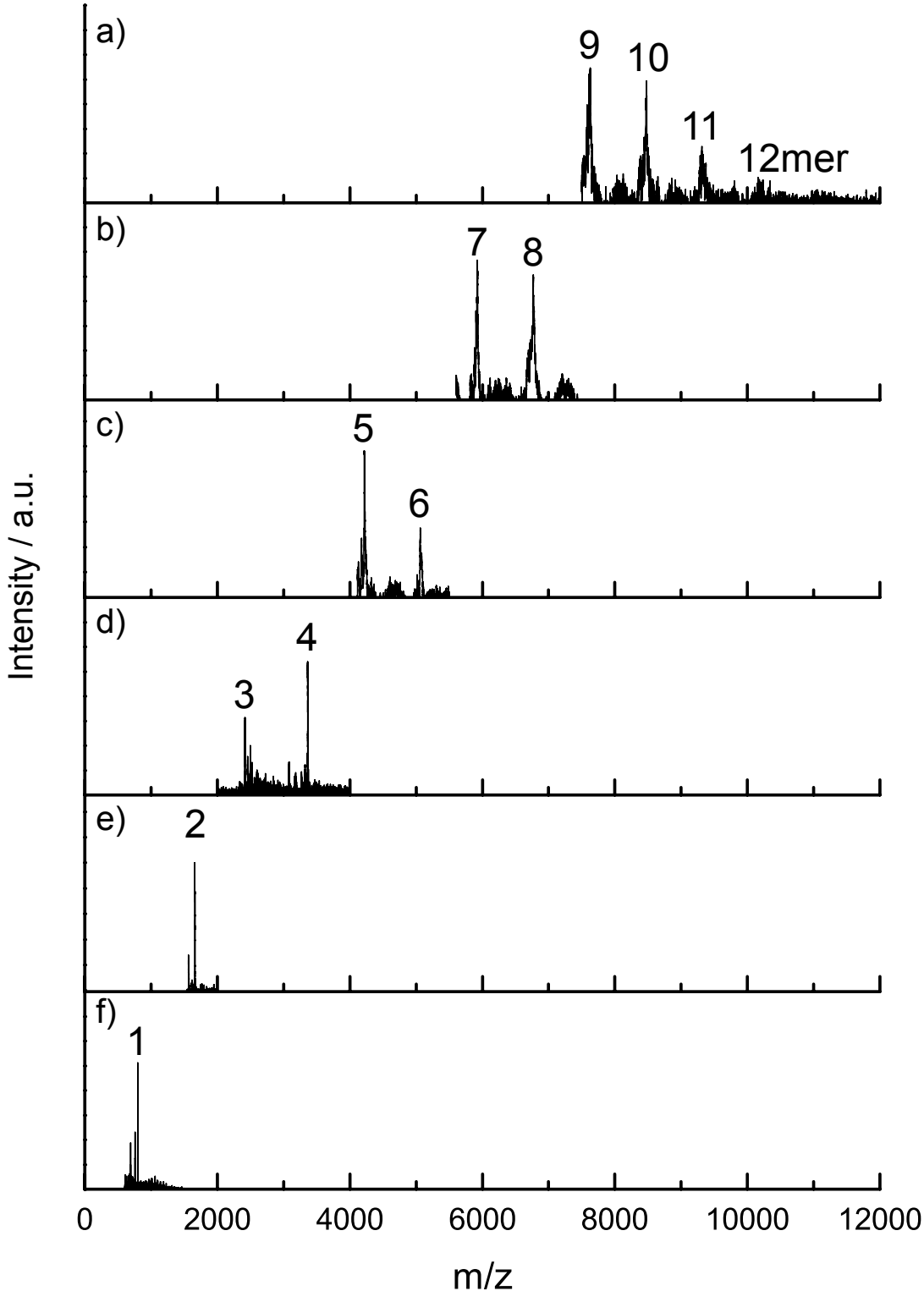


Fig. S6 MALDI-TOF mass spectra of crosslinked organic–inorganic hybrid lipid **1**. Numbers shown in the spectra represent the corresponding degree of oligomerization. Each spectrum was acquired in an m/z range of (a) 7500–12000, (b) 5500–7500, (c) 4000–5500, (d) 2000–4000, (e) 1500–2000, or (f) 600–1500. [**1**] = 33 μM at 25 °C.

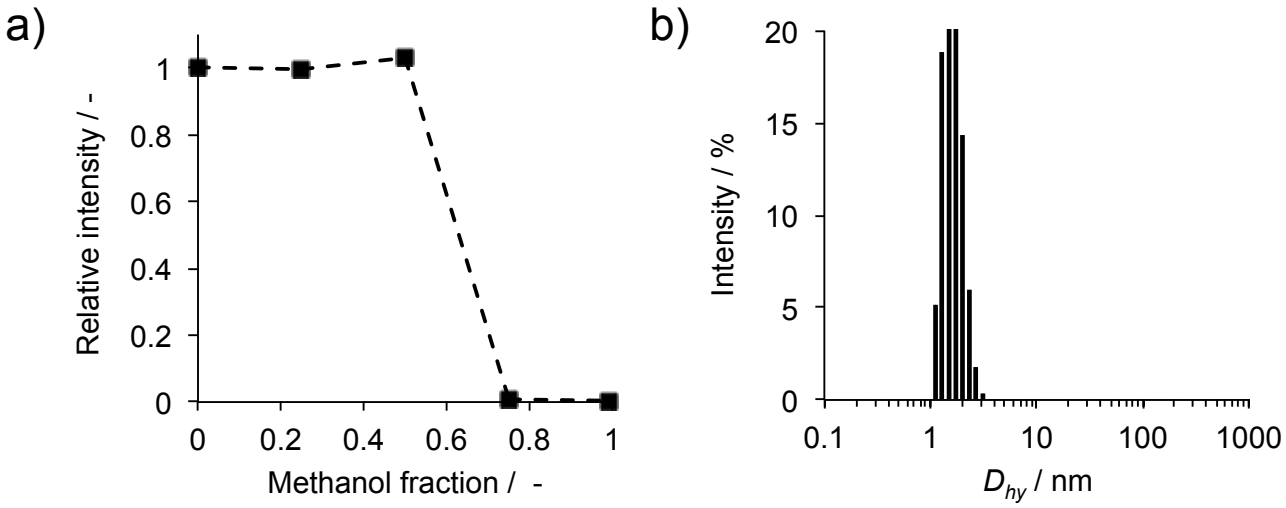


Fig. S7 Dissolution of the organic-inorganic hybrid vesicle in methanol. (a) Effect of methanol content on the scattering light intensty at 400 nm of the vesicular sample and (b) DLS data of the hybrid vesicle in methanol. [**1**] = 100 μM at 25 °C.