Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting Information

for

Intrinsically Permeable Polymer Vesicles Based on Carbohydrate-conjugated Poly(2-oxazoline)s Synthesized using a Carbohydrate-based Initiator System

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Materials

Materials for the synthesis of the glyco initiators

Maltotriose (Ma3; purity = 97.5%), maltopentaose (Ma5; purity > 97.4%), pyridine (dehydrated for organic synthesis), acetic anhydride (Ac_2O; purity > 99.5%), dichloromethane (CH_2Cl_2; super dehydrated for organic synthesis), and acetic acid (AcOH; purity > 99.7%) were purchased from Wako Pure Chemical Industries, Ltd. (Wako) and used as received. Hydrobromic acid solution (HBr, 33 wt% in acetic acid) was purchased from Sigma-Aldrich Co. LLC (Sigma-aldrich) and used as received.

Materials for the cationic ring-opening polymerization

2-*n*-Propyl-2-oxazoline (nPrOx; purity > 98.0%), 4,5-dihydro-2-phenyloxazole (PheOx; purity > 98.0%), and 1,2,3,4-tetrahydronaphtalene (tetralin; purity > 98.0%) were purchased from Tokyo Chemical Industry Co., Ltd.(TCI) and used after drying over activated molecular sieves (4 Å) overnight. 2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide (AcGluBr; purity > 98.0%), trifluoroacetic acid (TFA; purity > 99.0%), Silver trifluoromethanesulfonate (AgOTf; purity > 98.0%) were also purchased from TCI and used as received. Acetonitrile (CH_3CN; super dehydrated for organic synthesis) and ethyl acetate (EtOAc; super dehydrated for organic synthesis) were purchased from Wako and used after drying over activated molecular sieves (4 Å) overnight, followed by purging with argon prior to use.

Materials for the deprotection reaction

N, *N*-Dimethylformamide (DMF; super dehydrated for organic synthesis) and acetone (purity > 99.0%) were purchased from Wako and used as received. Hydrazine monohydrate ($N_2H_4 \cdot H_2O$; purity > 98.0%) was purchased from TCI and used as received.

Materials for the synthesis of rhodamine-labelled glyco polymers

Dimethyl sulfoxide (DMSO; super dehydrated for organic synthesis) was purchased from Wako and used as received. Rhodamine B isothiocyanate and dibutyltin dilaurate (purity > 95.0%) were purchased from Sigma-aldrich and used as received.

Materials for the preparation of FITC-labelled OVA

Albumin from chicken egg white (lyophilized powder; purity > 98.0%) and fluorescein isothiocyanate isomer (FITC; purity > 90.0%) were purchased from Sigma-aldrich and used as received.

Synthesis

NMR spectroscopy and mass spectrometry

Nuclear magnetic resonance spectra were recorded in chloroform-*d* or methanol-d_4 on a Bruker Avance III 400 MHz spectrometer to acquire ^{1}H and ^{13}C NMR spectra. Chemical shifts (δ) are expressed in parts per million and reported relative to trimethylsilane (TMS) as the internal standard in the ^{1}H and ^{13}C NMR spectra. Mass spectra were recorded on MALDI-TOF autoflex III (Bruker Daltonics) or Q-Exactive mass spectrometer (Thermo Fisher Scientific).

Size-exclusion chromatography (SEC)

Molecular-weight-distribution (MWD) curves, number-average molecular weight (M_n), and polymer dispersity index values (M_w/M_n) of the polymers were measured by size-exclusion chromatography (SEC) in DMF at 40 ºC (flow rate = 0.50 mL/min) on three linear-type polystyrene gel columns (TOSHO TSKgel SuperHM-M; exclusion limit: 4×10^6 g/mol; particle size: 3 μm; pore size: N/A; 6.0 mm i.d. × 15 cm) that were set in a HLC-8320GPC (TOSHO) machine equipped with refractive index and UV/Vis detectors (wavelength: 254 nm). The columns were calibrated against 12 poly(methyl methacrylate) standards (Fluka; M_p = 800 – 1,600,000 g/mol; M_w/M_n = 1.02 – 1.14).

Synthesis of the glyco-initiators

The glyco initiators were synthesized according to a literature procedure[S1]

Synthesis of the glyco polymers

The synthesis of the polymers was carried out using a syringe technique under an argon in a Schlenk tube equipped with a three-way stopcock. A representative synthetic procedure is given here:

AcMa3Br (1.33 g, 1.37 mmol), ethyl acetate (8.5 mL), *n*-propyl oxazoline (nPrOx, 41 mmol, 4.8 mL), tetralin (nPrOx/tetralin = 10/1, v/v), and a 200 mM EtOAc solution of AgOTf (1.37 mmol, 6.8 mL) were added to a dry Schlenk tube in this order under an atmosphere of argon (total volume: 20.6 mL). The mixture was then stirred for 8 hours at 70 ºC, before pheOx (0.2 mL, 1.51 mmol) was added. After stirring for 96 hours at 70 ºC, the reaction was quenched by addition of methanol (5.0 mL), whereupon stirring was continued for 3 days. The crude product was purified by dialysis (regenerated cellulose membrane; Spectra/Por® 7; MWCO 1000) against methanol for 4 days. Subsequently, the solvent was removed *in vacuo* to give a white solid (1.67 g). SEC (DMF; PMMA std.): M_n (SEC) = 7.3×10^3 g/mol; M_w/M_n (SEC) = 1.06. The ^{1}H-NMR spectrum in methanol-d_4 is shown in **Figure S2**.

^{1}H-NMR (400 MHz, methanol-d_4, r.t.), δ = 7.7–7.1 (aromatic), 4.0–3.0 (–N($COCH_2CH_3$)CH_2CH_2–, –N(COC_6H_5)CH_2CH_2–, sugar backbone), 2.6–2.2 (–COC$H_2$$CH_2CH_3$), 2.2–1.9 (OAc), 1.8–1.4 [–$COCH_2$C$H_2$$CH_3$], 1.2–0.6 [–$COCH_2CH_2CH_3$] Other glyco polymers were synthesized in the same way.

Deprotection of the peracetyl glycopolymers

A mixture of acetylated maltotriose polymer (416 mg, 9.1×10^{-5} mol), DMF (5.0 mL), and hydrazine monohydrate (0.5 mL, 16.1 mmol) was stirred for 24 hours at room temperature, before the reaction was stopped by the addition of acetone (0.5 mL). The crude product was purified by dialysis against doubly distilled water with a regenerated cellulose membrane (Spectra/Por® 7; MWCO 1000). The solvent was removed *in vacuo* to give a white solid (269 mg). The ^{1}H-NMR spectrum in methanol-d_4 is shown in **Figure S3**.

^{1}H-NMR (400 MHz, methanol-d_4, r.t.), δ = 7.6–7.1 (aromatic), 5.4–5.2 (anomeric protons), 3.8–3.3 (–N($COCH_2CH_3$)CH_2CH_2–, –N(COC_6H_5)CH_2CH_2–, sugar backbone), 2.6–2.2 (–COC$H_2$$CH_2CH_3$), 1.7–1.5 [–$COCH_2CH_2CH_3$], 1.1–0.8 [–$COCH_2CH_2CH_3$].

Synthesis of rhodamine-labelled glyco polymers

Rhodamine isothiocyanate (5.3 mg, 9.89 μmol) and dibutyltin dilaurate (95 mg, 15.0 μmol) were added to a DMF (5 mL) solution of the glyco polymer (31 mg, 7.40 μmol). The solution was stirred for 12 hours at 45 ºC under an atmosphere of argon. After cooling to room temperature, the reaction mixture was dialyzed (regenerated cellulose membrane; Spectra/Por® 7; MWCO 1000) for 2 days against DMSO and then for 12 days against distilled water. The resulting solution was lyophilized to yield a pale pink powder (9.0 mg). The degree of rhodamine modification (10 rhodamine groups per 100 carbohydrate-containing polymer) was determined using a rhodamine B calibration curve.

Synthesis of FITC-labelled OVA

A solution of OVA (20 mg) in 0.1 M sodium bicarbonate buffer (pH = 9.0) was treated with 50 μL of a DMSO solution of fluorescein isothiocyanate isomer (10 mg/mL). The resulting solution was allowed to stand for 17 hours at room temperature. Unreacted fluorescein isothiocyanate was removed using a PD-10 column (GE healthcare). After the FITC-modified OVA solution was concentrated by ultrafiltration (VIVASPIN 20, Sartorius) at 5000 *g* for 20 min, a degree of FITC modification of 2.1 FITC groups per 1 OVA molecule was determined.

Characterization of the polymer vesicles

Preparation of the polymer vesicle solutions

A glass tube was charged with 100 μL of the glyco polymer in methanol (10 mg/mL). The solvent was removed under a flow of argon gas to obtain a thin film. Subsequently, A 1000 μL of pre-warmed doubly distilled water or phosphate buffer saline (PBS) solution (pH = 7.4) were added to the glass tube at 40 ºC. The resulting solution was sonicated by a probe type sonicator (Qsonica, model Q500) at

40 ºC for 3 min and filtered over a 0.45 μm filter (PVDF; MILLEX-GV millipore). The final polymer concentration was 1 mg/mL.

Preparation of the giant polymer vesicle solutions

A glass tube was charged with 60 μL of the rhodamine functionalized glyco polymer in methanol (4 mg/mL) and 25 μL of glucose in methanol (20 mM). The solvent was removed under a flow of argon gas to obtain a thin film, which was further dried for 12 hours under reduced pressure. The resulting film was hydrated with doubly distilled water containing 10 vol% methanol (500 μL) at 40 ºC and allowed to stand for more than 24 hours at 40 ºC.

Dynamic light scattering (DLS)

DLS measurements were carried out on a Zetasizer Nano ZS instrument (Malvern Instruments, UK) operating at a wavelength of 632.8 nm and a 173° detection angle. The obtained data were collected at 40 ºC and analyzed using a cumulant method.

Nanoparticle tracking analysis (NTA)

The size distribution of the polymer vesicles was analyzed using a Nano Sight LM10 instrument equipped with NTA software (version 2.3). A PBS solution of the polymer vesicles (1 mg/mL) was diluted with PBS to 0.5 μg/mL for analysis. Measurements were performed at 40 ºC for a period of 60 s. The particle sizes and concentrations were evaluated using the nanoparticle tracking analysis (NTA) software.

Transmittance electron microscopy (TEM)

A PBS solution of the Polymer (10 μL, 1 mg/mL) were placed on a copper grid coated with an elastic carbon film (ELS-C10 STEM Cu100P, OKENSHOJI, Japan). Excess sample solution was removed using filter paper. Subsequently, a solution of 1 wt% phosphotungstic acid solution (10 μL, pH = 7.4) was added as the staining agent and removed again, before the sample was dried in a desiccator. The grid was placed in a HT-7700 (Hitachi, Tokyo, Japan) electron microscope operated at 100 kV.

Transmittance measurements

The transmittance of an aqueous solution of the polymer (1.0 to 15 mg/mL) was recorded on a UV-vis spectrophotometer (V-660, JASCO, Japan) at 500 nm equipped with a Peltier thermostat cell holder system (ETCS-761). The heating rate was 1 K/min between 25 and 45 or 60 ºC, and the optical path

length was 1.0 cm. The cloud point was defined as the temperature where the transmittance decreased to 50 % in the heating process.

Differential scanning calorimetry (DSC)

The thermoresponsiveness of the glyco polymer solutions was measured by DSC (DSC 204F1 phoenix, NETZSCH). For that purpose, the glyco polymers were dispersed in PBS solution (10 mg/mL), and heating and cooling rates of 3 K/min were applied.

Confocal laser scanning microscopy (CLSM) and observation of the giant polymer vesicles

Solutions of the giant polymer vesicles (200 μL) were poured into a glass dish (35 mm, AGC techno glass) and 50 μL of FITC-PEG ([FITC] = 0.21 mM) were added. After various time intervals, images of giant polymer vesicles were taken using a confocal laser scanning microscope (LSM780; Carl Zeiss, LLC, US) at a magnification of 40× lens (Plan-Apochromat 40×/1.4 Oil DIC, Carl Zeiss, LLC, US) and excitation wavelengths of 488 nm (FITC) and 561 nm (rhodamine).

Fluorescence recovery after photobleaching (FRAP)[S2]

FRAP analysis of the rhodamine-functionalized glyco polymers in the giant polymer vesicles was performed with a confocal laser scanning microscope (LSM780) at a magnification of the 40× lens using Zen software. A glass dish containing the giant vesicle solution was placed in a stage-top incubator (INU-ZILCS, Tokai Hit, Japan) at 40 ºC. A 3 μm circle was bleached using the maximum intensity of an argon laser (561 nm). The fluorescence recovery was recorded by taking time-lapse images at 1 s intervals. The results were collected from three independent measurements. Recovery curves were generated by plotting the average fluorescence intensity of the bleached area as a function of the time of recovery. The relationship $D = 0.224r^2/t_{1/2}$ was used to calculate the diffusion coefficients, where r is the radius of the bleached spot and $t_{1/2}$ is the half-life time of the recovery. The latter is defined by $[F(t_{1/2}) - F(0)]/[(F(\infty) - F(0)) = 0.5$, where $F(t)$ is the fluorescence intensity at the time t fitted to a single-exponential function.

FITC-OVA encapsulation and high-performance liquid chromatography (HPLC) analysis

500 μL of a PBS solution containing FITC-OVA were added to a thin film of the glyco polymer (polymer = 1.0 mg/mL; FITC-OVA= 0.45 mg/mL; total volume: 0.5 mL). The resulting solution was sonicated for 3 min using a probe-type sonicator. The resulting solution was analyzed by HPLC (Prominence, Shimazu, Japan), which was performed on a chromatography system using a refractive index detector (RID-10) connected to an UV-Vis detector (SPD-20A). Sephacryl S-500 HR (GE healthcare) was used as the column for the HPLC measurements. The polymer solutions (1 mg/mL) were eluted at 40 °C with PBS buffer (pH = 7.4) at a flow rate of 0.50 mL/min.

Fluorescence measurements

The fluorescence spectra were recorded on a fluorescence spectrophotometer (FP-8500, JASCO, Japan). A stock solution (25 μL) of pyrene (1×10^{-4} M) in ethanol was added to a vial. The ethanol was evaporated by flushing with nitrogen to form a thin film on the bottom of the vial. Polymer solutions (2.5 mL) were added to the thin films, and the resulting mixture was stirred overnight at room temperature. The final concentration of pyrene in the vial was 1×10^{-6} M. Pyrene was excited at 339 nm. The slit width was set to 5 nm for the excitation and to 5 nm for the emission. The measurement temperature was kept constant at 40 °C.

Cell culturing

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. Cells were incubated at 37 ºC in 5% CO_2.

Cytotoxicity.

HeLa cells were plated on a 96-well plate at 2×10^4 cells/well and cultured for 24 hours at 37 ºC in 5% CO_2. Different concentrations of the polymer solutions were added to the medium. After 24 hours of incubation, the medium was removed and replaced with fresh medium. 10 μL of the cell-counting-kit-8 reagent (Dojindo Laboratories, Japan) was then added to the medium, before incubation was continued for 2 hours. The UV absorbance at 450 nm was recorded with a plate reader (Enspire, PerkinElmer, USA). Untreated cells were used as the 100% cell viability control. The results are expressed as mean values and their corresponding standard deviations, which were obtained from six samples.

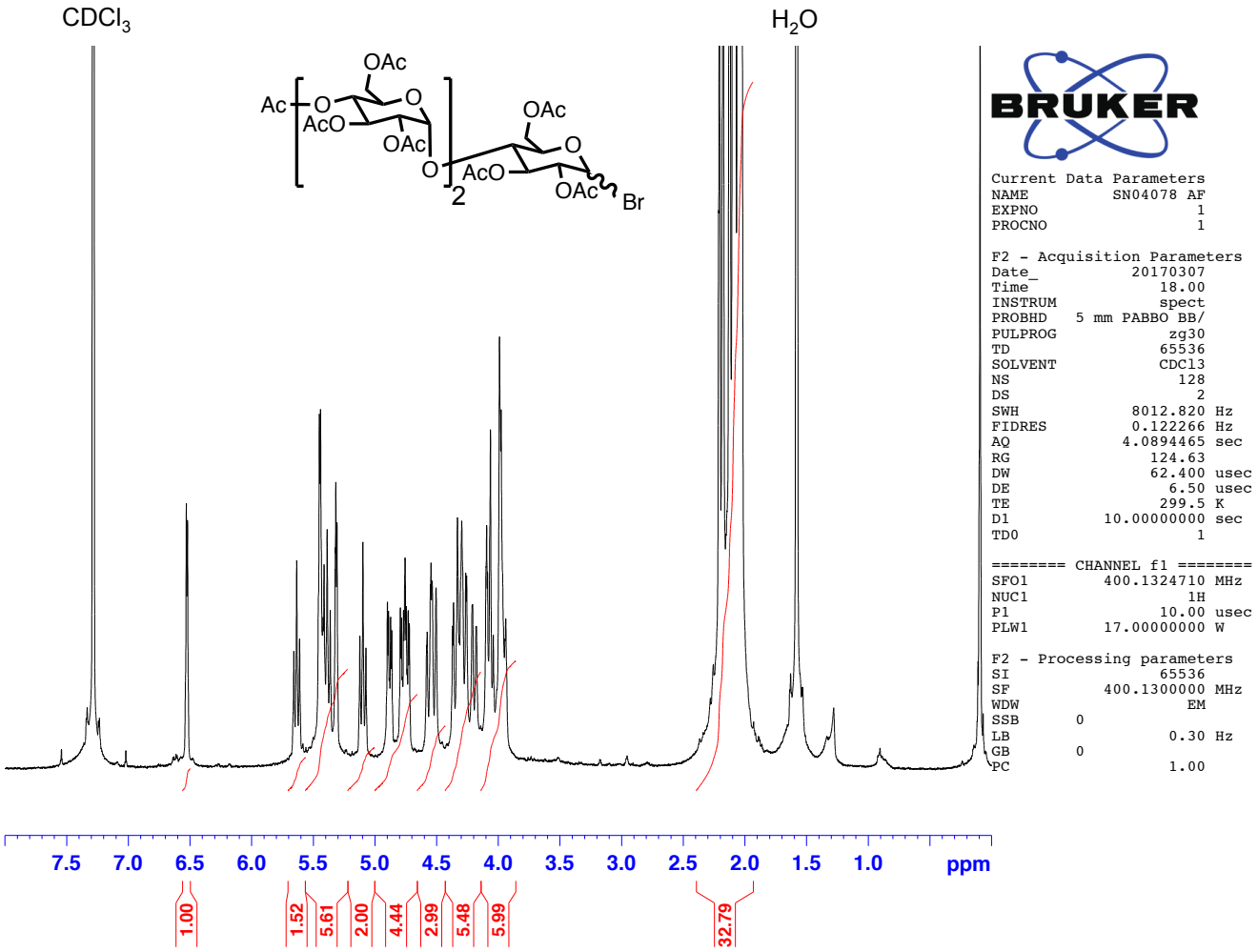


Fig. S1 ^{1}H-NMR spectrum of peracetylated maltotriosyl bromide in chloroform-d_1.

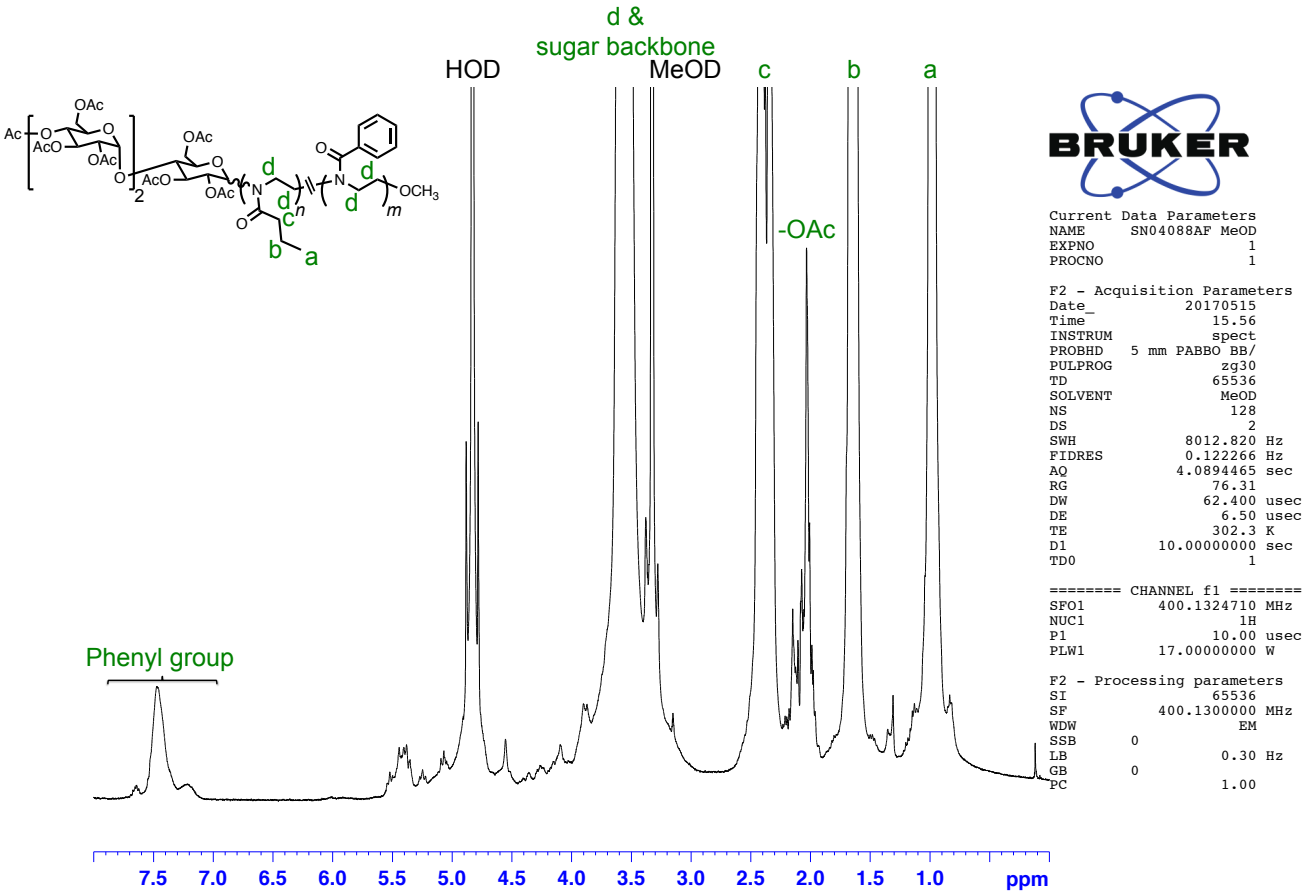


Fig. S2 ^{1}H-NMR spectrum of peracetylated maltotriose-*b*-poly(nPrOx$_{30}$-*co*-PheOx$_{1}$) in Methanol-d_4.

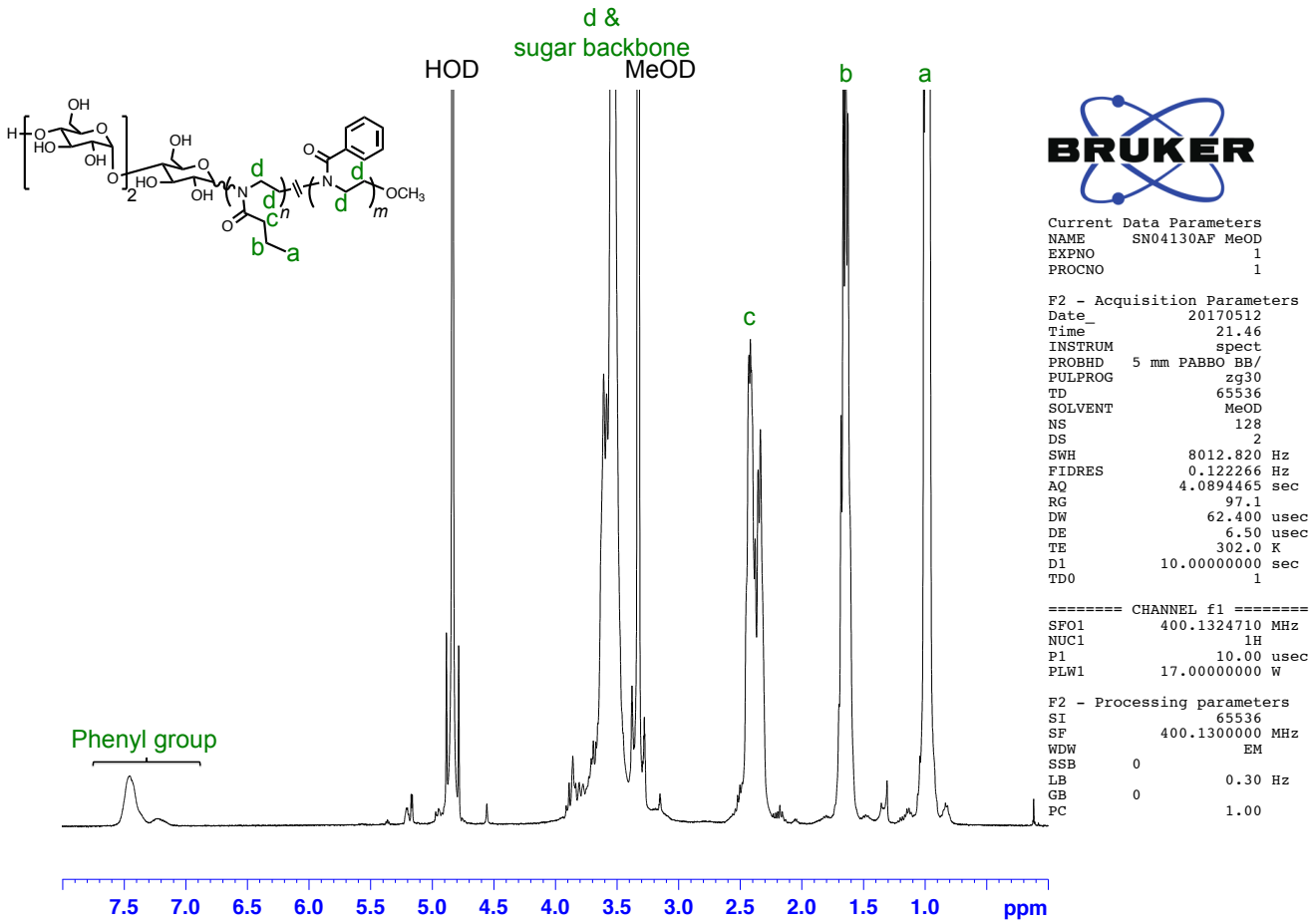


Fig. S3 ^{1}H-NMR spectrum of maltotriose-*b*-poly(nPrOx$_{30}$-*co*-PheOx$_{1}$) in Methanol-*d*$_{4}$.

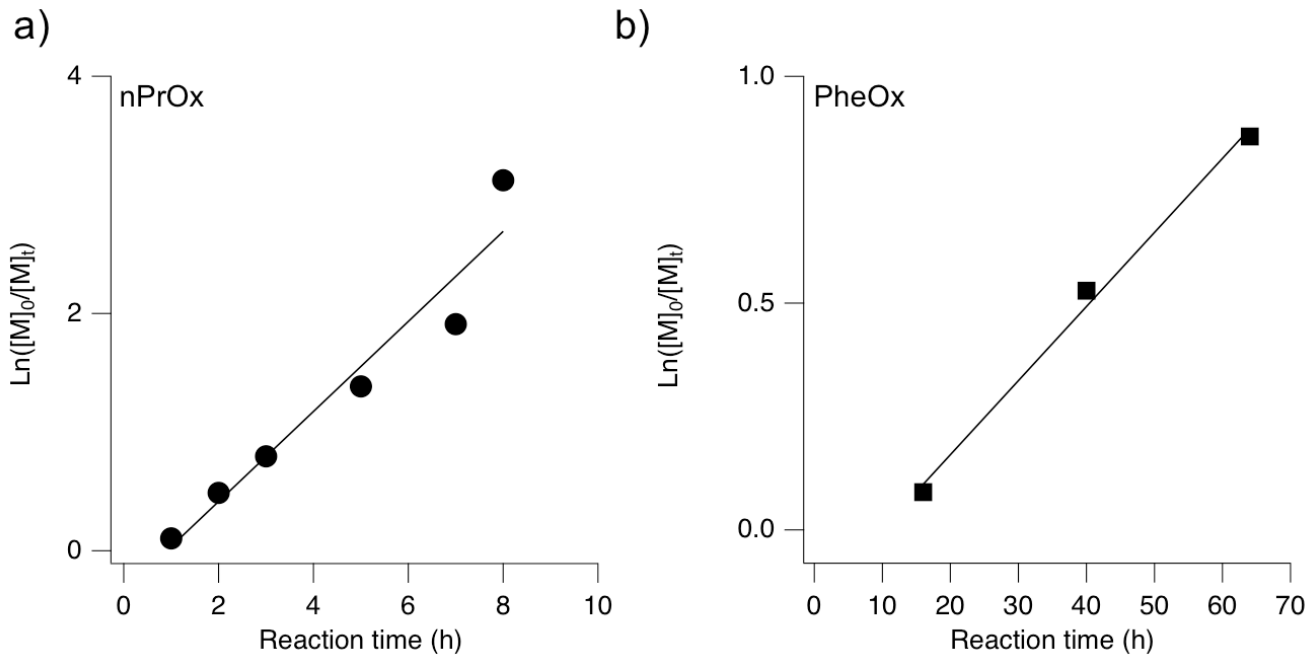


Fig. S4 Kinetic plots for the CROP of 2-oxazolines using peracetylated maltotriosyl bromide and AgOTf. $Ln([M]_0/[M]_t)$ plots as a function of time for a) nPrOx and b) PheOx.

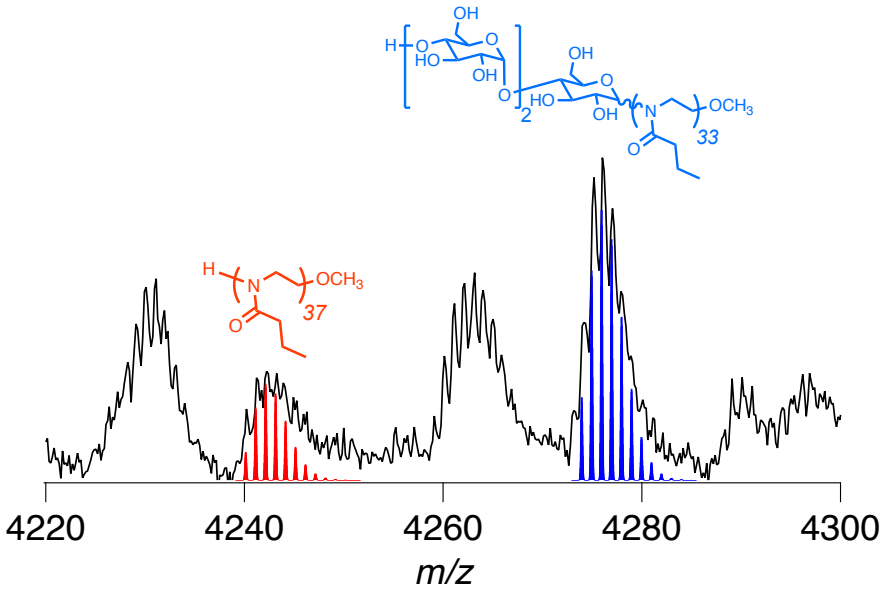
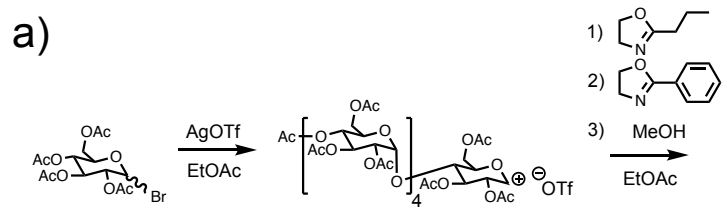
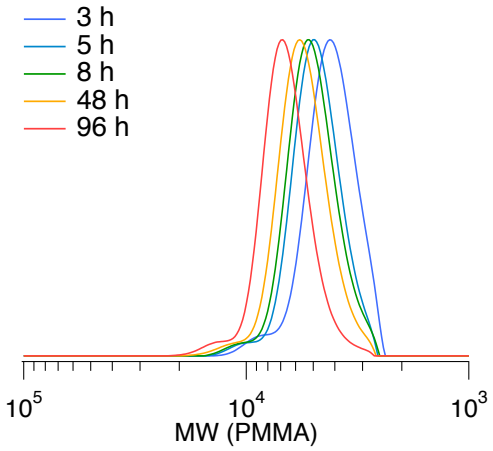


Fig. S5 MALDI-TOF mass spectrum of poly(*n*-propyl oxazoline) initiated by a maltotriose-based initiator. (black line: measured spectrum; colored lines: calculated isotopic mass patterns, which were calculated for the methanol and Na^+ adducted species).

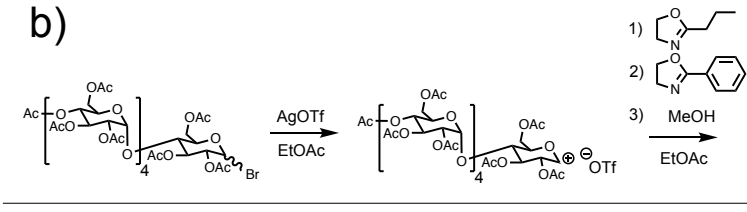
a)



Time, h	M_n, g/mol	M_w/M_n	Conversion, % (nPrOx)	Conversion, % (PheOx)
3	4.1×10^3	1.07	67	-
5	4.8×10^3	1.07	88	-
8	5.0×10^3	1.07	95	0
48	5.4×10^3	1.07	100	7.5
96	5.5×10^3	1.12	100	68



b)



Time, h	M_n	M_w/M_n	Conversion, % (nPrOx)	Conversion, % (PheOx)
3	5.1×10^3	1.09	79	-
5	5.7×10^3	1.08	88	-
8	6.6×10^3	1.06	96	0
48	6.6×10^3	1.08	100	52
96	6.7×10^3	1.07	100	66

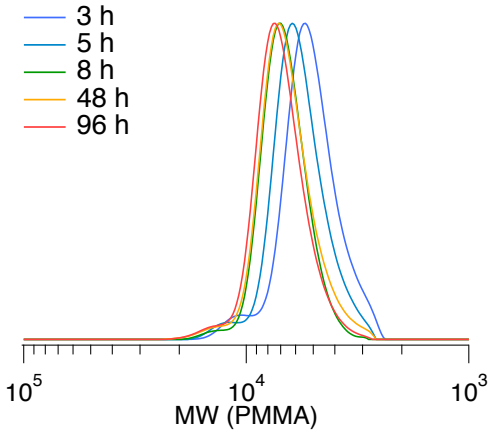


Fig. S6 a) Reaction scheme of the CROP of 2-oxazolines in the presence of peracetyl glucosyl bromide and AgOTf, together with SEC curves of the polymerization samples after 3, 5, 8, 48, and 96 hours. b) Reaction scheme of the CROP of 2-oxazolines in the presence of peracetyl maltopentaosyl bromide and AgOTf, together with SEC curves of the polymerization samples after 3, 5, 8, 48, and 96 hours.

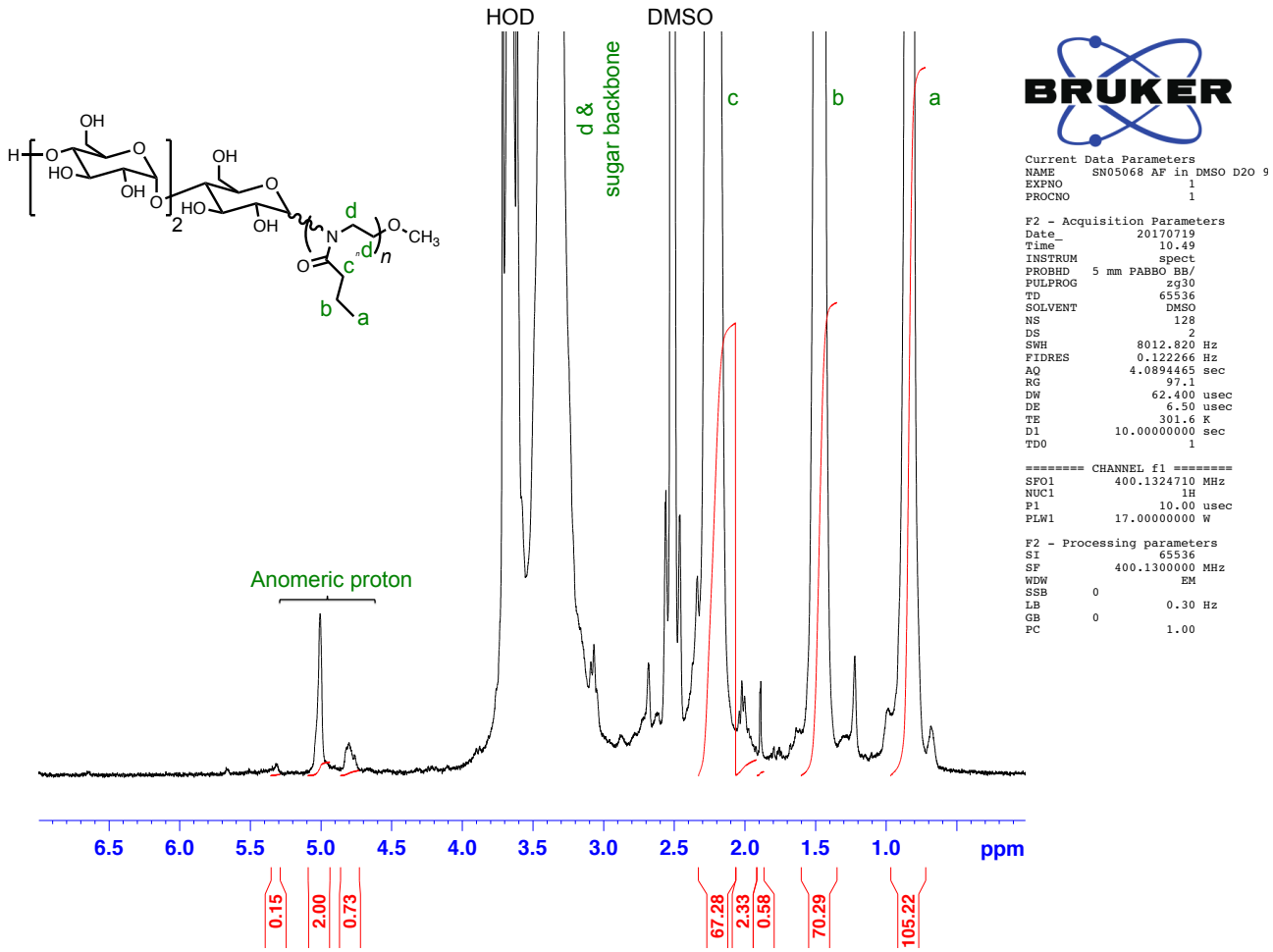


Fig. S7 ^{1}H-NMR spectrum of maltotriose-*b*-poly(nPrOx$_{35}$) in DMSO-d_6.

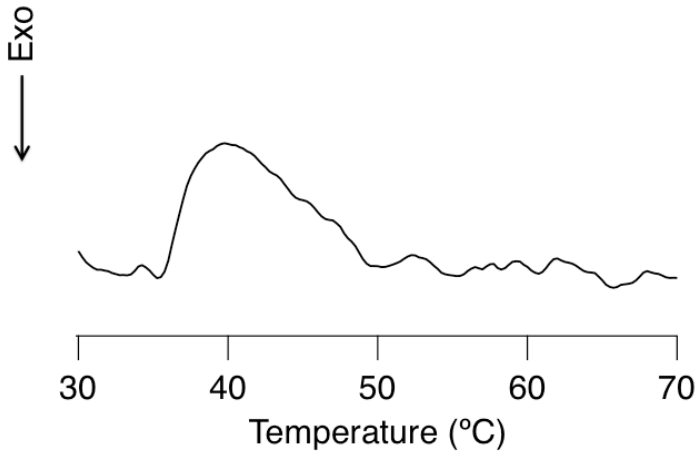


Fig. S8 DSC curve of a PBS solution of maltotriose-*b*-poly(nPrOx$_{35}$): [polymer] = 10 mg/mL; heating rate = 3 K/min.

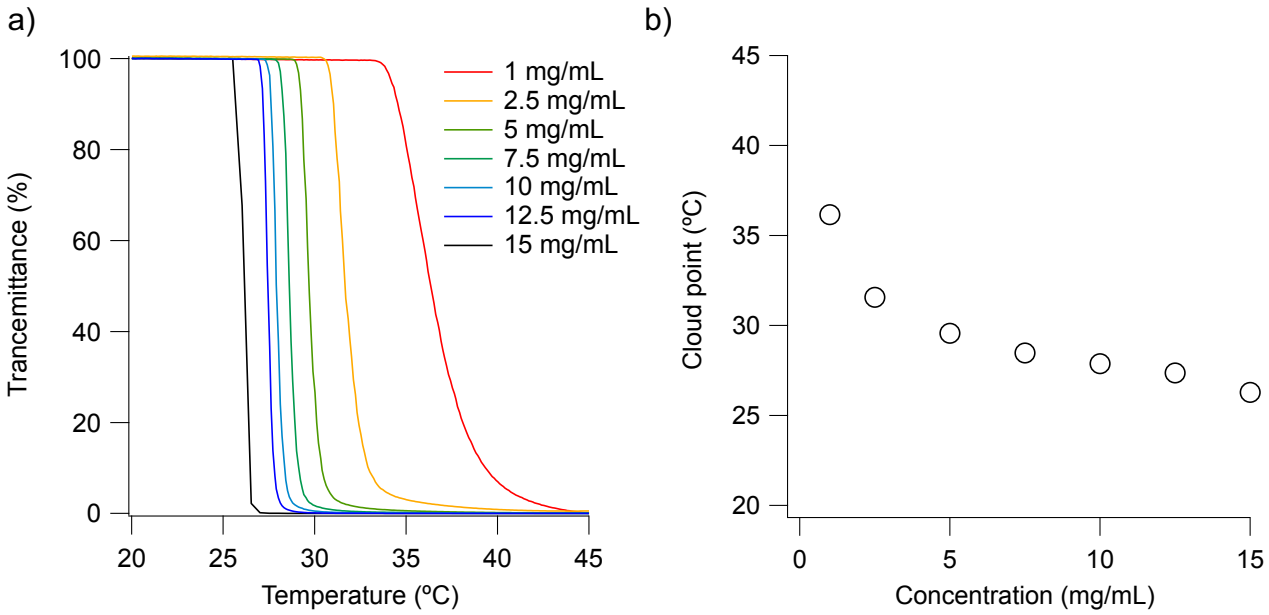


Fig. S9 a) The transmittance changes of maltotriose-*b*-poly(nPrOx$_{30}$-*co*-PheOx$_1$) solution with different polymer concentrations. b) Concentration dependence of the cloud points for maltotriose-b-poly(nPrOx$_{30}$-*co*-PheOx$_1$).

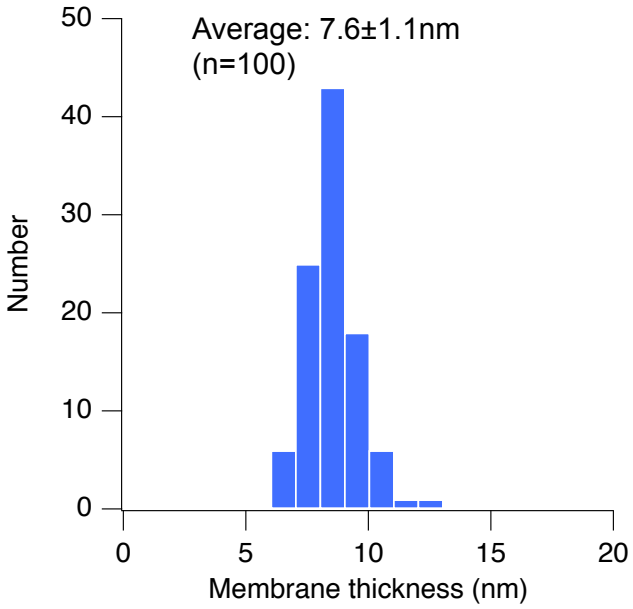


Fig. S10 TEM-derived membrane thickness distribution of maltotriose-*b*-poly(nPrOx$_{30}$-*co*-PheOx$_1$) vesicles.

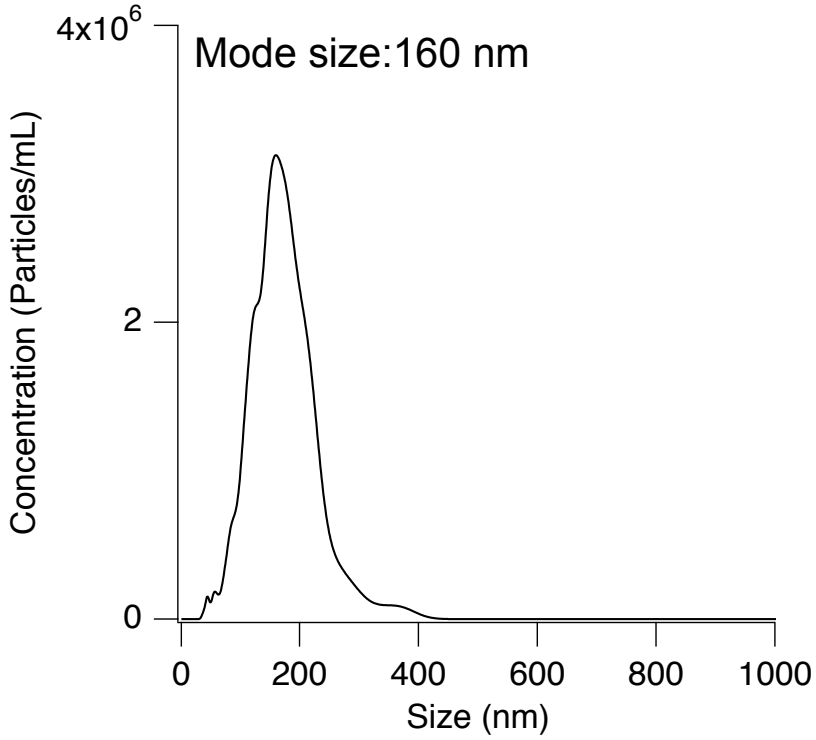


Fig. S11 NTA-derived size distribution of maltotriose-*b*-poly(nPrOx$_{30}$-*co*-PheOx$_1$) vesicles.

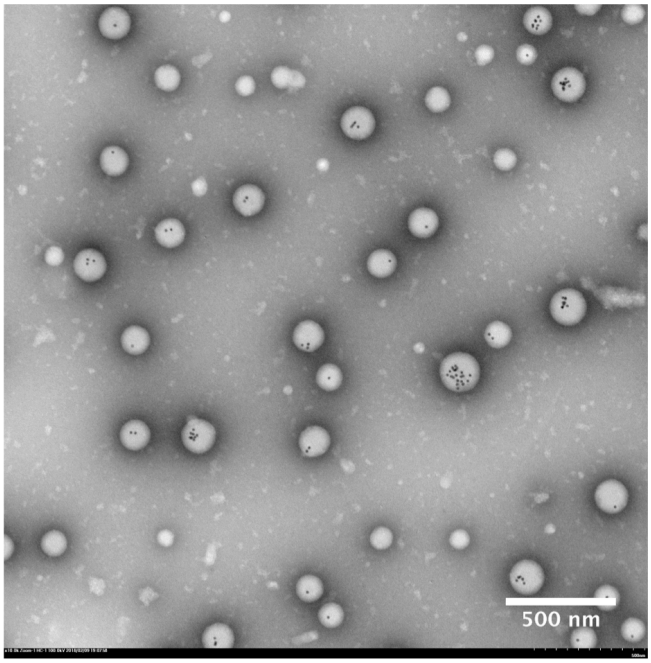
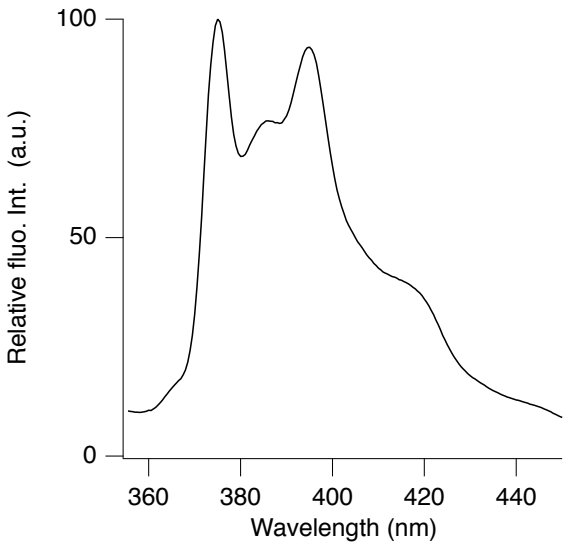


Fig. S12 TEM image of gold-nanoparticle-loaded polymer vesicles.



Sample	I_1	I_3	I_1/I_3
Maltotriose-Poly(2-oxazoline) vesicle	100	76.3	1.31

Fig. S13 Fluorescence spectra of pyrene in the presence of maltotriose-*b*-poly(nPrOx$_{30}$-*co*-PheOx$_1$) vesicles at 40 ºC.

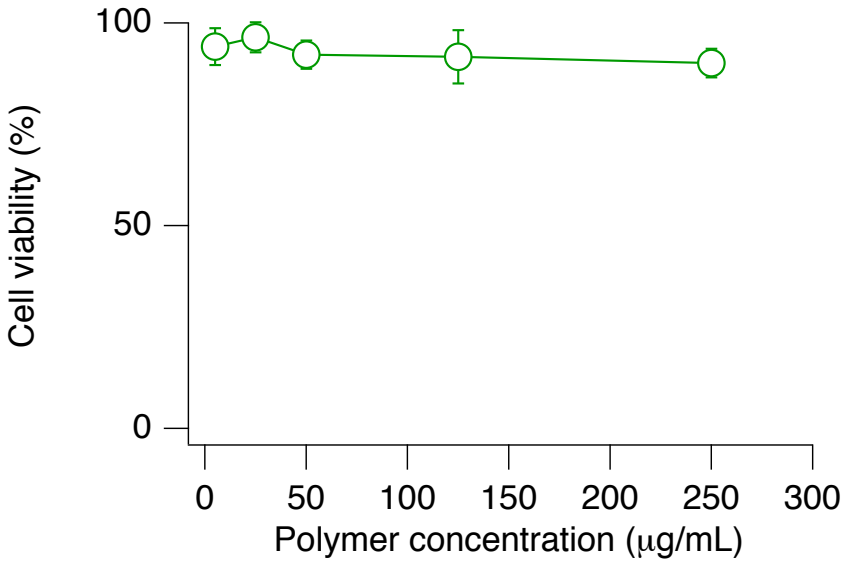


Fig. S14 Relative viability of HeLa cells treated with maltotriose-*b*-poly(nPrOx$_{30}$-*co*-PheOx$_1$) at various concentrations. The results show mean values ± standard deviation (n = 6).

References

S1 S. Garcia-Viñuales, I. Delso, P. Merino, T. Tejero, *Synthesis* **2016**, *48*, 3339-3351.

S2 a) Soumpasis, D. M., *Biophys. J.* **1983**, 41, 95-97 b) Vaz, W. L.; Kapitza, H. G.; Stumpel, J.; Sackmann, E.; Jovin, T. M., *Biochemistry* **1981**, 20, 1392-1396