Supporting Information

Mission ImPOxable – Or the Unknown Utilization of Non-toxic Poly(2-oxazoline)s as Cryoprotectant and Surfactant at the Same Time

Meike N. Leiske,[a,b] Anne-Kristin Trützschler,[a,b] Sabine Armoneit,[c] Pelin Sungur,[a,b] Stephanie Hoeppener,[a,b] Marc Lehmann,[c] Anja Traeger*[,a,b,c] & Ulrich S. Schubert*[,a,b,d]

[a]Laboratory of Organic and Macromolecular Chemistry (IOMC), Friedrich Schiller University Jena, Humboldtstrasse 10, 07743 Jena, Germany.
[b]Jena Center for Soft Matter (JCSM), Friedrich Schiller University Jena, Philosophenweg 7, 07743 Jena, Germany.
[c]SmartDyeLivery GmbH, Botzstrasse 5, 07743 Jena, Germany.
[d]Center for Sepsis Control & Care (CSCC), Jena University Hospital, Erlanger Allee 101, 07747 Jena, Germany.
*Correspondence to A. Traeger (anja.traeger@uni-jena.de) and U. S. Schubert (ulrich.schubert@uni-jena.de)

Experimental Section

Materials and instrumentation

Anhydrous triethylamine (TEA, Sigma Aldrich), 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPDB-COOH, Sigma Aldrich), neutral aluminium oxide (Sigma Aldrich), sulforhodamin B acid chloride (Sigma Aldrich), 2-(methylamino) ethanol (Sigma Aldrich), poly(D,L-lactide-*co*-glycolide) (Resomer® RG 502 H, PLGA, Sigma Aldrich), poly(vinyl alcohol) (PVA, Sigma Aldrich), PKC412 (Biomol), Eudragit RS100 (Evonik), poly(caprolactone) (PCL, Sigma Aldrich), di-*tert*-butyldicarbonate (Alfa Aesar), Nile red (Sigma Aldrich), Pluronic® F127 (Sigma Aldrich) were used as obtained. 2-Bisazobutyronitile (Acros) was recrystallized from methanol prior to use. Methyl methacrylate monomer (Sigma Aldrich) was treated with an inhibitor remover (Sigma Aldrich) prior to use. 2-Ethyl-2-oxazoline (EtOx, Sigma Aldrich), 2-methyl-2-oxazoline (MeOx, Sigma Aldrich) and methyl tosylate (MeTos, Sigma Aldrich) were distilled to dryness over calcium hydride (VWR) under argon atmosphere

prior to usage. The regenerated cellulose membrane cut-off 1000 was purchased from Spectralabs and cut-off 3500 from Carl Roth. Ethyl acetate (EtOAc) and acetone, hydrochloric acid, *N*,*N*-dimethylformamide (DMF) and tetrahydrofuran (THF) were purchased from VWR Chemicals. Acetonitrile was obtained from a solvent purification system (MB-SPS-800 by MBraun) and stored under argon. All other solvents used were obtained from standard suppliers.

CryoTEM investigations were conducted with a FEI Tecnai G2 20 at 200 kV acceleration voltage. Specimens were vitrified by a Vitrobot Mark V system on Quantifoil grids (R2/2). The blotting time was 1 s with an amount of solution of 8.5 µL. Samples were plunge frozen in liquid ethane and stored under liquid nitrogen until transferred to the Gatan cryo-holder and brought into the microscope. Images were acquired with an Olympus Mega View camera (Olympus Soft Imaging Solutions; 1376 × 1032 pixels).

SEC of the polymers was partwise performed on a Shimadzu system equipped with a SCL-10A system controller, a LC-10AD pump, a RID-10A refractive index detector and a PSS SDV column with $CHCl_3$-*i*-PrOH-NEt_3 (94:2:4) as eluent. The column oven was set to 50 °C and a polystyrene (PS) standard was used for calibration. SEC of the polymers was partwise performed on a Agilent 1200 series system equipped with a PSS degasser, a G1310A pump, a G1362A refractive index detector and a PSS GRAM guard/1000/30 Å column with *N,N*-dimethyl acetamide (DMAc) + 0.21% LiCl as eluent. The column oven was set to 50 °C. For the poly(2-oxazoline)s a PS standard and for the Boc-protected poly(methacrylate)s a PMMA standard was used. SEC of the cationic deprotected poly(methacrylate)s was performed on a Jasco system equipped with a DG-980-50 degasser, a PU-980 pump, a RI-930 refractive index detector and an AppliChrom ABOA CatPhil guard/200/350 Å column with 0.1% TFA + 0.1 M NaCl as eluent. The column oven was set to 50 °C and a dextrane standard was used.

Proton NMR spectroscopy (^{1}H-NMR) was performed at RT using a Bruker Avance I 300 MHz spectrometer, utilizing either $CDCl_3$ or D_2O as solvent. The chemical shifts are given in ppm relative to the signal from the residual non-deuterated solvent

Lyophilization of the nanoparticle suspensions was conducted using an Alpha 1-2 LDplus freeze dryer from Martin Christ Gefriertrocknungsanlagen GmbH (Germany). Nanoparticle suspensions were frozen in a -80 °C freezer and lyophilized overnight (p = 0.01 mbar, T = -50 °C).

Absorbance and fluorescence measurements were performed at RT using a TECAN Infinite M200 PRO.

Confocal laser scanning microscopy (CLSM) was performed with an LSM880 ELYRA PS.1 system (Zeiss, Oberkochen, Germany) using the λ_{Ex} = 514 nm laser for imaging of Rhodamine B.

Batch dynamic light scattering (DLS) was performed on a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany). All measurements were performed in folded capillary cells (DTS1071, Malvern Instruments, Herrenberg, Germany). After an equilibration time of 180 s, 3 × 30 s runs were carried out at 25 °C (λ_{Ex} = 633 nm). Scattered light was detected at an angle of 173°. Each measurement was performed in triplicates. Apparent hydrodynamic radii, R_h, were calculated according to the Stokes–Einstein equation (eq. 3):

$$R_h = \frac{kT}{6\pi\eta D} \qquad (3)$$

k: Boltzmann constant; T: absolute temperature: η: viscosity; D: diffusion coefficient, R_h: hydrodynamic radius

The density was determined at a temperature of 20 °C with a density meter DMA 4100 (Anton Paar, Graz, Austria).

Dynamic viscosities were measured on an AMVn microviscometer by Anton Paar, which is based on the approved and acknowledged rolling/falling ball principle according to DIN 53015 and ISO 12058. A Peltier thermostat was used to adjust the temperature to 20 °C. Measurements were conducted at 30°, 50° and 70°, 4 times each. All values are given as mean ± standard deviation.

The surface tension of the surfactant solutions was determined using a droplet shape measuring system (OCA 30, Dataphysics, Germany) with droplets of the surfactant solutions having a volume of 10 µL. The measurements were performed in five times each at room temperature and the surface tension was calculated using the Young-Laplace equation (Eq. 4):

$$\Delta p = \frac{2 \cdot \gamma}{r} \qquad (4)$$

r: radius; γ: surface tension; p: pressure

Synthesis and Characterization

Synthesis of N-methyl-N-tert-butyloxycarbonyl-(2-aminoethyl) methacrylate (BocMAEMA)

N-Methyl-*N*-*tert*-butyloxycarbonyl-(2-aminoethyl)-methacrylate was synthesized according to a procedure of Sinclair *et al*..[1] 10 g *N*-Methylaminoethanol was dissolved in 80 mL chloroform and cooled in an ice bath.Then, 29 g di-*tert*-butylcarbonate in 80 mL was added dropwise and stirred at room temperature for 1 hour. The solvent was removed under reduced pressure and the mixture was purified by distillation (30 mbar, 180 °C). Under schlenk conditions 21.4 g of *N*-methyl-*N*-*tert*-butyloxycarbonyl-2-aminoethanol were diluted with 100 mL dry dichloromethane, 49.4 mL triethylamine was added and the reaction mixture cooled in an ice bath. 17.7 mL methacryloyl chloride in 100 mL dichloromethane was added dropwise and the reaction was

stirred at room temperature overnight. The Mixture was washed with water and brine and dried over sodium sulfate. Further purification was done by column chromatography using a mixture of cyclohexane an ethyl acetate (9:1 to 3:1).

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.96 (s, 9H), 2.76 (m, 3H), 3.30 (m, 2H), 4.56 (m, 2H), 5.65 (s, 1H), 6.30 (s, 1H) ppm.

^{13}C NMR (300MHz, $CDCl_3$): δ = 18.2 (CH_3), 28.3 (CH_3), 35.2 (CH_2), 47.9 (CH_3), 62.7 (CH_2), 79.7(CH_2), 126.0 (C_{quart}), 136.1 (C_{quart}), 155.8 (C_{quart}), 167.1 (C_{quart}) ppm.

Synthesis of poly(methyl methacrylate)$_{97}$-co-N-methyl-N-tert-butyloxycarbonyl-(2-aminoethyl) methacrylate$_{32}$ (P(MMA$_{97}$-co-BocMAEMA$_{32}$))

In a typical synthesis 0.9 g methyl methacrylate (8.98 mmol), 0.55 g *N*-methyl-*N*-*tert*-butyloxycarbonyl-(2-aminoethyl)-methacrylate (2.24 mmol), 0.031 g CPDB-COOH (0.11 mmol), 0.004 g AIBN (0.03 mmol) and 0.97 g anisole as internal standard were dissolved in a reaction vial in 1.9 mL ethanol and degassed with argon for 30 min. Afterwards, the reaction mixture was heated to 70 °C for 6 h. The solvent was removed under reduced pressure and the residue was re-dissolved in THF and precipitated from n-hexane.

SEC (eluent: DMAc + 0.21% LiCl, PMMA-standard): M_n = 17,400 g mol^{-1}, M_w = 20,700 g mol^{-1}, Đ = 1.19.

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 0.83-1.26 (m, 6H), 1.45 (s, 9H), 1.76-2.08 (m, 2H), 2.92 (s, 3H), 3.44 (s, 2H), 3.58 (s, 3H), 4.02 (s, 2H) ppm.

Deprotection of boc-protected copolymers of P(MMA$_{97}$-co-BocMAEMA$_{32}$) to yield poly((methyl methacrylate)$_{97}$-co-N-methyl-(2-aminoethyl) methacrylate$_{32}$) (P(MMA$_{97}$-co-MAEMA$_{32}$))

Boc-protected copolymers were deprotected using diluted hydrochloric acid in methanol. In a typical procedure, 700 mg polymer was dissolved in 30 mL methanol and 3 mL of hydrochloric acid was added dropwise and stirred at room temperature overnight. The solvent was removed under reduced pressure, dissolved in water and freeze dried. The crude polymer was dissolved in water and a 0.1 M sodium hydroxide solution was added until the polymer precipitated, collected by centrifugation and washed thoroughly. The Polymer war dissolved in methanol and dialyzed against water with a regenerated cellulose membrane (cut-off 3500 g mol^{-1}) and lyophilized.

SEC (eluent: 0.1% TFA + 0.1 M NaCl, dextrane-standard): M_n = 21,600 g mol^{-1}, M_w = 25,900 g mol^{-1}, Đ = 1.20.

^{1}H-NMR (300 MHz, D_2O): δ = 0.85-1.04 (m, 6H), 2.00 (m, 2H), 2.80 (s, 3H), 3.42 (s, 2H), 3.66 (s, 3H), 4.31 (s, 2H) ppm.

Synthesis of P(Ox)s

Polymerization reactions of 2-oxazolines were performed under microwave irradiation, using an Initiator Sixty single-mode microwave synthesizer from Biotage, equipped with a noninvasive IR sensor (accuracy: 2%). Microwave vials were heated overnight at 100 °C under vacuum and allowed to cool to RT under argon before usage. Polymerizations were performed under temperature control. According to the polymer characteristics, size exclusion chromatography (SEC) of the polymers was performed on different systems and noted in the respective part.

The synthesis of P(Ox)s was accomplished as previously described.[2] As a consequence, the procedure is briefly described for $P(EtOx)_{61}$.

In a microwave vial, EtOx (6060 µL, 60.0 mmol), MeTos (150.0 µL, 0.1 mmol) and acetonitrile (8.79 mL) were mixed under inert conditions and the reaction mixture was heated to 140 °C for a predetermined time and subsequently quenched by either the addition of 500 µL of deionized H_2O or a twenty fold excess of ethylene diamine (EDA). The reaction mixtures were subsequently stirred at room temperature overnight. The resulting solution was purified *via* precipitation from ice cold diethyl ether. The polymer was filtered off and re-dissolved in CH_2Cl_2 and the solvent was evaporated under reduced pressure to obtain the product as a white crystalline solid.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 4.34 (0.1H, s, backbone-OH), 3.44 (4.0H, s, backbone), 3.02 (0.3H, s, CH_3-backbone), 2.4 (1.7H, m, CH_2 (EtOx)), 1.11 (2.5H, s, CH_3 (EtOx)) ppm.

SEC (eluent: DMAc, 0.21% LiCl, PS-standard): M_n = 11,200 g mol^{-1}, M_w = 12,200 g mol^{-1}, Đ = 1.09.

SEC (eluent: $CHCl_3$-*i*-PrOH-NEt_3 (94:2:4), PS-cal.): M_n = 6,100 g mol^{-1}, M_w = 7,000 g mol^{-1}, Đ = 1.15.

Amine functionalization of $P(EtOx)_{61}$

The amine functionalization of $P(EtOx)_{61}$ was performed according to the procedure of Nguyen *et al.* under standard schlenk conditions.[3] In a typical procedure 1 g $P(EtOx)_{61}$ (0.15 mmol) was dissolved in 12 mL *N,N*-dimethylformamide. The solution was cooled in an ice bath and 0.045 g NEt_3 (0.44 mmol) was added and stirred for 10 min. 0.089 g *p*-Nitrophenyl chloroformate (0.44 mmol) was dissolved in 2 mL dimethylacetamide, added

dropwise and the reaction mixture was stirred at room temperature overnight. 0.353 g ethylene diamine (5.9 mmol) was dissolved in 2.4 mL *N,N*-dimethylformamide and added dropwise to the reaction mixture at room temperature and stirred overnight. Afterwards the amine functionalized $P(EtOx)_{61}$-NH_2 was precipitated from cold diethyl ether, washed, dissolved in water and lyophilized.

SEC (eluent: $CHCl_3$-*i*-PrOH-NEt_3 (94:2:4), PS-cal.): M_n = 3,100 g mol^{-1}, M_w = 3,500 g mol^{-1}, Đ = 1.14.

Labeling of amine functionalized polymers with sulforhodamine B acid chloride

For a typical labeling reaction using sulforhodamin B acid chloride 1 equivalent polymer was dissolved in dimethylacetamide and 1 equivalent of a 20 mg mL^{-1} sulforhodamin B acid chloride solution was added at room temperature. The reaction mixture was stirred overnight at room temperature under light exclusion and then directly dialyzed against water in a regenerated cellulose membrane (cut-off 1,000 to 3,500 g mol^{-1}) and freeze dried. Afterwards, the polymer was re-dissolved in methanol and purified by preparative size-exclusion chromatography using Sephadex-LH 20.

$P(MeOx)_{57}$-Rhodamine B: SEC (eluent: DMAc, 0.21% LiCl, PS-cal.): M_n = 11,900 g mol^{-1}, M_w = 13,000 g mol^{-1}, Đ = 1.09.

$P(EtOx)_{61}$-Rhodamine B: SEC (eluent: $CHCl_3$-*i*-PrOH-NEt_3 (94:2:4), PS-cal.): M_n = 3,700 g mol^{-1}, M_w = 4,200 g mol^{-1}, Đ = 1.13.

Synthesis of diphenylmethyl-P(EO)$_{57}$ (P(EO)$_{57}$)

The synthesis of P(EO)$_{57}$ was carried out in the BüchiGlasUster PicoClave. Preparations of the initiator solutions for the reaction were exclusively performed in a glove box, using Schott Duran flasks with caps having special connectors for the introduction to the reactor. First, THF and diphenylmethyl potassium (DPMK) were mixed in a GL45 bottle and then transferred into the reactor. The solution was stirred and cooled to –20 °C. Afterwards, the exact amount of ethylene oxide (EO) was added by a mini CORI-FLOW, and the reaction mixture was heated stepwise to 45 °C. The polymerizations were carried out for 24 hours and terminated by the addition of EtOH/AcOH (95:5). The product was obtained by precipitation in cold diethyl ether and drying under vacuum, appearing as a white powder.

DPMK: 5.77 mL (c = 0.69 mmol mL^{-1}, 4.0 mmol), EO: 10 g, 11.76 mL (227 mmol). Yield: 4.76 g (48%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.41-6.98 (m, arom. CH DPM), 4.12 (t, CH DPM), 3.73-3.47 (PEO backbone) ppm.

M_n (^{1}H-NMR): 2,400 g mol^{-1}.

SEC ($CHCl_3$-*i*-PrOH-NEt_3 (94:2:4), P(EO)-cal.): M_n = 2,500 g mol^{-1}, M_w = 2,700 g mol^{-1}, Đ = 1.05.

Cytotoxicity

The cytotoxicity was tested with L929 cells, as this sensitive cell line is recommended by ISO10993-5. In detail, cells were seeded at 10^4 cells per well in a 96-well plate and incubated for 24 h. No cells were seeded in the outer wells. After exchanging the media with fresh one and 30 min incubation, polymers at the indicated end concentrations were added, and the cells were

incubated at 37 °C for further 24 h. Subsequently, the medium was replaced by fresh media and AlamarBlue (Life Technologies, Darmstadt, Germany) as recommended by the supplier. After incubation for 4 h, the fluorescence was measured at λ_{Ex} = 570 nm, λ_{Em} = 610 nm, with untreated cells on the same well plate serving as controls. The experiments were performed independently three times.

Hemolysis assay and erythrocyte aggregation

All animal husbandry is performed in compliance with the relevant European and German laws, institutional guidelines and to state the institutional animal committee. The sheep blood was taken for general veterinary management of the animal health.

To assess the hemolytic activity of the polymer solutions, blood from sheep, collected in heparinized-tubes (Institut für Versuchstierkunde und Tierschutz / Laboratory of Animal Science and Animal Welfare, Friedrich Schiller University Jena), was centrifuged at 4500 × g for 5 min, and the pellet was washed three times with cold 1.5 mmol L^{-1} phosphate buffered saline (PBS, pH 7.4). After dilution with PBS in a ratio of 1:7, aliquots of erythrocyte suspension were mixed 1:1 with the polymer solution and incubated in a water bath at 37 °C for 60 min. After centrifugation at 2400 × g for 5 min the hemoglobin release into the supernatant was determined spectrophotometrically using a microplate reader (TECAN Infinite M200 PRO) at λ_{Ex} = 544 nm wavelength. Complete hemolysis (100%) was achieved using 1% Triton X-100 serving as positive control. Thereby, PBS served as negative control (0%). A value less than 2% hemolysis rate was taken as non-hemolytic. Experiments were run in triplicates and were performed with three different blood donors.

For the examination of the erythrocyte aggregation, erythrocytes were isolated as described above. An erythrocytes suspension was mixed with the same volume of polymer solution in a clear flat bottomed 96-well plate. The cells were incubated at 37 °C for 2 h, and the absorbance was measured at λ_{Ex} = 645 nm in a microplate reader (TECAN Infinite M200 Pro). 25 kDa bPEI (50 µg mL^{-1}) was used as positive control and PBS treated cells served as the negative control. Absorbance values of the test solutions lower than negative control were regarded as aggregation. Experiments are the result of triplicates and were performed with three different donor blood batches.

Lactate dehydrogenase (LDH) assay

For the LDH assay the Lactate Dehydrogenase Activtity Assay Kit (Sigma Aldrich) was used. The assay was performed as recommended by the supplier. The samples were incubated for 4 h in growth medium with HEK-293 cells.

Cell culture

HEK-293 cells (CRL-1573, ATCC) were cultured in DMEM medium (Merck, Darmstadt, Germany) supplemented with 1 g L^{-1} glucose, 10% fetal calf serum (FCS, v/v), 100 µg mL^{-1} streptomycin, 100 IU mL^{-1} penicillin and 2 mM L-glutamine at 37 °C in a humidified 5% CO_2 atmosphere.

Cell uptake

For CLSM studies, 0.15 × 10^6 cells mL^{-1} were seeded on glass-bottomed dishes (CellView cell culture dishes with four compartments, Greiner bio-one) and cultivated for 24 h. One hour prior

to the nanoparticle addition, the medium was changed to 0.5 mL fresh growth media. 50µL of the medium were replaced by 50µL of the following Nile red containing solutions:

1. PLGA nanoparticles (1 mg mL^{-1}) with Nile red in 0.3wt% aq. $P(EtOx)_{61}$,
2. PLGA nanoparticles (1 mg mL^{-1}) with Nile red in 0.3wt% aq. $P(MeOx)_{57}$,
3. PLGA nanoparticles (1 mg mL^{-1}) with Nile red in MilliQ,
4. Nile red in 0.3wt% aq. $P(EtOx)_{61}$,
5. Nile red in 0.3wt% aq. $P(MeOx)_{57}$,
6. Nile red in MilliQ.

The nanoparticles were prepared as described below. In addition, a control sample was measured. Experiments were conducted once.

Nanoparticle formation via nanoemulsion

The particle formation *via* nanoemulsion technique was described elsewhere.[4] Briefly, the capsid polymer and the drug (Nile red) were dissolved in ethyl acetate in a certain concentration. Ultra-pure water bearing a predetermined amount of surfactant was added to a 15 mL falcon tube. The polymer-drug-solution was added carefully using an Eppendorf pipette. The solutions were sonicated for 10 s (power: 40 W, cycle: 100%, amplitude: 100%) using an ultra-sonicator (Hielscher, UP200St). After sonication, the solution was transferred to a 50 mL falcon tube equipped with a magnetic stir bar and ultra-pure water to achieve a 10-fold dilution. The particle suspension was stirred under flow overnight to evaporate the organic solvent. Subsequently, the size, PDI and zeta potential (ZP) were determined using 1 mL of the particle suspension. The remaining suspension was divided and purified as subscribed. All particles were then frozen in a -80 °C freezer and lyophilized overnight. All experiments were conducted in triplicates.

Nanoparticle formation via nanoprecipitation

The particle formation *via* nanoprecipitation technique was modified from literature.[5] Briefly, the capsid polymer and the drug (Nile red) were dissolved in acetone. Ultra-pure water bearing a predetermined amount of surfactant and a magnetic stir bar were placed in a 50 mL falcon tube. The polymer-drug-solution was added to *via* syringe pump (World Precision Instruments, AL-300) usage to the water under continuous magnetic stirring (750 rpm). The particle suspension was stirred under flow overnight to evaporate the organic solvent. Subsequently, the size, PDI value and zeta potential were determined using 1 mL of the particle suspension. The remaining suspension was divided and purified as subscribed. All particles were then frozen in a -80 °C freezer and lyophilized overnight. All experiments were conducted in triplicates.

For CLSM cell uptake studies, reference substances were prepared without the capsid polymer PLGA.

PKC 412 encapsulation via nanoprecipitation

Drug encapsulation experiments were performed *via* nanoprecipitation. 10 mg PLGA were dissolved in 1 mL acetone. 0.3 mg PKC 412 were dissolved in 30 µL DMSO and mixed with the PLGA solution. The drug-polymer mixture was then added to a 50 mL falcon tube containing 10 mL of either ultra-pure water or a 0.3wt% P(Ox) solution *via* syringe pump under continuous stirring (1000 rpm). The solvent was evaporated under in a fume hood overnight at room temperature in the dark. 1 mL of the particle suspension was separated for DLS measurements. The remaining suspension was centrifuged at 11,000 rpm for 45 min and resuspended in either 1 mL ultra-pure water or the corresponding 0.5wt% P(Ox) solution before freezing in a -80 °C freezer and lyophilization overnight. The encapsulation efficiency was determined *via* UV/Vis

measurements at λ_{Ex} = 280 nm. For this purpose, the nanoparticles were dissolved in DMSO. All experiments were conducted in triplicates.

Microparticle preparation

The preparation of microparticles was described before.[6] Briefly, 15 mg P(Ox) were dissolved in 1.5 mL ultra-pure water. 10 mg PLGA were dissolved in 1 mL EtOAc and added carefully to the surfactant solution. The two phases were emulsified (13,500 min^{-1}, Ultra-Turrax T25, IKA Labortechnik) and subsequently diluted using 13.5 mL ultra-pure water. The organic solvent was evaporated in a fume hood at room temperature overnight under continuous stirring. Centrifugation was conducted at 4 °C and 7,000 rpm for 10 min at 4 °C and the microparticles were resuspended using ultra-pure water after discarding the supernatant.

Results

Table S1. Key-properties of the synthesized P(Ox)s, determined by [a]^{1}H-NMR (300 MHz, $CDCl_3$), [b]SEC (DMAc, 0.05% LiCl; PS-cal.), [c]SEC ($CHCl_3$-*i*-PrOH-NEt_3; PS-cal.).

Polymer	**DP[a]**	**Mn[a]** [g mol^{-1}]	**Mn[b]** [g mol^{-1}]	**Mw[b]** [g mol^{-1}]	**Đ[b]**
$P(EtOx)_{25}$	25	2,500	5,800	6,300	1.09
$P(EtOx)_{61}$	61	6,000	11,200	12,200	1.09
			6,100[c]	7,000[c]	1.15[c]
$P(EtOx)_{61}$-NH_2	61	6,000	3,100[c]	3,500[c]	1.14[c]
$P(EtOx)_{61}$-Rhodamine B	61	6,000	3,700[c]	4,200[c]	1.13[c]
$P(EtOx)_{107}$	107	10,700	16,400	19,100	1.16
$P(EtOx)_{184}$	184	18,400	20,900	25,300	1.21
$P(MeOx)_{25}$	25	2,100	4,800	5,300	1.09
$P(MeOx)_{57}$	57	4,800	5,500	6,300	1.14
$P(MeOx)_{57}$-NH_2	57	4,800	12,000	12,900	1.07
$P(MeOx)_{57}$-Rhodamine B	57	4,800	11,900	13,000	1.09
$P(MeOx)_{98}$	98	8,300	15,100	18,000	1.19
$P(MeOx)_{211}$	211	17,900	21,800	30,100	1.38

Table S2. Key properties of the synthesized polymers. [a]^{1}H-NMR (300 MHz, $CDCl_3$), [b]SEC (DMAc, 0.05% LiCl; PMMA-cal.), [c]SEC($CHCl_3$-*i*-PrOH-NEt_3, P(EO)-cal.). n.a.: not available.

Polymer	**DP[a]**	**Mn[a]** [g mol^{-1}]	**Mn[b]** [g mol^{-1}]	**Mw[b]** [g mol^{-1}]	**Đ[b]**
P(MMA$_{97}$-*co*-BocMAEMA$_{32}$)	129[2]	n.a.	17,400	20,700	1.19
P(MMA$_{97}$-*co*-MAEMA$_{32}$)	129[2]	n.a.	21,600	25,900	1.20
DPM-P(EO)$_{57}$	57	2,400	2,500[c]	2,700[c]	1.05[c]

Table S3. Z-average and PDI ratios of PLGA nanoparticles using different cryoprotectants at various concentrations. Diameter size ratios of the z-average and PDI ratios of the nanoparticles were determined by DLS measurements. The ratios were calculated using Eq. 1 and 2. n.d.: not determined. Nanoparticles were prepared by nanoprecipitation (water and acetone). Data represents the mean of three samples and five measurements each.

Conc. [wt%]	**$P(EtOx)_{61}$**		**$P(EO)_{57}$**		**Saccharose**		**Glucose**		**Trehalose**	
	Z-average ratio	*PDI ratio*	*Z-average ratio*	*PDI ratio*	*Z-average ratio*	*PDI ratio*	*Z-average ratio*	*PDI ratio*	*Z-average ratio*	*PDI ratio*
10	n.d.	n.d.	n.d.	n.d.	1.0	1.8	1.0	2.1	1.8	3.3
5	1.7	2.5	3.0	6.2	1.0	2.2	2.1	4.3	1.4	2.7
3	n.d.	n.d.	n.d.	n.d.	1.3	3.6	2.4	5.1	2.1	4.6
2.5	1.6	2.4	2.7	5.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1	1.6	2.3	3.9	5.9	3.6	6.7	1.5	4.2	22.2	4.9
0.5	1.5	2.0	2.6	4.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0.1	1.6	2.9	15.2	9.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0.05	2.1	4.1	14.0	8.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0	26.4	7.0	26.4	7.0	26.4	7.0	26.4	7.0	26.4	7.0

Table S4. Properties of PLGA nanoparticles prepared *via* nanoprecipitation (water and acetone) or nanoemulsion (water and ethyl acetate) technique, using different surfactants determined by DLS measurements. Data represents the mean of three samples and five measurements each.

Conc. [wt%]	**Pluronic F127**		**PVA**		**$P(EtOx)_{61}$**		**$P(MeOx)_{57}$**	
	Z-average [d, nm]	*PDI*	*Z-average [d, nm]*	*PDI*	*Z-average [d, nm]*	*PDI*	*Z-average [d, nm]*	*PDI*
Nanoprecipitation								
1	n.d.	n.d.	n.d.	n.d.	116.7	0.086	195.8	0.068
0.5	119.2	0.106	153.9	0.114	122.2	0.153	n.d.	n.d.
0.3	113.7	0.105	149.7	0.099	137.6	0.093	n.d.	n.d.
0	97.9	0.103	97.9	0.103	97.9	0.103	97.9	0.103
Nanoemulsion								
1	n.d.	n.d.	n.d.	n.d.	220.0	0.141	159.7	0.086
0.5	88.6	0.230	105.7	0.166	329.0	0.419	n.d.	n.d.
0.3	99.0	0.219	122.2	0.104	550.5	0.587	n.d.	n.d.
0	231.1	0.353	231.1	0.353	231.1	0.353	231.1	0.353

Table S5. Properties of PLGA nanoparticles prepared nanoprecipitation (water and acetone) or nanoemulsion (water and ethyl acetate), using hydrophilic P(Ox)s with varying DP as surfactants (nanoprecipitation: 0.3wt%, nanoemulsion: 1wt%). Z-average and PDI values were determined *via* DLS measurements. Data represents the mean of three samples and five measurements each.

Nanoprecipitation				
DP	**P(EtOx)**		**P(MeOx)**	
	Z-average [d, nm]	*PDI*	*Z-average [d, nm]*	*PDI*
25	157.6	0.071	169.8	0.059
60	137.6	0.093	195.8	0.069
100	164.4	0.076	172.9	0.069
200	172.5	0.074	181.8	0.124
Nanoemulsion				
DP	**P(EtOx)**		**P(MeOx)**	
	Z-average [d, nm]	*PDI*	*Z-average [d, nm]*	*PDI*
25	808.1	0.754	803.7	0.289
60	217.4	0.164	159.7	0.086
100	230.2	0.146	273.2	0.165
200	194.2	0.086	200.5	0.090

Table S6. Z-average and PDI ratios of PLGA nanoparticles prepared by nanoprecipitation (water and acetone) technique using 0.3wt% P(Ox). The particles were lyophilized without further purification and the ratio was calculated using Eq. 1 and 2. Data represents the mean of three samples and five measurements each.

DP	**P(EtOx)**		**P(MeOx)**	
	Z-average ratio	*PDI ratio*	*Z-average ratio*	*PDI ratio*
25	1.7	3.7	1.7	5.7
60	1.0	1.6	1.1	2.1
100	1.0	1.5	1.1	2.1
200	1.0	1.5	1.0	1.1

Table S7. Z-average and PDI values of PLGA nanoparticles after lyophilization, dependent on the purification method determined by DLS measurements. Nanoparticles were prepared by nanoprecipitation (water and acetone). Data represents the mean of three samples and five measurements each.

Additive	**$P(EtOx)_{61}$**		**$P(MeOx)_{57}$**	
Purification method before lyophilization	*Z-average [d, nm]*	*PDI*	*Z-average [d, nm]*	*PDI*
After preparation	157.7 ± 1.3	0.102 ± 0.017	201.7 ± 4.2	0.062 ± 0.029
centrifugation and resuspension in 0.5% P(Ox)	898 ± 1062	0.549 ± 0.305	206.7 ± 3.1	0.111 ± 0.034
Syringe filtration and addition of 0.5% P(Ox) solution	165.1 ± 1.0	0.172 ± 0.044	189.6 ± 1.2	0.068 ± 0.018
Syringe filtration	1075 ± 111.6	0.948 ± 0.100	190.6 ± 1.5	0.101 ± 0.019

Table S8. Key properties of particles of various capsid polymers using different surfactants (P(Ox): 1%, PVA: 0.3%, Pluronic F127: 0.5%) prepared *via* nanoemulsion (water and ethyl acetate) determined by DLS measurements. n.a.: not available because of particle aggregation. Data represents the mean of three samples and five measurements each.

Hydrophobic polymer	Surfactant	Z-average [d, nm]	PDI	ZP [mV]
PLGA	$P(EtOx)_{61}$	214.9 ± 1.5	0.147 ± 0.030	-33.0 ± 0.9
	$P(MeOx)_{57}$	155.8 ± 0.9	0.087 ± 0.023	-37.9 ± 0.5
	PVA	120.8 ± 1.3	0.104 ± 0.029	-6.9 ± 0.3
	Pluronic® F127	83.5 ± 1.7	0.214 ± 0.012	-30.6 ± 3.6
	None	n.a.	n.a.	n.a.
PCL	$P(EtOx)_{61}$	n.a.	n.a.	n.a.
	$P(MeOx)_{57}$	n.a.	n.a.	n.a.
	None	n.a.	n.a.	n.a.
Eudragit® RS 100	$P(EtOx)_{61}$	214.0 ± 0.7	0.063 ± 0.018	35.7 ± 0.4
	$P(MeOx)_{57}$	244.3 ± 2.5	0.081 ± 0.012	42.1 ± 1.7
	None	101.6 ± 0.5	0.246 ± 0.020	58.6 ± 0.4
$P(MMA_{97}$-co-$MAEMA_{32})$	$P(EtOx)_{61}$	155.2 ± 2.8	0.165 ± 0.012	32.3 ± 0.1
	$P(MeOx)_{57}$	162.9 ± 1.3	0.138 ± 0.028	33.6 ± 1.4
	PVA	127.1 ± 1.7	0.198 ± 0.010	31.6 ± 0.5
	None	176.2 2.4	0.191 ± 0.012	49.1 ± 0.4

Table S9. Z-average and PDI values of nanoparticles consisting of P(MMA_{80}-*co*-$MAEMA_{20}$) or Eudragit RS100 depending on the purification method determined by DLS investigations. Nanoparticles were prepared by nanoprecipitation (water and acetone). Data represents the mean of three samples and five measurements each. n.a.: not available because of particle aggregation. *$P(MeOx)_{57}$ was used.

$P(MMA_{97}$-*co*-$MAEMA_{32})$						
Additive	**$P(EtOx)_{61}$**		**$P(MeOx)_{57}$**		**None**	
Purification method	*Z-average [d, nm]*	*PDI*	*Z-average [d, nm]*	*PDI*	*Z-average [d, nm]*	*PDI*
After preparation	155.2 ± 2.8	0.165 ± 0.012	162.9 ± 1.3	0.138 ± 0.028	176.2 ± 2.4	0.191 ± 0.012
None	172.2 ± 2.7	0.215 ± 0.021	157.5 ± 1.9	0.109 ± 0.026	n.a.	n.a.
Centrifugation and resuspension in 1 mL ultra-pure water	229.2 ± 14.9	0.330 ± 0.019	294.7 ± 67.1	0.375 ± 0.033	325.8 ± 88.1	0.362 ± 0.034
Centrifugation and resuspension in 1 mL 0.5wt% P(Ox) solution	175.4 ± 2.3	0.258 ± 0.035	179.7 ± 3.8	0.128 ± 0.083	253.4 ± 4.1*	0.278 ± 0.042*
Syringe filtration	n.a.	n.a.	211.6 ± 3.1	0.269 ± 0.032	n.a.	n.a.
Eudragit® RS100						
Additive	**$P(EtOx)_{61}$**		**$P(MeOx)_{57}$**		**None**	
Purification method	*Z-average [d, nm]*	*PDI*	*Z-average [d, nm]*	*PDI*	*Z-average [d, nm]*	*PDI*
After preparation	*214.0* ± 0.7	*0.063* ± 0.018	*244.3* ± 2.5	*0.081* ± 0.012	*101.6* ± 0.5	*0.246* ± 0.020
None	217.2 ± 2.2	0.080 ± 0.024	247.3 ± 4.3	0.086 ± 0.038	n.a.	n.a.
Centrifugation and resuspension in 1 mL ultra-pure water	239.3 ± 1.7	0.105 ± 0.037	315.9 ± 8.3	0.220 ± 0.044	n.a.	n.a.

Centrifugation and resuspension in 1 mL 0.5wt% P(Ox) solution	223.3 ± 1.5	0.069 ± 0.026	252.0 ± 3.4	0.105 ± 0.039	n.a.*	n.a.*
Syringe filtration	220.4 ± 4.4	0.062 ± 0.042	257.5 ± 3.7	0.068 ± 0.033	n.a.	n.a.

Table S10. Z-average and PDI ratios of nanoparticles consisting of P(MMA_{80}-*co*-$MAEMA_{20}$) or Eudragit® RS100 depending on the purification method determined by DLS investigations. Nanoparticles were prepared by nanoprecipitation (water and acetone). Data represents the mean of three samples and five measurements each. Ratios were calculated using Eq. 1 and 2. Actual size and PDI values can be found in Table S9. n.a.: not available because of particle aggregation. *$P(MeOx)_{57}$ was used.

$P(MMA_{97}$-*co*-$MAEMA_{32})$						
Additive	**$P(EtOx)_{61}$**		**$P(MeOx)_{57}$**		**None**	
Purification method	*Size ratio*	*PDI ratio*	*Size ratio*	*PDI ratio*	*Size ratio*	*PDI ratio*
None	1.11	1.30	0.97	0.79	n.a.	n.a.
Centrifugation and resuspension in 1 mL ultra-pure water	1.48	2.00	1.01	1.06	1.85	1.90
Centrifugation and resuspension in 0.5wt% P(Ox)	1.13	1.56	1.10	0.93	1.44*	1.46*
Syringe filtration	n.a.	n.a.	1.30	1.95	n.a.	n.a.
Eudragit® RS100						
Additive	**$P(EtOx)_{61}$**		**$P(MeOx)_{57}$**		**None**	
Purification method	*Size ratio*	*PDI ratio*	*Size ratio*	*PDI ratio*	*Size ratio*	*PDI ratio*
None	1.01	1.27	1.01	1.06	n.a.	n.a.
Centrifugation and resuspension in 1 mL ddH_2O	1.12	1.67	1.29	2.71	n.a.	n.a.
Centrifugation and resuspension in 0.5% P(Ox)	1.04	1.10	1.03	1.30	n.a.*	n.a.*
Syringe filtration	1.03	0.98	1.05	0.84	n.a.	n.a.

Table S11. Key properties of the PLGA nanoparticles using the model drug Nile red, prepared by either nanoprecipitation (water and acetone) or nanoemulsion (water and ethyl acetate) technique (after preparation) determined by DLS measurements. Data represents the mean of three samples and five measurements each. n.a.: not available because of particle aggregation.

Surfactant	**Preparation method**	**Size [d, nm]**	**PDI**
$P(EtOx)_{61}$	Nanoprecipitation	160.8 ± 1.5	0.053 ± 0.028
$P(EtOx)_{61}$	Nanoemulsion	190.7 ± 1.9	0.124 ± 0.013
$P(MeOx)_{57}$	Nanoprecipitation	151.2 ± 0.8	0.065 ± 0.021
$P(MeOx)_{57}$	Nanoemulsion	180.0 ± 0.8	0.099 ± 0.014
None	Nanoprecipitation	145.2 ± 2.5	0.075 ± 0.018
None	Nanoemulsion	n.a.	n.a.

Table S12. Actual z-average and PDI values of Nile red containing PLGA nanoparticles after lyophilization, dependent on the purification method (1 mL each) determined by DLS investigations. Nanoparticles were prepared by nanoprecipitation (water and acetone). Data represents the mean of three samples and five measurements each. n.r.: not resuspendable; n.a.: not available because of particle aggregation.

Additive	**$P(EtOx)_{61}$**		**$P(MeOx)_{57}$**		**None**	
Purification method before lyophilization	*Size [d, nm]*	*PDI*	*Size [d, nm]*	*PDI*	*Size [d, nm]*	*PDI*
After preparation	160.8 ± 1.5	0.053 ± 0.028	169.0 ± 2.5	0.069 ± 0.021	145.2 ± 2.5	0.075 ± 0.018
None	168.0 ± 1.6	0.087 ± 0.031	n.a.	n.a.	n.a.	n.a.
Centrifugation and resuspension in 1 mL 0.5wt% P(Ox) solution	184.3 ± 1.6	0.145 ± 0.028	187.4 ± 3.2	0.123 ± 0.052	n.r.	n.r.
Syringe filtration and addition of 1 mL 0.5wt% P(Ox) solution	167.7 ± 3.2	0.148 ± 0.045	233.1 ± 8.4	0.314 ± 0.022	180.5 ± 1.6	0.169 ± 0.020
Syringe filtration	161.7 ± 3.4	0.157 ± 0.011	n.a.	n.a.	n.a.	n.a.

Table S13. Influence of the particle purification of Nile red containing nanoparticles before lyophilization (1 mL each) on z-average, PDI and EE. Z-average and PDI determined by DLS measurements. Data represents the mean of three samples and five measurements each. Ratios describe the results after lyophilization divided by the initial results after preparation. Particles were prepared *via* nanoprecipitation (water and acetone). n.r.: not resuspendable; n.a.: not available because of particle aggregation. [a]Calculated from UV/vis absorption measurements at λ_{Ex} = 630 nm, mean value of n = 3.

Additive	**P(EtOx)$_{61}$**			**P(MeOx)$_{57}$**			**None**		
Purification method before lyophilization	*Size ratio*	*PDI ratio*	*EE[a] [µg mg^{-1}]*	*Size ratio*	*PDI ratio*	*EE[a] [µg mg^{-1}]*	*Size ratio*	*PDI ratio*	*EE[a] [µg mg^{-1}]*
None	1.04	1.64	0.52	n.a.	n.a.	n.a.	28.21	12.23	1.53
45 min centrifugation and resuspension in 1 mL 0.5% P(Ox) solution	1.14	2.74	0.28	1.15	1.71	0.32	n.a.	n.a.	n.a.
Syringe filtration and addition of 1 mL 0.5% P(Ox) solution	1.04	2.79	0.21	n.a.	n.a.	n.a.	1.24	2.25	0.28
Syringe filtration	1.01	2.96	0.51	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Table S14. Key properties of the PLGA nanoparticles using PKC 412 as the encapsulated drug, prepared by nanoprecipitation (water and acetone) determined by DLS investigations. Data represents the mean of three samples and five measurements each. n.a.: not available because of particle aggregation. [a]Ratios describe the results after lyophilization divided by the initial results after preparation. [b]Calculated from UV/Vis absorption measurements at λ_{Ex} = 293 nm, mean value of n = 3.

Additive	**After preparation**		**After lyophilization**		**Ratio[a]**		
	Size [d, nm]	*PDI*	*Size [d, nm]*	*PDI*	*Size*	*PDI*	*EE[b] [µg mg^{-1}]*
P(EtOx)$_{61}$	168.6 ± 2.4	0.061 ± 0.019	178.9 ± 2.0	0.058 ± 0.025	1.06	0.95	0
P(MeOx)$_{57}$	184.6 ± 1.1	0.063 ± 0.034	190.4 ± 2.4	0.086 ± 0.022	1.03	1.36	0.92 ± 0.08
None	156.1 ± 0.9	0.077 ± 0.024	7272 ± 2494	0.740 ± 0.383	n.a.	n.a.	0.98 ± 0.31

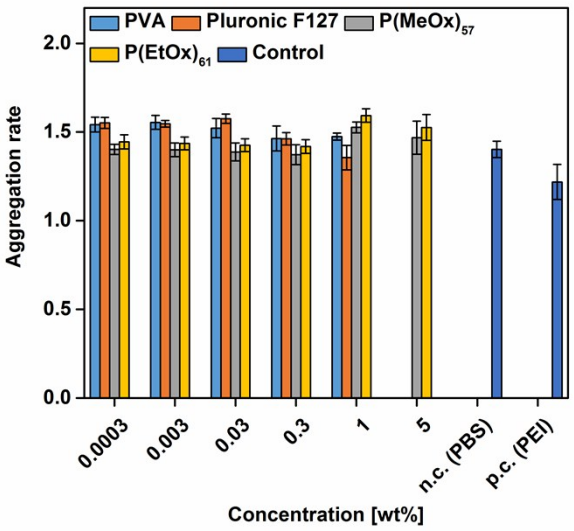

Figure S1. Concentration dependent erythrocyte aggregation of surfactants. PEI represents the positive control (p.c.) and PBS the negative control (n.c.). Values represent the mean ± SD of three measurements.

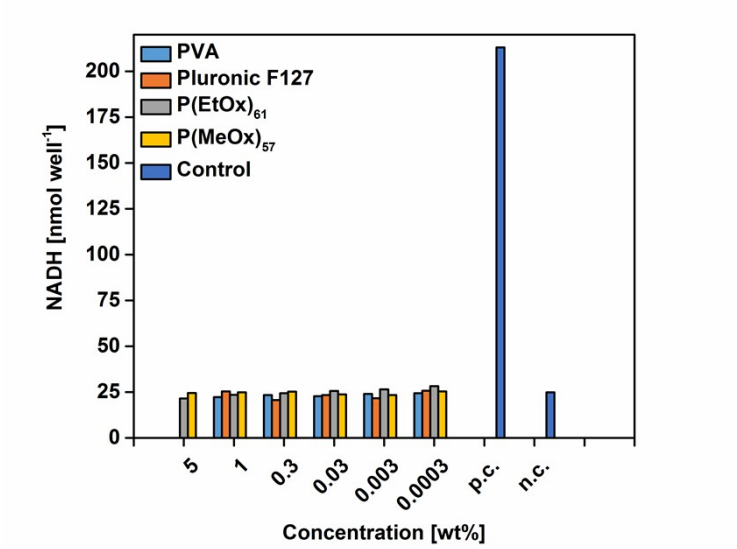

Figure S2. LDH assay of HEK cells. Cells were incubated with surfactants of indicated concentrations for 4 h. Media represent the negative control (n.c.), Triton-X 100 serves as positive control (p.c.).

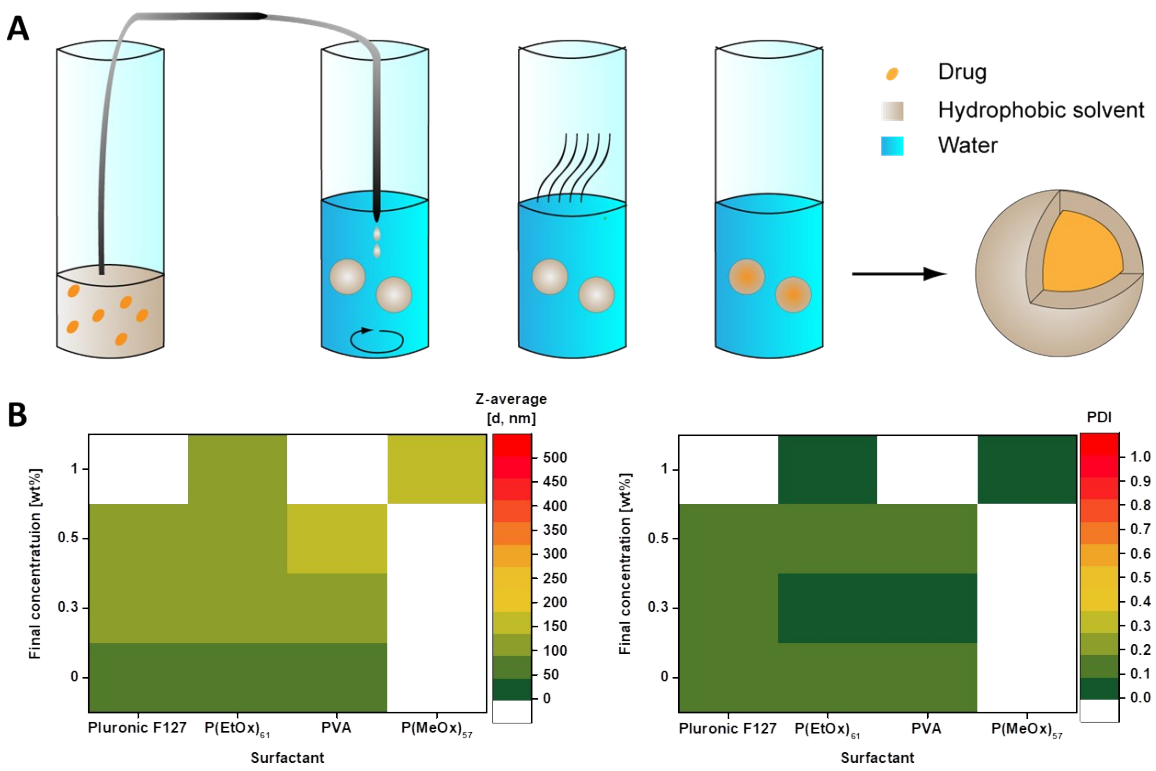

Figure S3: A: Schematic representation of nanoparticle preparation *via* the nanoprecipitation technique. A hydrophobic polymer (and a hydrophobic drug) are dissolved in an organic solvent and dropwise added to water under continuous stirring. After evaporation of the

organic solvent, nanoparticles are obtained. **B**: Properties of PLGA nanoparticles prepared *via* nanoprecipitation (water and acetone), using different surfactants as determined by DLS measurements. Data represents the mean of three samples and five measurements each. Values colored in white were not investigated. See values in Table S4.

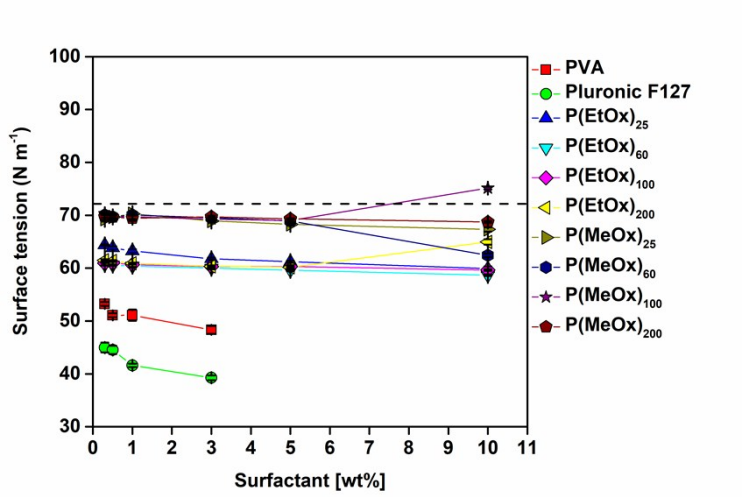

Figure S4. Surface tension of different aqueous surfactant solutions, determined at 20 °C. Dashed line symbolizes the surface tension of pure water. Data represents the mean ± SD of 5 measurements.

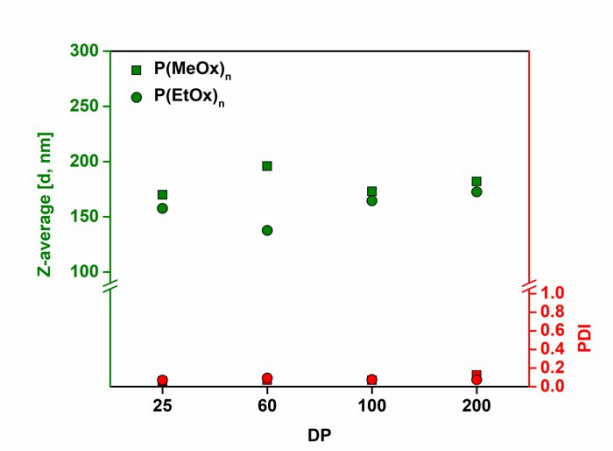

Figure S5: Properties of PLGA nanoparticles prepared by nanoprecipitation, using hydrophilic P(Ox)s with varying DP as surfactants (0.3wt%). Z-average and PDI values were determined *via* DLS investigations. Data represents the mean of three samples and five measurements each. See values in Table S5.

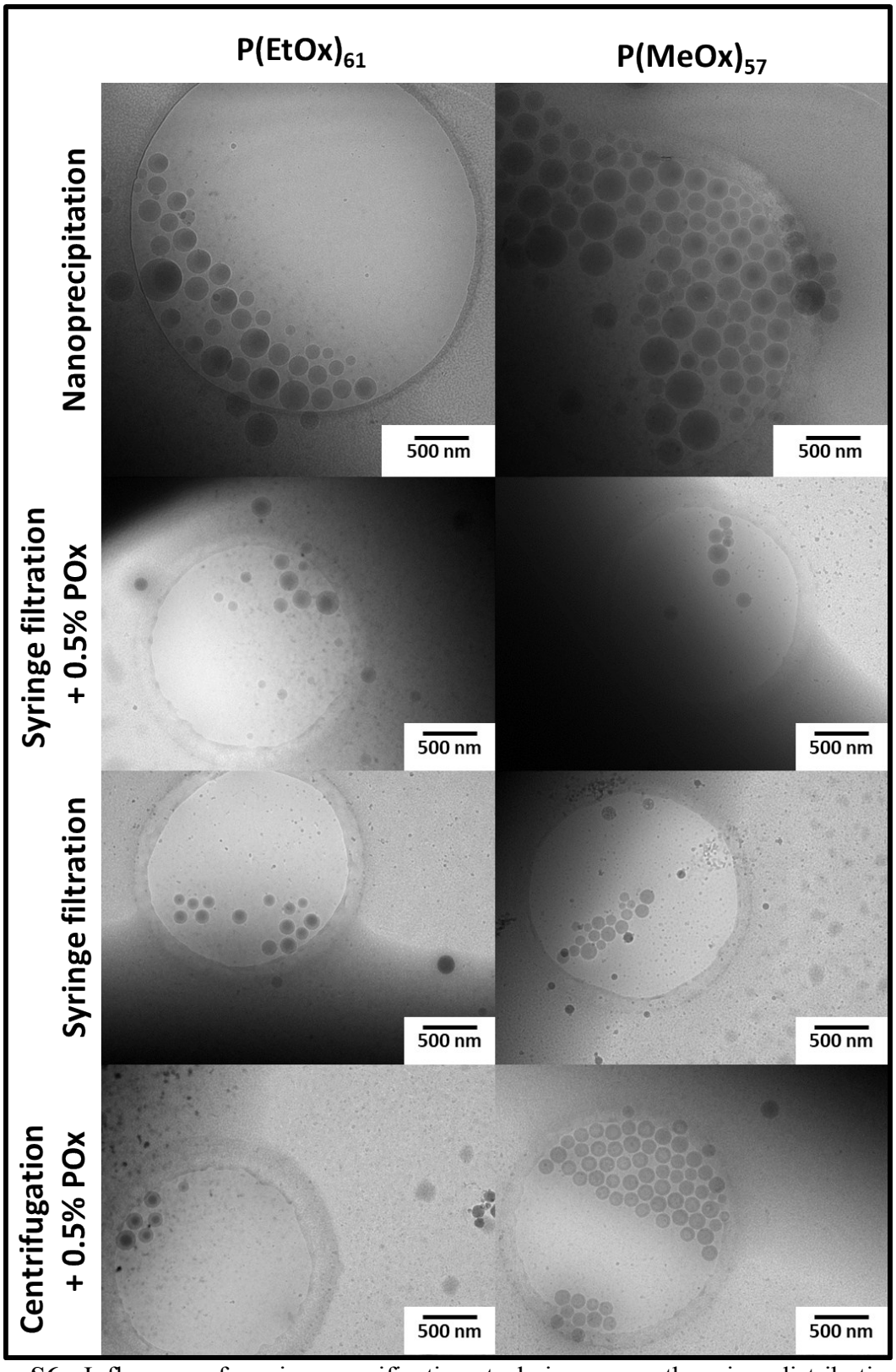

Figure S6. Influence of various purification techniques on the size distribution and morphology of PLGA nanoparticles that were prepared by nanoprecipitation, determined by cryoTEM measurements.

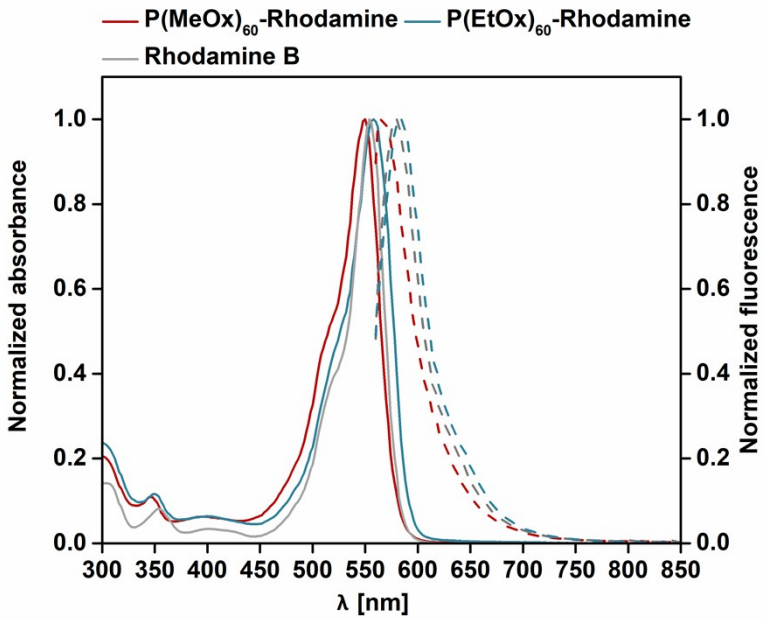

Figure S7. Absorbance (solid lines) and emission (dashed lines) spectra of rhodamine B labelled P(Ox)s and rhodamine B in ultra-pure water. Fluorescence spectra were excited at λ_{Ex}=520 nm. For all samples, a reference spectrum of ultra-pure water was recorded and subtracted from the measurement.

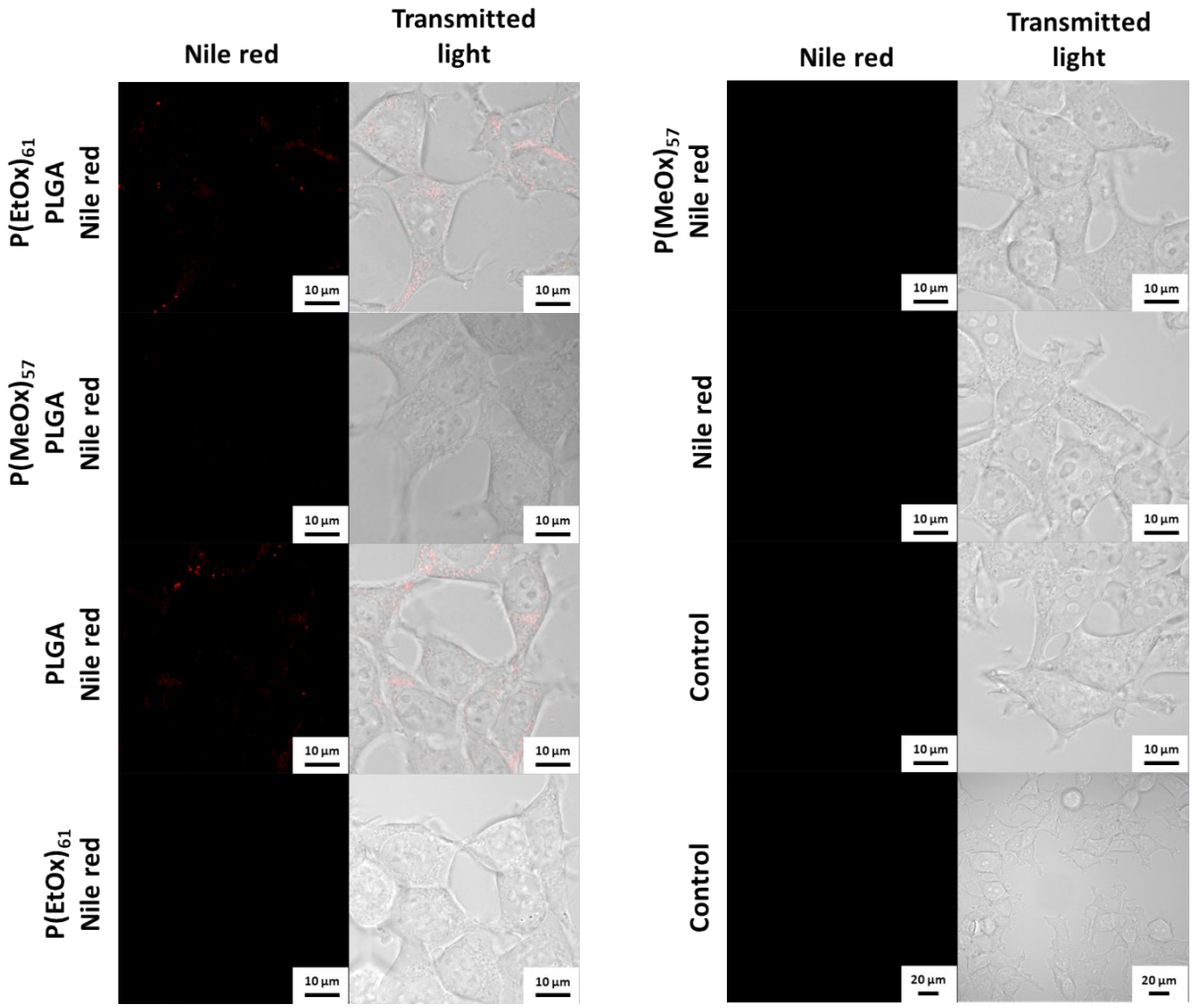

Figure S8. Cellular uptake study of different Nile red formulations. HEK-293 cells were treated with formulations for 30 min in growth media and analyzed *via* CLSM (red: Nile red). See Figure 12 for overview images.

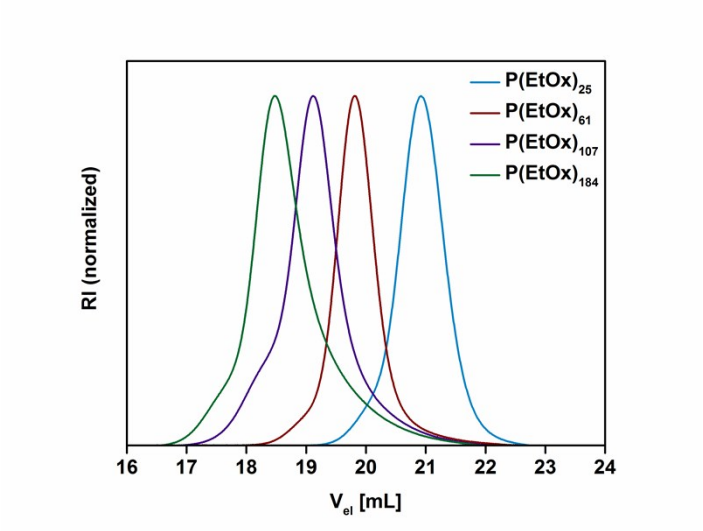

Figure S9. SEC-traces (DMAc, 0.21% LiCl; PS-cal.) of the purified $P(EtOx)_n$.

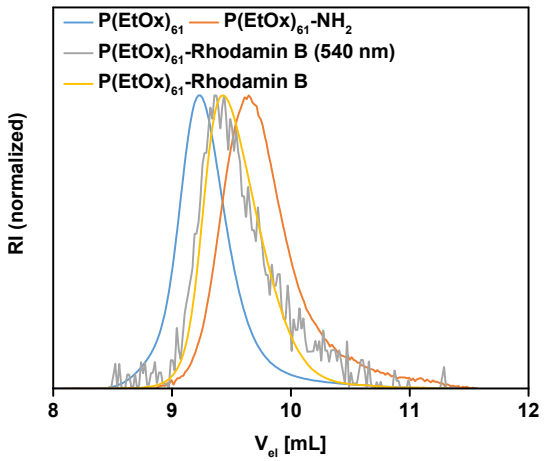

Figure S10. SEC-traces ($CHCl_3$-*i*-PrOH-NEt_3) of the purified $P(EtOx)_{61}$, $P(EtOx)_{61}$-NH_2 and $P(EtOx)_{61}$-Rhodamine B.

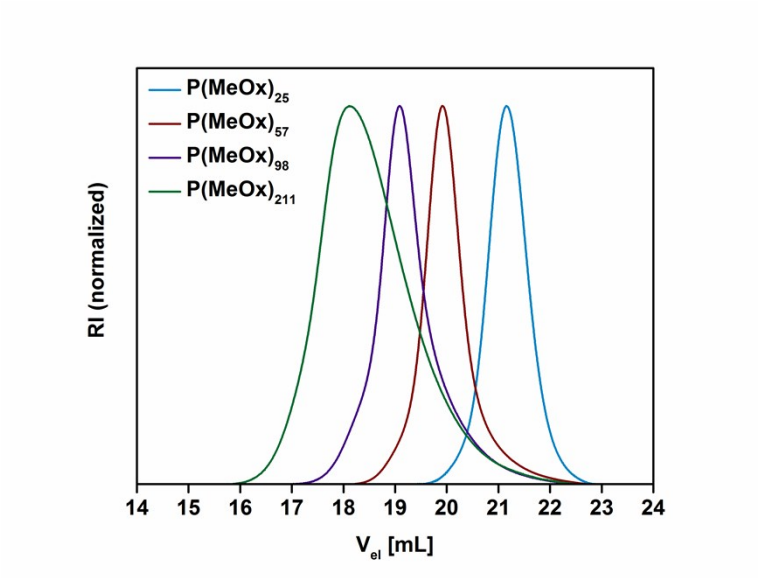

Figure S11. SEC-traces (DMAc, 0.21% LiCl) of the purified $P(MeOx)_n$.

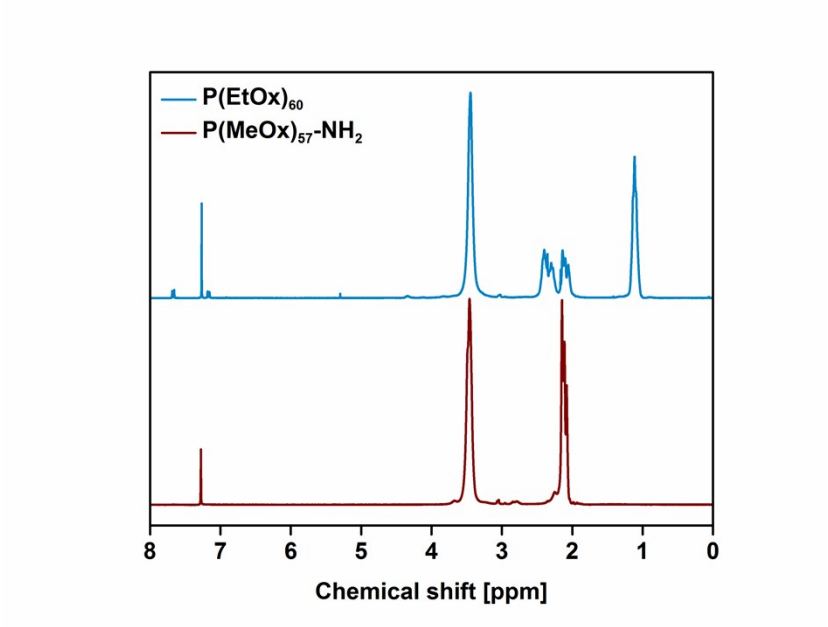

Figure S12. Representative ^{1}H-NMR spectra of the used P(Ox)s, showing the characteristic traces for $P(EtOx)_n$ and $P(MeOx)_n$.

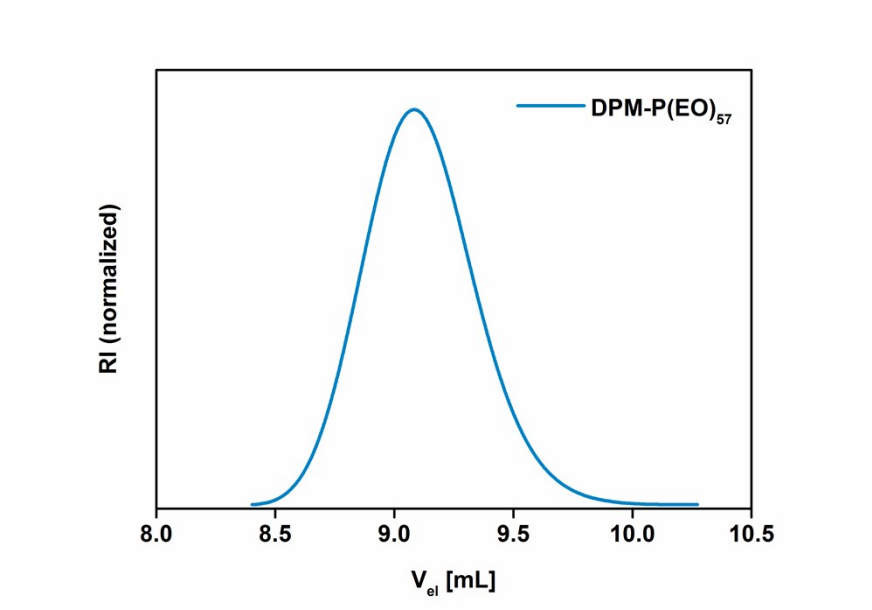

Figure S13. SEC-trace ($CHCl_3$-*i*-PrOH-TEA; P(EO)-cal.) of the purified DPM-$P(EO)_{57}$.

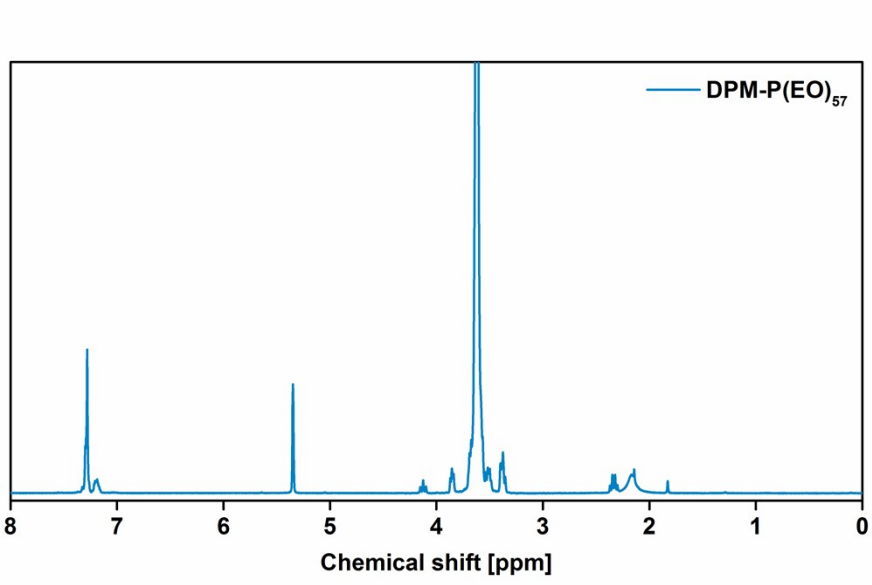

Figure S14. ^{1}H-NMR (300 MHz, $CDCl_3$) of the purified $P(EO)_{57}$.

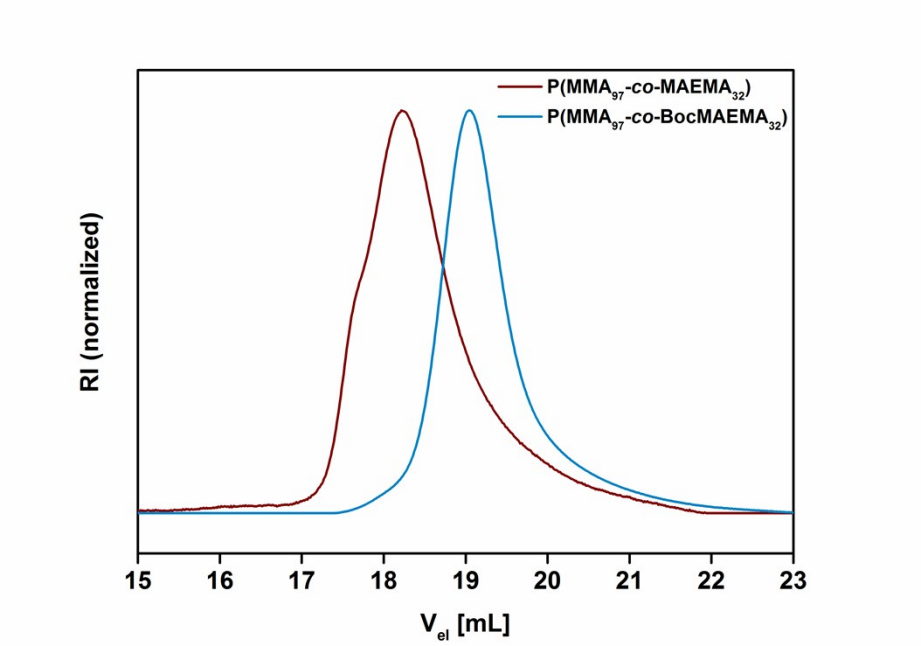

Figure S15. Normalized SEC traces (DMAc, 0.21% LiCl, PMMA-cal.) of P(MMA_{97}-*co*-BocMAEMA_{32}) and P(MMA_{97}-*co*-MAEMA_{32}).

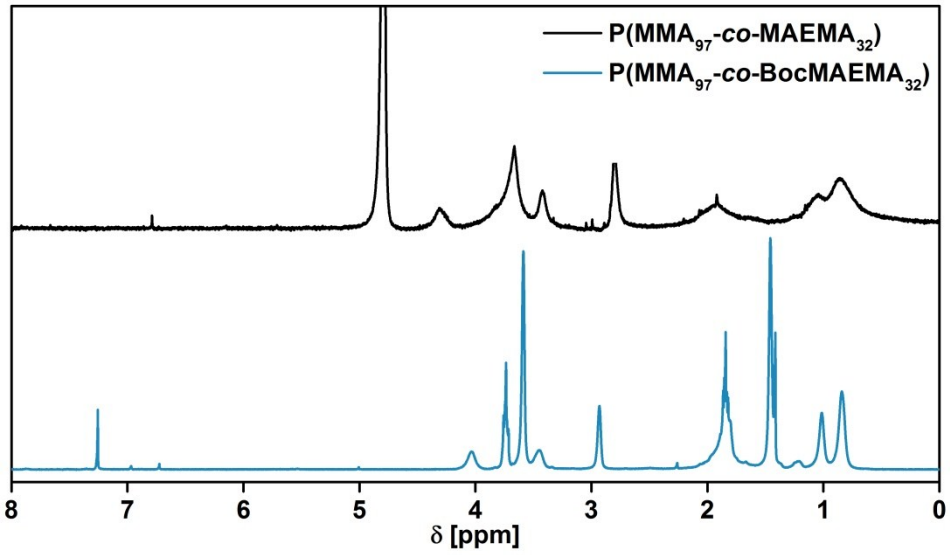

Figure S16. ^{1}H-NMR (300 MHz, $CDCl_3$) of P(MMA_{97}-*co*-BocMAEMA_{32}) and P(MMA_{97}-*co*-MAEMA_{32}).

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