SUPPORTING INFORMATION

Interaction of pH-Responsive Polyanions with Phospholipid Membranes

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Table S1. Overview of the polymers used in this study

Name	Mn [a] (kDa)	Mn[b] (kDa)	Mw[b] (kDa)	PDI
PCEA	10.4	16	17.5	1.03
PCEA#	-	10.2	11.12	1.08
PCEA[f]	14.4	34.0	41.9	1.17
PCPA1	7.7	21.8	23.2	1.06
PCPA2	16	37.1	41.1	1.11
PCPA[f]	18	20.1	20.7	1.03
PPAA	12.8	-	-	-
PMAA[c]	-	-	15	-
PMAA[f]	-	21.5	22.3	1.04
PEI[c]	-	-	18	

[a]Based on ^{1}H NMR, [b]Based on GPC, [c]commercial product.

The difference between Mn by ^{1}H NMR and GPC is probably due to hydrolysis of the NHS group used as reference peak. Also, ^{1}H NMR measurements cannot estimate the correct Mw above 5 kDa.

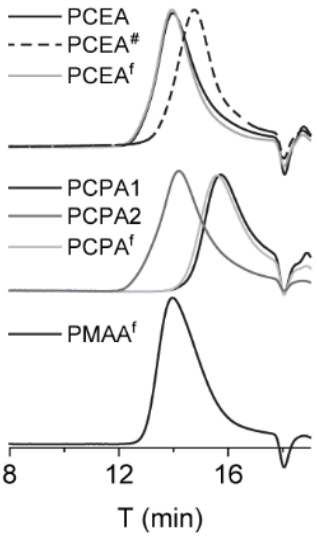


Fig. S1 GPC traces of the synthesized polymers in PBS buffer.

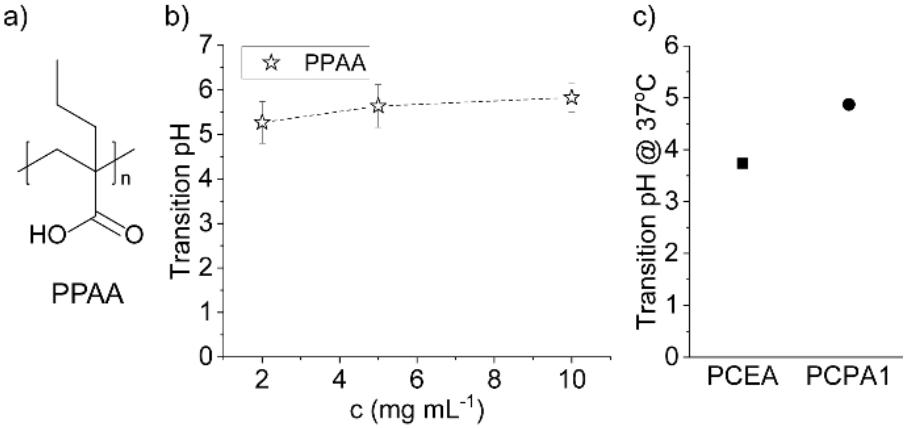


Fig S2 a) Chemical structure of poly(propylic acid) (PPAA). b) Hydrophilic-to-hydrophobic phase transition: The concentration dependent pH transition points for PPAA determined from titration curves in ultrapure water. c) Hydrophilic-to-hydrophobic phase transition: pH transition points for 2 mg mL^{-1} PCPA1 and PCEA in HEPES buffer at 37°C.

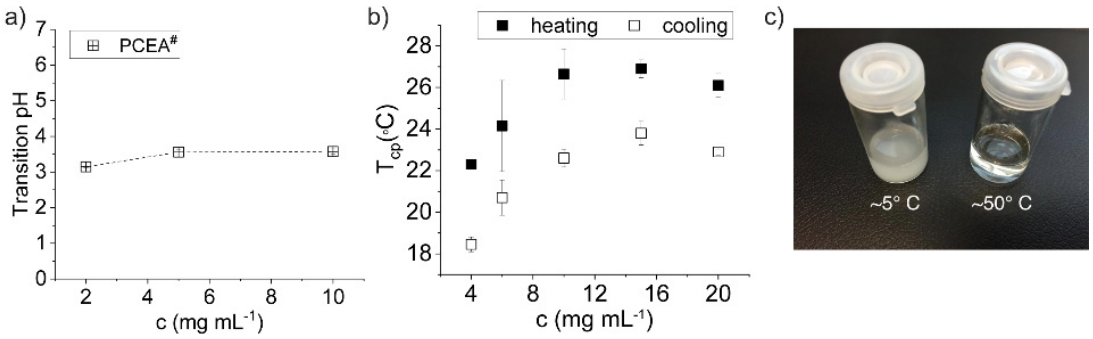


Fig. S3 Upper critical solution temperature (UCST) of PCEA$^{\#}$ (with traces of DMF): a) The concentration dependent pH transition point of PCEA$^{\#}$. b) Cloud point measurement of PCEA$^{\#}$ in water/DMF (n_{CEA}:n_{DMF} ~ 1:1). c) Photograph of PCEA$^{\#}$ solution at 5 °C and 50 °C.

We would like to note that similar experiments could not be performed with PCPA, since this polymer did not dissolve in ultrapure water.

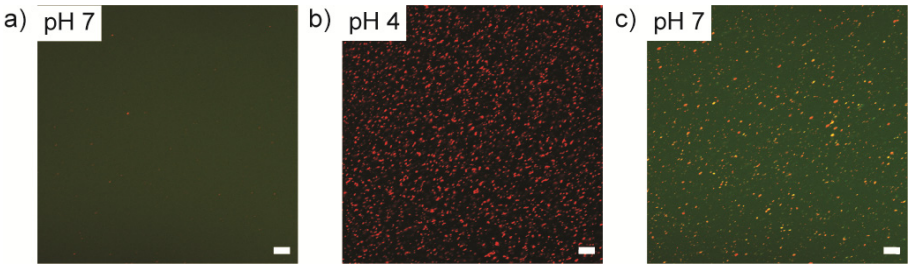


Fig. S4 CLSM images of pH-dependent aggregation of Liss Rhod PE labeled (red) POPC SUV in the presence of PCPA^{f} (green). a) pH 7 no interaction between the polymer and the SUV was detectable. b) ~1μm aggregates of SUVs were visible (the green fluorescence is not detectable due to the pH dependence of fluorescein.) c) The aggregates partially separated when the pH returned to 7. The remaining aggregates are largely orange, indicating the presence of both, PCPA^{f} and PE-Rh labeled lipids. Scale bars are 20 μm.

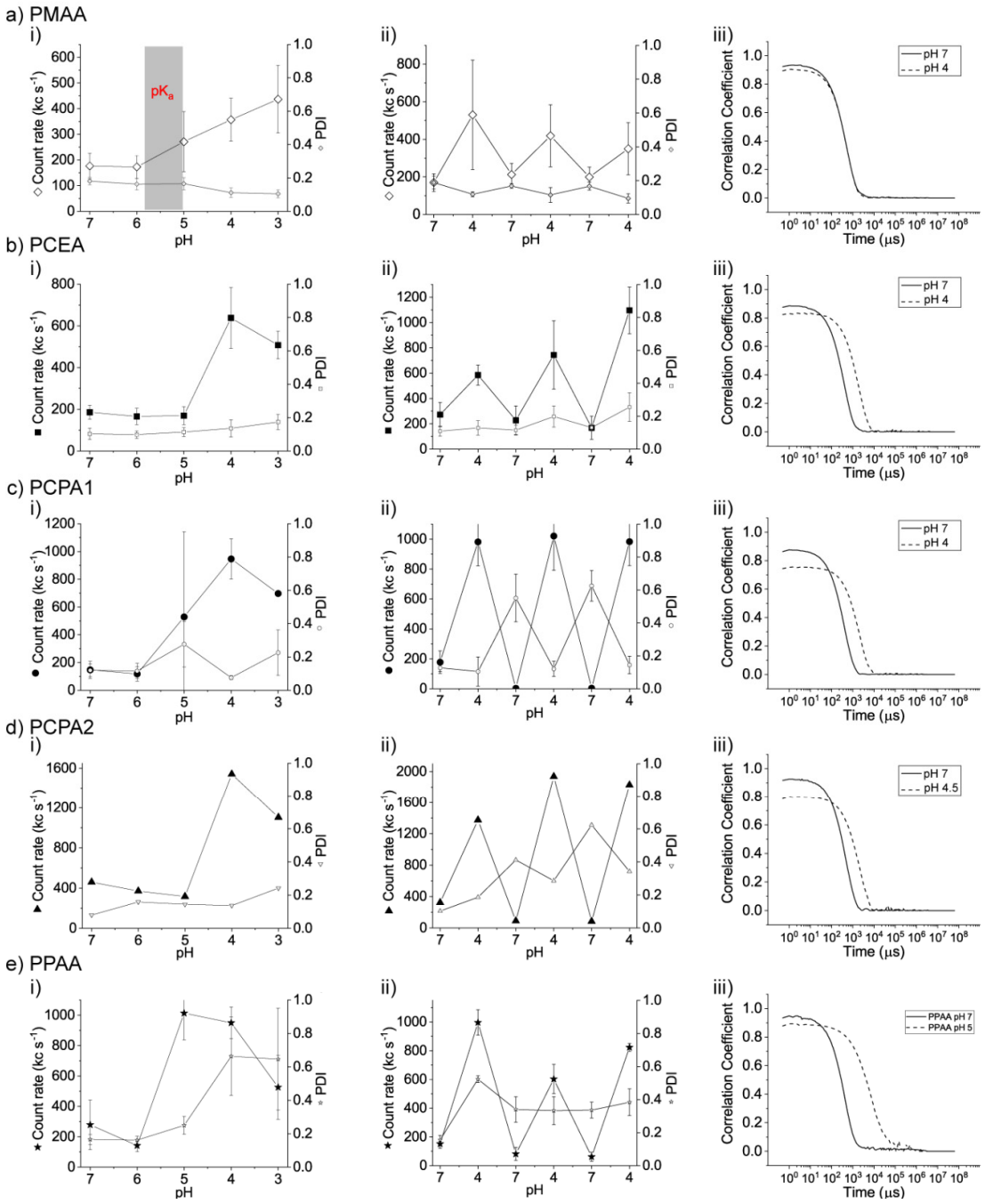


Fig. S5 Count rate of a mixtures of SUVs and polyanions when decreasing the solution pH (left row) or when switching the pH between 7 – 4—7 (middle row) as monitored by DLS. The correlation curve at pH 7 and pH 4 of the SUVs and polyanion mixtures. The pKa for PMAA is indicated in ai). PMAA (a), PCEA (b), PCPA1 (c), PCPA2 (d) and PPAA (e).

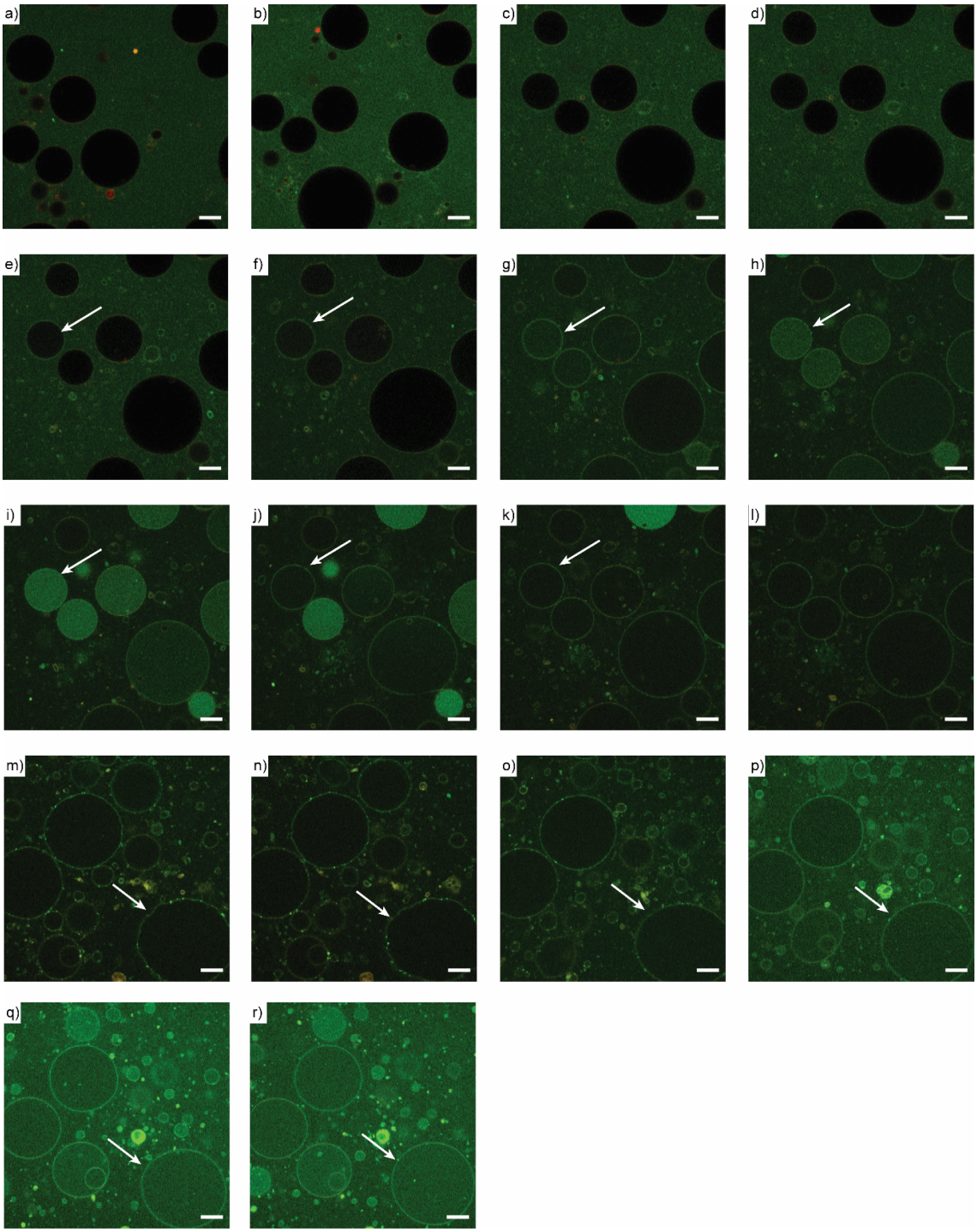


Fig. S6 Series of CLSM images of DOPC/PE-Rh GUVs (red) and Oregon green-labeled $PCPA^{OG}$ (green) when the solution pH decreased from 7 to 5 (a) to l)). Initially, the $PCPA^{OG}$ surrounded the GUVs. In time, 'green rings' appeared, indicating the association of $PCPA^{OG}$ with the lipid membrane of the GUVs. Furthermore, green signal appeared inside of the GUVs (white arrow), indicating the presence of the polymer. The background faints as the pH drops due to the pH sensitivity of OG. The initial bright signal inside of the structures was likely due to the higher pH inside the lipid structures that also fainted when pH dropped in the GUV's void as well. The intensity of the green signal increased both outside and inside the GUVs upon increasing pH from 5 to 7 (m) – r)). Scale bars are 10 μm.

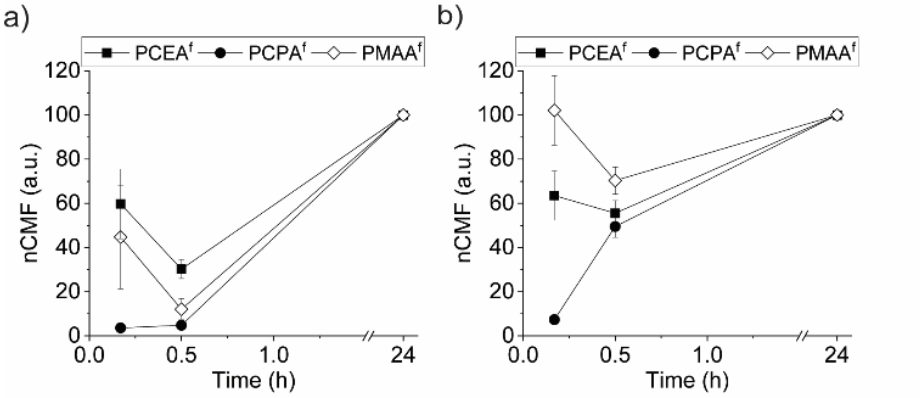


Fig. S7 Normalized CMF of a) Raw264.7 cells, b) HepG2 cells incubated PCEA^f (1 mg mL^{-1}) PCPA^f (0.05 mg mL^{-1}) and PMAA^f (0.5 mg mL^{-1}) for 10 min, 30 min and 24 h. All data are normalized to the CMF fluorescent monitored after 24 h by flow cytometry. All data represent mean ± StD (n=3).

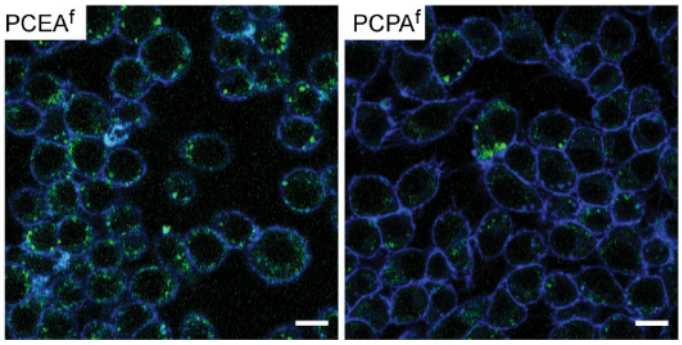


Fig. S8 Representative CLSM images of Raw 264.7 cells 2 h after replacing of polymer-containing media with regular media (Green: fluorescently labeled polymers, blue: Cell Mask^{TM} stained cell membrane). Scale bars are 10 μm.

EXPERIMENTAL SECTION

1. Materials

2-carboxyethyl acrylate, acryloyl chloride, ε-caprolactone, fluorescein-o-acrylate (flMA), fluorescein-o-methacrylate (flA), ethanol, 2-(Dodecylthiocarbonothioylthio)-2-methylpropionic acid N-hydroxysuccinimide ester (CTA-NHS), 4-Cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (CTA-PA), 2,2'-azobis(2-methylpropionitrile) (AIBN), dichloromethane, trimethylamine, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium chloride, polyethylenimine (PEI) (Mw ~25 000), *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC), *N*-Hydroxysulfosuccinimide sodium salt (NHSNa), Phosphate buffered saline, 0.25% trypsin EDTA and cell counting kit-8 solution were purchased from Sigma-Aldrich. Methacryloyl chloride, sodium hydroxide, magnesium sulfate, and silica gel 60 (0.032 – 0.063 mm) was purchased from Alfa Aesar. Dialysis tubing 3.5 kDa (Spectra/POR 3) were obtained from Spectrumlabs. Acetone, ethyl acetate, chloroform, hydrochloric acid 2M, diethyl ether, dimethyl sulfoxide-*d*6, deuterated chloroform and dimethyl formamide was purchased from VWR. AIBN was recrystallized from methanol. Oregon Green™ 488 Cadaverine, 5-isomer and CellMask Deep Red Plasma Membrane Stain was obtained from Thermo Fisher (Denmark).
1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (PE-Rh) and 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids, Inc, (USA).

2. Instruments

Size-exclusion chromatography (SEC) was performed using a system comprising a LC-20AD Shimadzu HPLC pump, a Shimadzu RID-10A refractive index detector and a Wyatt DAWN HELEOS 8 light scattering detector along with a SPD-M20A PDA detector, equipped with a HEMA-Bio Linear column with 10 µm particles, a length of 300mm and an internal diameter of 8mm from MZ-Analysentechnik in series with a OHpak SB- 803 HQ Shodex column with the dimensions 8.0×300 mm a particle size of 6 µm. The solvent used was 0.01 M PBS filtered through a 0.1 µm filter with 300 ppm sodium azide at 1.0 mL min^{-1} at 40 °C. Values of dn/dc used for molar mass calculations were established by Wyatt MALS/Astra software assuming full mass recovery.

^{1}H NMR and ^{13}C NMR were recorded with a Bruker 400 MHz NMR spectrometer.

Confocal laser scanning microscope (CLSM) measurements were performed on a Zeiss LSM700 instrument (Carl Zeiss, Germany).

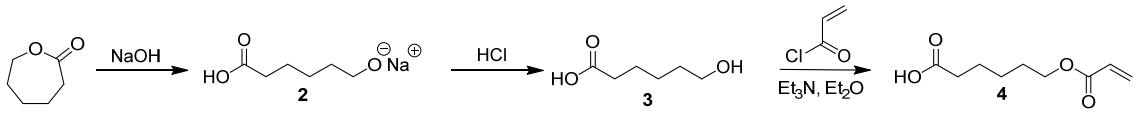
3. Synthetic procedures

3.1 Synthesis of benzyl (2-hydroxyehtyl)carbonotrithioate (CTA-OH)

Mercaptoethanol (1 g, 12.8 mmol) was dissolved in a suspension of K_3PO_4 (2.7 g, 2.52 mmol) in 20 mL acetone. After stirring for 10 min CS_2 (2.92g, 38.5 mmol) was added dropwise and stirred for 10 min. Then benzylbromide (2.20 g, 12.84 mmol) was added and after an additional 10 min stirring the solution was filtered off. The organic solvent was removed under reduced pressure and the product was purified by column chromatography in hexane:ethylacetate (3:1) as an eluent. Yield: 2.35 g, 75%.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 3.54 (t, -S-CH_2-CH_2), 3.82 (t, CH_2-CH_2-OH), 4.55 (s, Ar-CH_2-S-), 7.22 (m, Ar)

3.2 Monomer synthesis



Scheme S1. Synthesis steps to obtain 5-carboxypentyl acrylate (**4**).

Synthesis of sodium-6-hydroxyhexanoate (**2**)

Sodium hydroxide (4.00 g, 0.100 mol) was dissolved in water (10 mL) and ε-caprolactone (10.00 g, 87.6 mmol) was added over a 30-minute period and stirred for one hour until pH = 7 was reached. Water was evaporated, and the product was washed with diethyl ether to give sodium-6-hydroxyhexanoate as a white powder. Yield: 9.67 g, 62.7 mmol, 71.6 %.

^{1}H NMR (400 MHz, D_2O) δ (ppm) 1.34 (m, -CH_2-*CH_2*-CH_2-), 1.58 (m, (-*CH_2*-CH_2-*CH_2*-), 2.19 (t, -*CH_2*-COOH), 3.61(t, -*CH_2*-O).

Synthesis of 5-carboxypentyl acrylate (**4**)

2 (4.00 g, 23.4 mmol) was dissolved in water (10 mL) and hydrochloric acid (30 mL, 1 M) was added while stirring. After four hours of stirring at room temperature the aqueous phase was extracted twice with chloroform and twice with ethyl acetate and the organic phase was dried with magnesium sulphate. The organic solvent was removed under reduced pressure to yield 6-hydroxyhexanoic acid (**3**). Yield: 2.87 g, 21.7 mmol, 72.0 %

^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 1.28 (m, -CH_2-*CH_2*-CH_2-), 1.30-1.80 (m, -*CH_2*-CH_2-*CH_2*-), 2.18 (t, -*CH_2*-COOH), 3.37 ppm (t, -*CH_2*-OH), 4.35 (s, -*OH*), 11.93 (s, br, -*COOH)*.

3 (2.87 g, 21.7 mmol) was dissolved in diethyl ether (100 mL) together with triethyl amine (2.4 ml) and acryloyl chloride (1.77, 21.2 mmol) was added dropwise. The organic phase was washed with water three times, dried with magnesium sulphate, filtered and the diethyl ether was removed under reduced pressure to yield 5-carboxypentyl acrylate (2.45g, 13.2 mmol, 67.7 %)

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.43 (m, -CH_2-*CH_2*-CH_2-), 1.60-1.80 (m, -*CH_2*-CH_2-*CH_2*-), 2.36 (m, -C*H_2*-COOH), 4.00-4.20 (t, -C*H_2*-OH), 5.93 – 6.35 (m, -C*H*=C*H_2*), 9.67 (s, -C*H*O).

3.3 Polymer synthesis

General polymerization procedure

Monomer, flMA (if applicable), AIBN and CTA-NHS was dissolved in DMF. The mixture was bubbled with argon for one hour and placed into a preheated oil bath at 75 °C for overnight. The mixture was then precipitated into diethyl ether, dissolved in water and dialyzed two days against water to yield the polymers. For details, see Table S2.

Monomer, flMA (if applicable), AIBN and CTA-PA or CTA-OH was dissolved in DMF. After purging the solution for 2 min with argon, the reaction vial was subjected to four evacuation cycles and subsequently refilled with argon after each cycle. The polymerization tube was placed in a preheated oil bath at 75 °C and stirred overnight. The polymerization was quenched by exposure to oxygen The polymer was purified by three consecutive precipitation steps in diethyl ether and re-dissolving in DMF or ethanol. The polymer was dried under vacuum.

^{1}H NMR (PCEA) (400 Mhz, DMSO-*d*6) δ (ppm) 1.3-1.6 (br, -*CH*$_2$-, backbone), 1.70 (br, -*CH*-, backbone), 2.2 (b, -*CH*$_2$-COOH), 4.08 ppm (br, -*CH*$_2$-COO-).

^{1}H NMR (PCPA) (400 Mhz, DMSO-*d*6) δ (ppm) 1.3 & 1.53 ppm (m, -*CH*$_2$- *CH*-), 2.1-2.3, 2.37 (-(C*H*$_2$)$_2$-*CH*-COO-), 3.33 (br, *CH*$_2$-COOH), 4.08 ppm (br, -*CH*$_2$-COO-).

Table S2. Polymerization details of the polymers used in this study.

Name	$m_{Monomer}$ / g	$m_{monomer}$[f] / mg	m_{AIBN} / mg	$m_{NHS-CTA}$ / mg	m_{CTA-PA} / mg	m_{CTA-OH} / mg	Solvent / ml
PCPA1	1.00		1.7	47.0			4
PCPA2	1.00		0.8	23			2
PCPA[f]	0.20	39[a]	0.16	2.8			1
PCEA	1.00		0.8	23			2
PCEA2	1.00		3.3		56.6		2
PCEA[f]	0.93	66[b]	0.8	24			2
PMAA[f]	1.00	116[c]	1.0			30.7	3

[a] FIA, [b] FIMA

Labeling with Oregon Green (PCEA^{OG}, PCPA^{OG}, PMAA^{OG})

PCEA (46 mg), PCPA2 (65 mg) and PMAA (100 mg) were dissolved in 1 mL 0.05M $NaHCO_3$ (pH=8) each. 5.8 mg EDC and 4.8 mg NHSNa was added and each solution were stirred for a few minutes before adding 2.8 mg Oregon Green-cadevarin dye dissolved in 100 μL DMSO. The reaction flasks were protected from light and stirred for 48 h. The labeled polymers were dialyzed against ultrapure water for 2 d and subsequently freezedried.

4. Transition pH measurements

Coil-to-globe transition of the polymer solution was followed by absorbance measurements (Nanodrop 2000c, Thermo Fisher Scientific). 1 mL polymer solution (2, 5 and 10 mg mL^{-1} in alkaline water, adjusted to pH above 8 by sodium hydroxide, and 2 mg mL^{-1} in HEPES2) was placed in a quartz cuvette. The absorbance was measured by a UV/vis spectrophotometer at λ = 500 nm while incrementally lowering the pH using 1M hydrochloric acid (HCl). The pH was determined after each HCl addition with a pH-meter (FiveEasy, Mettler Toledo) and the corresponding absorbance was measured. Each measurement was repeated twice. The data obtained can be fitted using equation 1 to determine the transition point.

$$A(pH) = \frac{a}{1+e^{b(pH-c)}} \qquad \text{eq. 1}$$

The parameter *c* is the inflection point of the fitting and is used as the transition pH. a is the maximum absorbance, and *b* is responsiveness of absorbance with respect to pH near the inflection point. A non-linier least square function fit algorithm was used to identify the parameters.

5. Polymer/small unilamellar vesicle (SUV) interaction

5.1 SUV Assembly

100 μl DOPC or POPC (25 mg mL^{-1} in chloroform) was charged into a 25 mL round bottom flask and dried while slowly turning the flask under a steady stream of N_2 until a visibly dry film was left. The flasks were put on a vacuum line for at least 1 h followed by rehydration with 1 mL HEPES2 buffer. The solutions were extruded through 100 nm porous polycarbonate membrane (11×, Whatman) using a mini-extruder (Avanti Polar Lipids, Inc.).

Dynamic light scattering (DLS) measurements were carried out using a Malvern Zeta sizer Nano-590 at λ = 632 nm laser at 25 °C. The Zetasizer program was used to calculate the Z average size and the polydispersity (PDI). Each experiment was repeated twice with different liposomes solutions.

5.2 Polymer/SUV aggregate formation

700 μL polymer/SUV solution (pH=7.4, 670 μL of 0.25 mg mL^{-1} polymer solution and 30 μL freshly made liposome solution) were placed into a disposable cuvette and the pH was adjusted by adding 1 M HCl solution to obtain solutions with pH= 7, 6, 5, 4, 3. The pH was confirmed with pH paper. The Z average size and the polydispersity (PDI) was measured after each HCl addition by DLS.

The minimal PCPA2 concentration to induced detectable aggregation formation with SUV was determined by mixing 670 μL of PCPA2 solution (with a concentration between 0.001 – 0.01 mg mL^{-1}) and 30 μL freshly made liposome solution. The Z average size and the polydispersity (PDI) was measured at pH=7 and at pH=4.

5.3 Cycling experiment

700 μl polymer/SUV solution (pH = 7) (same ratio as above) were placed into a disposable cuvette. The pH was switched between 4 and 7 by adding 1 M HCl and 1 M NaOH solution, respectively, through four cycles. The Z average size and the polydispersity (PDI) was measured after each HCl or NaOH addition by DLS.

5.4 Visualization

67 μL labelled polymer (0.25 mg mL^{-1} in HEPES buffer) solution was placed on a glass coverslip and 30 μL SUV solution was added. CLSM images were at two different area were recorded. The pH was lowered by adding HCl solution and again images were taken, then the pH was increased by NaOH and new images were taken.

6. Polymer/giant unilamellar vesicle (GUV) interaction

30 μL DOPC in chloroform (10 mg mL^{-1}) and 3 μL PE-Rh in chloroform (1 mg mL^{-1}) were mixed in a vial. 10 μL of the lipid mixture was evenly spread to a thin layer of an indium tin oxide (ITO)-coated glass coverslips (VesiclePrepChamber, Nanion Technologies GmbH, Munchen). A 16×1 mm O-ring was placed on this coverslip and another ITO-coated coverslip was placed on top. The space between the coverslips was filled with 250 μL buffer solution (300 mM sucrose and 1 mM HEPES) to rehydrate the

lipid film. An AC electric field (3 V, 10 Hz) was applied for 2 h at 35 °C to generate the GUVs. The GUVs were transferred to a vial and stored at 4 °C before imaging.

50-60 µl fluorescently-labeled polymer solution in HEPES buffer (0.25 mg mL^{-1}) was placed on a glass coverslip and 5 µL of GUV solution was added. CLSM images at three different areas were recorded. Then, the pH was lowered to pH 4 - 5 by adding 30 µL Sodium acetate buffer solution (20mM and 130 mM NaCL) or 1 mL HCl solution and at least three different areas were chosen for imaging. To increase the pH 10 mL HEPES solution (pH12) was added.

7. Evaluation in cell culture

The human liver cancer cell line HepG2 and RAW 264.7 mouse macrophage cell line were purchased from European Collection of Cell Cultures. HepG2 cells were cultured in 75 cm^2 culture flasks in Minimum Essential Medium Eagle with Earle's Salts and sodium bicarbonate (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 2 mM L-glutamine and 1% MEM Non-essential Amino Acid Solution (Sigma-Aldrich), 100 µg mL^{-1} streptomycin and 100 u mL^{-1} penicillin (Thermo Fisher Scientific) at 37 °C and 5% CO_2. RAW 264.7 cells were cultured in 75 cm^2 culture flasks in Dulbecco's Modified Eagle's Medium with 4500 mg L^{-1} glucose, sodium pyruvate and sodium bicarbonate (Sigma-Aldrich) supplemented with 10% FBS, 2 mM L-glutamine (from Sigma-Aldrich), 100 µg mL^{-1} streptomycin and 100 u mL^{-1} penicillin at 37 °C and 5% CO_2.

7.1 Cell viability in Cell Proliferation Assays

RAW 264.7 cells and HepG2 cells were seeded in a 96-well plate (20 000 cells per well) and allowed to adhere overnight at 37 °C in 5% CO_2. The polymers was dissolved in HEPES buffer and added to the cell media not exceeding 10 vol% per well. The cells were incubated for 24 h at 37 °C in 5% CO_2. HepG2 cells were exposed to polymer concentrations between 0.1 and 0.001 mg L^{-1} of PEI, 2 and 0.1 mg L^{-1} of PCPA and 1.5 and 0.1 mg L^{-1} of PCEA, PPAA and PMAA. RAW 264.7cells were exposed to concentrations between 0.0001 and 0. 1 mg L^{-1} of PEI and from 0.1 to 1.5 mg L^{-1} of PCPA, PCEA, PPAA and PMAA. Then, the cells were washed twice with PBS buffer, and 110 µL of cell medium containing 10 µL of cell counting kit-8 solution (CCK-8) was added to each well. (CCK-8 monitors the dehydrogenase activities in cells.) The cells were incubated for 2 h at 37 °C in 5% CO_2. Then, 100 µL of the solution from each well was transferred to a new 96-well plate and analyzed using a multimode plate reader by measuring the absorbance at λ = 450 nm. Three independent repeats were performed for all experiments.

7.2 Uptake experiments - flow cytometry

RAW 264.7 cells and HepG2 cells were seeded in a 96-well plate (40 000 and 50 000 cells per well, respectively) and allowed to adhere overnight at 37 °C in 5% CO_2. The cells were incubated with fluorescently labeled polymers for 2 h at 37 °C or 4 °C, and for 10min, 30 min, 6 h and 24 h at 37 °C. The highest concentration of the polymers, which did not show an effect on the cell viability was chosen. The cells were incubated with 0.05 mg L^{-1} $PCPA^f$, 1 mg L^{-1} $PCEA^f$, 0.5 mg L^{-1} $PMAA^f$, or 0.005 mg L^{-1} calcein and washed twice with PBS buffer after the incubation time. For the HepG2 cells 30 µL of trypsin-EDTA (5 min at 37 °C) was used to detach the cells. The trypsin was neutralized with 125 µL cell medium. For RAW 264.7 cells, 100 µL medium was added and the cells were scraped off the plate with a needle. Cell mean fluorescence (CMF) was recorded by flow cytometry (Guava® easyCyte Single Sample Flow Cytometer, Merck) using an excitation wavelength of λ = 488 nm. The autofluorescence of the cells was subtracted by analyzing control cells where no polymer was added. The CMF of the

cells with 2 h incubation with polymers at 4 °C was additionally normalized to the CMF value of 2 h incubation with the polymers at 37 °C, which was set to 100%., resulting in nCMF. At least 5000 cells were analyzed. Three independent repeats were performed for all reported flow cytometry results. The statistical significance used to compare the distribution was determined using a one-way ANOVA followed by a Tukey's multiple comparison posthoc test (*$p < 0.05$).

7.3 Uptake experiments – CLSM

RAW 264.7 cells were seeded in a 24-well plate (50 000 cells per well) and allowed to adhere on the cover glass slides inserted in each well overnight at 37 °C in 5% CO_2. The glass slides were cleaned by sonication in water and ethanol and sterilized in PBS buffer under UV light overnight. The cells were incubated at 37 °C in 5% CO_2 for 24 h exposed to either 0.05 mg L^{-1} $PCPA^f$, or 0.1 mg L^{-1} $PCEA^f$. Then, the cells were washed twice with PBS buffer and fixed with 4% paraformaldehyde in PBS buffer for 15 min at room temperature. The fixing solution was removed and each well was washed 3× with PBS buffer and immersed into a 0.1 vol% Triton-X 100 in PBS buffer solution (T-PBS) for 15 min. The cells were then incubated with DAPI (1 μg mL^{-1}) for 15 min followed by a washing step with three times T-PBS solution and one time PBS buffer. Finally, PBS buffer was added for storage.
HepG2 cells were seeded in a confocal dish (500 000 cells per dish) and allowed to adhere overnight at 37 °C in 5% CO_2. Then, the cells were incubated with either 0.05 mg L^{-1} $PCPA^f$, or 0.1 mg L^{-1} $PCEA^f$ for 6 h and washed with clean medium. Cell Mask™ Deep red (5 μg ml^{-1}) in fresh medium was added for 10 min, before recording CLSM images.