

# Supporting Information for

## Synthesis and Biophysical Characterization of an

### Odd-numbered 1,3-Diamidophospholipid

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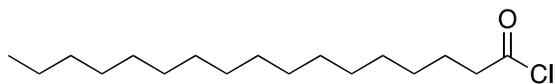
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## 1. General information

Text adopted from.<sup>25</sup> The starting compounds and solvents were purchased from Sigma-Aldrich/Fluka, ABCR, TCI Chemicals or Acros and were used without further purification. For reactions under inert gas conditions, the solvents (dichloromethane and chloroform) were dried over molecular sieves 4 Å and degassed afterwards. Column chromatography was carried out using 230–400 mesh, 60 Å silica gel (Brunschwig). TLC plates (Merck, Silica gel 60 F254) were developed with KMnO<sub>4</sub>-solution. <sup>1</sup>H, <sup>13</sup>C and <sup>31</sup>P NMR spectra were recorded (as indicated) on either a Bruker 300 or 400 MHz spectrometer and are reported as chemical shifts in ppm relative to TMS, calibrated to the signal of the deuterated NMR-solvent. Spin multiplicities are reported as singlet (*s*), doublet (*d*), triplet (*t*), with coupling constants (*J*) given in Hz, or multiplets (*m*). Broad peaks are marked as *br*. HRESI-MS was performed on a QSTAR Pulsar (AB/MDS Sciex) spectrometer and are reported as mass-per-charge ratio *m/z*. IR spectra were recorded on a PerkinElmer Spectrum One FT-IR spectrometer (ATR, Golden Gate).

## 2. Synthesis of heptadecanoyl chloride (6)



Heptadecanoic acid (8.114 g, 30 mmol) and thionyl chloride (7.138 g, 60 mmol) were placed in a round bottom flask, 3 drops of DMF were added and the mixture was heated to reflux for 4 h. The solvents were removed under reduced pressure. The crude product was distilled ( $1.0 \times 10^{-1}$  mbar, 155 °C) yielding 6.24 g (21.6 mmol, 72 %) of the product.

**$^1\text{H-NMR}$ :** (300 MHz;  $\text{CDCl}_3$ ,  $\delta$ ): 2.88 (t, 2H), 1.71 (quin, 2H), 1.26 (s br, 26H), 0.88 (t, 3H).

**$^{13}\text{C-NMR}$ :** (75 MHz;  $\text{CDCl}_3$ ,  $\delta$ ): 174.1, 47.3, 32.1, 29.8, 29.7, 29.5, 29.2, 28.5, 25.2, 22.8, 14.3.

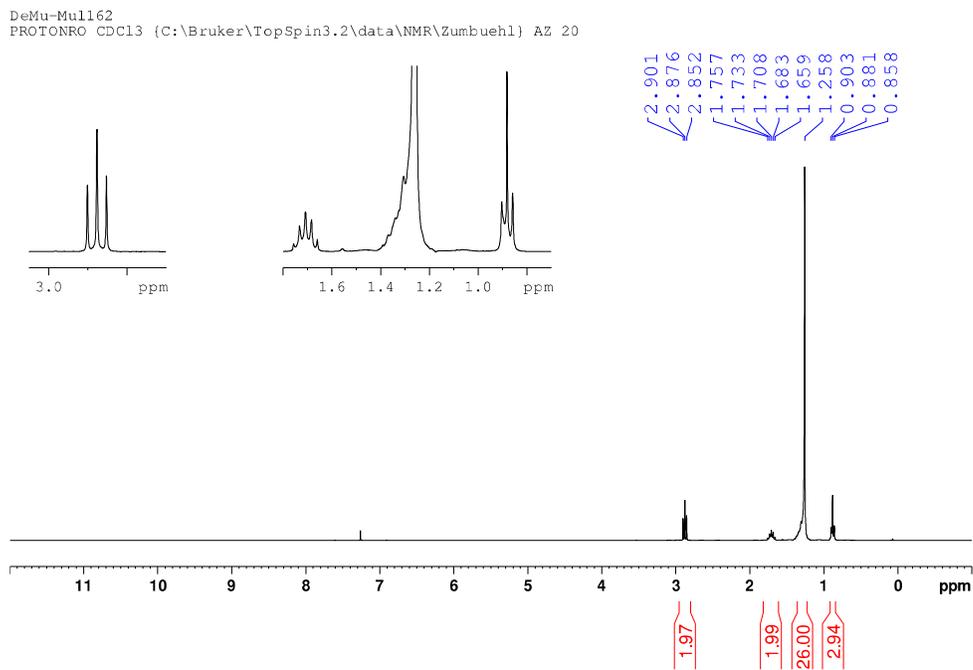


Figure S1:  $^1\text{H}$ -NMR of heptadecanoyl acid chloride.

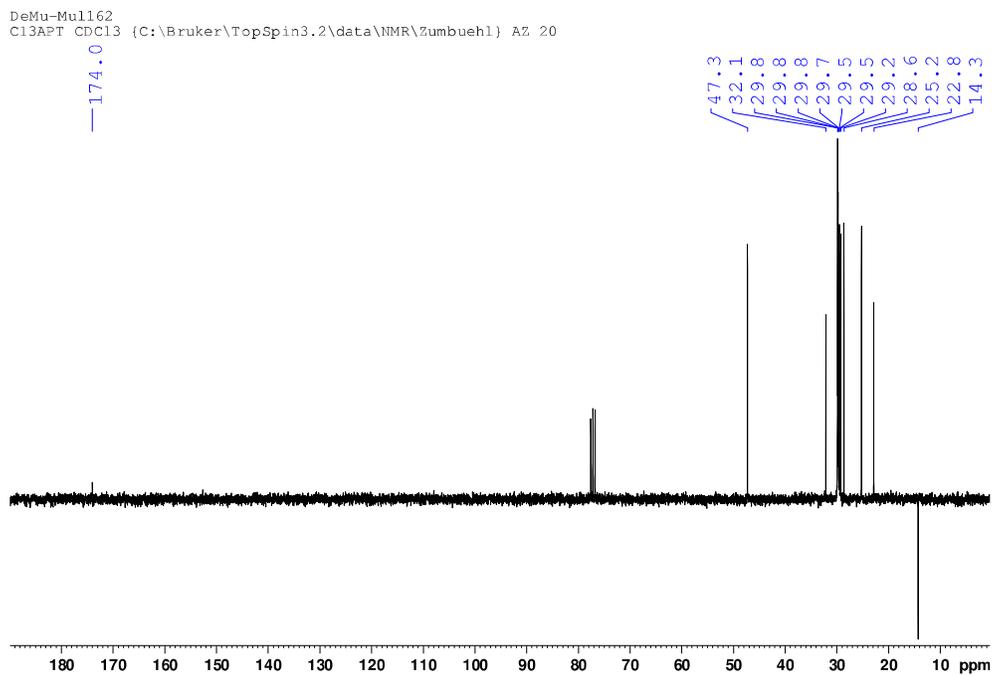
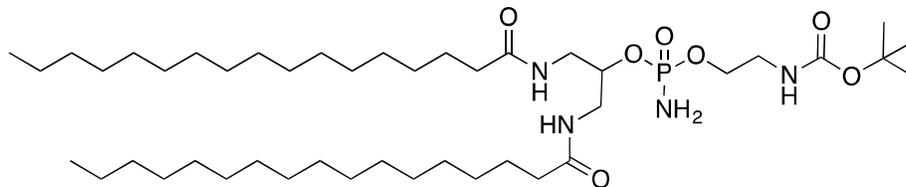


Figure S2:  $^{13}\text{C}$ -NMR of heptadecanoyl acid chloride.

### 3. Synthesis of *tert*-butyl (2-((amino((1,3-diheptadecan- amidopropan-2-yl)oxy)phosphoryl)oxy)ethyl)carbamate (7)



To the dichlorophosphoramidite (1.0 g, 2.9 mmol) dissolved in DMSO (50 mL)  $\text{NaN}_3$  (0.5 g, 7.7 mmol) was added and it was heated to 100 °C for 15 h. Afterwards it was cooled to RT. Brine (60 mL) was added and the solution was extracted with  $\text{Et}_2\text{O}$  (4 x 50 mL). The combined organic phases were dried over  $\text{Na}_2\text{SO}_4$ . MeOH (100 mL) was added and  $\text{Et}_2\text{O}$  was removed under reduced pressure. The crude diazide, still solved in MeOH, was used without further purification. Palladium on charcoal (150 mg) was added to the solution of the crude product in MeOH.  $\text{H}_2$  was bubbled through the solution (one filled balloon). Afterwards, a second balloon filled with  $\text{H}_2$  was added and the solution was stirred overnight. To remove the Pd-catalyst the solution was filtered over celite and washed with MeOH (3 x 30 mL). The solvent was removed under reduced pressure. The resulting crude diamine was used without further purification.

The crude product was dissolved in DCM (20 mL) and  $\text{NEt}_3$  (1.4 mL, 9.8 mmol) was added. The reaction mixture was cooled to 0 °C and heptadecanoyl chloride (2.83 g, 9.8 mmol) was added and the mixture was stirred over night at RT. The solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography (EtOAc). The product was isolated as a white wax (1.256 g, 1.54 mmol, 53 %).

**$^1\text{H-NMR}$** : (300 MHz;  $\text{CDCl}_3$ ,  $\delta$ ): 7.00 (br s, 1H), 6.86 (br s, 1H), 5.25 – 5.50 (m, 1H), 4.34 – 4.46 (m, 1H), 4.02 – 4.17 (m, 1H), 3.51 – 3.63 (m, 2H), 3.31 – 3.50 (m, 4H), 2.26 (t,  $^3J_{\text{HH}} = 7.6$  Hz, 4H), 1.65 (quin,  $^3J_{\text{HH}} = 7.5$  Hz, 4H), 1.46 (s, 9H), 1.34 (br s, 2H), 1.28 (s, 52H), 0.90 (t,  $^3J_{\text{HH}} = 13.4$  Hz, 6H).

**$^{13}\text{C NMR}$**  (75 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 175.3, 160.7, 66.1, 65.6, 47.7, 40.9, 36.7, 31.9, 29.7, 29.5, 29.4, 29.3, 28.4, 25.8, 22.7, 14.1.

**$^{31}\text{P-NMR}$** : (121 MHz;  $\text{CDCl}_3$ ,  $\delta$ ): 9.4.

**HRMS (ESI $_+$ )**:  $m/z$   $[\text{M}+\text{Na}]^+$  calcd for  $[\text{M}+\text{Na}]^+$  839.6361; found, 839.6361.

**IR** 2927, 2855, 1659, 1550, 1252, 1240, 1069, 1006, 979  $\text{cm}^{-1}$ .

**$R_f$** (DCM / MeOH /  $\text{H}_2\text{O}$ , 65 : 25 : 4): 0.83.

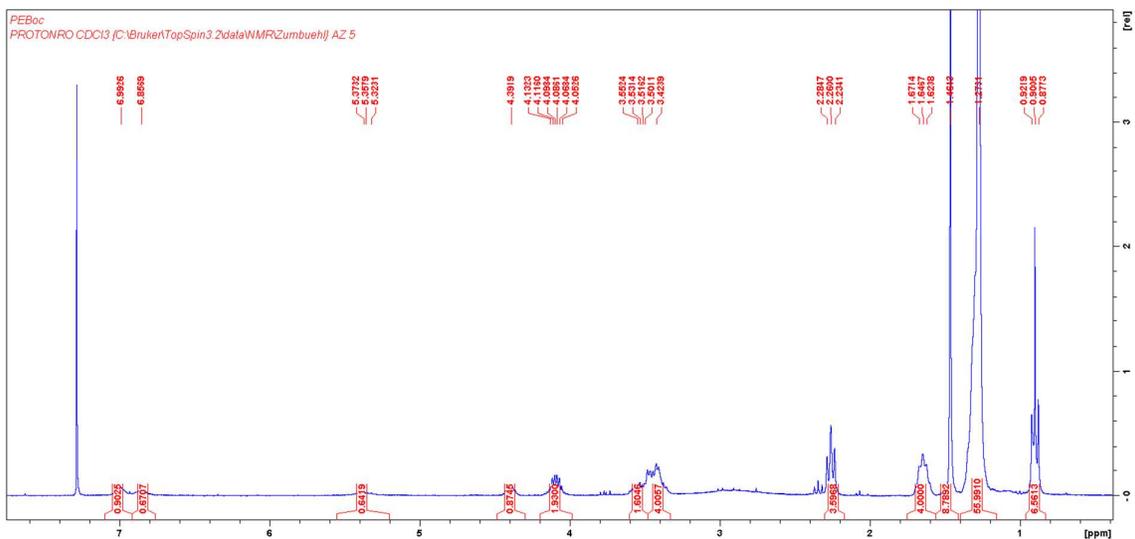


Figure S3:  $^1\text{H-NMR}$  of *tert*-butyl (2-((amino((1,3-diheptadecan-amidopropan-2-yl)oxy)phosphoryl)oxy)ethyl)carbamate.

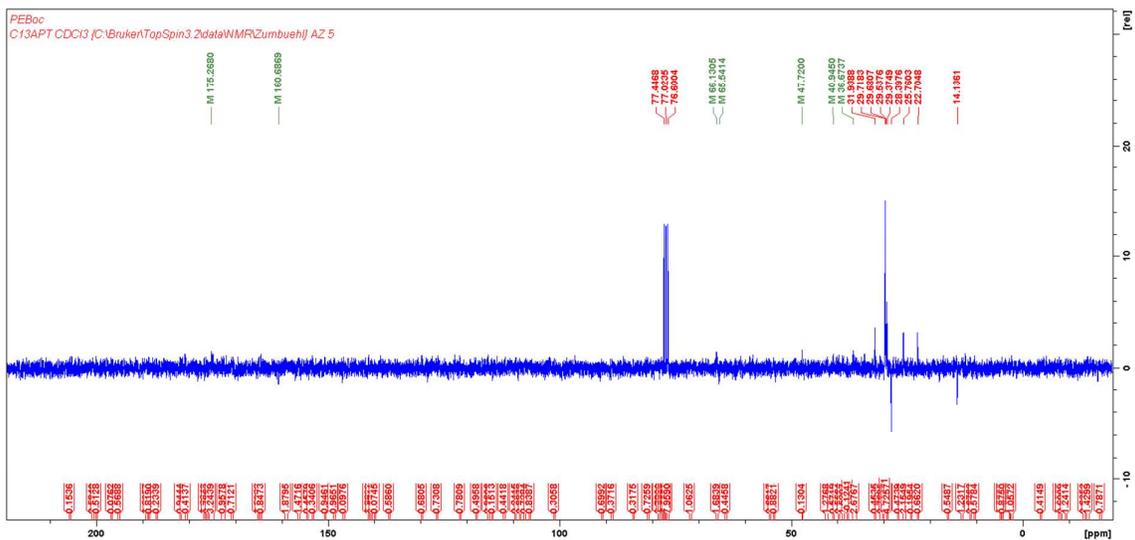
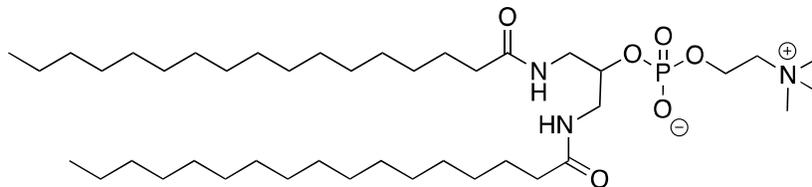


Figure S4:  $^{13}\text{C-NMR}$  of *tert*-butyl (2-((amino((1,3-diheptadecan-amidopropan-2-yl)oxy)phosphoryl)oxy)ethyl)carbamate.



#### 4. Synthesis of 1,3-diheptadecanamidopropan-2-yl (2-(trimethylammonio)ethyl) phosphate (Rad-PC-Rad, 4)



*N*-Boc protected Rad-phosphatidyl ethanolamine-Rad (1.13 g, 1.4 mmol) was dissolved in HCl in Et<sub>2</sub>O (1 M, 20 mL + 0.5 mL water). The solution was stirred for 2 h and afterwards the solvents were removed under reduced pressure.

The deprotected product was used without any further purification and was dissolved in MeOH (100 mL) and dimethyl sulfate (1.0 mL, 1.33 g, 10.5 mmol) was added. The mixture was heated to 40 °C and then a solution of potassium carbonate (1.45 g, 10.5 mmol, in H<sub>2</sub>O (20 mL)) was added, while it was stirred heavily and afterwards the solution was stirred for 1 h at 40 °C. Afterwards the solvents were removed under reduced pressure. The product was purified by silica column chromatography (DCM / MeOH / H<sub>2</sub>O, 65:25:4), yielding a white powder (0.76 g, 1.0 mmol). The crude product was further purified by precipitation from CHCl<sub>3</sub> / MeOH (1:1) with acetone and by sephadex LH20 column chromatography (DCM / MeOH / H<sub>2</sub>O, 65 : 25 : 4). A white powder was obtained (0.76 g, 0.99 mmol, 71 %).

<sup>1</sup>H-NMR (400 MHz; CDCl<sub>3</sub>, δ): 7.85 (s, 2H), 4.57 (s, 2H), 4.29 (s, 1H), 4.01 (s, 2H), 3.49 (s, 2H) 3.40 (s, 9H), 3.26 (s, 2H), 2.24-2.21 (m, 4H), 1.60-1.55 (m, 4H), 1.25 (s, 52H), 0.87 (t, <sup>3</sup>J<sub>HH</sub> = 6.7 Hz, 6H).

<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>, δ): 175.5, 72.8, 66.1, 59.8, 54.2, 40.1, 36.5, 32.0, 30.0, 30.0, 29.9, 29.9, 29.8, 29.7, 29.6, 29.5, 26.2, 22.9, 22.7, 14.1.

<sup>31</sup>P-NMR (121 MHz; CDCl<sub>3</sub>, δ): -1.6.

HRMS (ESI<sub>+</sub>) : m/z [M+H<sup>+</sup>] : calcd for [M+H] : 760.6333; found: 760.6331.

IR 3130, 3042, 2915, 2848, 1645, 1402, 1224, 1044 cm<sup>-1</sup>.

R<sub>f</sub>(DCM / MeOH / H<sub>2</sub>O, 65 : 25 : 4): 0.48.

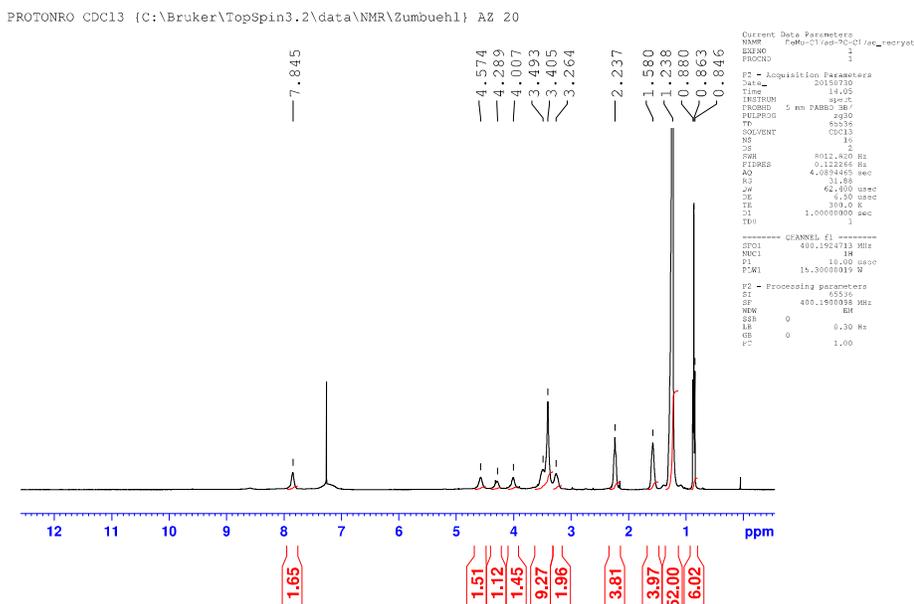


Figure S6: <sup>1</sup>H-NMR of Rad-PC-Rad (4).

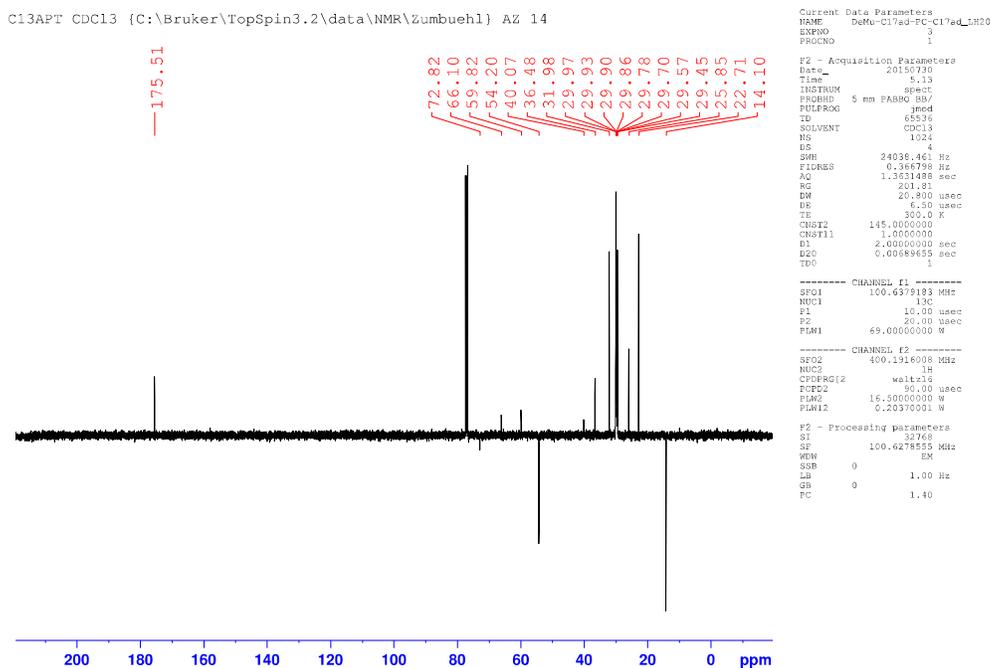


Figure S7: <sup>13</sup>C-NMR of Rad-PC-Rad (4).

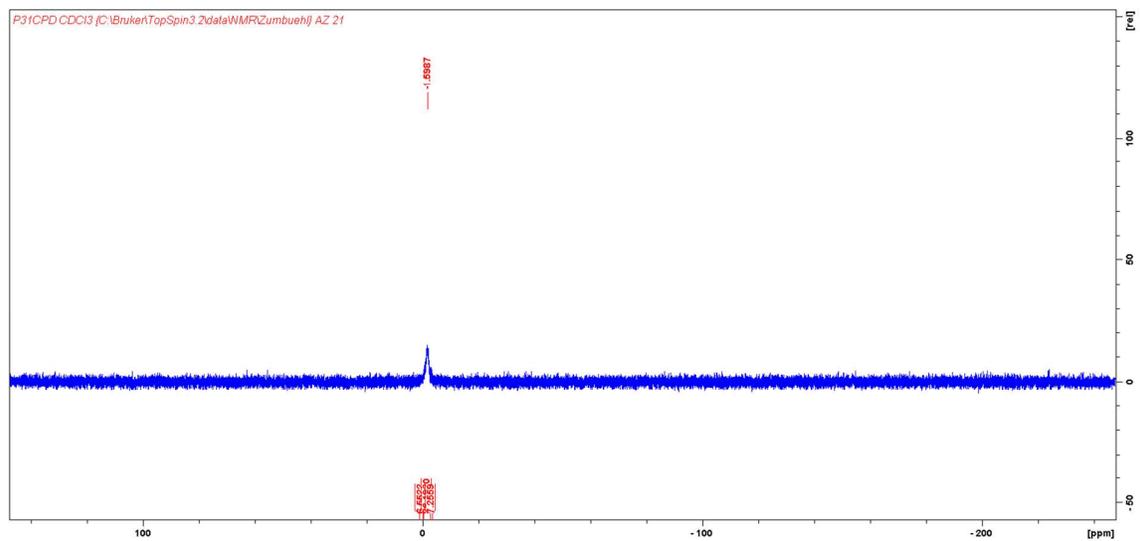


Figure S8:  $^{31}\text{P}$ -NMR of Rad-PC-Rad (4).

## 5. Differential scanning calorimetry

Text adopted from.<sup>25</sup> Multilamellar vesicle suspensions were prepared by hydration of 1 mg of phospholipid powder with 1 mL of ultra pure water (18.2 M $\Omega$ ·m). The unextruded vesicle suspensions were degassed for 30 minutes using a TA degassing station. The alternative heating-cooling scans were recorded on a TA Nano DSC (TA Instruments, USA) from 5 °C to 90 °C with a scanning speed of 0.5 K/min. The experiment was performed twice, starting with new suspensions, in order to ensure reproducibility. The scans of the second heating-cooling scans are reported. Raw data was base-line corrected and converted to molar heat capacity (MHC) using the NanoAnalyze software (TA Instruments, USA). The main phase transition and enthalpy were determined with the same software and verified using the Origin software (OriginLab, Northampton, USA).

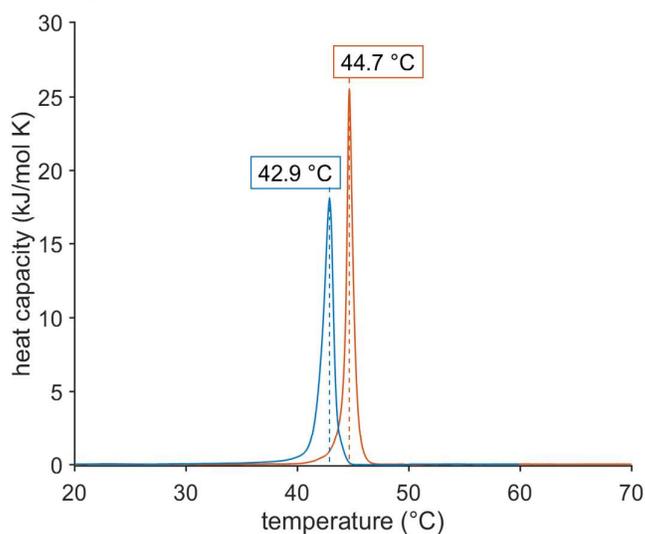


Figure S9: Differential scanning calorimetry heating- (orange) and cooling-curve (blue) of MLV suspensions with a heating-/cooling-rate of 0.5 K/min. The main phase transition temperature of  $T_m = 44.7$  °C with an enthalpy change of  $\Delta H$ : 22.6 kJ/mol; cooling  $T_m = 42.9$  °C with an enthalpy change of  $\Delta H$ : 24.1 kJ/mol.

## 6. Vesicle preparation

Text adapted from.<sup>25</sup> LUVET<sub>100</sub> of Rad-PC-Rad (4) were prepared following the standard extrusion protocol: In a 25 ml round bottom flask, 10 mg of the lipid were dissolved in CH<sub>2</sub>Cl<sub>2</sub>. After evaporation of the organic solvent, the film was further dried under high vacuum (40 mbar) overnight. Then the film was hydrated with the internal buffer for 30 min. (1 ml, 50 mM 5(6)-carboxyfluorescein, 10 mM HEPES buffer (AppliChem), 10 mM NaCl dissolved in pure water, pH 7.4 (NaOH)). Now at least 5 freeze-thaw cycles (liquid N<sub>2</sub> to 65 °C) were carried out before the suspension was extruded 11 times using a Mini Extruder (Avanti Polar Lipids, USA) using track-edged filters with a mesh size of 100 nm (Whatman, USA). The external buffer was then exchanged on a size exclusion column (1.5 × 20 cm Sephadex G-50 column) in external buffer (107 mM NaCl, 10 mM HEPES dissolved in ultrapure water, pH 7.4 (NaOH)).

## 7. Cryogenic transition electron microscopy

Text adopted from.<sup>9</sup> LUVET<sub>100</sub> of Rad-PC-Rad (4) were prepared by the extrusion protocol described above at a concentration of 10 mg/ml. The liposome suspensions were let to reach room temperature and were diluted 1:1 with isotonic saline and were mounted on glow-discharged holey carbon grids, quickly frozen by a Cryoplunge 3 system (Gatan, USA) and transferred to a JEM2200FS transmission electron microscope (JEOL, Japan) using a Gatan626 cryo-holder. Cryo-transmission electron micrographs were recorded at the acceleration voltage of 200 kV, x 20,000 magnification, 4-8  $\mu\text{m}$  underfocus and a dose of 10 electrons/ $\text{\AA}^2$ , using a F416 CMOS detector (TVIPS, Germany). The final concentration of the liposomal suspension used was 5 mg/ml or 6.3 mM. The thickness of the bilayer was measured using the open source software Fiji.<sup>30-32</sup> The membrane thickness for spherical vesicles is  $5.324 \text{ nm} \pm 0.447 \text{ nm}$  (N=502) and for two stacked bilayers  $8.491 \text{ nm} \pm 0.624 \text{ nm}$  (N=148).

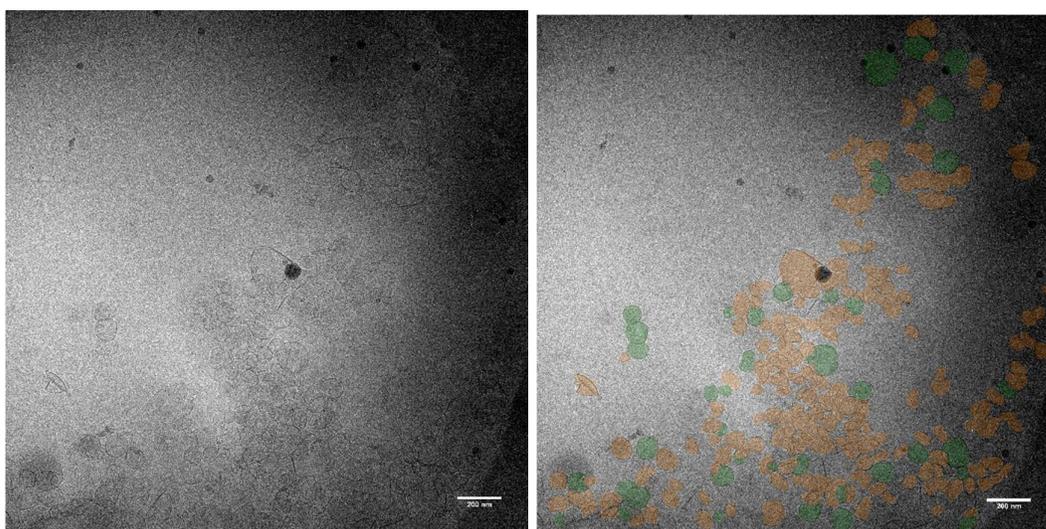


Figure S10: Overview cryoTEM image and colored vesicles for statistical evaluation of the spherical to faceted ratios (orange faceted; green spherical) (scale bars are 200 nm long).

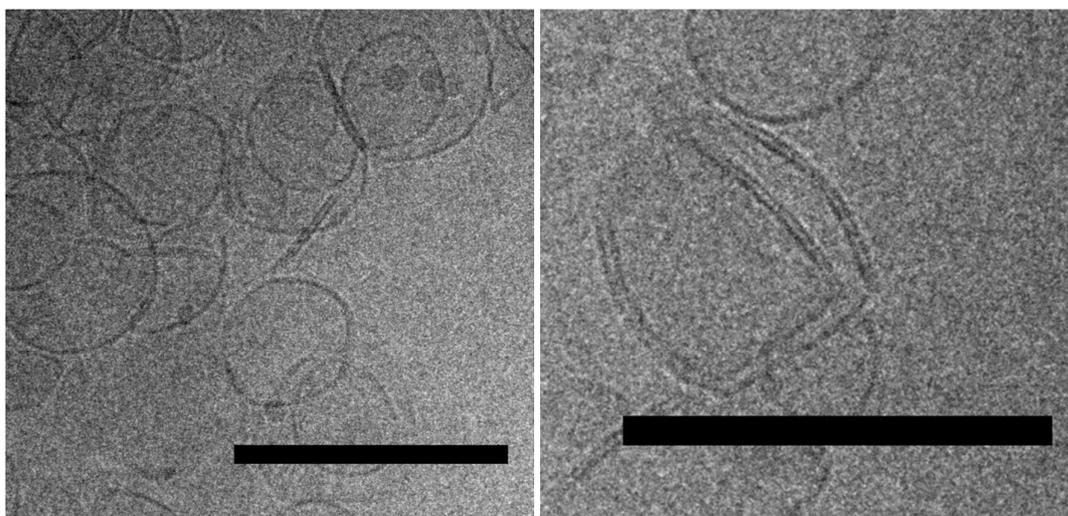


Figure S11: CryoTEM images zoomed in to different sections of the full image to highlight some observed vesicle structures (scale bars are 200 nm long).

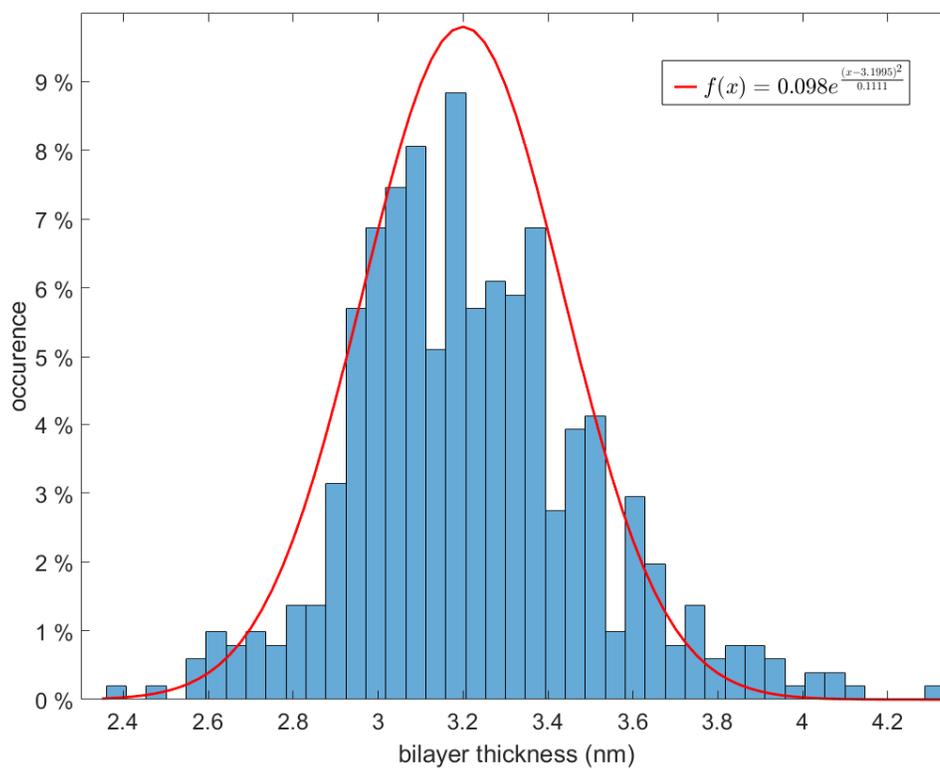


Figure S12: Membrane thickness distribution of N = 509 membrane cuts through Rad-PC-Rad containing vesicle membranes (see Figure S10) and a Gaussian fit.

## 8. Release experiments

Text adopted from.<sup>25</sup> The inner buffer (CF buffer) was prepared from 50 mM 5(6)-carboxyfluorescein (powder, Sigma-Aldrich) and 10 mM HEPES buffer (powder, Sigma-Aldrich) dissolved in ultra-pure water (18.2 M $\Omega$ ·cm). The pH was adjusted to 7.4 (NaOH) and the osmotic concentration to 200 mOsmL<sup>-1</sup> (NaCl). The outer buffer was prepared from 10 mM HEPES buffer (powder, Sigma-Aldrich) dissolved in ultra-pure water (18.2 M $\Omega$ ·cm). The pH was adjusted to 7.4 (NaOH) and the osmotic concentration to 200 mOsmL<sup>-1</sup> (NaCl). Rad-PC-Rad (4) (2 mg) was weighed into a round-bottomed flask (25 mL) and dissolved in CHCl<sub>3</sub> (1 mL, amylene stabilized, Sigma-Aldrich, USA). The solvent was removed by low-pressure rotatory evaporation. The thin film was dried under high vacuum overnight, to ensure the removal of residual water and prevent cholesterol oxidation. Inner buffer (1 mL) was added to the round-bottomed flask and the film was hydrated for 30 minutes at 65 °C. The film was subjected to five cycles of freeze/thaw using liquid nitrogen and a 65 °C water bath. The resulting MLV suspension was extruded 11 times through a track-etched filter membrane at 65 °C (100 nm, Whatman, USA) placed in a Mini Extruder (Avanti Polar Lipids, USA). The vesicles were left standing at room temperature overnight. The residual non-encapsulated CF buffer, in the LUVET<sub>100</sub> suspension, was exchanged with the outer buffer using size exclusion chromatography (PD-10 desalting columns, GE Healthcare, UK). The size exclusion chromatography was carried out after 24 hours storage, in the dark at room temperature, of the LUVET<sub>100</sub> suspension.

The purified LUVET<sub>100</sub> suspension was diluted, in a volumetric flask, to 100 mL using additional outer buffer. Six aliquots (2 mL) were separated into vials (5 mL, PE caps) and vortex mixed for different amounts of time (0, 5, 10, 20, 30, 60 s) at 2500 rpm. The 5(6)-carboxyfluorescein release was quantified using a fluorospectrometer (Sense 425-301, Hidex, Finland). For each sample, five microplate wells were filled with 200  $\mu$ l of the vortex 20 mixed vesicular suspension. The wavelengths used for measurements were 485 nm (excitation) and 535 nm (emission). As a control for the maximum dye release (F<sub>100</sub>), a Triton X-100 solution (2  $\mu$ l of a 10 vol% solution) was added to additional five microplate wells, filled with 200  $\mu$ l of vesicular suspension, for each sample. The release fraction at time X was calculated with the formula:

$$Release (\%) = \frac{F_X - F_0}{F_{100} - F_0}$$

where  $F_X$  is the fluorescence at time X,  $F_0$  the fluorescence at time zero and  $F_{100}$  the maximum fluorescence recorded after treatment with Triton X-100.

The data were fitted according to simple release kinetics, using the software pro Fit 7.0.11 (QuantumSoft, Uetikon am See, Switzerland). The time dependence of the drug release  $R = R(t)$  can be perfectly described by the use of the time constant  $\tau$  and the release amplitude  $R_0$  as the two fitting parameters in the following equation:

$$R(t) = R_0(1 - e^{-t/\tau})$$

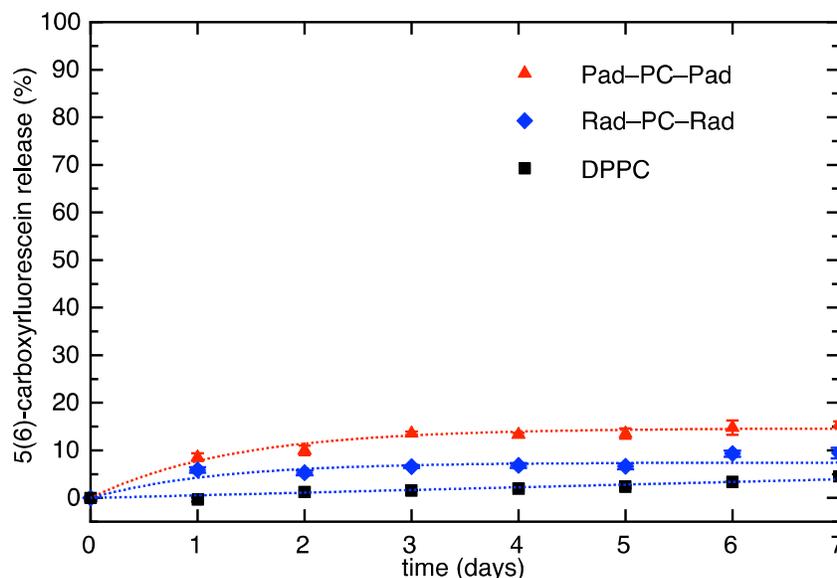


Figure S13: Long-term release of 5(6)-Carboxyfluorescein containing vesicles formulated from Pad-PC-Pad (2), Rad-PC-Rad (4) and DPPC (1) respectively. The formulations have been stored at 22 °C for 7 days.

## 9. Film balance measurements

Text adapted from.<sup>25</sup> Pressure-area isotherms were recorded on a computer interfaced Langmuir trough with a surface area of 194 cm<sup>2</sup> (Riegler & Kierstein, Potsdam, Germany). The paper plate Wilhelmy method was used to measure the surface tension with an accuracy of  $\pm 0.1$  mN/m. Each measurement was repeated at least three times. The Langmuir trough was filled with ultrapure water (specific resistance of 18.2 M $\Omega$ ·cm). The lipid solution (1 mg/ml) in chloroform was spread and the measurement was started 5–15 min after spreading. The compression speed was 3.12 cm<sup>2</sup>min<sup>-1</sup>.

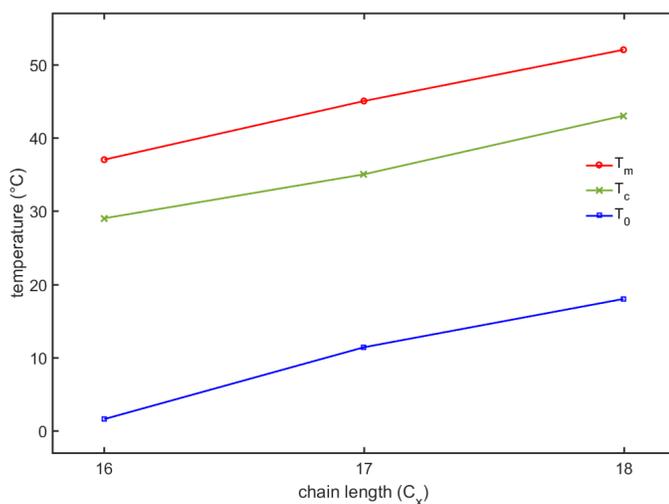


Figure S14: T<sub>m</sub> (main phase transition temperature for a bilayer), T<sub>c</sub> (critical temperature for a monolayer) and T<sub>0</sub> (minimum transition temperature for a monolayer) versus fatty acyl chain length of 1,3-disubstituted diamido phospholipids Pad-PC-Pad (2), Rad-PC-Rad (4) and Sad-PC-Sad (3).

## 10. Grazing incidence X-ray diffraction measurements

Text adapted from.<sup>25</sup> The monolayer's lattice structure was investigated by grazing incidence x-ray diffraction (GIXD) measurements performed at the PETRA III beamline P08 at the German Synchrotron DESY-Hamburg, Germany. A photon source with an energy of 15 keV was used for the measurements. The monolayers were prepared on a Langmuir-Pockels trough on an antivibrational table with a fully extended area of 450 cm<sup>2</sup> and 300 mL subphase volume at 295 K air temperature and different subphase temperatures between 10–25 °C. Throughout the whole measurement, the trough chamber was flushed with wet Helium. A linear position-sensitive MYTHEN detector system (PSI, Villigen, Switzerland) measured the diffracted signal and was rotated around the trough to scan the in-plane  $Q_{xy}$  component values of the scattering vector. To restrict the in-plane divergence of the diffracted beam a Soller collimator was placed in front of the MYTHEN detector, the resulting divergence was 0.09°. The vertically distributed pixels of the MYTHEN measured the out-of-plane  $Q_z$  component of the scattering vector between 0.0 and 1.2 Å<sup>-1</sup>. The intensities of the scattered radiation were corrected for polarization, footprint area, and powder averaging. Model peaks taken to be Lorentzians in the in-plane direction (Bragg peaks) and Gaussians in the out-of-plane direction (Bragg rods) were fitted to the measured intensities. The in-plane lattice repeat distances  $d$  of the ordered structures in the monolayer were calculated from the Bragg peak positions,  $d = 2\pi/Q_{xy}$ .

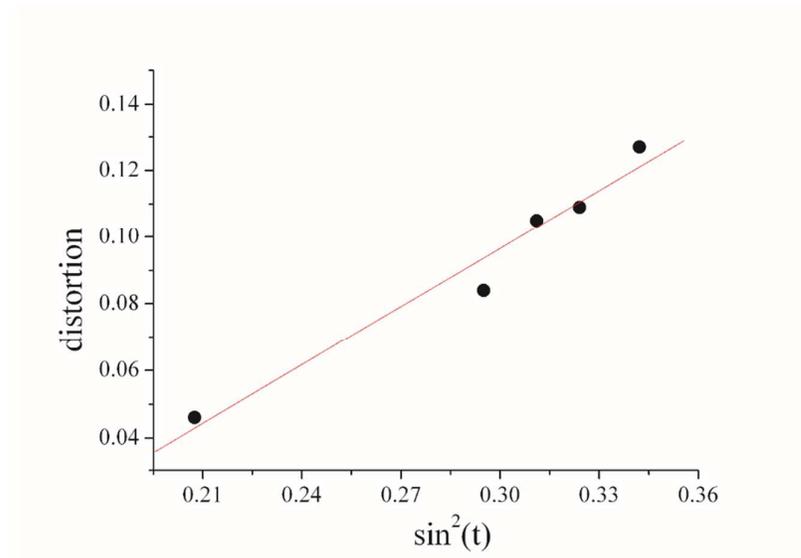


Figure S15: Lattice distortion  $d$  as function of  $\sin^2(\theta)$  of Rad-PC-Rad (4) monolayers at 20 °C.

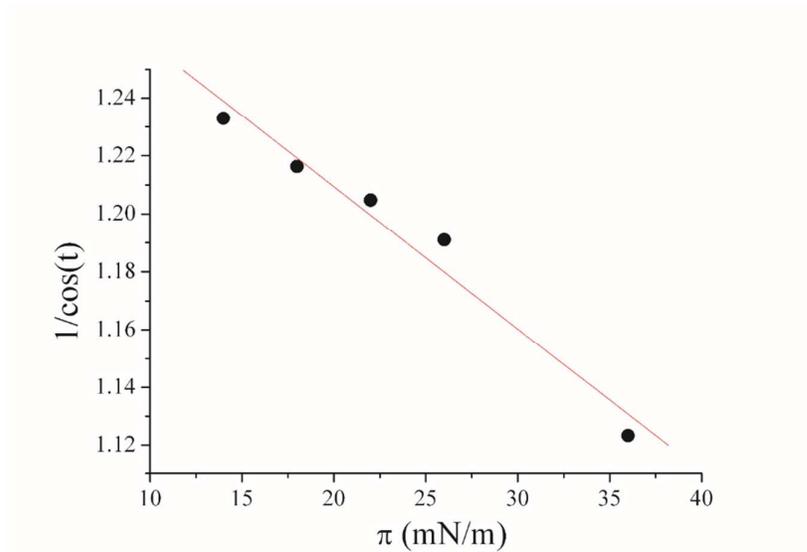


Figure S16: Dependence of the tilt angle of the alkyl chains ( $\theta$ ) represented as  $1/\cos(\theta)$  on the lateral surface pressure ( $\pi$ ) at 20 °C.

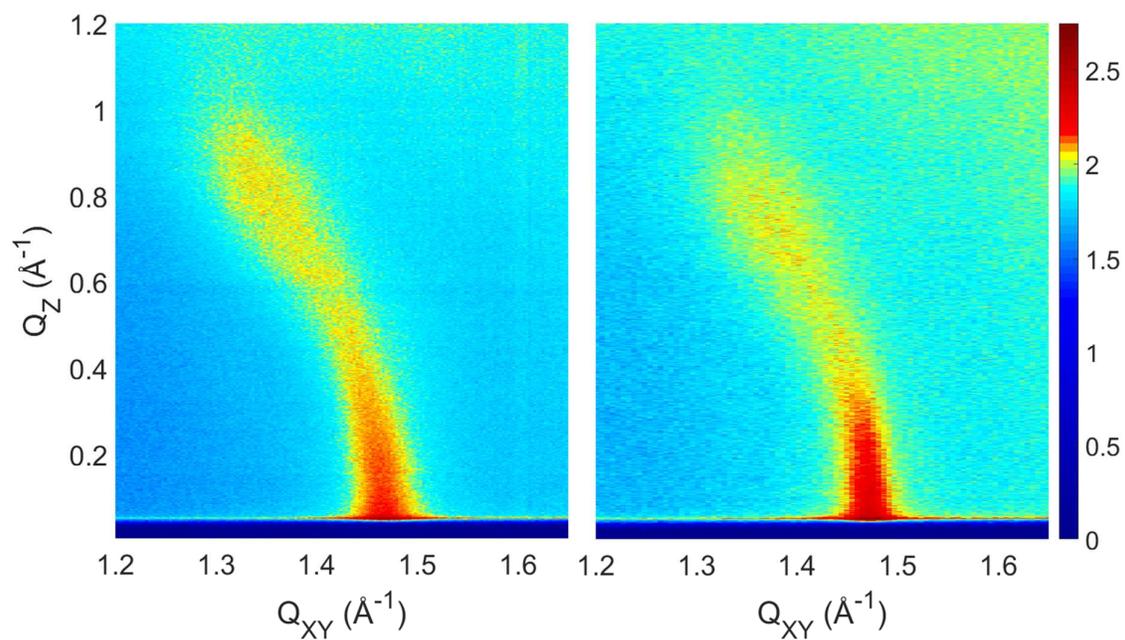


Figure S17: Selected GIXD heatmaps of the diffraction intensities as a function of the in-plane  $Q_y$  and out-of-plane  $Q_x$  components of the scattering vector  $Q$  for Rad-PC-Rad (**4**) at 10 °C and 30 mN/m (left) as well as 20 °C and 30 mN/m (right).