Supplementary information for:

Processing-Structure Relationships of Poly(ethylene glycol)-Modified Liposomes

K. P. Mineart,[a] E. G. Kelley,[b] M. Nagao,[b,c] and V. M. Prabhu[a*]

[a]Materials Science and Engineering Division, and [b]Center for Neutron Research, National Institute of Standards and Technology, Gaithersburg, MD, 20899, [c]Center for Exploration of Energy and Matter, Department of Physics, Indiana University, Bloomington, IN 47408

Materials and Liposome Preparation‡

Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), alkyl tail-deuterated DPPC (*i.e.* DPPC-d62, denoted dDPPC), and dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[mPEG-5000] (DPPE-PEG) were each purchased in powder form from Avanti Lipids, Inc. Chloroform ($CHCl_3$, anhydrous 99.8 %) and heavy water (D_2O, 99.8 %) were purchased from Fisher Scientific and Cambridge Isotopes, respectively.

Liposomes were prepared using the film rehydration and extrusion procedure. In short, the desired amounts of DPPC, dDPPC, and DPPE-PEG were dissolved in $CHCl_3$ (~0.5 mL total). Then, the solvent was removed using a steady stream of ultra-high purity N_2, forming a thin, solid film. The films were further dried under vacuum at 55 °C for 18 hr. For rehydration, D_2O was added to films and they were held at 55 °C with periodic agitation over the period of an hour followed by aging at 55 °C overnight. The resulting dispersions were extruded through 400-nm (20 passes), 200-nm (20 passes), and 100-nm (41 passes) polycarbonate filters (Whatman) using an Avanti Mini-Extruder mounted with a programmable syringe pump (Harvard Apparatus PHD

2000) and maintained at 55 °C. Extrusions were conducted using 1-mL glass syringes and a flow rate of 3.1 mL/min in both directions.

PEGylated liposomes were prepared following two unique protocols referred to as co-extrusion (CX) and post-insertion (PI) based on the route of DPPE-PEG incorporation. CX liposomes were prepared as described above (*i.e.* DPPE-PEG was incorporated in the dry film). Alternatively, PI liposomes were produced by mixing extruded DPPC/dDPPC liposomes with varying amounts of a 12 mM (*i.e.* mmol/L) DPPE-PEG solution (in D_2O). These freshly mixed samples were maintained at 55 °C for 1 hr with frequent agitation.

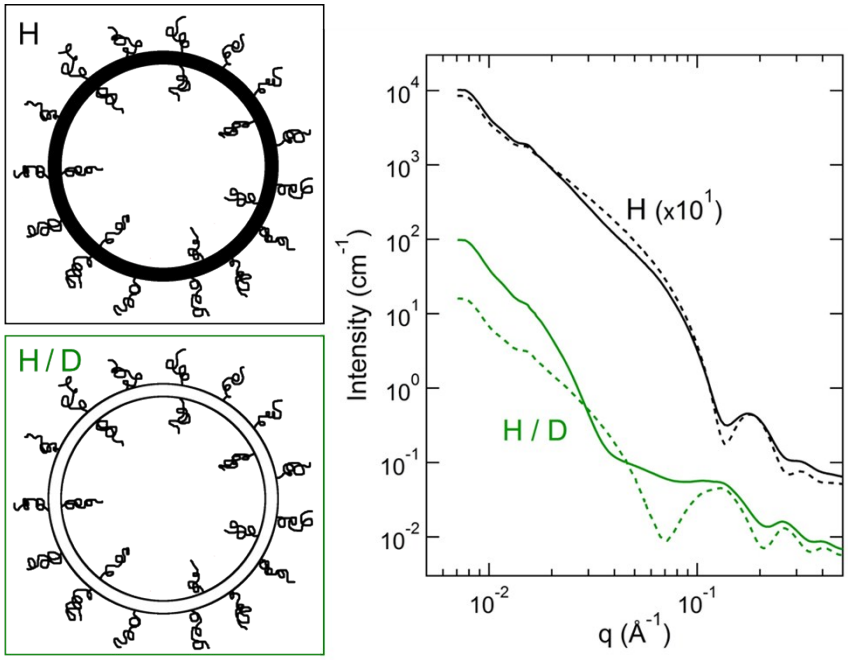

Figure S1. Schematic depictions (left) of PEGylated liposomes in absence (fully protonated DPPC tails) and presence (mixed protonated/deuterated DPPC tails) of contrast matching labeled by H and H/D, respectively. Corresponding model SANS profiles (right) for each case where dashed and solid lines are for liposomes with 0 and 5 mol % DPPE-PEG, respectively (where mol % represents mole percentage). Model profiles are for liposomes with R = 550 Å, σ_R/R = 0.25, d = 50.5 Å, σ_d/d = 0.05, R_g = 31 Å, and f_{out} = 0.5. The H profiles are shifted x10^1 for clarity. Further model details and variable definitions can be found below.

All solutions were prepared with a deuterated-to-protonated tail molar ratio of 6.5:1 to achieve contrast matching between the bilayer core and D_2O, which enhances the scattering sensitivity to PEG chain contributions (Figure S1). The total concentration of all components (DPPC + dDPPC + DPPE-PEG) was approximately 25 mM for all samples.

Liposome Characterization

Small angle neutron scattering (SANS) experiments were performed at ambient temperature (*i.e.* 25 °C) on the NGB 10m (initial) and NGB 30m (10-week aging) beamlines at the National Institute of Standards and Technology Center for Neutron Research (NIST-CNR). On NGB 10m, three neutron wavelength/sample-to-detector distance (λ/l_{sd}) configurations (10.0 Å/5.2 m : 5 Å/5.2 m : 5 Å/1.2 m [low-*q* : mid-*q* : high-*q*]) were utilized to provide a sufficiently wide *q*-range (~0.005 $Å^{-1}$ to 0.5 $Å^{-1}$). All three configurations had wavelength spreads ($\Delta\lambda/\lambda$) of 0.15, and the mid-*q* configuration used an offset detector position. On NGB 30m, three instrument configurations were used to access a similar q-range (6 Å/13.2 m : 6 Å/4.0 m : 6 Å/1.3 m [low-*q* : mid-*q* : high-*q*]). The $\Delta\lambda/\lambda$ was 0.14 and the high-q configuration used an offset detector position. Sample solutions were measured in Hellma 1-mm quartz cells.

The scattered intensity, $I(q)$, was collected as a function of the scattering vector, q, where $q = 4\pi\sin(\theta/2)/\lambda$ and θ is the scattering angle. The collected intensities for each sample were corrected for the detector dark current and reduced to the scattering solely from the sample by subtracting the cell contribution. Sample intensities were placed on an absolute scale using the direct beam intensity. All data reduction and low-, mid-, and high-*q* data merging was completed using the NIST-CNR SANS package.[1] Scattering from D_2O (Hellma 2-mm quartz cell) was subtracted from dispersions to isolate liposome scattering.[2]

Dynamic light scattering (DLS) was conducted with a Malvern Nano ZS maintained at 25 °C and equipped with a 532-nm laser. The instrument-standard backscattering detector (173°) was utilized for all data collection. Solutions diluted 10-fold with D_2O (2.5 mM) were analyzed in disposable, polystyrene cuvettes. A D_2O viscosity (η) and refractive index (n_0) of 1.1×10^{-3} Pa·s and 1.33, respectively, were used to determine the hydrodynamic diameter ($2R_H$) distribution of liposomes.[3] Cryo-transmission electron microscopy (cryo-TEM) was conducted on an FEI Titan G2 microscope operated at an accelerating voltage of 300 kV. Samples were prepared on plasma treated (10 sec) EMS C-flat holey carbon grids. A Leica EM GP plunge freezer was used to prepare cryo-specimens of appropriate thickness for imaging.

SANS Modeling (PEGylated Liposome Form Factor)

Arleth and Vermehren[4] showed that an analytical, Pedersen-type[5] model could be derived for surface functionalized liposomes. Here, a slight modification to their model is made to capture the partitioning of PEG chains inside and outside of liposomes. The scattering intensity for PEG-modified liposomes ($I_{\mathrm{PEGlip}}(q)$) calculated using the modified model is assumed to be independent of liposome-liposome structure factor contributions and is therefore described by:

$$I_{PEGlip}(q) = n_{PEGlip} P_{PEGlip}(q) + bkg$$

where n_{PEGlip} is the number density of liposomes in solution, $P_{\mathrm{PEGlip}}(q)$ is the PEGylated liposome form factor, and *bkg* is the incoherent background. The number density can be estimated based on sample formulation and $P_{\mathrm{PEGlip}}(q)$ is further defined by:

$$\begin{aligned} P_{PEGlip}(q) &= \beta_{bil}{}^2 P_{bil}(q) + N_{PEG}(V_{PEG}\Delta\rho_{PEG})^2 P_{chain}(qR_g) + (f_{in}N_{PEG})(f_{in}N_{PEG}-1)(V_{PEG}\Delta\rho_{PEG} \\ &(f_{out}N_{PEG}-1)(V_{PEG}\Delta\rho_{PEG})^2 S_{c_o-c_o}(q) + 2(f_{in}N_{PEG})(f_{out}N_{PEG})(V_{PEG}\Delta\rho_{PEG})^2 S_{c_i-c_o}(q \\ &(f_{in}N_{PEG})V_{PEG}\Delta\rho_{PEG}\beta_{bil}S_{bil-c_i}(q) + 2(f_{out}N_{PEG})V_{PEG}\Delta\rho_{PEG}\beta_{bil}S_{bil-c_o}(q) \end{aligned}$$

All variable definitions are given in Table S1 for brevity, and the individual form factor ($P_i(q)$) and cross correlation ($S_{i\text{-}i}(q)$) terms are defined by the equations that follow.

The bilayer form factor ($P_{bil}(q)$) is that for three, concentric spherical shells:[6]

$$P_{bil}(q) = \Phi_{bil}(q)^2 = \left(\frac{\Delta\rho_{HG}\left\{\left[\frac{4}{3}\pi r_o^3\right]\Phi_s(qr_o) - \left[\frac{4}{3}\pi r_i^3\right]\Phi_s(qr_i)\right\} + (\Delta\rho_C - \Delta\rho_{HG})\left\{\left[\frac{4}{3}\pi r_2^3\right]\Phi_s(qr_2) - \left[\frac{4}{3}\pi r_1^3\right]\Phi_s(qr_1)\right\}}{\Delta\rho_{HG}\left\{\left[\frac{4}{3}\pi r_o^3\right] - \left[\frac{4}{3}\pi r_i^3\right]\right\} + (\Delta\rho_C - \Delta\rho_{HG})\left\{\left[\frac{4}{3}\pi r_2^3\right] - \left[\frac{4}{3}\pi r_1^3\right]\right\}} \right)^2$$

where $\Phi_{bil}(q)$ and $\Phi_s(qr_j)$ are the form factor amplitudes for the 3-shell bilayer and a sphere, respectively, and the latter is given by:

$$\Phi_s(qr_j) = \frac{3\left(\sin\left(qr_j\right) - qr\cos\left(qr_j\right)\right)}{\left(qr_j\right)^3}$$

The chain form factor ($P_{\text{chain}}(qR_g)$) that applies to PEG chains is:

$$P_{chain}(qR_g) = \frac{2\left(e^{-\left(qR_g\right)^2} + \left(qR_g\right)^2 - 1\right)}{\left(qR_g\right)^4}$$

where R_g is the PEG radius of gyration.

The interior-interior, exterior-exterior, and interior-exterior chain cross correlations terms ($S_{ci\text{-}ci}(q)$, $S_{co\text{-}co}(q)$, $S_{ci\text{-}co}(q)$, respectively) are:

$$S_{c_i - c_i}(q) = \psi\left(q^2{R_g}^2\right)^2\left[\frac{\sin\left(q[r_i - R_g]\right)}{q[r_i - R_g]}\right]^2$$

$$S_{c_o - c_o}(q) = \psi\left(q^2{R_g}^2\right)^2\left[\frac{\sin\left(q[r_o + R_g]\right)}{q[r_o + R_g]}\right]^2$$

$$S_{c_i - c_o}(q) = \psi\left(q^2{R_g}^2\right)^2\left[\frac{\sin\left(q[r_i - R_g]\right)}{q[r_i - R_g]}\right]\left[\frac{\sin\left(q[r_o + R_g]\right)}{q[r_o + R_g]}\right]$$

where $\psi\left(q^2{R_g}^2\right) = \left[1 - e^{-q^2{R_g}^2}\right]/q^2{R_g}^2$.

The bilayer-interior chain and bilayer-exterior chain cross correlation terms ($S_{bil\text{-}ci}(q)$ and $S_{bil\text{-}co}(q)$, respectively) are:

$$S_{bil-c_i}(q) = \psi\left(q^2 {R_g}^2\right)\left[\frac{\sin\left(q[r_i - R_g]\right)}{q[r_i - R_g]}\right]\Phi_{bil}(q)$$

$$S_{bil-c_o}(q) = \psi\left(q^2 {R_g}^2\right)\left[\frac{\sin\left(q[r_o + R_g]\right)}{q[r_o + R_g]}\right]\Phi_{bil}(q)$$

The number of PEG chains on a single liposome (N_{PEG}) was determined using the formulated PEG mole fraction (x_{PEG}) along with volumes of DPPC and DPPE (V_{DPPC} and V_{DPPE}, respectively) and the total bilayer volume (V_{bil}):

$$N_{PEG} = \frac{x_{PEG} V_{bil}}{x_{PEG} V_{DPPE} + (1 - x_{PEG}) V_{DPPC}}$$

We determined that accounting for thermal fluctuations[7] in bilayer thickness (d) using a Gaussian distribution was necessary to best represent our data (Figure S2d). Liposome radius polydispersity (σ_{R}/R) was also considered using a Gaussian population distribution. Instrument resolution was accounted for in the modeling of SANS data.

Because $R >> d$, liposome radius does not have a major impact on scattering features at $q \geq 0.02$ Å^{-1}, which is clearly captured in the present model as shown in the liposome radius sensitivity plot (Figure S2a). The scattering information stemming from the bilayer and PEG chain features, on the other hand, is present at $q > 0.02$ Å^{-1}. Therefore, R and σ_{R} have minimal impact on fitting the primary features of interest.

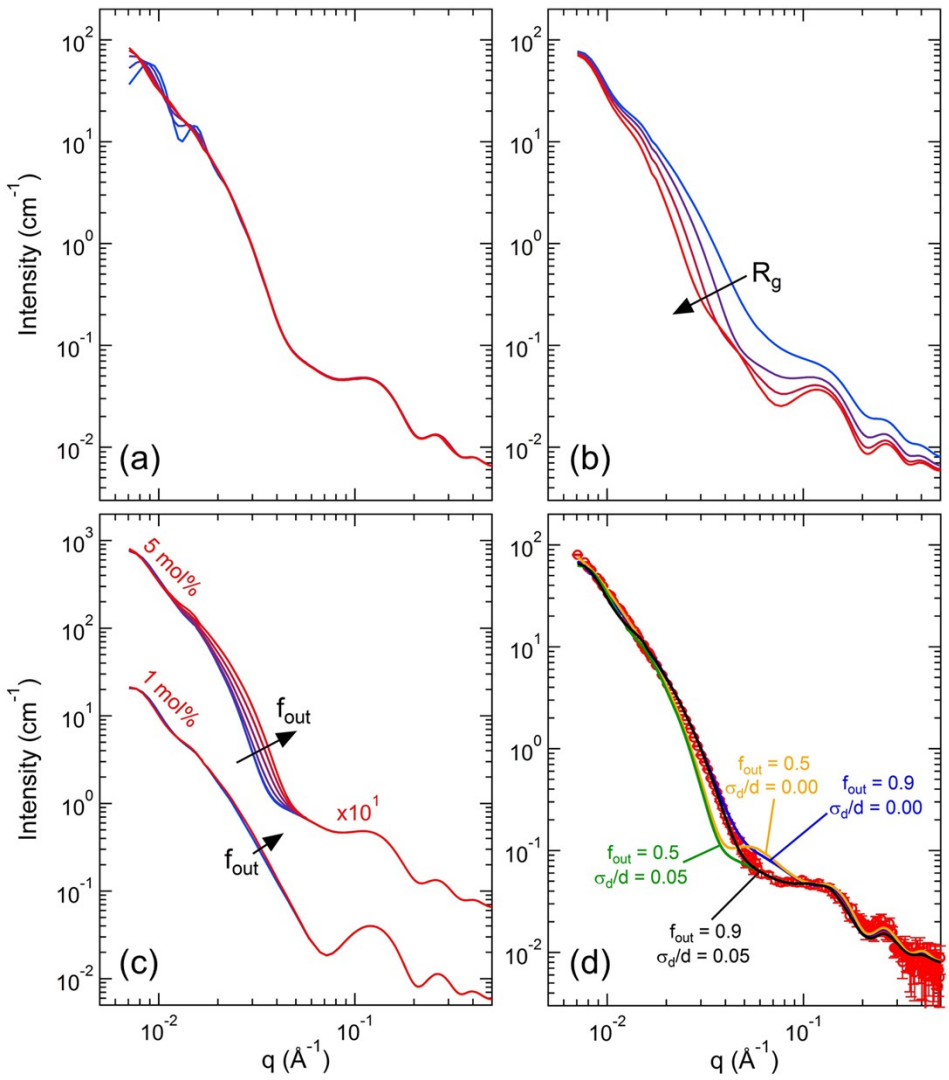

Figure S2. SANS model data indicating the sensitivity to various geometric parameters including (a) liposome radius, R, (b) PEG R_g, and (c) exterior fraction of PEG, f_{out}. The parameter ranges displayed are 300 nm to 700 nm (100 nm increments), 20 nm to 50 nm (10 nm increments), and 0.5 to 1.0 (0.1 increments), respectively, all displayed in gradient colors from blue to red for increasing values. All three figures have 5 mol % PEG, $\sigma_R/R = 0.25$, $d = 50.5$ Å, and $\sigma_d/d = 0.05$; (a) and (b) have $f_{out} = 0.9$; (b) and (c) have $R = 550$ Å; and (a) and (c) have $R_g = 31$ Å. (c) Also includes model data for 1 mol % PEG (labeled) for comparison. (d) Experimental data from 5 mol % DPPE-PEG liposomes prepared by PI (red points) fitted with the PEGylated liposome form factor model with and without σ_d/d and at f_{out} values of 0.5 and 0.9 (labeled).

As indicated in the model equations above, the polymer R_g contribution to overall scattering extends beyond the chain form factor, $P_{chain}(qR_g)$, since it is also included in several cross-correlation scattering terms ($S_{ci\text{-}ci}(q)$, $S_{co\text{-}co}(q)$, $S_{ci\text{-}co}(q)$, $S_{bil\text{-}ci}(q)$ and $S_{bil\text{-}co}(q)$). The overall

scattering contribution of R_g is depicted by the R_g sensitivity plot shown in Figure S2b. Of highest interest in the current work, f_{out} has an impact on the model's power law slope within the range of 0.02 $Å^{-1}$ to 0.04 $Å^{-1}$ (Figure S2c); higher PEG chain partitioning to the exterior liposome surface results in a more gradual decrease in I with increasing q.

Table S1. A comprehensive summary of PEGylated liposome form factor parameters including values fixed during fitting and inter-parameter relationships.

Parameter	Units	Description (fixed value)	Relation
R	Å	Liposome radius	-
σ_R	-	Liposome radius deviation ($\sigma_R/R = 0.25$)	-
d	Å	Liposome bilayer total shell thickness	-
σ_d	-	Liposome bilayer thickness deviation ($\sigma_d/d = 0.05$)	-
t	Å	Liposome bilayer head group thickness (4.9 Å)[8]	-
r_o	Å	Radius to liposome bilayer exterior	$R+d/2$
r_2	Å	Radius to bilayer exterior C/HG interface	$R+d/2-t$
r_1	Å	Radius to bilayer interior HG/C interface	$R-d/2+t$
r_i	Å	Radius to liposome bilayer interior	$R-d/2$
V_o	cm^3	Volume within r_o	$4\pi r_o^3/3$
V_2	cm^3	Volume within r_2	$4\pi r_2^3/3$
V_1	cm^3	Volume within r_1	$4\pi r_1^3/3$
V_i	cm^3	Volume within r_i	$4\pi r_i^3/3$
V_{bil}	cm^3	Volume within liposome bilayer	$V_o - V_i$
$\Delta\rho_{HG}$	cm^{-2}	Lipid head group neutron SLD contrast (-4.50×10^{10})	-
$\Delta\rho_C$	cm^{-2}	Lipid methyl tail neutron SLD contrast (0.00×10^{10})	-
β_{bil}	cm	Scattering volume of lipid bilayer	$\Delta\rho_{HG}(V_o - V_i - V_2 + V_1) + \Delta\rho_C(V_2 - V_1)$
R_g	Å	PEG chain radius of gyration (30±2)	-
V_{PEG}	cm^3	Single PEG chain volume (6.940×10^{-21})[4]	-
N_{PEG}	-	Number of PEG chains on a single liposome	-
f_{out}	-	Fraction of PEG outside liposomes	-
f_{in}	-	Fraction of PEG inside liposomes	$1-f_{out}$
$\Delta\rho_{PEG}$	cm^{-2}	PEG neutron SLD contrast (-5.71×10^{10})	-
x_{PEG}	-	Macroscopic PEG mole fraction	-
V_{DPPC}	cm^3	DPPC molecular volume (1.144×10^{-21})[4]	-

V_{DPPE}	cm^3	DPPE molecular volume $(1.114x10^{-21})^4$	-

Supporting Experimental Data

The distributions of pure DPPC liposomes before and after SANS and those of PEGylated liposomes before and after SANS and following a 10-week aging period measured via DLS are displayed in Figure S3.

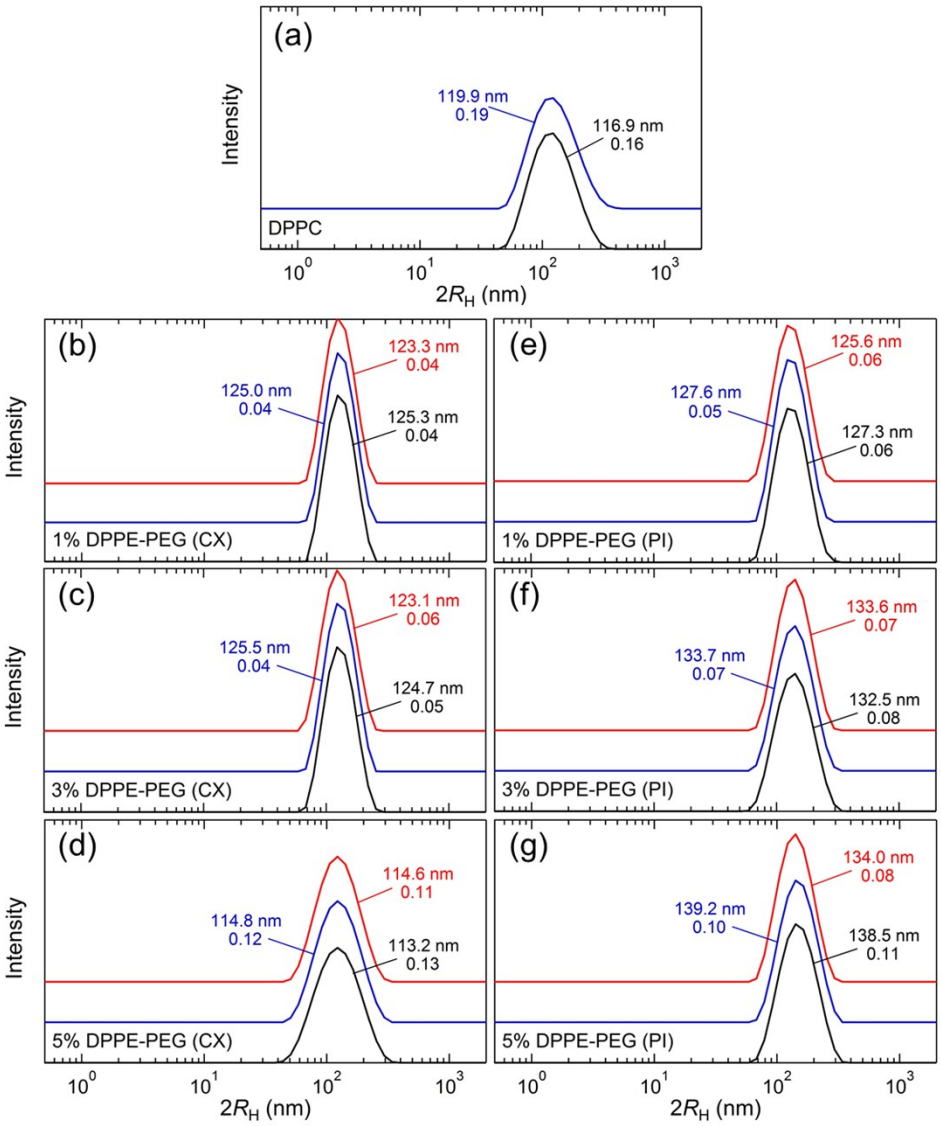

Figure S3. Liposome hydrodynamic size distributions obtained before (black) and after (blue) initial SANS experiments and after 10-week SANS experiments (red) for the control (DPPC) liposomes (a); 1 (b), 3 (c), and 5 (d) mol % DPPE-PEG CX liposomes; and 1 (e), 3 (f), and 5 (g) mol % DPPE-PEG PI liposomes. Average $2R_H$ and polydispersity are indicated.

In general, the average size and distribution breadth are unchanged ($|\Delta 2R_H| < 2$ nm) over the entire experimental period (~48 h for DPPC liposomes and ~10 weeks for DPPC/DPPE-PEG liposomes). The only exceptions are the reduction in size of PI liposomes containing 5 mol % DPPE-PEG ($\Delta 2R_H \approx$ -5 nm), which is discussed in the main text, and the increase in size of pure DPPC liposomes ($\Delta 2R_H \approx$ +3 nm), which may be an early sign of liposome fusion.

Liposomes prepared by each method, *i.e.* CX and PI, were imaged using cryoTEM (Figure S4a and b). The micrographs display the liposomes as dark, circular features that can be semi-

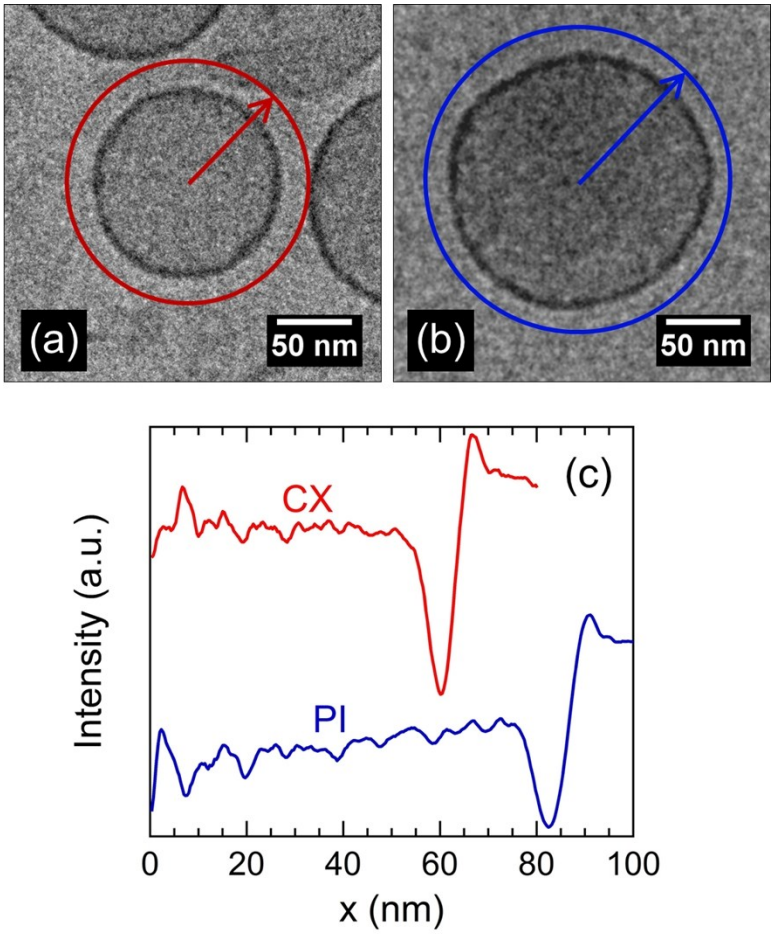

Figure S4. Representative cryoTEM images for CX (a) and PI (b) 5 mol % DPPE-PEG liposomes with radial profile areas indicated. (c) Radial profiles from the images displayed in (a) and (b). The dark regions in (a) and (b) and corresponding low intensities in (c) indicate the bilayer. The CX profile in (c) is vertically shifted for clarity.

quantitatively assessed using radial intensity profiles (Figure S4c). The bilayer is easily identified as a downward peak in the radial profile with the liposome radius and bilayer thickness specifically measured by the peak position and breadth, respectively. The existence of imperfect circularity leads to smearing of the bilayer thickness and, therefore, slight overestimation of the bilayer thickness (more prominent in PI example).

The effect of aging on liposome structure was probed by reconducting SANS experiments following a 10-week period (Figure S5). The primary change noted following the aging period was a decrease in the fraction of PEG chains on the surface of PI liposomes with 5 mol % DPPE-PEG from 0.90 to 0.87. All other samples had negligible changes in structure independent of DPPE-PEG composition and processing route.

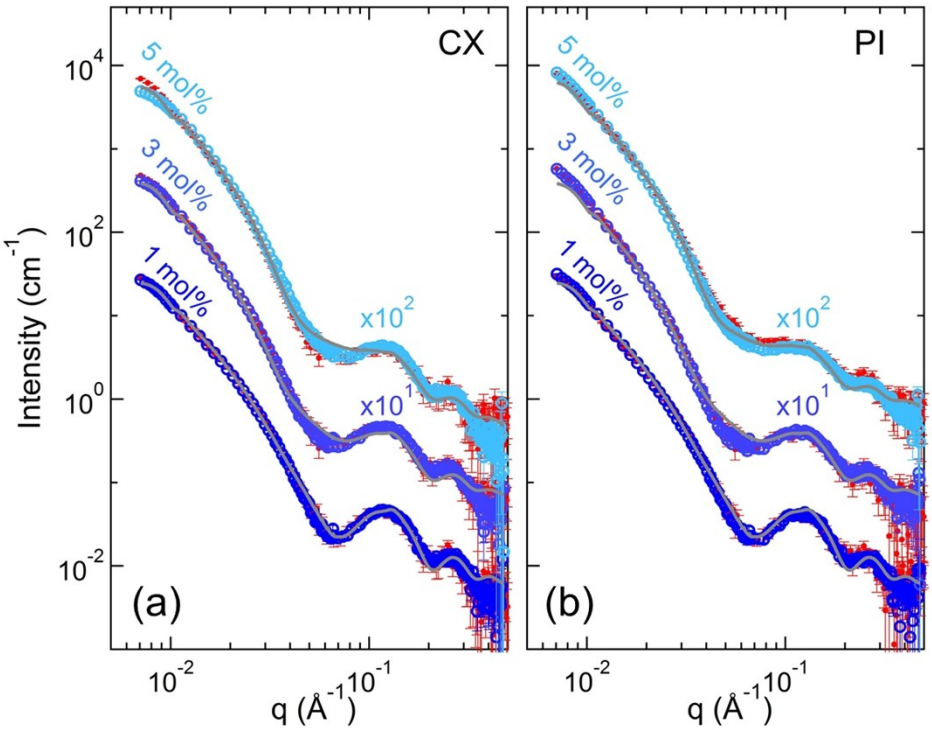

Figure S5. SANS profiles collected for CX (a) and PI (b) liposomes following a 10-week aging period at ambient conditions (blue) overlaid on data from initial measurements (red). The solid lines indicate fits for the samples aged for 10 weeks. DPPE-PEG composition and vertical shift factors are indicated.

‡ Certain commercial equipment and materials are identified in this paper to adequately specify the experimental procedure. In no case does such identification imply recommendation by the National Institute of Standards and Technology (NIST) nor does it imply that the material or equipment identified is necessarily the best available for this purpose.

References

1. S. Kline. *J. Appl. Cryst.* **39**, 895 (2006).
2. C. J. Glinka, J. G. Barker, B. Hammouda, S. Krueger, J. J. Moyer, W. J. Orts. *J. Appl. Cryst.* **31**, 430 (1998).
3. C. H. Cho, J. Urquidi, S. Singh, G. W. Robinson. *J. Phys. Chem. B* **103**, 191 (1999).
4. L. Arleth, C. Vermehren. *J. Appl. Cryst.* **43**, 1084 (2010).
5. (a) J. S. Pedersen, M. C. Gerstenberg. *Macromolecules* **29**, 1363 (1996); (b) J. S. Pedersen. *J. Appl. Cryst.* **33**, 637 (2000).
6. J. S. Pedersen, in *Neutrons, X-rays, and Light: Scattering Methods Applied to Soft Condensed Matter*, edited by Th. Zemb, Lindner (North Holland, The Netherlands, 2002).
7. A. C. Woodka, P. D. Butler, L. Porcar, B. Farago, M. Nagao. *PRL* **109**, 58102 (2012).
8. (a) J. F. Nagle, S. Tristram-Nagle. *Biochim. Biophys. Acta.* **1469**, 159 (2000); (b) D. Uhríková, N. Kučerka, J. Teixeira, V. Gordeliy, P. Balgavý. *Chem. Phys. Lipids*, **155**, 80 (2008).