Structural Evidence for a Reinforcing Response and Retention of Hydration during Confinement of Cartilage Lipids.

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Supporting Information

Materials and methods

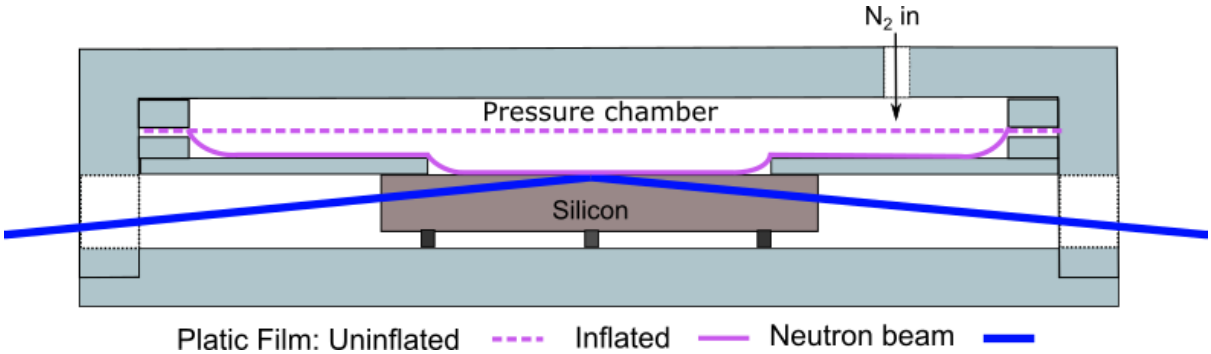

Figure S1. Schematic of the confinement sample environment used in combination with neutron reflectometry. The stack of lipid bilayers is deposited on top of the silicon block and is confined by the inflated plastic film.[1]

Silicon block cleaning:

Polished silicon blocks of 75 mm diameter and 10 mm thickness were first cleaned using piranha solution and when changing samples they were cleaned using a three solvent process, in an ultrasonic bath with acetone, followed by chloroform and then water and finally ten minutes in a UV/ozone cleaner. The silicon blocks were warmed to approximately 40°C before spin coating.

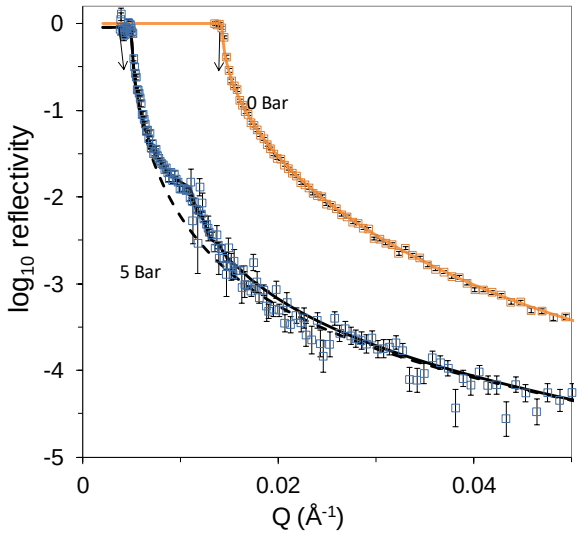

Figure S2. Neutron reflectivity curves for the Melinex membrane inflated against a block of silicon as schematically depicted in Figure S1 with D_2O between the surfaces. Points are data, lines are fits. The deviation from the dashed line that represents the case where there is no mixed signal from bulk D_2O. In the fit the contribution of dust is estimated to be 10% of the sample area. Reproduced with permission from AIP Publishing.[1]

Model details

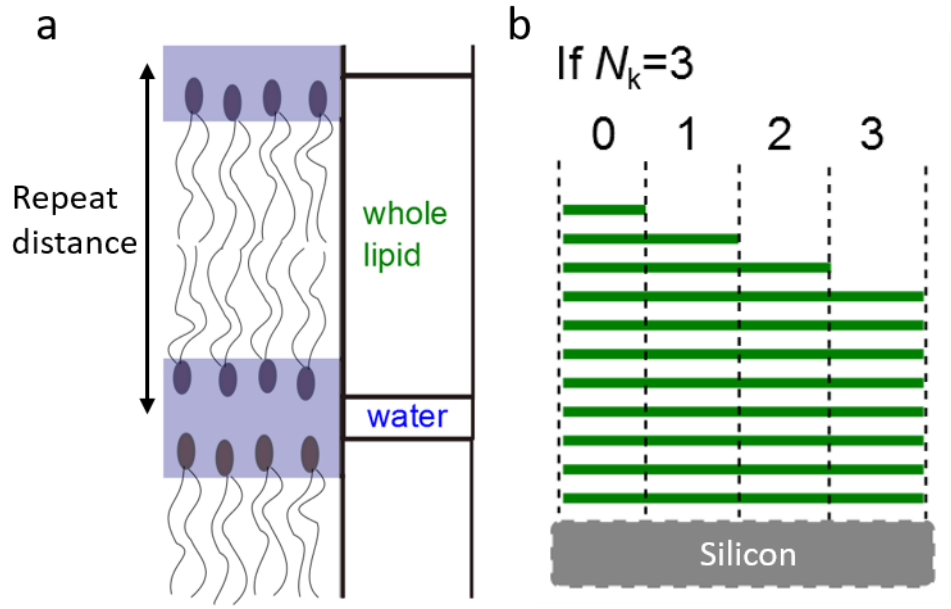

Figure S3. a Schematic to illustrate the model layer structure, alongside a cartoon depiction of the lipid molecules. **b** Schematic to show how the N_k parameter removes layers to form a distribution of numbers of layers, which are averaged as illustrated to obtain the best fit to the data.

Different model forms were tested before the final model structure was chosen. However, the outermost layers of the model always remained the same, a fixed, standard SLD for bulk SiO_2 was used, 3.41×10^{-6} Å^{-2} and the thickness was allowed to vary as a fitting parameter. Next to the oxide was always a lipid head group layer. The final layer was a complete bilayer with the head group at the interface with air for 0 bar measurements, whereas under confinement, at the hydrophobic Melinex surface, a half bilayer was used so that the hydrocarbon chains were at the interface. The final bulk medium was therefore air (SLD 0 Å^{-2}) for the 0 bar measurements and Melinex (SLD 2.56×10^{-6} Å^{-2}) for the others (as confirmed previously[1]). Between these start and end points were multiple occurrences of a repeat unit representing the hydrated stack of lipid bilayers, Figure S3a. The SLD for those lipids were used as given in Table S1.

Table S1 Scattering length densities used for the lipid section of the model fit the volumes used for the calculations are from Armen *et al*.[2]

Lipid	Total tail volume ($Å^3$)	Total lipid volume ($Å^3$)	SLD from the volume ratios (10^{-6} $Å^{-2}$)
DLPC	668	1012	0.34
DMPC	780	1124	0.28
DPPC	892	1236	0.22
DSPC	1005	1349	0.18
DOPC	984	1328	0.19

The simple two-layer model was selected for the analysis after trials. It gave physically possible values of the lipid layer thickness and it did not over-parameterise the data so the important trends were seen clearly. The repeat unit of the model used (Figure S3a) consisted of two layers. Both the head groups and the hydrocarbon chains form one layer to represent the whole lipid bilayer with the rest of the repeat unit formed by a layer of D_2O, in a similar way to the slab model of Gutberlet *et al*.[3] The roughness between the two layers was allowed to increase and thus smooth the interface to provide the same effect as penetration of water into the head groups.

The number of repeat units used to fit each individual data set was a fitting parameter. The spacing of the interference fringes can be used directly to determine the overall thickness, hence the number of bilayers in the sample, while the intensity of the 'Fresnel decay' in the same region is dependent on the overall scattering length density of the sample, hence the amount of water within the whole stack. The size and shape of the Bragg peak indicates both the thickness of the repeating set of bilayers[4] and the ratio of the amount of lipid to water through the difference in SLDs between them. A parameter, N_k, was defined to specify a range in the number of repeats in the bilayer stacks. The overall reflectivity was calculated by taking an average for stacks with between zero and N_k repeats removed from the maximum value, as illustrated by the schematic in Figure S3b. In the diagram an average of 9.5 bilayers is achieved by applying $N_k = 3$ to a maximum number of layers of 11 leading to an equal weighting of the signal from a stack of N=11, 10, 9 and 8 bilayers. Non-integer values of Nk were accommodated by suitable weighting. The final model fit applied represents the averaged signal thus accounting for regions of the sample where the number of bilayers varies slightly.

Supplementary Discussion

During the initial measurement of each sample, for those hydrated using D_2O vapor (Figures S4, S5), there could be a small amount of exchange between the D_2O vapor and H_2O in the

atmosphere of the experimental hall. The low temperature DMPC sample used for the fully hydrated study (Figure S6a) had the water condensed onto the surface prior to confinement. This process provided a bulk layer of water on the surface and hence a critical edge in the reflectivity profile where the water on the surface acted as a bulk medium of higher SLD than the silicon block. Therefore, this provided a good estimate for the maximum amount of exchange and therefore the value of 4.18×10^{-6} Å^{-2}, which equates to 68% D_2O. Thus, 4.18×10^{-6} Å^{-2} was used as the lower limit for the SLD of the hydrating water, while 6.2×10^{-6} Å^{-2} was used as the upper limit (a small amount of exchange is always expected with the atmosphere during assembly of the cell). Data from each vapor hydrated sample were fitted with a water SLD of 4.18×10^{-6} Å^{-2}, 5.2×10^{-6} Å^{-2} and 6.2×10^{-6} Å^{-2} where the extremes provided the estimated uncertainties on the fitting parameters, the mean values and fits shown used 5.2×10^{-6} Å^{-2} as the water SLD. Good quality fits were achievable for the samples in this range of hydrating SLDs (Figure S4). Further, once the sample has been put under a confining pressure the SLD will remain constant as the water is pushed out and there will be vary little contact with the environment, stopping any further exchange. Therefore, with any uncertainty in the SLD the points fitted for lipid thickness and water layer thickness for the different confining pressures would all shift consistently making the confidence in the trends shown higher than the error bars would indicate.

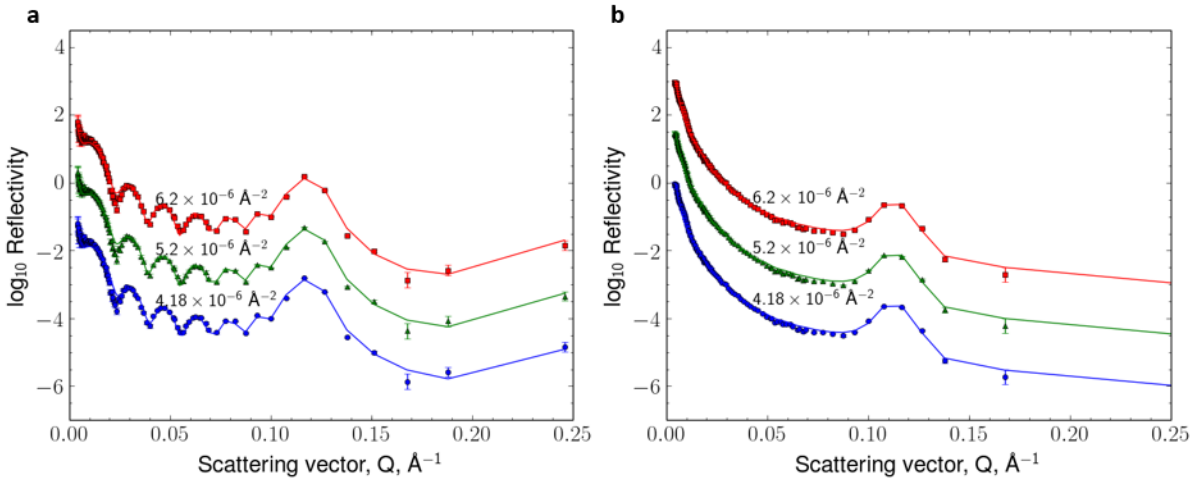

Figure S4. Reflectivity profiles and model fits for vapor hydrated samples of DPPC at 50°C **a** under 0 bar and **b** under 1 bar confinement, showing the good quality fits that can be achieved over the range of hydrating SLDs from 4.18×10^{-6} Å^{-2} to 6.2×10^{-6} Å^{-2}. These are examples of the fits used to estimate the uncertainties on the fitting parameters. Data are off-set vertically for clarity.

Another experimental factor which affected the quality of the fit for a small number of samples was the presence of dust on the sample. The size of the samples and nature of the reflectivity experiments makes it impractical to eliminate all the dust, however its effects are usually small.[1] The dust may provide a place for water to be held within the sample and it is known that the lipids have a large increase in repeat spacing between 99 and 100% relative humidity. In certain samples this leads to an additional, weaker peak or shoulder to the Bragg peak that cannot be captured by the model fit (DMPC fully hydrated at 1 bar 40°C, Figure

S6b and DLPC vapor hydrated 1 bar 20°C, Figure S5a) and therefore only the parts of the reflectivity profile relating to the primary Bragg peak position were fitted. On a small number of occasions it was not possible to fit those data sets, however the unfitted data are included as the trends exhibited by the main Bragg peak position can still be interpreted (DSPC 0 bar vapor hydrated 20°C, Figure S5d and DMPC fully hydrated 0 bar 12°C, Figure S6a).

As discussed above there can be some variation in the number of bilayers present across the sample area. The distribution applied using the N_k parameter has been effective in almost all data sets, however it is possible to have an uneven distribution across the sample area which will impact on the smoothing of the interference fringes and the shape of the Bragg peak in subtly different ways.[5] This is the most likely cause of the small deviation from the model fit of the Bragg peak in the DSPC 75°C vapor hydrated data, Figure S5f.

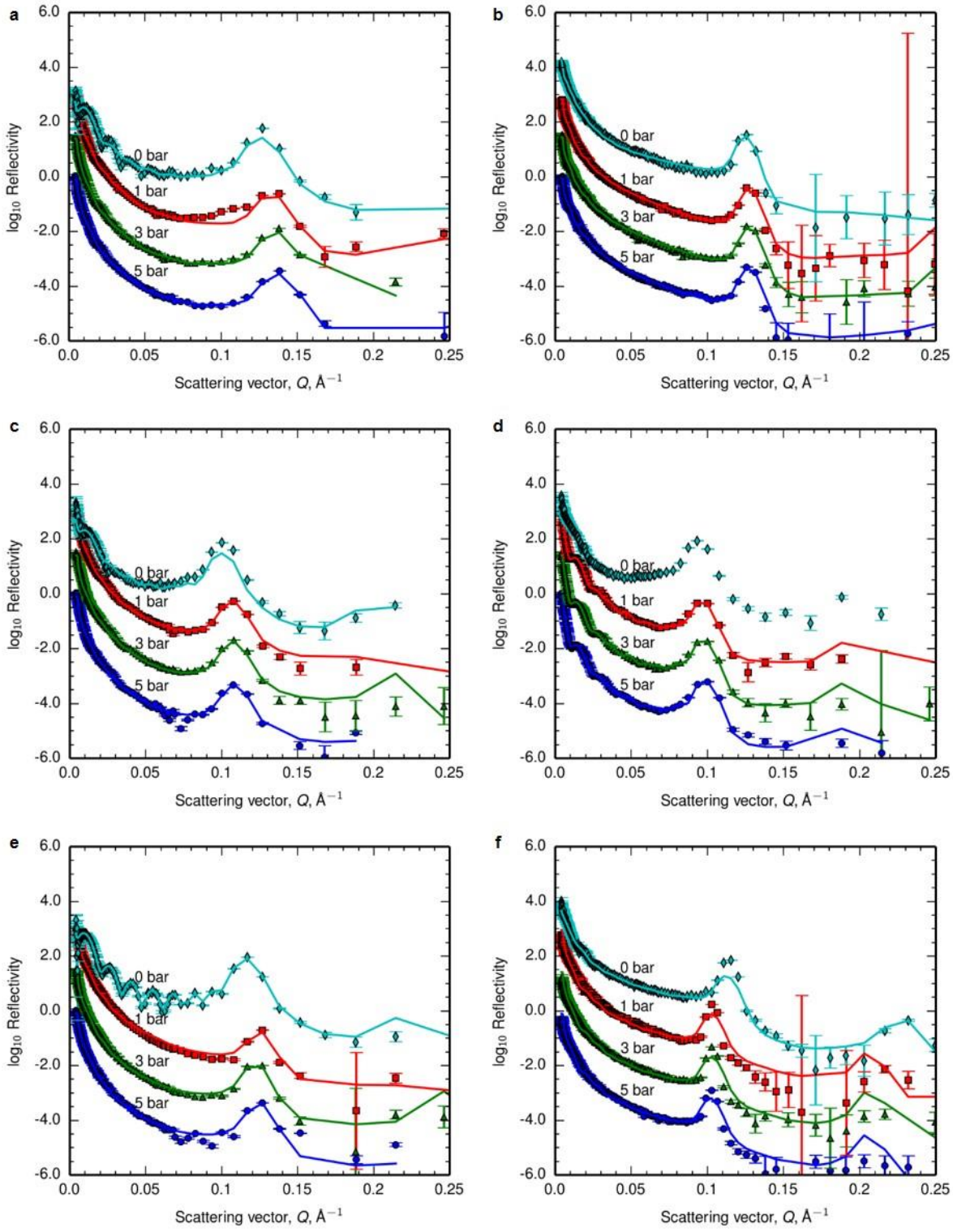

Figure S5. Reflectivity profiles and model fits for vapor hydrated samples of lipids **a** DLPC at 20°C, **b** DMPC at 40°C, **c** DPPC at 20°C, **d** DSPC at 20°C, **e** DOPC at 20°C, **f** DSPC at 75°C. Data are off-set vertically for clarity.

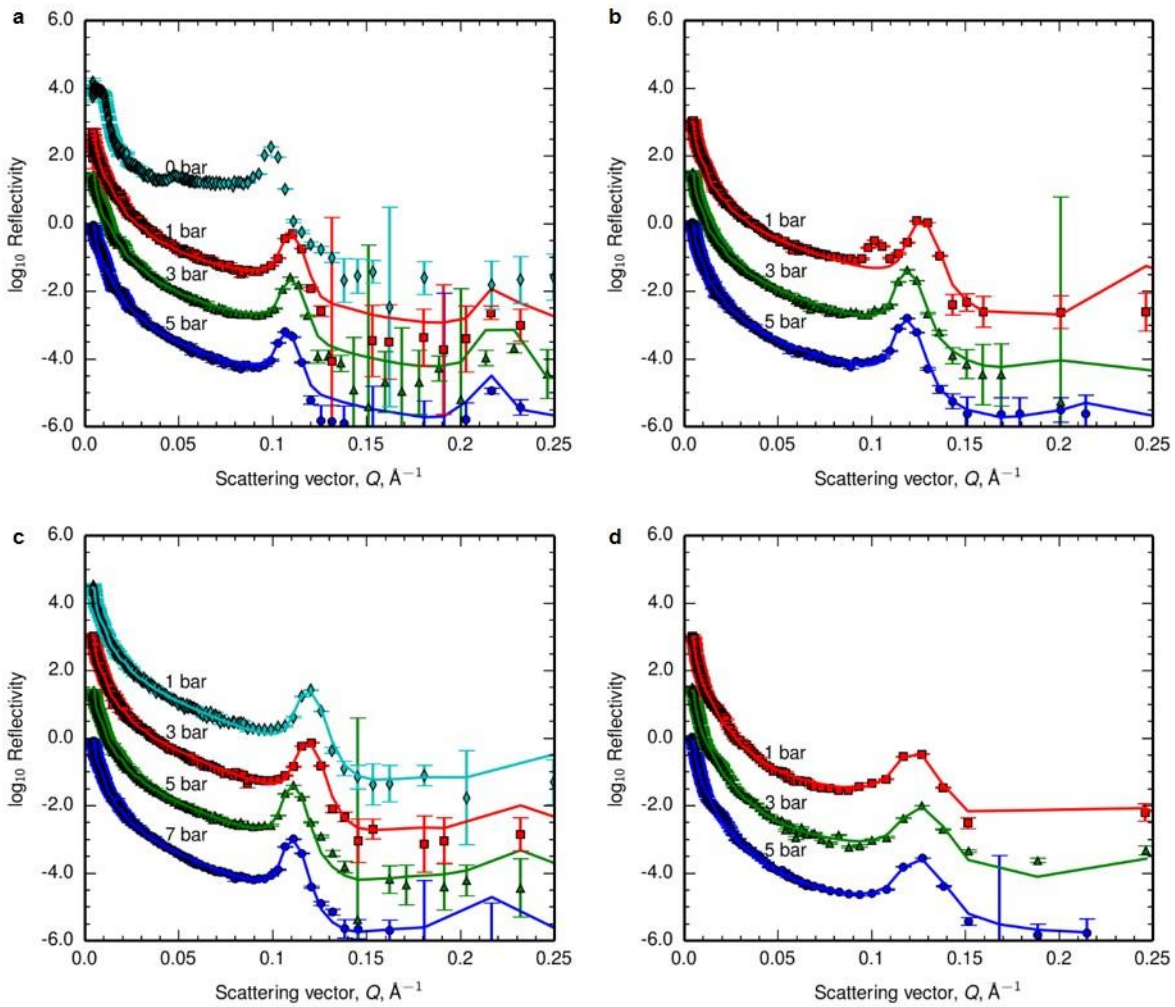

Figure S6. Reflectivity profiles and model fits for fully hydrated samples of the lipids **a** DMPC 12°C condensation hydrated, **b** DMPC 40°C, **c** DPPC 60°C, **d** DOPC 20°C. Data are off-set vertically for clarity.

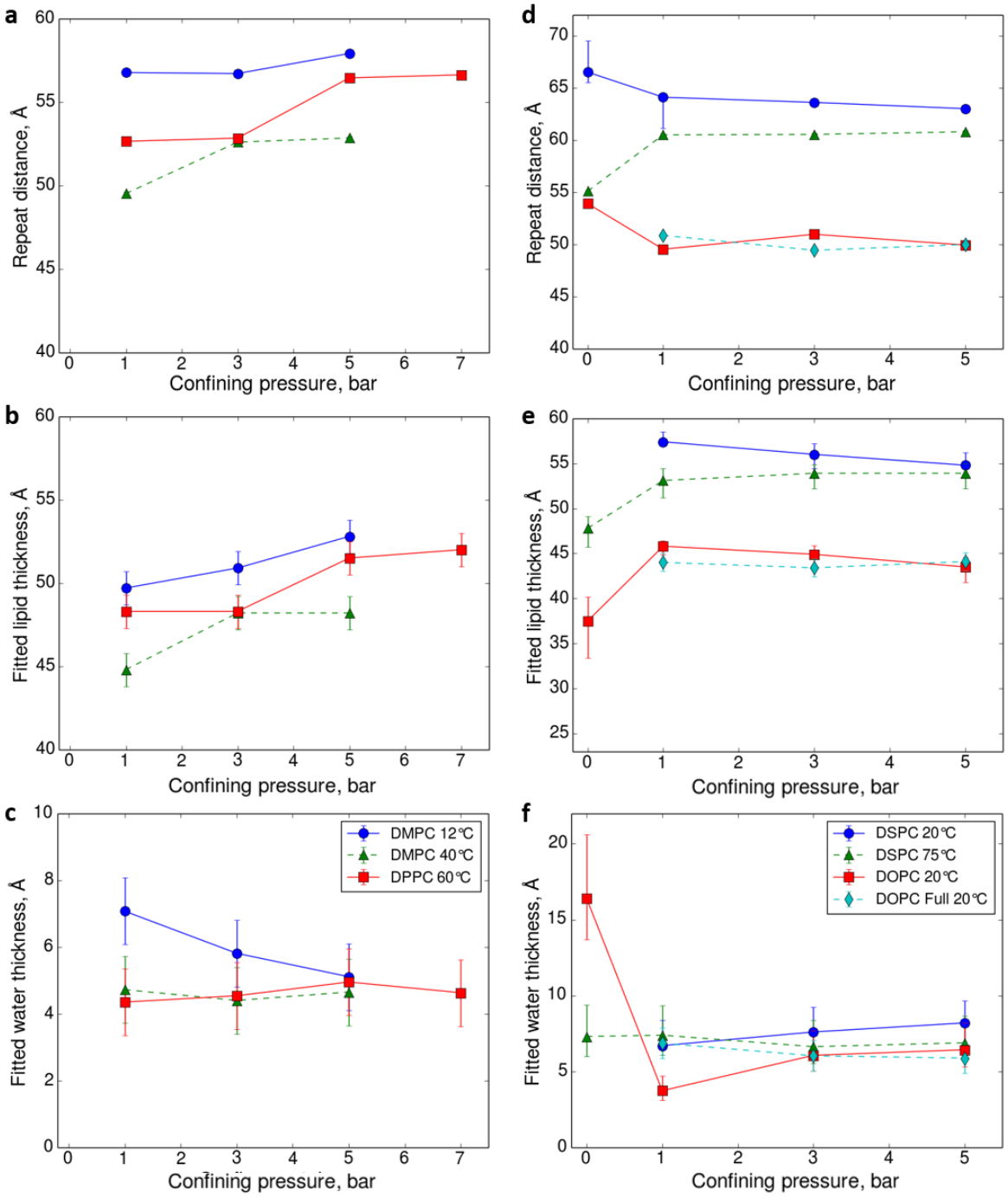

Figure S7. Plots of the trends for the overall repeat spacing, the fitted lipid thickness and the fitted water layer thickness for **a-c** the fully hydrated DMPC at 12°C condensation hydrated and 40°C and DPPC at 60°C. **d-f** show the same for the comparison of DSPC vapor hydrated and DOPC both vapor and fully hydrated. The legend in **c** applies to plots **a-c** and the one in **f** applies to plots **d-f**.

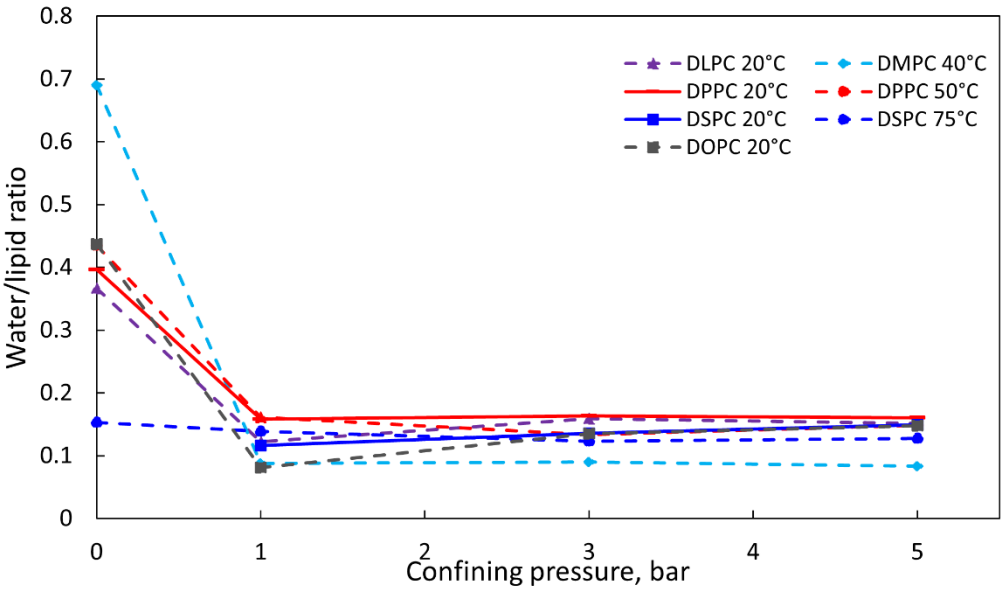

Figure S8. Plot of the ratio of the fitted water layer thickness parameter over the fitted lipid layer thickness parameter for the vapor hydrated lipids at different temperatures, as presented in the legend.

Table S2. Parameters used in the model fitting of the reflectivity data for the vapor hydrated samples. These are the parameters that change only between the unconfined and confined states, apart from the SiO_2 thickness, which is fitted simultaneously for each separate sample. The error from fitting with higher and lower hydrating SLDs for the SiO_2 thickness is ±1 Å. N_average is the mean number of lipid bilayers including the repeats and the terminating full bilayer (for unconfined samples) or half bilayer (for the confined samples).

Lipid	Temperature (°C)		N_{rep}	N_k	$N_{average}$	SiO_2 thickness (Å)
DLPC	20	Unconfined	8	2.4	7.8	13
		Confined	12	9	8.5	13
DMPC	40	Unconfined	26	14	20	44
		Confined	26	14	20	44
DPPC	20	Unconfined	6	2.5	5.8	16
		Confined	11	11	6.5	16
DPPC	50	Unconfined	6	0	7	16
		Confined	9	8	6	16
DSPC	20	Confined	8	2.1	7.9	15
DSPC	75	Unconfined	11	7	8.5	40

DOPC	20	Confined	11	7	8.5	40
		Unconfined	7	0	8	19
		Confined	12	10	8	19

Table S3. Parameters used in the model fitting of the reflectivity data from the vapor hydrated samples, which change with increasing pressure. The errors from fitting with higher and lower hydrating SLDs for the scale factor is ±0.01 and for the roughnesses the maximum error was 1 Å. N_average is the mean number of lipid bilayers including the repeats and the terminating full bilayer (for unconfined samples) or half bilayer (for the confined samples).

Lipid	Temperature (°C)	Confining pressure	Scale factor	Internal roughness (Å)	Outer roughness (Å)
DLPC	20	0	0.84	3	26
		1	0.81	3	20
		3	0.84	3	19
		5	1.00	13	3
DMPC	40	0	0.68	5	18
		1	0.88	5	18
		3	0.87	4	18
		5	0.84	3	26
DPPC	20	0	0.41	6	50
		1	0.99	6	5
		3	0.86	5	5
		5	0.85	6	5
DPPC	50	0	0.85	5	50
		1	1.00	9	8
		3	0.94	3	8
		5	0.95	6	8
DSPC	20	1	0.84	7	12
		3	0.80	7	13
		5	0.78	7	12
DSPC	75	0	0.99	7	50
		1	0.90	7	3
		3	0.83	6	8
		5	0.78	6	8
DOPC	20	0	0.89	3	50
		1	0.84	3	13
		3	0.76	3	13
		5	0.80	3	13

Table S4. Parameters used in the model fitting of the reflectivity data for the fully hydrated samples. These are the parameters that change only between the unconfined and confined states, apart from the SiO_2 thickness, which is fitted simultaneously for each separate sample. Only the DPPC sample at 60°C was measured in the unconfined state, vapor hydrated before D_2O was added to fully hydrate the sample. The error from fitting with higher and lower hydrating SLDs for the SiO_2 thickness is ±1 Å. N_average is the mean number of lipid bilayers including the repeats and the terminating full bilayer (for unconfined samples) or half bilayer (for the confined samples).

Lipid	Temperature (°C)		N_{rep}	N_k	$N_{average}$	SiO_2 thickness (Å)
DMPC	12	Confined	18	11	13.5	33
	40	Confined	24	12	19	45
DPPC	60	Unconfined(vapour)	23	13	17.5	51
		Confined	23	12	18	51
DOPC	20	Confined	11	10	7	17

Table S5. Parameters used in the model fitting of the reflectivity data from the fully hydrated samples, which change with increasing pressure. The DMPC at 12°C was fully hydrated via condensation and the DPPC 60°C 0 bar was measured via vapor hydration prior to full hydration. The errors from fitting with higher and lower hydrating SLDs for the scale factor is ±0.01 and for the roughnesses the maximum error was 1 Å.

Lipid	Temperature (°C)	Confining pressure	Scale factor	Internal roughness (Å)	Outer roughness (Å)
DMPC	12	1	0.57	6	11
		3	1.00	5	11
		5	1.00	5	11
DMPC	40	1	1.0	4	15
		3	1.0	3	15
		5	1.0	3	15
DPPC	60	0 (vapour)	0.89	9	19
		1	0.90	6	17
		3	0.99	7	17
		5	0.93	6	17
		7	0.89	7	17
DOPC	20	1	0.90	7	6
		3	0.81	6	6
		5	0.81	7	6

References

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(2) Armen, R. S.; Uitto, O. D.; Feller, S. E. Phospholipid Component Volumes: Determination and Application to Bilayer Structure Calculations. *Biophys. J.* **1998**, *75* (2), 734–744.

(3) Gutberlet, T.; Steitz, R.; Fragneto, G.; Klösgen, B. Phospholipid Bilayer Formation at a Bare Si Surface: A Time-Resolved Neutron Reflectivity Study. *J. Phys. Condens. Matter* **2004**, *16* (26), S2469–S2476.

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