Supplementary material:
Fast cholesterol flip-flop and lack of swelling in skin lipid multilayers

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S1. ANALYSIS DETAILS AND ADDITIONAL RESULTS

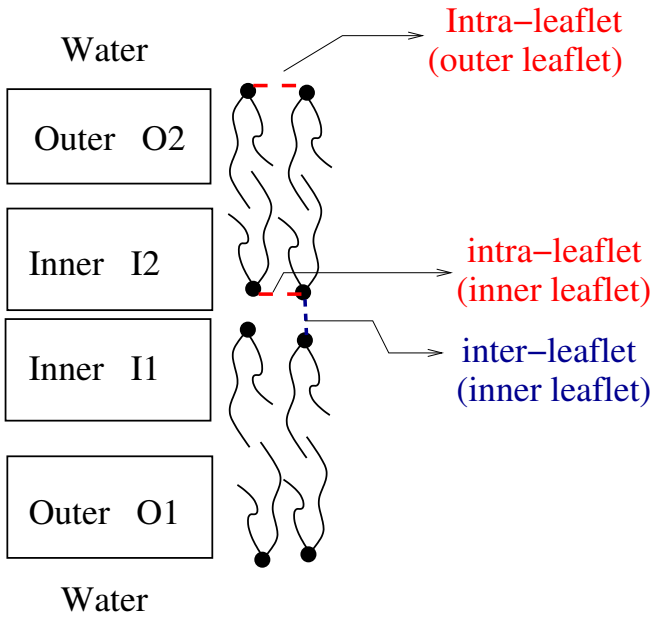

FIG. S1. Naming convention for leaflets and inter-lipid hydrogen bonds.

In this supplementary material, we provide the methods used in calculating the hydrogen bonds, flip-flop time-scales, and the excess chemical potential of water. In our simulation of double bilayer in excess water, only two of the leaflets are in contact with water (Fig. S1). We term these two leaflets as the 'outer leaflets'. The other two 'inner' leaflets face each other and are not in contact with water. After an equilibration time of 20 ns, we stored 5000 configurations separated by 0.2 ns spanning a total of 1 μs. Unless otherwise stated, the results below are averaged over these 5000 configurations.

A. Hydrogen bonds

We use a geometric criteria [1–4] to define a hydrogen bond if the distance between the donor and the acceptor atoms is less than 3.5Å and simultaneously the absolute angle between the vectors $\vec{r}_{DH}$ and $\vec{r}_{AH}$ is less than 30° (Fig. S2).

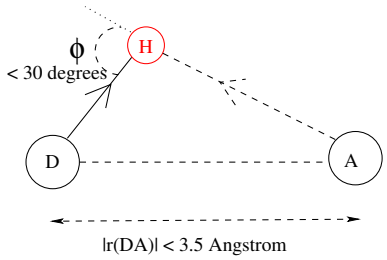

FIG. S2. Geometric criteria used to identify hydrogen bonds.

B. Tail order

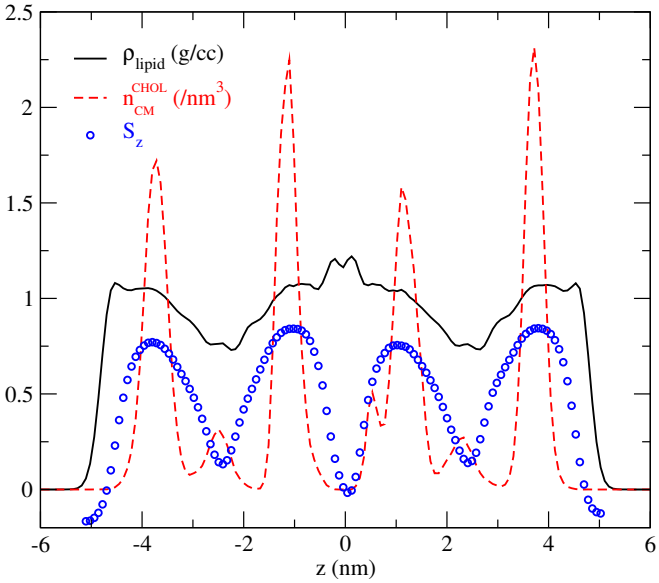

FIG. S3. Total lipid mass density $\rho_{\rm lipid}$ (black solid line), number density of CHOL center of mass $n_{\rm CM}^{\rm CHOL}$ (red dashed lines) and tail order parameter S_z (blue symbols) as a function of distance from lipid center of mass.

To investigate the alignment of the lipid tails, for any three consecutive CH_2 groups (C_{i-1}, C_i and C_{i+1}) in the CER or FFA molecules, we consider the angle θ_z of the vector (C_{i+1} - C_{i-1}) with respect to the z-axis (normal direction to the lipid layers). We define an order parameter [5]

$$S_z(z) = \left\langle \frac{3\cos^2\theta_z - 1}{2} \right\rangle, \qquad \text{(S1)}$$

where the angular bracket denotes averaging over all CH_2 triplets with the central group being at a distance z from

the lipid center of mass. The usual order parameter is calculated (or measured) as a function of carbon number along the lipid tails [5], while we consider it as a function of z. For perfect tail alignment along the z-direction, $S_z = 1$; random order gives $S_z = 0$; and perfect aligment perpendicular to the z-direction gives $S_z = -0.5$. Fig. S3 shows that S_z becomes close to zero in the tail-tail interface region ($z \simeq \pm 2.4$nm) signifying a disordered region. The same region shows lower total mass density and a subpopulation of CHOL center of mass.

C. Density profile and CHOL flip-flop

We unfold the molecules in the saved configurations and fix the lipid center of mass at the origin. The mass density of the lipids and the number density of the center of masses of the lipids were calculated in this lipid center of mass reference frame.

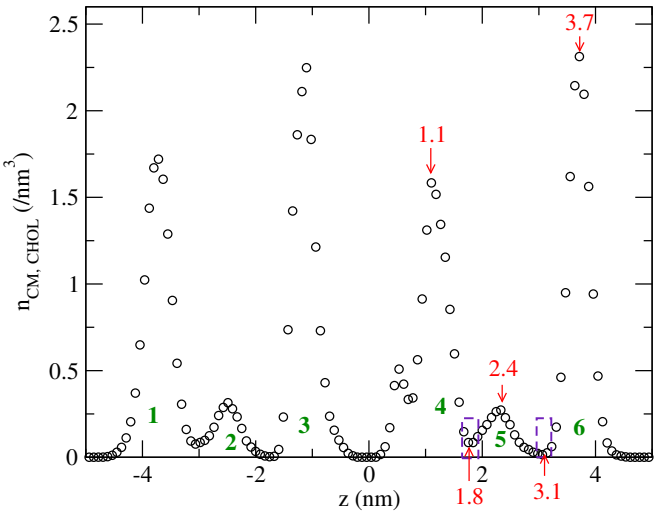

FIG. S4. Average density of center of mass of CHOL and identification of zones (1-6). The (red) arrows show the boundaries and local density maximum of zone 5, while the (violet) dashed boxes show the criteria for a 'flip', which is a transfer between adjacent zones.

From the distribution of the centers of mass of CHOL, we identify two inter-leaflet liquid like regions centered at $z = \pm 2.4$ nm. For the peak at $z = 2.4$ nm, the minima in the distribution of CM are at $z = 1.8$ nm and $z = 3.1$ nm. In the first configuration, we assign a zone index to a given CHOL depending on the z-coordinate of its center of mass:

$$\begin{aligned}
\text{(ordered) zone 1:} &\quad z_{CM} \le -3.1 \\
\text{(disordered) zone 2:} &\quad -3.1 \le z_{CM} < -1.8 \\
\text{(ordered) zone 3:} &\quad -1.8 \le z_{CM} < 0 \\
\text{(ordered) zone 4:} &\quad 0 \le z_{CM} < 1.8 \\
\text{(disordered) zone 5:} &\quad 1.8 \le z_{CM} < 3.1 \\
\text{(ordered) zone 6:} &\quad 3.1 \le z_{CM}.
\end{aligned}$$

In subsequent configurations we assign a new provisional zone index to a given CHOL only if has moved into the next zone by at least 10% of width of the next zone. This criteria is indicated by dashed boxes in Fig. S4. Thus, a CHOL initially belonging to zone 5 is considered to have moved to zone 4 only if its $z_{CM} < 1.64$.

We define a *flip* event as a transit between that does not revert to the original zone within two frames (0.4ns). There are nearly 3 times more flip events involving the outer leaflets than involving the inner leaflets. We define a *flip-flop* event to be when a CHOL enters a ordered zone after having entered the disordered zone from a different ordered zone. In our trajectory we did not find any lipid exchange between the bilayers, so that all flip-flop events involve CHOL exchange between an inner leaflet and an outer leaflet. Table S1 shows the data used in calculating the flip-flop time scales.

D. Force fluctuations for constrained water molecules

We perform simulations in which a single water molecule is constrained to at a given z-separation from the lipid center of mass. This constraint induces a rapidly fluctuating force $F_z(z,t)$ in the z direction. However, we find a slowly decaying time correlation in $F_z(z,t)$, particularly in the ordered regions. At a fixed z, we fit the autocorrelation of $F_z(z,t)$ to a sum of two generalized exponentials:

$$C(z,t) \equiv \langle F_z(z,t) F_z(z,0) \rangle \tag{S2}$$

$$= \sum_{i=1}^{2} A_i(z) \exp\left[-\left(\frac{t}{\tau_i(z)} \right)^{\beta_i(z)} \right], \tag{S3}$$

where the parameters A_i, τ_i, and β_i are fitted with the Levenberg-Marquardt damped least-square method. Fig. S5 shows $C(t)$ in the ordered leaflet region close to bilayer-bilayer interface ($z = 0.3$ nm) along with the fit with two stretched/compressed exponentials. Assigning an average decay time $\tau_{av,i} = \frac{\tau_i}{\beta_i} \Gamma\left(\frac{1}{\beta_i}\right)$, the fit gives a fast decay time of 0.03 ps and a slow decay time of 7.9 ns. At each z, we ensure that the simulations are longer than the slow decay time.

Fig. S6 shows the variation of the average decay times and the exponents with z. There is a fast decay in $C(z,t)$ at all z with $\tau_{av} \simeq 0.03$ ps and exponent $\beta \simeq 1.5$ (open circles in Fig. S6). Inside the lipid double bilayer, there is an additional slowly decaying component (filled squares in Fig. S6) that follows a stretched exponential with $\tau_{av} \sim 20$ ns in the most ordered part of the leaflets, with a stretching exponent $\beta \simeq 0.2$.

Inside the ordered lipid leaflets, the $x - y$ diffusion of the constrained water molecule is limited to 40 ns timescale (the longest time simulated for the constrained water simulations). We use six separate simulations with a different randomly chosen water molecules to calculate

Transition	Starting zone		$n_{\rm start}$	Finishing Zone		events	τ/μs
	region	zone #	region		zone #		
flip	outer ordered	(1, 6)	102.7	disordered	(2, 5)	241	0.4
	inner ordered	(3, 4)	102.7	disordered	(2, 5)	81	1.2
	ordered	(1, 3, 4, 6)	102.7	disordered	(2, 5)	322	0.64
	disordered	(2,5)	9.3	ordered	(1,3,4,6)	322	0.06
flip-flop	outer or inner leaflets	(1,6) or (3,4)	112	inner or outer leaflets	(3,4) or (1,6)	6	0.64

TABLE S1. Statistics for transitions of CHOL molecules between different regions in 1 μs, for calculations of flip times and flip-flop times. For flip-flop, CHOL begin in the outer (inner) ordered region of a leaflet, and explore the outer (inner) ordered and disordered regions until it enters the inner (outer) ordered regime of the other leaflet of the same bilayer.

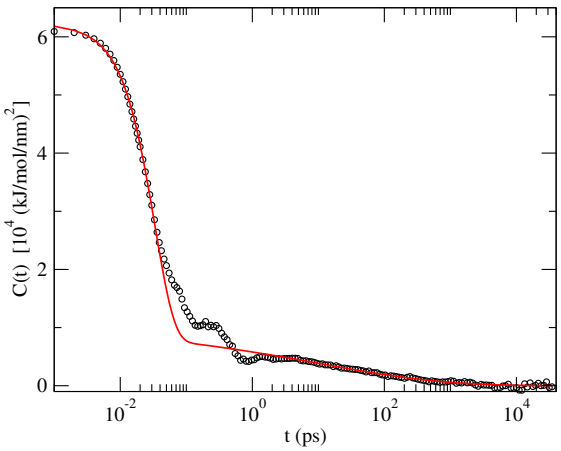

FIG. S5. Autocorrelation of $F_z(z,t)$ (symbols) at $z = 0.3$ nm with a fit (line) to Eq. S3, with parameters $\tau_i = 0.03$ ps and 7.9 ns for the fast and slow times. The corresponding exponents are $\beta = 1.3$ (fast: compressed exponential) and $\beta = 0.18$ (slow: stretched exponential).

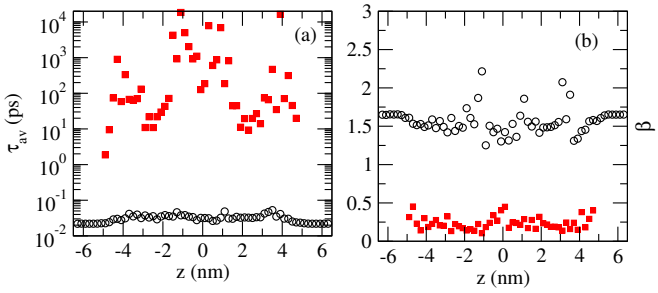

FIG. S6. Average decay times of $C(z,t)$ and exponents from fitting of autocorrelation of $F_z(z,t)$, at different distances z from the double bilayer center.

$\langle F_z(z)\rangle$ at a fixed z. Furthermore, we use the antisymmetric property of $\langle F_z(z)\rangle$ to get 12 independent estimates of $\langle F_z(z)\rangle$ at a given z. The error-bars in the excess chemical potential are calculated from error of mean from these 12 estimates of $\langle F_z(z)\rangle$ at each z.

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