Supplementary Information

Computational Insights into the Role of Cholesterol in Inverted Hexagonal Phase Stabilization and Endosomal Drug Release

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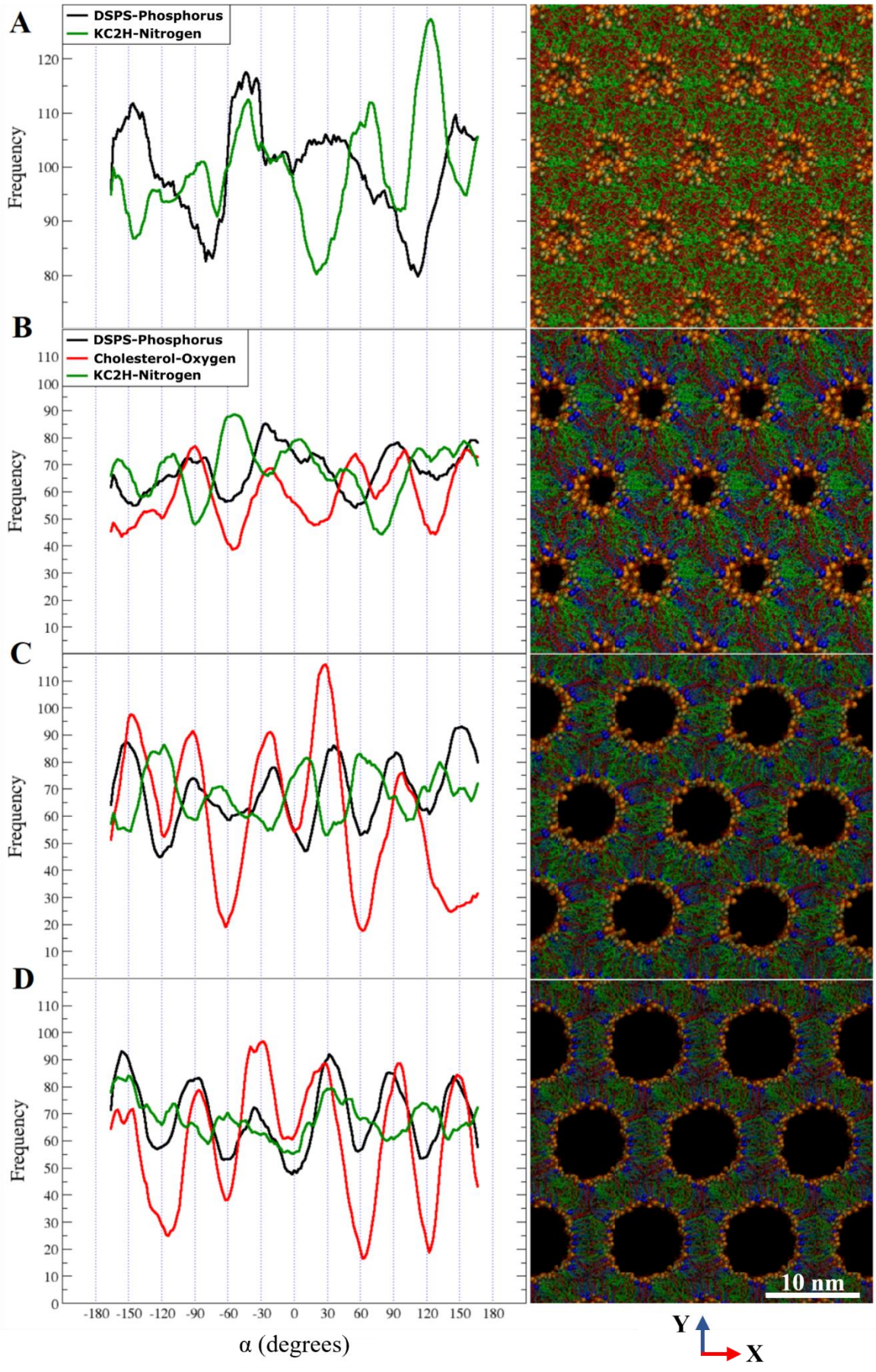
A
DSPS-Phosphorus
KC2H-Nitrogen
Frequency
B
DSPS-Phosphorus
Cholesterol-Oxygen
KC2H-Nitrogen
Frequency
C
Frequency
D
Frequency
-180 -150 -120 -90 -60 -30 0 30 60 90 120 150 180
α (degrees)
10 nm
Y
X

Figure S1. Lipid angular distribution around the water core

The angular distributions of each lipid type in DSPS/KC2H/cholesterol systems at 313 K are shown in the left column. These correspond to the same systems with (50/50/0) mixing ratio with 10 n_w (**A**), and (35/35/30) system with 10 n_w (**B**), 20 n_w (**C**), and 30 n_w (**D**). Black, red, and green lines are running averages over histograms of DSPS, cholesterols, and KC2H. The Y axis shows the frequency for a lipid of certain type to be found in a certain angle. Snapshots for the corresponding systems are shown in the right column. The horizontal white bar shows the scale, is the same for all the figures on the right column and can be used for comparison. The lipid tubules point in and out of the page along the Z direction. Water and hydrogens are not shown for clarity. The color code for snapshots is the same as in **Figure 1B** and **Figure 2**.

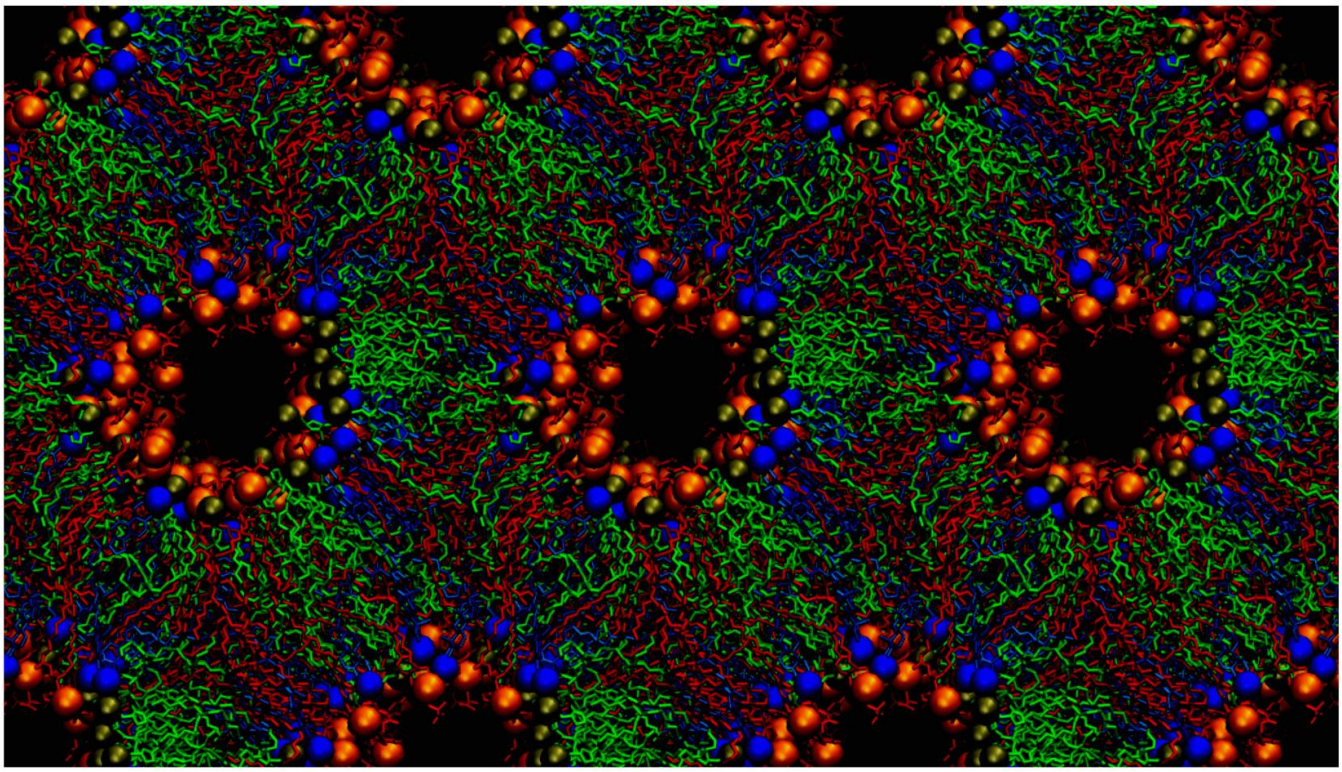

Figure S2. Lipid angular distribution for DSPS/KC2H/cholesterol at 10 n_w

A snapshot at 300 ns of the DSPS/KC2H/cholesterol (35/35/30) system with 10 n_w at 313 K. The color code and definitions are the same as in **Figure 1B** and **Figure 2**.

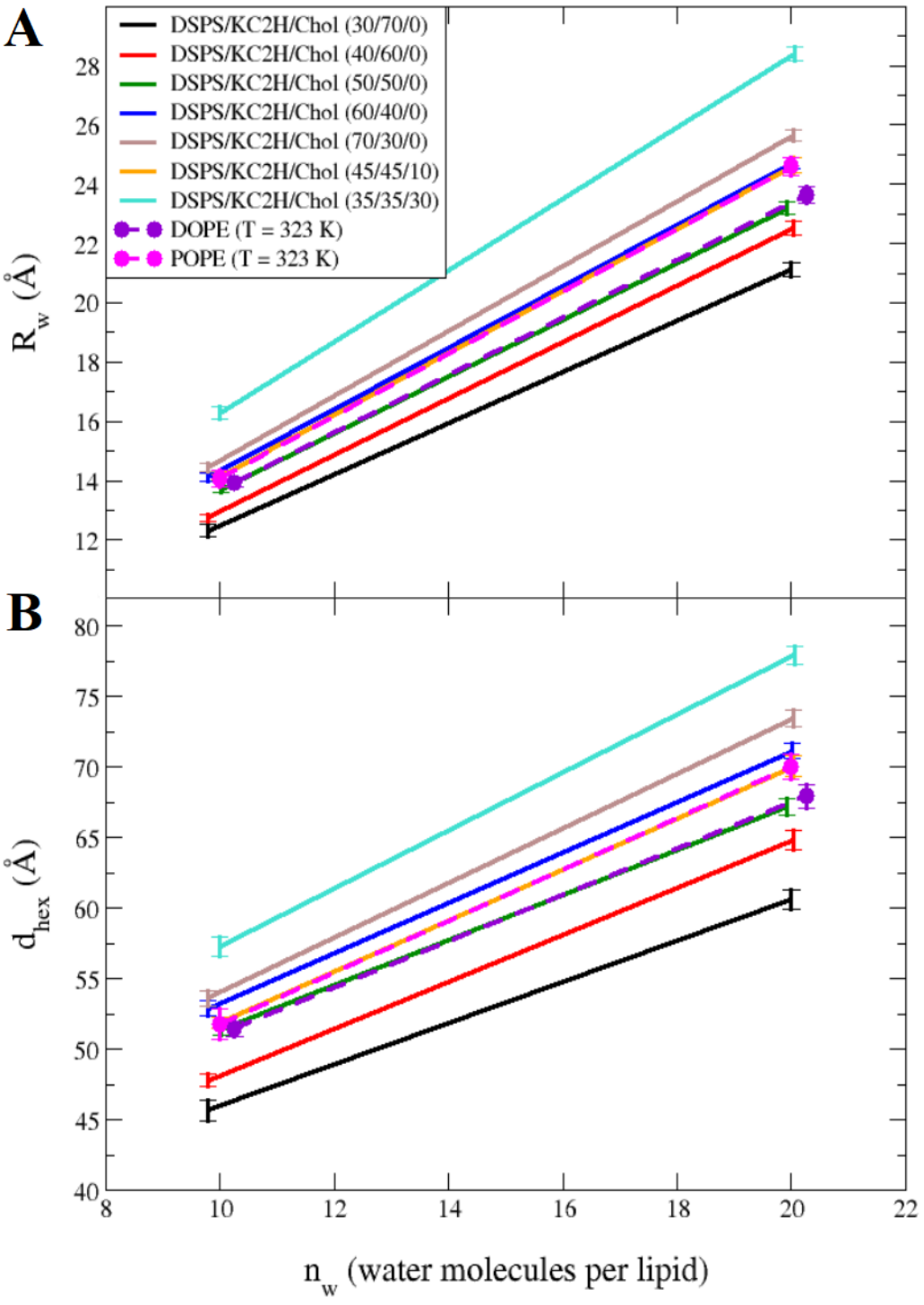

Figure S3. Structural parameters of DSPS/KC2H/cholesterol *versus* PE H_{II} systems

A) Radius of water core (R_w), and **B)** lattice distance (d_{hex}) of DSPS/KC2H/cholesterol H_{II} systems with different mixing ratios at 313 K are plotted versus hydration level (n_w). Calculated parameters for two hydration levels (10 n_w and 20 n_w) are shown and connected with a line (it is well supported and documented in Ramezanpour et al. (1) that both d_{hex} and R_w are linearly dependent on n_w. Therefore, for better visualization, only two values corresponding to 10 n_w and 20 n_w hydration levels are shown and connected using a line. Values for 30 n_w, are provided in **Table S1**. Data for DOPE and POPE at 323 K are taken from Ramezanpour et al. (1) and shown for comparison. The data points are the mean values, and error bars represent the standard deviations of the mean.

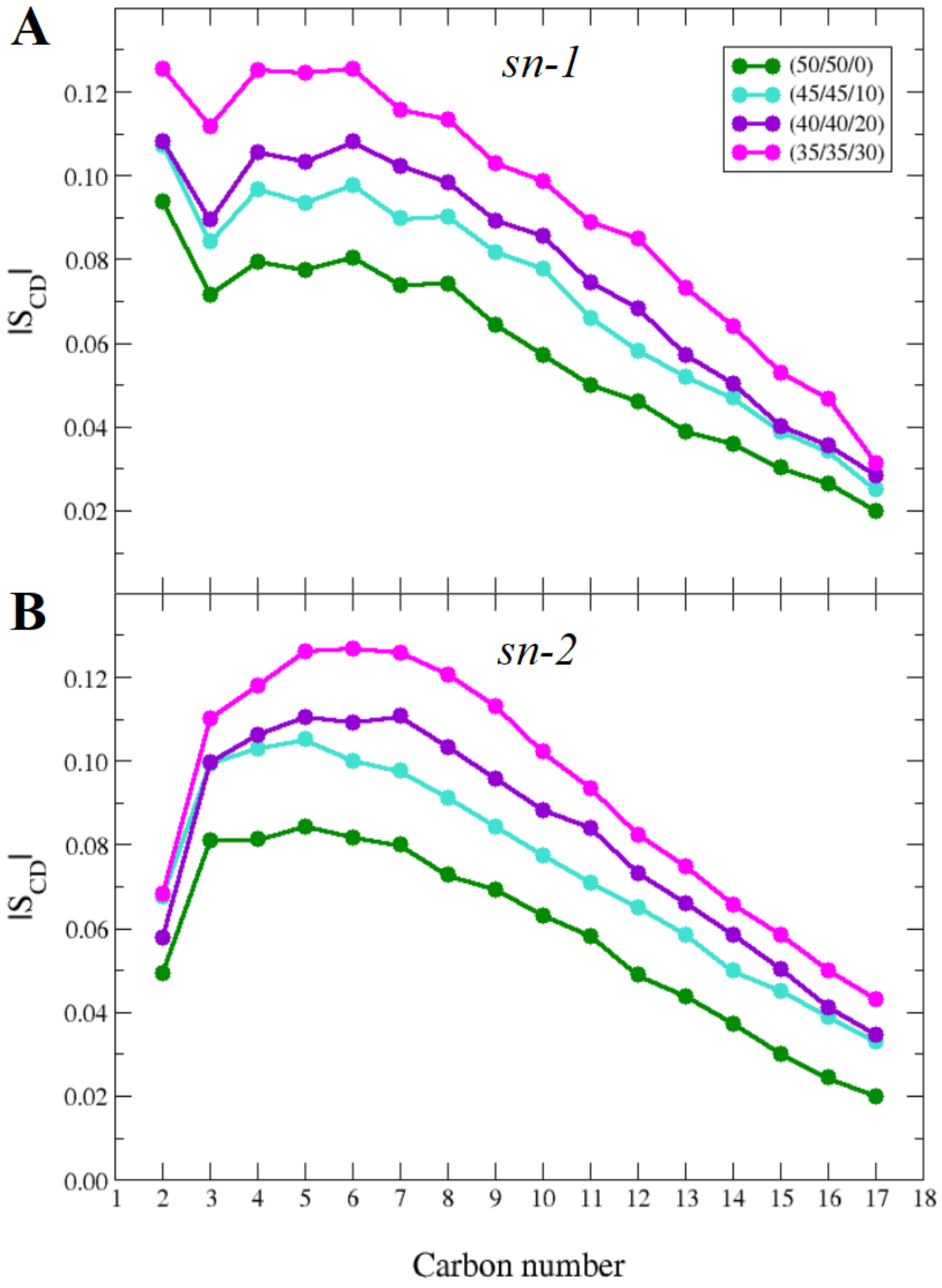

Figure S4. S_{CD} parameters of DSPS acyl chains in DSPS/KC2H/cholesterol H_{II} systems

Computational S_{CD} parameters for **A)** *sn-1*, and **B)** *sn-2* acyl chains of DSPS in DSPS/KC2H/cholesterol H_{II} system with 10 n_w at 313 K. Only systems with an equimolar concentration of DSPS and KC2H are shown here to highlight the effect of different cholesterol concentration. Systems with a higher concentration of cholesterol had higher S_{CD} parameters.

METHODS

Simulation setup validation

The force field (FF) parameters for these simulations were taken from the CHARMM36 (C36 FF) (See the **METHODS** section in main text for details). These parameters have been shown to be valid for simulation of systems where cholesterol is both minority and majority component in lipid bilayers (2), and for simulation of non-lamellar phases, e.g., H_{II} phase (3).

To confirm that the simulated DSPS/KC2H mixtures are valid, we checked whether this system follow the same trends and fulfils the expectations previously reported for H_{II} phase (1,4,5). MD simulations of DOPE and POPE H_{II} systems conducted at multiple hydrations but at the same temperature showed that lipids in H_{II} systems with higher hydrations (n_w) have higher S_{CD} parameters (1). An increase in temperature is also known to decrease the S_{CD} parameter in H_{II} phase (4,5). Furthermore, increasing mol% of unsaturated lipids in the mixture is expected to reduce the S_{CD} in the saturated lipids acyl chains in system (6,7). All these expectations are met for the simulated DSPS/KC2H/cholesterol (50/50/0) H_{II} systems [**Figure S5, Figure S6**].

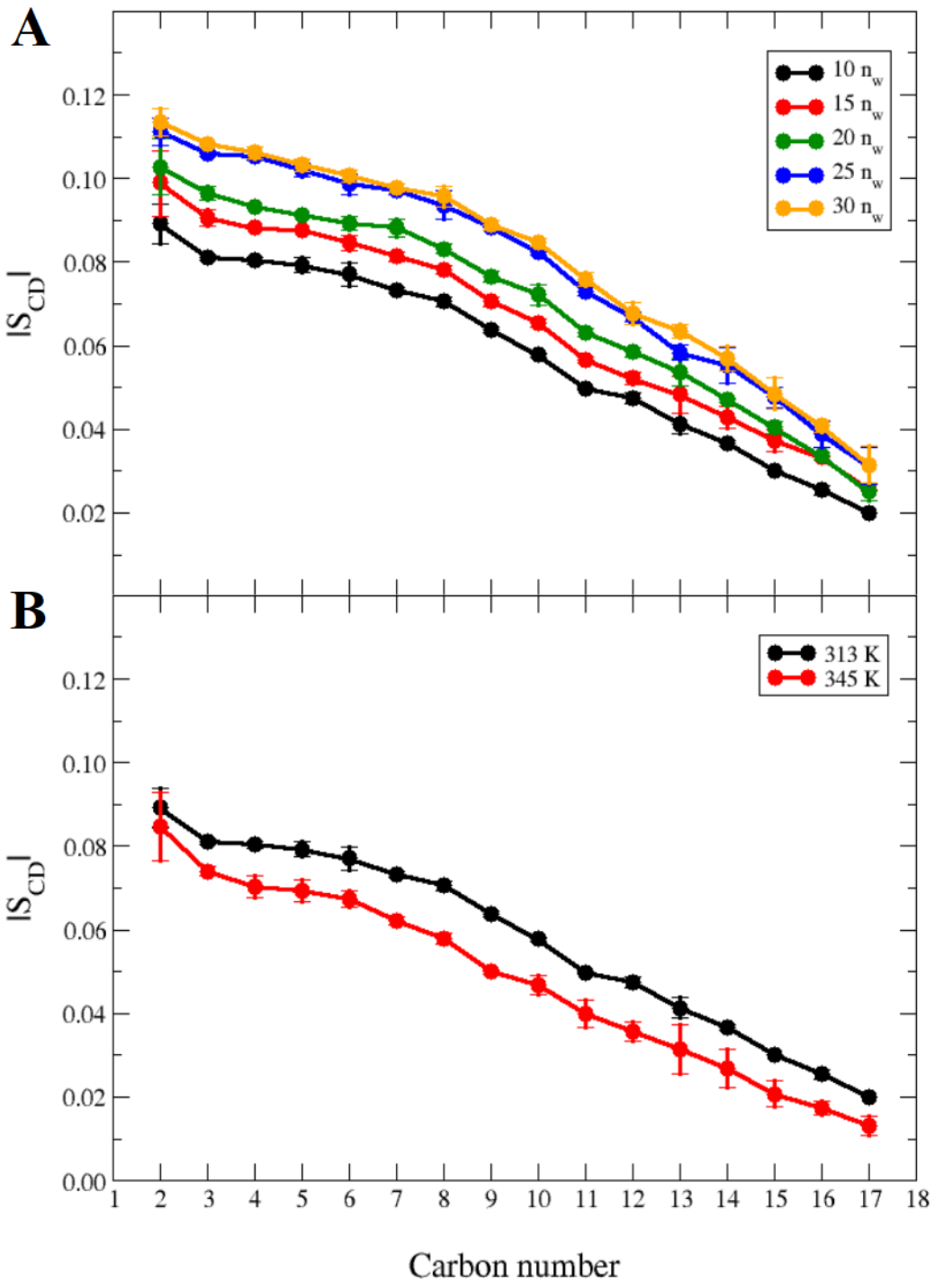

Figure S5. Effect of hydration level and temperature on DSPS S_{CD} parameters

S_{CD} parameters for DSPS lipid in DSPS/KC2H/cholesterol (50/50/0) H_{II} system sorted in descending order (aka smoothed S_{CD} parameters). **A)** Effect of hydration level at 313 K. **B)** Effect of temperature for the

system with 15 n_w. The data points are the average values over both chains, and error bars represent the standard deviation of the mean.

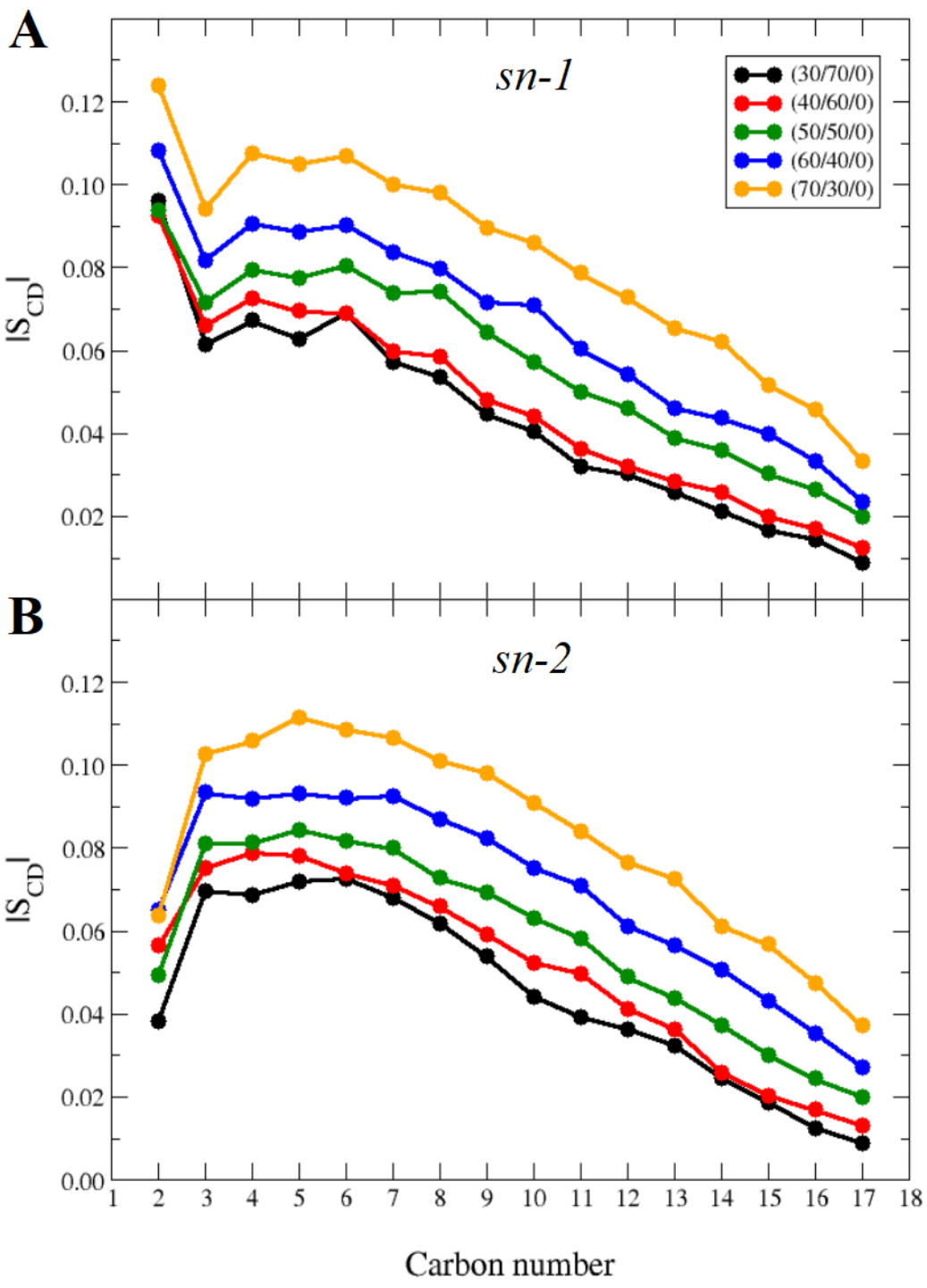

Figure S6. Effect of mixing ratio on DSPS S_{CD} parameters

The S_{CD} parameters for **A)** *sn-1*, and **B)** *sn-2* acyl chain of DSPS in DSPS/KC2H/cholesterol H_{II} systems with 10 n_w at 313 K as a function of mixing ratio. The cholesterol concentration is zero and only the DSPS and KC2H molar concentrations is different in the compared systems.

Estimation of maximum hydration for DSPS/KC2H/cholesterol (45/45/10) at 310 K

Both ^{2}H-NMR and SAXS experiments suggest that the DSPS/KC2H/cholesterol (45/45/10) system form H_{II} phase at 310K (8). The d_{hex} value measured using SAXS experiments in the presence of excess water was 53.8 Å at this temperature. This, in addition to three d_{hex} values (only the computational d_{hex} values for 10 n_w and 20 n_w are shown and connected by a line) calculated from MD simulations (simulations with the same lipid composition and temperature but with 10, 20, and 30 n_w hydration level) were used to estimate the maximum hydration for this system using ref (1) [**Figure S7-A**]. The maximum hydration is estimated to be about 11.1 n_w at 310 K. Since the d_{hex} values can be measured with ca. 0.5 angstrom uncertainty (9), the uncertainty in the maximum hydration - estimated from the slope of the diagonal line (1.817), is about 0.3 n_w (1).

To further validate the estimated hydration level, the smoothed ^{2}H-NMR S_{CD} parameters for the DSPS stearoyl chains in this system was calculated from the MD simulation of the same system with 10 n_w and was compared with the experimental data available at ref (1) [**Figure S7-B**]. Experimentally, both chains in DSPS lipids were deuterated. Due to the low resolution of ^{2}H-NMR spectra in the H_{II} phase, the reported S_{CD} values for each methylene group are the averaged S_{CD} over both acyl chains (1). In simulations, the S_{CD} parameters for this system were also averaged in the same way, sorted descending, and are shown in **Figure S7-B** to compare. The shape, maximum, and minimum S_{CD} values are in reasonable agreement between simulation and ^{2}H-NMR. Considering that the hydration level is known to affect the S_{CD} parameters, this suggests and further supports that our estimated maximum hydration for this system is reasonable.

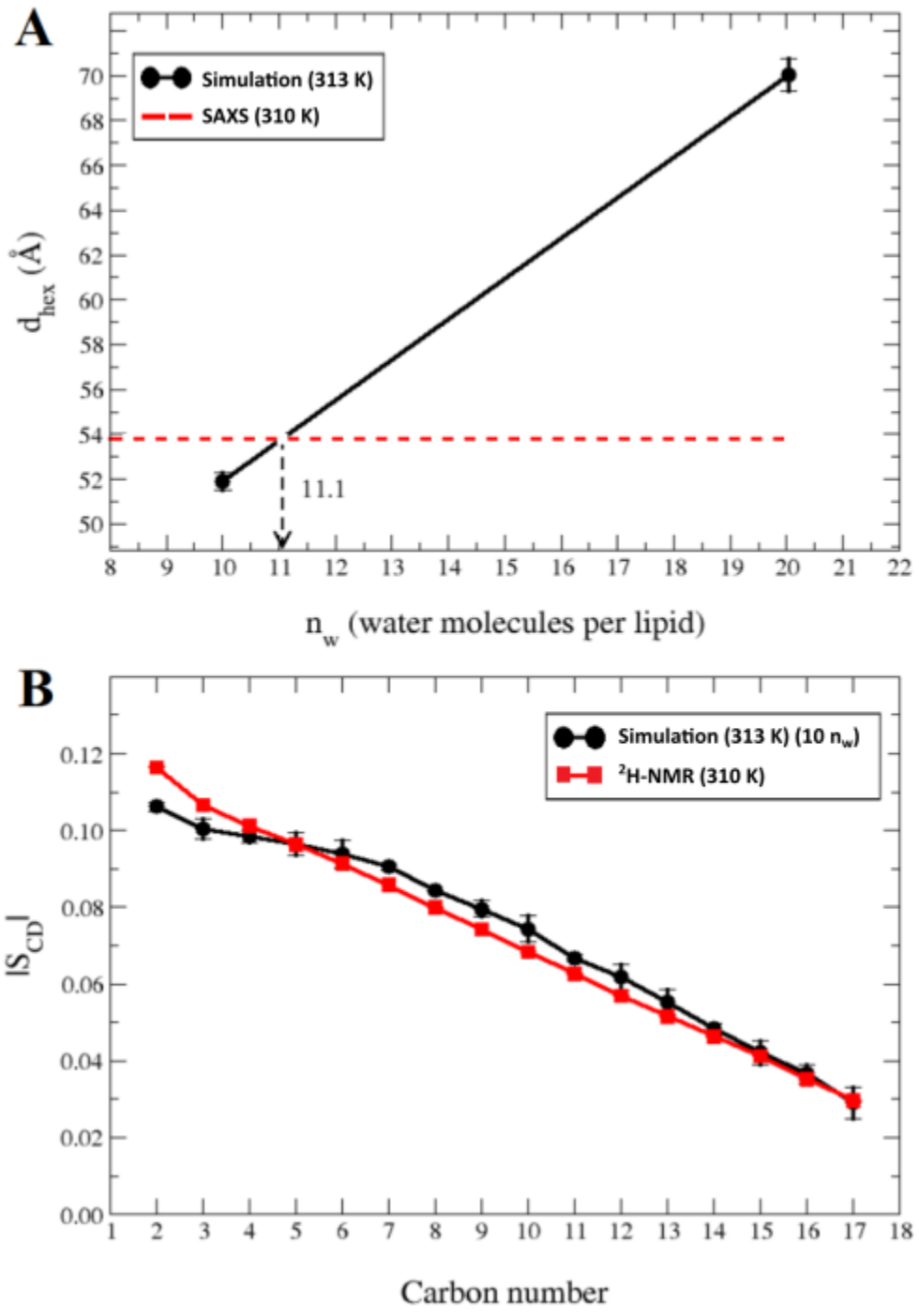

Figure S7. Maximum hydration for DSPS/KC2H/cholesterol (45/45/10) H_{II} system at 310 K

A) Maximum hydration for DSPS/KC2H/cholesterol (45/45/10) system at 310 K estimated using the hybrid protocol proposed in ref (1) . The solid black line corresponds to the expected d_{hex} - n_w line for low hydration levels obtained from two simulations done on the same mixing ratio at 10 n_w and 20 n_w at 313 K. The horizontal dotted line corresponds to the single d_{hex} value measured by SAXS in excess water at

310 K (8). **B)** Smoothed averaged S_{CD} parameters for DSPS acyl chains measured using ^{2}H-NMR (310 K) (8) and calculated from MD simulation of system with 10 n_w and 313 K. Data points are the mean values and error bars represent the standard deviations of means.

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