SUPPLEMENTARY TABLES:

Table S1: Comparison of average sterol tilts and bilayer bending rigidities for 30% DMPC/sterol mixtures calculated from SIM1 and SIM2 trajectories.

	30% DMPC/Chol (**SIM1**)	30% DMPC/7DHC (**SIM1**)	30% DMPC/Chol (**SIM2**)	30% DMPC/7DHC (**SIM2**)
Average sterol tilt, °	13.6	15.7	16.5	18.8
K_c, k_BT	776	420	28	19

Table S2: Determined form factors, F_h, and d-spacings of DMPC with mol% 7DHC at 35 °C.

mol%	**d (Å)**	**F_1**	**F_2**	**F_3**	**F_4**	**F_5**
0	61.0	-1.00	-1.24	+0.07	+0.10	-
1	61.4	-1.00	-1.23	+0.12	+0.14	-
2	61.9	-1.00	-1.23	>0.00	+0.17	-
5	61.9	-1.00	-1.23	+0.15	+0.15	-
7	62.4	-1.00	-1.21	+0.11	+0.11	-
10	63.9	-1.00	-1.22	+0.10	+0.00	-
20	63.8	-1.00	-1.13	+0.16	-0.10	-0.22
30	63.2	-1.00	-1.19	+0.13	-0.20	-0.28
40	62.9	-1.00	-1.15	+0.17	-0.36	-0.45

Table S3: Determined form factors, F_h, and d-spacings of DMPC with mol% cholesterol at 35°C.

mol%	**d (Å)**	**F_1**	**F_2**	**F_3**	**F_4**	**F_5**
0	61.0	-1.00	-1.24	+0.07	+0.10	-
20	63.6	-1.00	-1.05	+0.23	-0.16	-0.28
40	63.2	-1.00	-1.07	+0.21	-0.25	-0.29

SUPPLEMENTARY FIGURE CAPTIONS:

Figure S1: Potential energy landscape of the torsion angle C=C-C=C in cis,cis-2,4-hexadiene molecule obtained from *ab initio* calculations (*black line*) and molecular dynamics (*red line*) simulations.

Figure S2: Free energy perturbation (FEP) calculations performed in 30% DMPC/Cholesterol system from SIM1 simulations. From the last frame of 30% DMPC/Cholesterol trajectory, four sterols at low tilt angles (*Chols 9, 19, 90, 38*) and four – at high tilt angles (*Chols 110, 54, 37, 5*) were selected for mutations. Subsequently, using FEP, eight separate Chol → 7DHC *in silico* mutations were performed. For each transformation, the calculations were carried out in the forward (*red*) and backward (*green*) directions, employing 60ps or 120ps time intervals between successive iterations in λ parameter. The panels show the free energies $\Delta\mu(\lambda)$ accumulated in the iteration interval of [0; λ] (*for forward calculations*) or [1; λ] (*for backward calculations*).

Figure S3: Convergence of simulation box size dimensions for DMPC/sterol membranes from SIM1 simulations. Time evolution of x, y, and z coordinates during the last 20ns intervals of DMPC/Chol (*left*) and DMPC/7DHC (*right*) trajectories are shown. For completeness, the convergence of pure DMPC membrane is also illustrated.

Figure S4: Convergence of simulation box size dimensions for large DMPC/sterol membranes from SIM2 simulations. Time evolution of x, y, and z coordinates during the last 14ns interval of 30% DMPC/Chol (*red*), 30% DMPC/7DHC (*green*), and pure DMPC (*black*) membranes are shown.

Figure S5: Comparison of $P(\theta)$ distributions computed from the MD simulations (*red plots*) and from the analytical expression $P(\theta) = \sin\theta\, e^{-(\chi/2k_BT)\theta^2} \Big/ \int_{\theta=0}^{\pi/2} \sin\theta\, e^{-(\chi/2k_BT)\theta^2} d\theta$ (*green plots*). To achieve the latter, for each system the value of the tilt modulus χ obtained from the low-angle fits to the $P(\theta)$ densities from the respective MD simulations was used (see the main text). For illustration, the comparison is only demonstrated for 5% and 20% DMPC/7DHC, and 20% and 30% DMPC/Cholesterol mixtures.

Figure S6: Normalized probability densities $P_{12}(\alpha)$ of finding pairs of sterol molecules at angle α with respect to each other. α angle is defined as the angle between the vectors C3-C17 on two sterols. To limit the analysis to near neighbors, for these calculations, only sterol molecules within 1nm distance of each other were considered (see Figure 7 and Discussion in the main text) from 20% DMPC/Cholesterol (*red curve*) and 20% DMPC/7DHC mixtures (*green curve*).

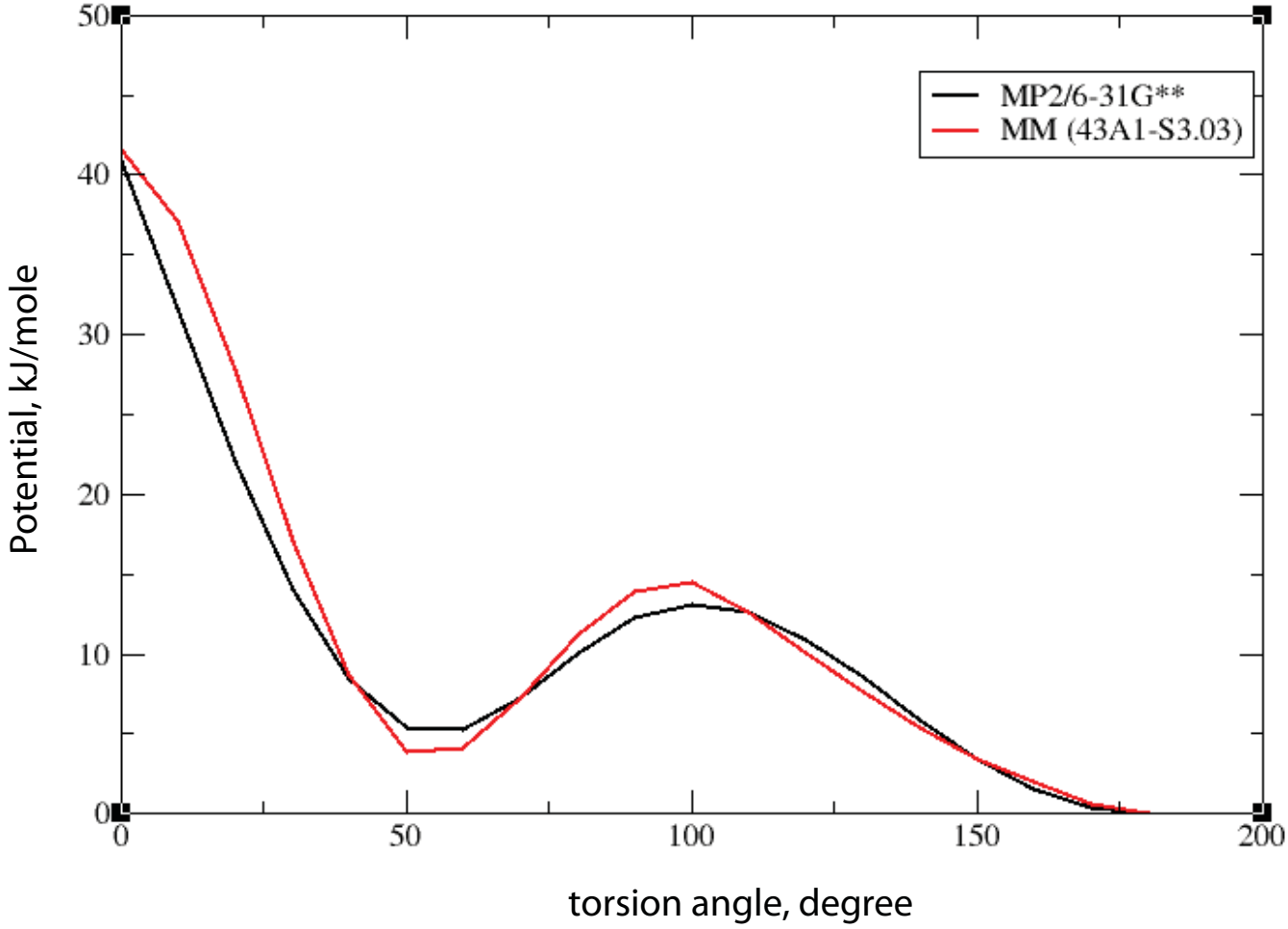
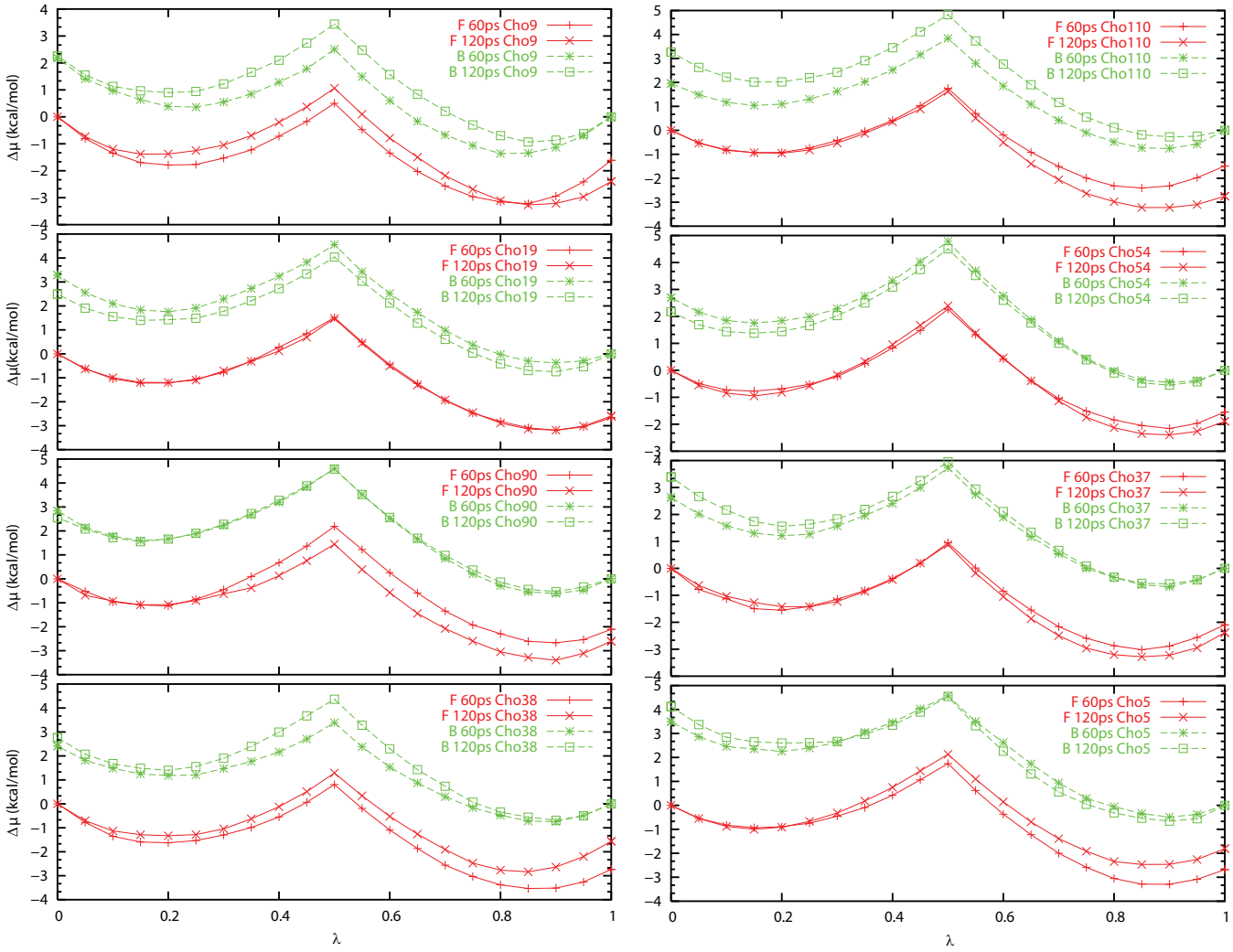

MP2/6-31G**
MM (43A1-S3.03)
Potential, kJ/mole
torsion angle, degree
0
10
20
30
40
50
0
50
100
150
200

Figure S1


F 60ps Cho9
F 120ps Cho9
B 60ps Cho9
B 120ps Cho9
F 60ps Cho110
F 120ps Cho110
B 60ps Cho110
B 120ps Cho110
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F 120ps Cho19
B 60ps Cho19
B 120ps Cho19
F 60ps Cho54
F 120ps Cho54
B 60ps Cho54
B 120ps Cho54
F 60ps Cho90
F 120ps Cho90
B 60ps Cho90
B 120ps Cho90
F 60ps Cho37
F 120ps Cho37
B 60ps Cho37
B 120ps Cho37
F 60ps Cho38
F 120ps Cho38
B 60ps Cho38
B 120ps Cho38
F 60ps Cho5
F 120ps Cho5
B 60ps Cho5
B 120ps Cho5
Δμ (kcal/mol)
λ

Figure S2

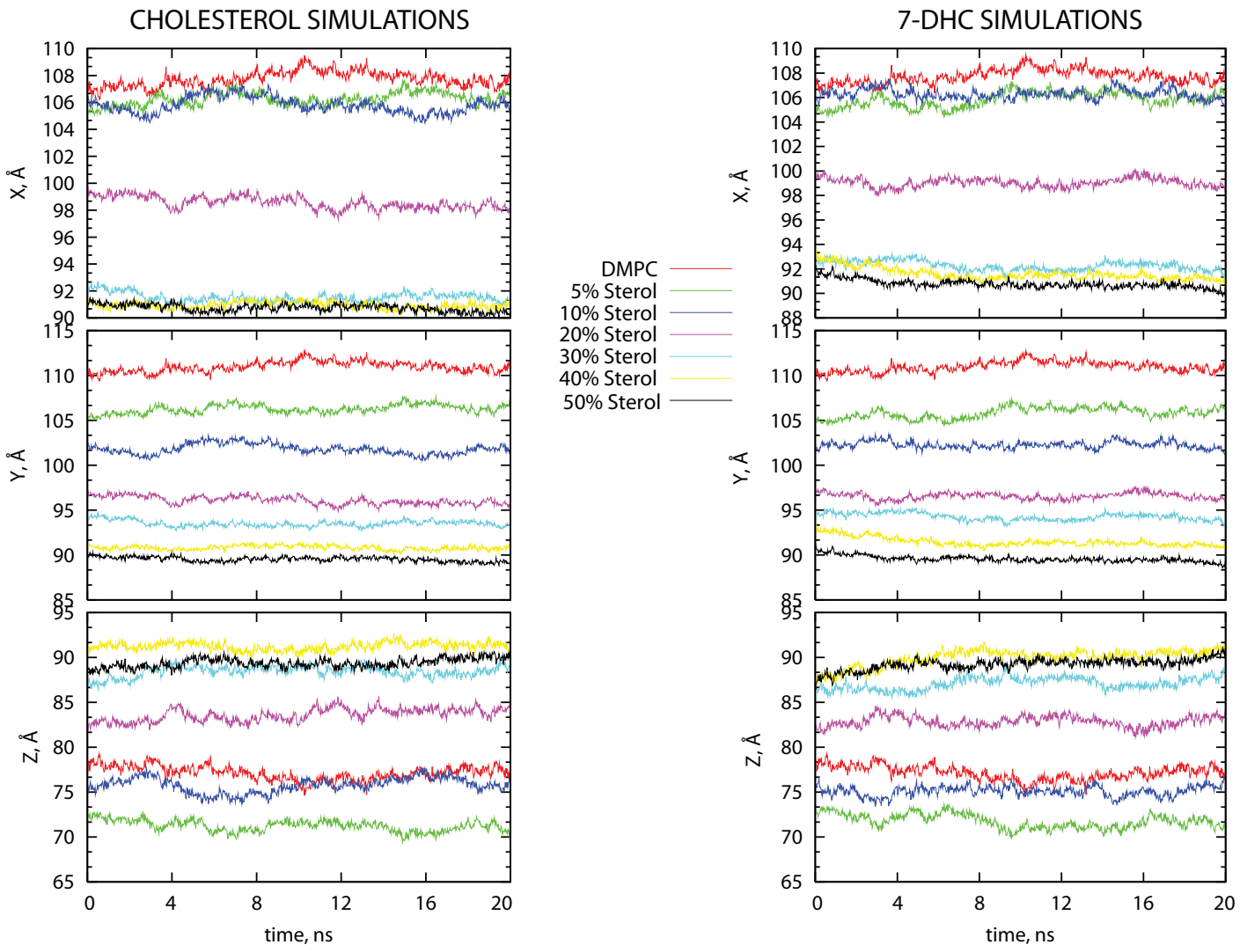
CHOLESTEROL SIMULATIONS
7-DHC SIMULATIONS
X, Å
Y, Å
Z, Å
time, ns
DMPC
5% Sterol
10% Sterol
20% Sterol
30% Sterol
40% Sterol
50% Sterol

Figure S3

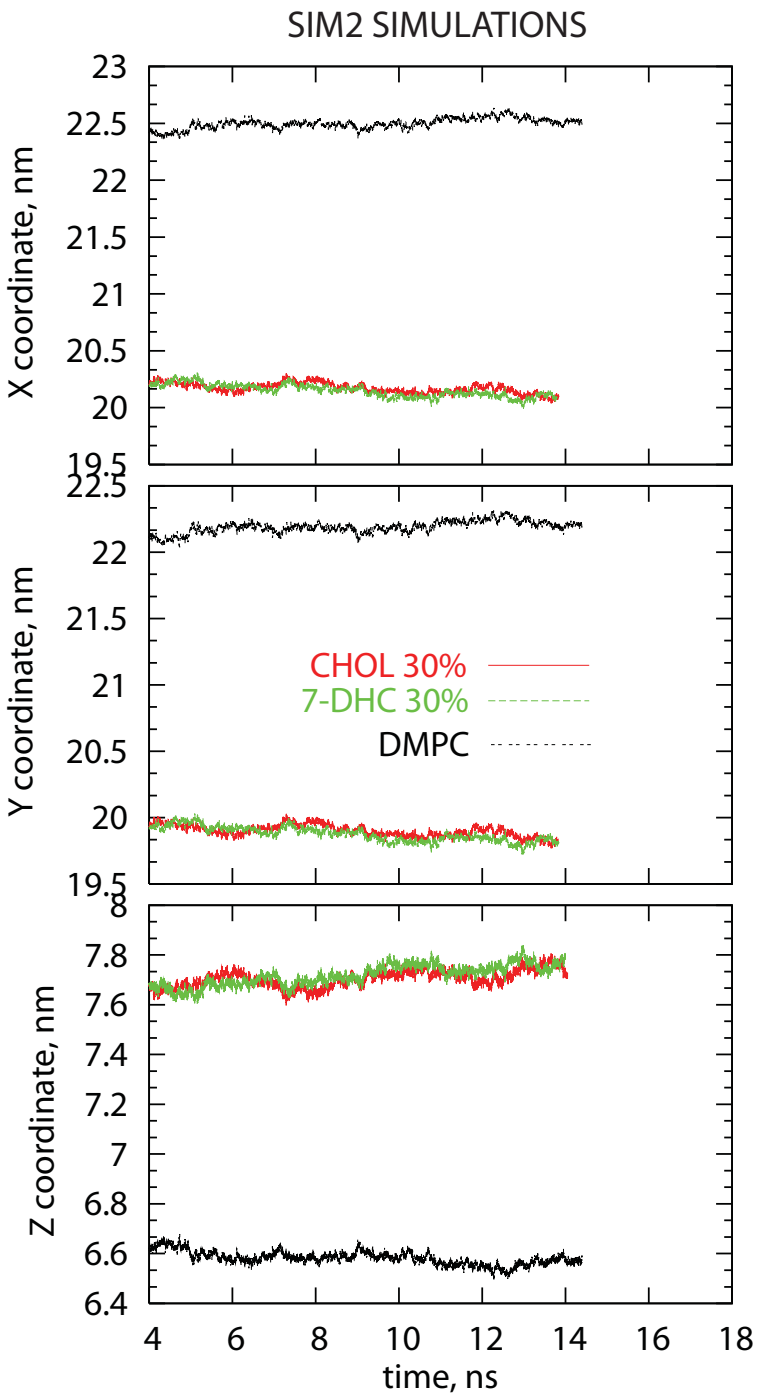
SIM2 SIMULATIONS
X coordinate, nm
Y coordinate, nm
Z coordinate, nm
time, ns
CHOL 30%
7-DHC 30%
DMPC

Figure S4

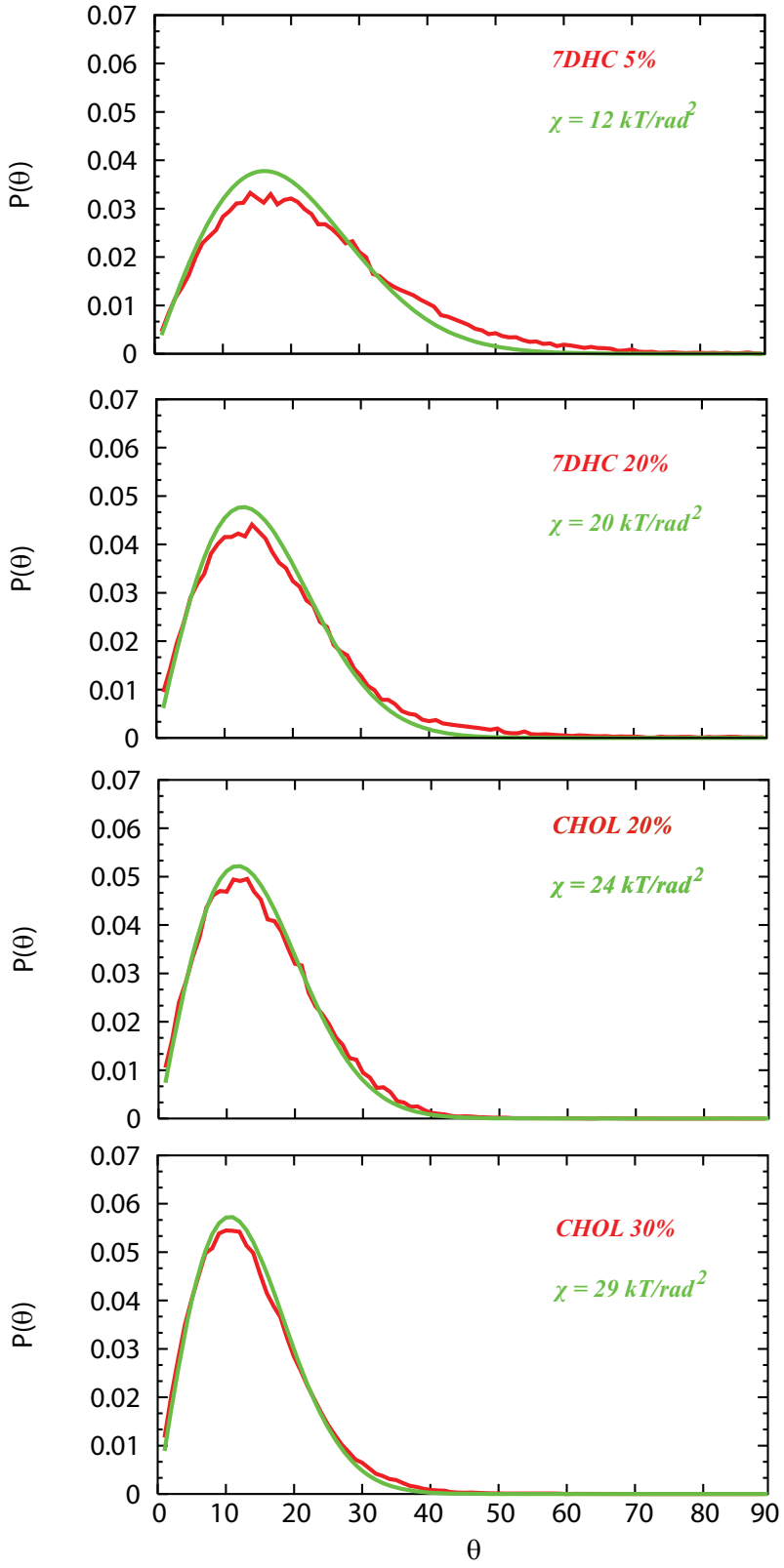
7DHC 5%
χ = 12 kT/rad²
7DHC 20%
χ = 20 kT/rad²
CHOL 20%
χ = 24 kT/rad²
CHOL 30%
χ = 29 kT/rad²
P(θ)
θ
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0 10 20 30 40 50 60 70 80 90

Figure S5

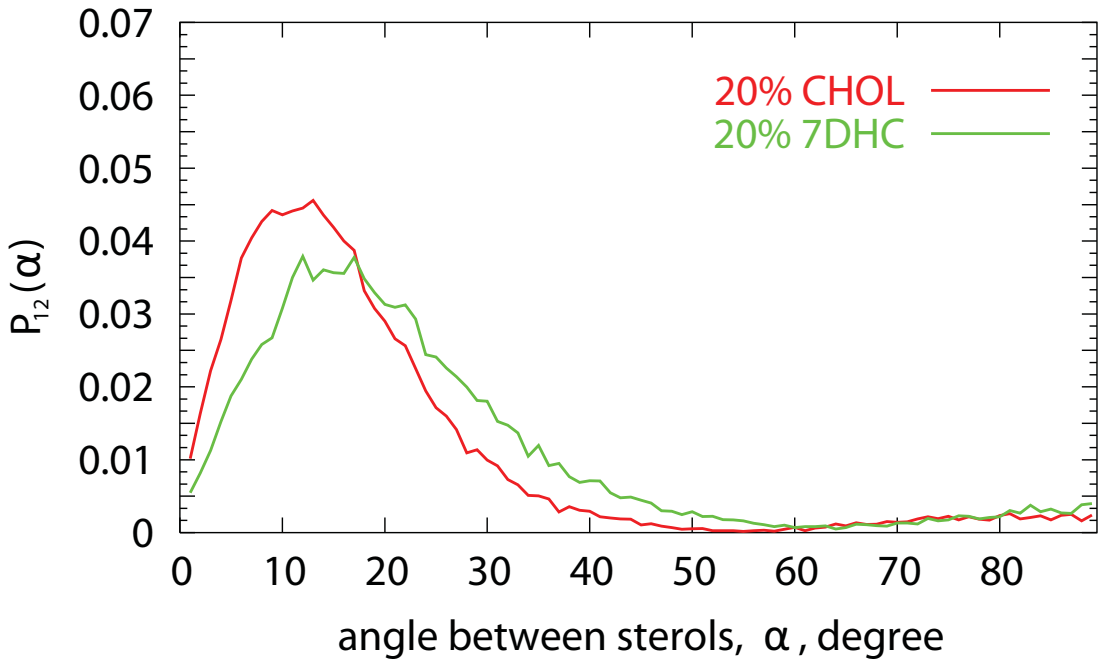
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
$P_{12}(\alpha)$
20% CHOL
20% 7DHC
0
10
20
30
40
50
60
70
80
angle between sterols, α , degree

Figure S6