Eliminating the Roughness in Cholesterol’s β-Face: Does it Matter?

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1. General Information

Fluorescence measurements were made using a Perkin Elmer LS50B Luminescence Spectrometer employing a temperature controlled cell holder. Determination of the exchangeable dimer content in NNR reactions was made by HPLC analysis using a 5 µm, 80 Å, 4.6 x 250 mm Ultrasphere ODS C18 column (Hichrom, Reading, England) and a Waters Breeze HPLC system consisting of a Waters 717 plus Autosampler and a Waters 2487 Dual λ Absorbance Detector. The effect of NNR reactions and vesicle composition on vesicle size was investigated using a Nicomp Model 270 Submicron Particle Sizer. All 1H NMR spectra were recorded on a Bruker Avance 500 MHz instrument. Chemical shifts are reported in parts per million relative to residual solvent.

1,2-Dipalmitoyl-snglcero-3-phosphocholine (DPPC) was obtained from Avanti Polar Lipids (Alabaster, AL, USA) and used as obtained. Cholesterol was obtained from Sigma-Aldrich (St. Louis, MO, USA). House-deionized water was purified using a Millipore Milli-Q filtering system containing one carbon and two ion-exchange stages. Other chemicals and solvents used in this study were obtained commercially from various vendors and used without further purification.

2. Experimental Procedures

2.1 Synthesis

The exchangeable dimers \{1-1\}, \{2-2\}\(^1\) and \{1-2\}\(^2\) were prepared as described elsewhere. Dchol was prepared as shown in Scheme 1 of the main text and as described elsewhere.\(^3\)

2.2 Monolayer experiments

Surface pressure-area isotherms were recorded for varying mixtures of 1,2-dimyristoyl-snglcero-3-phosphocholine (DMPC)/cholesterol and DMPC/Dchol at the air/Tris buffer interface by a Nima 612D film balance. In brief, approximately 550 mL of Tris buffer (pH 7.4 at 25°C) was added to the film balance as the subphase. The surface of the subphase was then aspirated to ensure the absence of surface-active contaminants. A precise amount (typically 35 uL of a 1.0 mg/mL solution) of the surfactants mixture in a mixed organic solvent (i.e., CHCl\(_3\)/MeOH 10:1 mixture) was then spread onto the surface of the subphase when the barriers of the film balance produced a maximum area. The residual organic solvent at the air/Tris buffer interface was then allowed to evaporate for 25 min to form the organic solvent-free surfactant monolayer. The surface pressure-area isotherm was obtained by compressing the barriers at a constant speed, typically 25 cm\(^2\)/min, until the monolayer collapsed, as indicated by a sudden loss of surface pressure. All the data were recorded by computer, and these data then used for making surface pressure versus molecular area plots. The molecular areas at different surface pressures were obtained from such plots.
2.3 Fluorescence experiments

Liposomes made of 37.5/2.5/57.5/2.5 Dchol/cholesterol/DPPC/DPPG (mol/mol/mol/mol, 12 µmol total lipid) plus Laurdan (0.5 mol% with respect to total lipid) were prepared from thin lipid films using methods similar to those used in NNR experiments (see below). Liposomal dispersions were placed in sealed fluorescence cuvettes and the fluorescence of each sample then measured as a function of temperature using a Perkin Elmer LS50B Luminescence Spectrometer employing a temperature controlled cell holder. An excitation wavelength of 350 nm was used, along with an excitation slit width of 5.0 nm. Fluorescence emissions were recorded from 350 to 600 nm using an emission slit width of 5.0 nm. To correct for light scattering, a vertical polarizer was placed on the excitation beam and a horizontal polarizer was placed on the emission beam. Generalized Polarization (GP) values were calculated using the equation: \[ GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \], where \( I_{440} \) and \( I_{490} \) are fluorescence emission intensities at 440 and 490 nm respectively.

2.4 Nearest-Neighbor Recognition Analysis.

Thin films of lipid were prepared by evaporating a chloroform solution containing 0.30 µmol \{1-2\} and varying amounts of DPPC and cholesterol (for exact composition see Table SI-1) under a stream of argon. After drying the thin film overnight under reduced pressure (0.4 mm Hg), 2.0 mL of a 10 mM Tris-HCl buffer (10 mM Tris, 150 mM NaCl, 2 mM NaN₃, 1 mM EDTA, pH = 7.4) was added to each of the dried films. The mixtures were then vortexed every 5 min for 30 s over a time span of 30 min with intermittent incubation at 60 °C. Following this, the dispersions were subjected to six freeze/thaw cycles (liquid nitrogen/60 °C water bath) and extruded 20 times through a 200 nm pore diameter polycarbonate filter (Nuclepore, Whatman Inc.) using argon at a pressure of ~100 psi. Afterwards, a 60 µL aliquot of 1.68 µM monesin in TRIS-HCl buffer was added to aid in pH equilibration across the membrane during NNR reactions. The vesicle dispersions (1600 µL) were heated to 45 °C, oxygen was removed by purging with argon for 10 min., thiolate-disulfide interchange reactions were initiated by adding threo - dithiothreitol (15 µL of a 19.8 mM solution in pH 7.4 Tris buffer, 1 eq. with respect to disulfide content) and sufficient amounts of 0.1 M NaOH (10 µL) to bring the pH to 7.4 at 45 °C.

Aliquots (250 µL) were withdrawn as a function of time and the exchange reactions quenched by adding 25 µL of 8.3 M acetic acid with vortexing to the test tubes containing these aliquots. Aliquots were quickly frozen, using liquid nitrogen and stored at -20 °C until HPLC analysis was carried out. For HPLC analysis to each thawed aliquot was added 1000 µL of CHCl₃/MeOH (2/1, v/v) and aldrithiol-2 (2,2´-dipyridyldisulfide, 37 µL of a 10 mM solution in CHCl₃). The tubes were vortexed, centrifuged, and the aqueous phases removed using a Pasteur pipette. The organic phase was then concentrated under reduced pressure using a Savant SVC-100 SpeedVac concentrator equipped with a cold trap and vacuum pump (~1 hr at ~ 0.4 torr). The residual lipids were dissolved in 20 µL of CHCl₃ and 80 µL of the HPLC mobile phase. These samples were subsequently analyzed by HPLC using a C18 reversed phase column. The analysis was done in an isocratic mode using a mobile phase consisting of
760 mL of EtOH, 120 mL of deionized H₂O, 100 mL of hexane, and 10 mL 1 M aq. N(n-Bu)₄OAc. The flow-rate was 0.9 ml/min, the column temperature was 31 °C, and detection was done at 203 nm. Values of \( K (K = [\text{1-2}]^2 / ([\text{1-1}] | \text{2-2}]) \) were calculated from peak areas obtained from the HPLC chromatograms using appropriate calibration curves.

Table SI-1: Compositions of liposomal dispersions

|        | Dchol mol% | n [µmol] | Cholesterol mol% | n [µmol] | DPPC mol% | n [µmol] | \{1-2\} mol% | n [µmol] |
|--------|------------|----------|------------------|----------|------------|----------|--------------|----------|
| Exp. 1 | 37.5       | 4.5      | -                | -        | 57.5       | 6.9      | 2.5          | 0.3      |
| Exp. 2 | 20         | 2.4      | 17.5             | 2.1      | 57.5       | 6.9      | 2.5          | 0.3      |

3. Calibration of Chromatographic System

The chromatographic system was calibrated and the system was found to respond as follows:

For \{\text{1-1}\}, \( 478140 \times n_{\text{1-1}} - 518 = \text{Signal} \) (\( R = 0.9984 \)); for \{\text{1-2}\} \( 533520 \times n_{\text{1-2}} - 12634 = \text{Signal} \) \( n \) (\( R = 0.9988 \)); for \{\text{2-2}\} \( 591890 \times n_{\text{2-2}} - 90867 = \text{Signal} \) \( R = 0.9988 \), where \text{signal} is the area of the chromatographic peaks for the dimers and \( n_{\text{1-1}}, n_{\text{1-2}}, \) and \( n_{\text{2-2}} \) are the numbers of moles of dimers.

4. Data for Monolayer experiments

Table SI-2: Molecular Areas of cholesterol/DMPC mixture at various surface pressures

| Mixture (Cholesterol/DMPC) | Molecular Area (nm²) | Nominal Molecular Weight |
|---------------------------|----------------------|--------------------------|
|                           | 10 mN/m 15 mN/m 20 mN/m 25 mN/m |                          |
| 100/0                     | 0.420 ± 0.017 0.409 ± 0.016 0.401 ± 0.015 0.396 ± 0.015 | 386.65                   |
| 90/10                     | 0.428 ± 0.004 0.420 ± 0.003 0.412 ± 0.004 0.407 ± 0.003 | 415.79                   |
| 80/20                     | 0.438 ± 0.023 0.431 ± 0.020 0.425 ± 0.018 0.420 ± 0.017 | 444.92                   |
| 50/50                     | 0.452 ± 0.012 0.443 ± 0.012 0.436 ± 0.013 0.431 ± 0.014 | 532.33                   |
| 40/60                     | 0.465 ± 0.003 0.449 ± 0.003 0.439 ± 0.003 0.431 ± 0.003 | 561.46                   |
| 80/20                     | 0.634 ± 0.010 0.584 ± 0.009 0.549 ± 0.008 0.523 ± 0.008 | 619.73                   |
| 90/10                     | 0.712 ± 0.008 0.656 ± 0.007 0.614 ± 0.007 0.581 ± 0.007 | 648.87                   |
| 0/100                     | 0.790 ± 0.011 0.721 ± 0.010 0.671 ± 0.008 0.631 ± 0.008 | 678.00                   |
Table SI-3: Molecular Areas of Dchol/DMPC mixture at various surface pressures

| Mixture (Dchol/DMPC) | Molecular Area (nm²) | Nominal Molecular Weight |
|----------------------|-----------------------|--------------------------|
|                      | 10 mN/m   | 15 mN/m   | 20 mN/m   | 25 mN/m   |                        |
| 100/0                | 0.395 ± 0.006 | 0.386 ± 0.008 | 0.380 ± 0.008 | 0.374 ± 0.009 | 358.50                 |
| 90/10                | 0.426 ± 0.018 | 0.415 ± 0.015 | 0.408 ± 0.014 | 0.402 ± 0.013 | 390.45                 |
| 80/20                | 0.447 ± 0.033 | 0.430 ± 0.028 | 0.419 ± 0.024 | 0.410 ± 0.022 | 422.4                  |
| 50/50                | 0.472 ± 0.008 | 0.446 ± 0.008 | 0.429 ± 0.009 | 0.417 ± 0.008 | 518.25                 |
| 40/60                | 0.484 ± 0.030 | 0.454 ± 0.028 | 0.431 ± 0.026 | 0.415 ± 0.026 | 550.20                 |
| 80/20                | 0.665 ± 0.031 | 0.622 ± 0.037 | 0.583 ± 0.034 | 0.551 ± 0.031 | 614.10                 |
| 90/10                | 0.766 ± 0.031 | 0.703 ± 0.029 | 0.656 ± 0.028 | 0.610 ± 0.026 | 646.05                 |
| 0/100                | 0.790 ± 0.011 | 0.721 ± 0.010 | 0.671 ± 0.008 | 0.631 ± 0.008 | 678.00                 |

Figure SI-1: Molecular area-additivity curves for (red circle) DMPC/cholesterol and (black square) DMPC/DChol with a surface pressure of 25 mN/m. Ideal additivities are indicated by linear plots. Error bars that are not visible lie within the symbols themselves.
Figure SI-2: Molecular area-additivity curves for (red circle) DMPC/cholesterol and (black square) DMPC/DChol with a surface pressure of 20 mN/m. Ideal additivities are indicated by linear plots. Error bars that are not visible lie within the symbols themselves.

Figure SI-3: Molecular area-additivity curves for (red circle) DMPC/cholesterol and (black square) DMPC/DChol with a surface pressure of 15 mN/m. Ideal additivities are indicated by linear plots. Error bars that are not visible lie within the symbols themselves.
5. Data for Laurdan General Polarization (GP) Measurements

5.1. Emission Spectra

**Figure SI-4:** Emission spectra for 37.5/2.5/57.5/2.5 (mol/mol/mol/mol) Dchol/Cholesterol/DPPC/DPPG membranes.

**Figure SI-5:** Emission spectra for 20/20/57.5/2.5 (mol/mol/mol/mol) Dchol/Cholesterol/DPPC/DPPG membranes.
5.2. GP Values

Table SI-4: Laurdan GP values for all liposomes investigated in this study.

|                        | 2.5 mol% Cholesterol | 40 mol% Cholesterol |
|------------------------|-----------------------|----------------------|
|                        | 95 mol% DPPC          | 57.5 mol% DPPC       |
|                        | 2.5 mol% DPPG         | 2.5 mol% DPPG        |
| T (°C)                 | GP                    | T (°C)               | GP                  |
| 24.7                   | 0.49                  | 30.5                 | 0.49                |
| 27.7                   | 0.49                  | 34.5                 | 0.47                |
| 34.5                   | 0.45                  | 37.9                 | 0.45                |
| 38.0                   | 0.41                  | 40.3                 | 0.44                |
| 39.9                   | 0.28                  | 42.3                 | 0.43                |
| 41.9                   | 0.02                  | 44.5                 | 0.40                |
| 44.1                   | -0.05                 | 46.4                 | 0.39                |
| 46.1                   | -0.10                 | 50.2                 | 0.34                |
| 49.9                   | -0.17                 | 54.4                 | 0.30                |
| 54.4                   | -0.22                 |                      |                     |

|                        | 37.5 mol% Dchol       | 20 mol% Dchol        |
|                        | 2.5 mol% Cholesterol  | 20 mol% Choletsreol  |
|                        | 57.5 mol% DPPC        | 57.5 mol% DPPC       |
|                        | 2.5 mol% DPPG         | 2.5 mol% DPPG        |
| T (°C)                 | GP                    | T (°C)               | GP                  |
| 28.4                   | 0.47                  | 28.8                 | 0.47                |
| 32.8                   | 0.46                  | 33.2                 | 0.46                |
| 37.0                   | 0.45                  | 36.7                 | 0.45                |
| 40.5                   | 0.41                  | 40.5                 | 0.41                |
| 43.3                   | 0.38                  | 43.9                 | 0.40                |
| 46.8                   | 0.33                  | 46.6                 | 0.36                |
| 50.1                   | 0.27                  | 49.9                 | 0.31                |
| 53.4                   | 0.23                  | 53.7                 | 0.27                |
| 57.3                   | 0.18                  | 57.6                 | 0.23                |
6. Data for Nearest Neighbor Recognition (NNR) measurements

6.1. HPLC traces

Figure SI-6: A sample chromatogram at $t = 0h$. 
Figure SI-7: A sample chromatogram at $t = 1\text{h}$.

Figure SI-8: A sample chromatogram at $t = 48\text{h}$. 
### 6.2. NNR Data

**Table SI-5**: Data for \{1-2\} equilibration in 37.5 mol% Dchol + 2.5 mol% Cholesterol LUVs at 45 °C using 1 equivalent of DTT.

| Experiment | Reaction Time [h] | Dimer | \(R_f\) [min] | Peak Area | \(N\) [nmol] | \(K\) |
|------------|-------------------|-------|--------------|-----------|-------------|------|
| 1          | 24                | (1-1) | 15.61        | 1405438   | 2.94        | 7.89 |
|            |                   | (1-2) | 23.28        | 4570483   | 8.59        |      |
|            |                   | (2-2) | 40.62        | 1791338   | 3.17        |      |
| 1          | 48                | (1-1) | 15.62        | 1799928   | 3.76        |      |
|            |                   | (1-2) | 23.29        | 5978843   | 11.23       | 9.34 |
|            |                   | (2-2) | 40.84        | 2030760   | 3.58        |      |
| 2          | 24                | (1-1) | 16.07        | 1129449   | 2.36        |      |
|            |                   | (1-2) | 23.95        | 3712049   | 6.98        | 7.51 |
|            |                   | (2-2) | 41.42        | 1534930   | 2.75        |      |
| 2          | 48                | (1-1) | 16.14        | 1163153   | 2.43        |      |
|            |                   | (1-2) | 24.13        | 3943073   | 7.41        | 9.13 |
|            |                   | (2-2) | 41.49        | 1374270   | 2.48        |      |
| 3          | 10                | (1-1) | 16.90        | 762814    | 1.59        |      |
|            |                   | (1-2) | 25.28        | 2586219   | 4.87        | 8.22 |
|            |                   | (2-2) | 42.84        | 979948    | 1.81        |      |
| 3          | 24                | (1-1) | 17.20        | 895510    | 1.87        |      |
|            |                   | (1-2) | 25.64        | 3089824   | 5.82        | 7.96 |
|            |                   | (2-2) | 43.57        | 1250281   | 2.27        |      |

**Table SI-6**: Data for \{1-2\} equilibration in 20 mol% Dchol + 20 mol% Cholesterol LUVs at 45 °C using 1 equivalent of DTT.

| Experiment | Reaction Time [h] | Dimer | \(R_f\) [min] | Peak Area | \(N\) [nmol] | \(K\) |
|------------|-------------------|-------|--------------|-----------|-------------|------|
| 1          | 24                | (1-1) | 13.98        | 1964380   | 4.11        | 7.07 |
|            |                   | (1-2) | 20.87        | 5694117   | 10.70       |      |
|            |                   | (2-2) | 37.35        | 2238969   | 3.94        |      |
| 1          | 48                | (1-1) | 13.98        | 2332579   | 4.88        |      |
|            |                   | (1-2) | 20.93        | 7063047   | 13.26       | 8.22 |
|            |                   | (2-2) | 37.62        | 2506205   | 4.39        |      |
| 2          | 24                | (1-1) | 14.34        | 1721352   | 3.60        |      |
|            |                   | (1-2) | 21.49        | 5639088   | 10.59       | 8.61 |
|            |                   | (2-2) | 38.08        | 2055142   | 3.62        |      |
| 2          | 48                | (1-1) | 14.40        | 2087002   | 4.37        |      |
|            |                   | (1-2) | 21.51        | 6527716   | 12.26       | 8.08 |
|            |                   | (2-2) | 38.22        | 2429564   | 4.26        |      |
| 3          | 24                | (1-1) | 14.94        | 1793048   | 3.75        |      |
|            |                   | (1-2) | 22.35        | 6151314   | 11.55       | 9.02 |
|            |                   | (2-2) | 39.20        | 2243523   | 3.94        |      |
| 3          | 48                | (1-1) | 15.20        | 1887550   | 3.95        |      |
|            |                   | (1-2) | 22.67        | 6147601   | 11.55       | 7.73 |
|            |                   | (2-2) | 39.69        | 2494508   | 4.37        |      |
7. DLS Data

Typically, 50 µL aliquots were taken from each vessel before and after the exchange reaction. These aliquots were diluted with 450 µL of Tris buffer (10 mM Tris HCl, 150 mM NaCl, 2 mM NaN₃ and 1 mM EDTA, pH 7.4) and analyzed at 23 °C assuming a sample viscosity of 0.9325 centipoise. The photopulse rate was adjusted to ~300 kHz. Vesicle size was evaluated through Gaussian analysis. At least 100000 scans were performed.

Table SI-7: DLS data for vesicles before and after NNR reactions. Monomodal size distribution was observed in all cases.

| mol% Dchol | Experiment | Time [h] | Diam. (nm) |
|------------|------------|----------|------------|
| 40         | 1          | 0        | 193.2 ± 64 |
|            |            | 48       | 187.5 ± 45 |
|            | 2          | 0        | 194.6 ± 62 |
|            |            | 48       | 209.9 ± 97 |
|            | 3          | 0        | 184.3 ± 79 |
|            |            | 24       | 195.5 ± 94 |
| 20         | 1          | 0        | 193.3 ± 73 |
|            |            | 48       | 194.6 ± 82 |
|            | 2          | 0        | 198.3 ± 83 |
|            |            | 48       | 207.7 ± 77 |
|            | 3          | 0        | 209.8 ± 67 |
|            |            | 48       | 193.8 ± 76 |

8. References

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