Supporting Information for:

Mechanical properties determination of DMPC, DPPC, DSPC and HSPC solid-ordered bilayers

Dominik Drabik,* Grzegorz Chodaczek, Sebastian Kraszewski, Marek Langner

a Department of Biomedical Engineering, Faculty of Fundamental Technical Problems, Wroclaw University of Science and Technology, 50-377 Wroclaw, Pl. Grunwaldzki 13, Poland.

b PORT – Polish Center for Technology Development, Stabłowicka 147, 54-066 Wroclaw, Poland.

* Corresponding author: Dominik.Drabik@pwr.edu.pl

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1. Molecular Dynamics simulation details of vesicle systems

All full-atomic simulation simulations were performed with the NAMD\textsuperscript{1} software and united-atom CHARMM36 force field under NPT conditions. Each of vesicles was modelled with 10nm radius. United atom chain models were used for lipids\textsuperscript{2}. The system was hydrated with TIP3P water molecules giving a final simulation box of 30 nm\textsuperscript{3}. Ions were not added to system. It was our aim to recreate experimental setup, where deionized water is used, at accurately as possible. Furthermore ions are charged particles which increase significantly simulations time, as they require calculation of additional electrostatic interactions. Due to significant volume of systems such option was choose in order to avoid prolonged simulation time. Three dimensional periodic boundary conditions were applied in the simulations. Vesicle system was created using custom script in Matlab. Starting area per lipid (APL) value was assumed based on literature data\textsuperscript{3}. APL was adopted to account for the effect of vesicle’s curvature – multiplied by respectively 0.95 and 1.05 for inner and outer leaflet. Simulations were analysed for at least the last 10ns of equilibrated system. In order to determine equilibration six selected parameters were monitored. Those were vesicle radius, thickness of lipid bilayer, mean values and standard deviations of both inner and outer leaflets. All calculation of this parameters is based on location of phosphorus atom in lipid molecules. The simulations were run with time-step equal to 2fs. The simulated POPC system consisted of 3637 lipid molecules (269 139 atoms) and 748 344 water molecules (2 245 032 atoms). Adopted Area per lipid (APL) was equal to 68.1 Å\textsuperscript{2}. There were 1521 lipid molecules in inner leaflet and 2 116 in outer leaflet. As a reference simulation it was carried out for the longest time - total 163 ns simulation time. Final simulation unit cell was equal to 298Å in each of xyz axis. The equilibration parameters are presented in figure S1.A. Last 65 ns of simulation were used for bending rigidity determination. The simulated DMPC system consisted of 3556 lipid molecules (241877 atoms) and 720 421 water molecules (2 161 263 atoms). Adopted APL was equal to 70 Å\textsuperscript{2}. There were 1156 lipids in inner leaflet and 2 400 in outer. The simulation was carried out for 32 ns. Final simulation unit cell was equal to 293Å in each of xyz axis. The equilibration parameters are presented in figure S1.B. At the beginning of equilibration spontaneous water pore has opened. It closed itself at the end of equilibration time. Last 13 ns of simulation were used for bending rigidity determination. The simulated DSPC system consisted of 4251 lipid molecules (326 949 atoms) and 682 785 water molecules (2 048 355 atoms). From those lipid molecules 495 of them was DPPC and 3833 DSPC. Adopted APL was equal to weighted average of DPPC and DSPC APLs. There were 1520 lipids in inner and 2808 it outer leaflet. The simulation was carried out for 31 ns. Final simulation unit cell was equal to 294Å in each of xyz axis. The equilibration parameters are presented in figure S3.A. Last 15 ns of simulation were used for bending rigidity determination. The simulated DPPC system consisted of 4251 lipids (306 073 atoms) and 693 076 water molecules (2 079 228 atoms). Adopted APL was equal to 65.7 Å\textsuperscript{2}. There were 1443 lipid molecules in inner leaflet and 2 808 in outer. The simulation was carried out for 35 ns. Final simulation unit cell was equal to 294Å in each of xyz axis. The equilibration parameters are presented in figure S3.B. Last 12 ns of simulation were used for bending rigidity determination. The simulated HSPC system, as a mixture of 11.4% of DPPC and 88.6% DSPC, consisted of 4328 lipid molecules (326 949 atoms) and 682 785 water molecules (2 048 355 atoms). From those lipid molecules 495 of them was DPPC and 3833 DSPC. Adopted APL was equal to weighted average of DPPC and DSPC APLs. There were 1520 lipids in inner and 2808 it outer leaflet. The simulation was carried out for 31 ns. Final simulation unit cell was equal to 294Å in each of xyz axis. The equilibration parameters are presented in figure S3.C. Last 12 ns of simulation were used for bending rigidity determination.
Figure S3. Visualization of six parameters used for equilibration determination for (A) DSPC, (B) DPPC and (C) HSPC vesicle systems. Red line represents the starting point of data used for bending rigidity determination.

2. Calculation of order parameter for vesicle systems
Carbon-hydrogen order parameter of lipid tails is often used to assess force field accuracy. In this section we are presenting the change of order parameter throughout whole simulation for both inner and outer membranes. Three carbons were selected on both tails — second (C22, C32), eighth (C28, C38) and fifteenth (C215, C315). For DSPC vesicle system thirteen carbon (C213, C313) was selected instead of fifteenth for obvious reasons. Order parameter was calculated by established protocol for united-atom with slight adaptation for vesicle systems. Namely, the vector between vesicle center and position of phosphorus atom for given lipid is treated as membrane normal.

Figure S4. Order parameter for selected carbon atoms in function of simulation time for POPC vesicle system.
Carbon atoms for POPC system located closer to lipid head (namely C22, C32, C28 and C38) became stable after 20 ns of simulations. For C215 and C315 order parameter semi-stabilized after 20 ns, after which constant small drift could be observed. However such drift is to be expected in carbon atoms at the end of carbon tails. Parameter evolution is presented in Figure S4.
Carbon atoms in DMPC vesicle system located closer to lipid head (namely C22, C32, C28 and C38) became stable after 10 ns of simulations for sn-2 tail and after 8 ns for sn-1 tail. For carbon atoms at the end of tails the order parameter semi-stabilized almost instantly after less than 2 ns, after which remain constant when a small drift could be observed around 28 ns. Additionally, significant difference was observed in parameter value between the inner and outer leaflets. This was not observed only for middle carbon atom. However it can be concluded that system is thermally stable. Order parameter evolution in time is presented in Figure S5.

For DSPC system atoms closest to head, namely C22 and C23, obtained stability after 10 ns for both inner and outer leaflets. However the fluctuation of the parameter in time was much higher than observed for POPC or DMPC systems. Similar tendency was observed for C28 and C38 atoms. Additionally it takes longer for inner leaflet to obtain stability. A constant drift was observed for last carbon atoms in tails, the drift was higher in inner leaflet. Except for C22 atom, order parameter values were different between the inner and outer leaflet. Despite that the parameters and stable, therefore system can be treated as thermally stable as well. Order parameter evolution in time is presented in Figure S6.
Figure S7. Order parameter for selected carbon atoms in function of simulation time for DPPC vesicle system.

For DPPC system and carbon atoms closest to head, stability was obtained after 15 ns for inner leaflet and after 5ns for outer leaflet. For C28 and C38 stability was obtained relatively quick after 5 ns. However, either small drift or high fluctuation can be observed for carbon atom in inner leaflet. A constant drift was observed for last carbon atoms in tails, after obtaining semi-stability at around 3 ns. It can be concluded that system can be treated as thermally stable after 15 ns. Order parameter evolution in time is presented in Figure S7.

Figure S8. Order parameter for selected carbon atoms in function of simulation time for HSPC vesicle system.

For HSPC system two different lipid types can be found. It can be observed that order parameter value is the same for both DSPC and DPPC lipid molecules. Only exception from this tendency can be observed in C213 and C313 atoms in outer leaflet. For carbon atoms closes to head as well as for middle carbon atoms stability was observed after 8ns. For carbon atoms at the end of tail semi-stability was observed after 4 ns for outer and 10 ns for inner leaflet. However constant small drift was present in both cases. Nevertheless, it can be safely assumed that systems are thermally stable after 10 ns of simulations. Order parameter evolution in time is presented in Figure S8.
3. Flicker-noise analysis approach for Molecular Dynamics simulation

For bending rigidity determination in case of molecular dynamics study the fluctuation analysis is done on whole liposome. However, in case of flicker-noise spectroscopy, only cross-section is analysed and used to mathematically re-establish fluctuations on whole vesicle. To compare accuracy of such approach, fluctuation contour of cross-section of vesicle in molecular dynamics studies was extracted and analysed similarly to flicker-noise images. Fluctuation spectra was collected for range of 3 nm and under five different angles as visualized in Figure S9. Bending rigidity calculated using Braun and Sachs approach was equal to \( \kappa = 17.86 \times k_B T \). When analysed the slice under 0 degree angle it was equal to 17.9±0.6 \( k_B T \) and 17±3 \( k_B T \) from average-based and statistical approaches respectively. In other angles result were within margin of error: for 30 degree slice it was equal to 17.2±0.7 \( k_B T \) and 16±4 \( k_B T \) and for 90 degree slice - 17.7±0.7 \( k_B T \) and 17±4 \( k_B T \). In each case first result from average-based approach is presented followed by statistical approach. Only in case of 60 degree angle slice result was slightly different - 13.9±0.5 \( k_B T \) and 14.3±3 \( k_B T \). Despite this single discrepancy, it can be concluded that determination of fluctuation spectra from cross section is accurate.

Figure S9. Liposomes used for contour determination in (a) 0 (b) 45 and (c) 90 degree cross-sections. Images were rendered using Blender software.

4. Planar bilayer MD simulations details and mechanical parameters determination

Mechanical parameters for investigated lipids were also determined in planar lipid bilayer configuration. All full-atomic simulation simulations were performed with the NAMD software and united-atom CHARMM36 force field under NPT conditions. Specifically, planar lipid bilayers were generated using CHARMM-GUI membrane builder, which was followed with hydrogen removal to reflect united-atom force field. Each investigated bilayer consisted of 648 lipids (324 for each leaflet). For HSPC system the bilayer consisted of 574 DSPC molecules and 74 DPPC molecules. Other options were the same as in vesicle system simulations. Planar bilayer simulations were run for at least 100 ns. Last 50 ns were used for mechanical parameter determination.

To determine mechanical parameters (focusing on bending rigidity coefficient, but also tilt modulus) a real-space fluctuation (RSF) method was used. Specifically, a probability distribution for both tilt and splay is determined for all lipids over all analyzed time steps. Tilt \( \theta \) is defined as an angle between the lipid director (vector between lipid head – midpoint between C2 and P atoms – and lipid tail – midpoint between last carbon atoms) and bilayer normal. Lipid splay \( S_r \) is defined as divergence of an angle formed by the directors of neighboring lipids providing that they are weakly correlated. The method, along with equations and calculations, is thoroughly described in given references. APL in planar bilayer simulation is simply determined by dividing box area over number of lipids, which is followed by averaging over analyzed time steps. In table 1 obtained parameters are presented. In figures S10-S14 obtained probabilities \( P(\theta) \) and \( P(S_r) \) along with model fit to the potential of mean force (PMF) for each bilayers are presented. Area compressibility is determined using same algorithm as for vesicle systems.

Table S1. Mechanical parameter determination from planar lipid bilayer simulations using RSF method.

| Lipid type | APL [Å^2] | \( \kappa \) [J] | \( \kappa_s \) [J] | \( K_A \) [N/m] |
|------------|-----------|----------------|----------------|-----------|
| POPC       | 62.4 ± 0.6 | (10.8 ± 0.3) \times 10^{-20} | (2.80 ± 0.04) \times 10^{-20} | 0.18 |
| DMPC       | 59.4 ± 0.5 | (11.7 ± 0.4) \times 10^{-20} | (2.69 ± 0.06) \times 10^{-20} | 0.30 |
| DPPC       | 51.3 ± 0.5 | (23 ± 1) \times 10^{-20} | (3.8 ± 0.3) \times 10^{-20} | 0.55 |
| DSPC       | 49.2 ± 0.4 | (19.9 ± 1.1) \times 10^{-20} | (5.3 ± 0.1) \times 10^{-20} | 0.88 |
| HSPC       | 49.6 ± 0.2 | (23.2 ± 1.8) \times 10^{-20} | (6.61 ± 0.32) \times 10^{-20} | 0.48 |

APL, Area per lipid; \( \kappa \), bending rigidity coefficient; \( \kappa_s \), thermodynamic tilt modulus; \( K_A \), area compressibility;
Figure S10. Visualization of the fitting procedure used for the determination of the tilt and splay moduli on example of POPC bilayer. Both probability distributions and PMFs of tilt angle $\theta$ and lipid splay $S$ are presented. A quadratic function is fitted to PMF in range of $[\mu-\sigma, \mu+\sigma]$ to obtain either tilt of bending moduli in low tilt/splay region. Values of tilt and splay modulus in function of fold $k_BT$ are also included.

Figure S11. Visualization of the fitting procedure used for the determination of the tilt and splay moduli on example of DMPC bilayer. Both probability distributions and PMFs of tilt angle $\theta$ and lipid splay $S$ are presented. A quadratic function is fitted to PMF in range of $[\mu-\sigma, \mu+\sigma]$ to obtain either tilt of bending moduli in low tilt/splay region. Values of tilt and splay modulus in function of fold $k_BT$ are also included.
Figure S12. Visualization of the fitting procedure used for the determination of the tilt and splay moduli on example of DPPC bilayer. Both probability distributions and PMFs of tilt angle $\theta$ and lipid splay $S$ are presented. A quadratic function is fitted to PMF in range of $[\mu-\sigma, \mu+\sigma]$ to obtain either tilt of bending moduli in low tilt/splay region. Values of tilt and splay modulus in function of fold $k_B T$ are also included.

Figure S13. Visualization of the fitting procedure used for the determination of the tilt and splay moduli on example of DSPC bilayer. Both probability distributions and PMFs of tilt angle $\theta$ and lipid splay $S$ are presented. A quadratic function is fitted to PMF in range of $[\mu-\sigma, \mu+\sigma]$ to obtain either tilt of bending moduli in low tilt/splay region. Values of tilt and splay modulus in function of fold $k_B T$ are also included.
Figure S14. Visualization of the fitting procedure used for the determination of the tilt and splay moduli on example of HSPC bilayer. Both probability distributions and PMFs of tilt angle $\theta$ and lipid splay $S$ are presented. A quadratic function is fitted to PMF in range of $[\mu - \sigma, \mu + \sigma]$ to obtain either tilt of bending moduli in low tilt/splay region. Values of tilt and splay modulus in function of fold $k_B T$ for individual lipid types are also included, as well as final parameters calculated according to phenomenological dependency established to heterogeneous bilayers.

5. Flicker-noise detailed results

Presented in main paper bending rigidity coefficients determined in flicker noise spectroscopy were averaged values. They are averaged over at least 10 individual vesicles. However average value do not fully show the diversity of individual measurements. To this end the values of bending rigidity coefficient for individual vesicles as well as their average values are presented in this paragraph (Figures S15-18). They are presented for both average-based approach and statistical one. Furthermore image of vesicles are shown to further emphasize the difference in their shape, which is stated in main paper.

Figure S15. (A) Image of DMPC vesicle and (B) bending rigid coefficient values for individual measurements.

Figure S16. (A) Image of DPPC vesicle and (B) bending rigid coefficient values for individual measurements.
6. Time-stability of solid-ordered vesicles shapes

Vesicles created from lipids with \( T_m \) higher than room temperature exhibited oddly-rectangular shape rather than typical quasi-spherical one. This was more visible the higher was the \( T_m \) of lipid – namely observed ‘bilayer wrinkles’ were common view in DSPC, DPPC and HSPC, while they were less visible in DMPC. In this section time stability of this peculiar bilayer shape are shown. As one can see in Figures S9 and S10, visible ‘wrinkles’ can be seen stable for over a minute. Furthermore, the changes in the vesicle shape is mostly due to rotation of vesicle rather than change of individual wrinkles.
Figure S19. Time evolution of selected DPPC vesicle shape.
Figure S20. Time evolution of selected HSPC vesicle shape.

6. Bibliography

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