

Comment on “Capturing Phase Behavior of Ternary Lipid Mixtures with a Refined Martini Coarse-Grained Force Field”

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We report here on the pitfalls of the simulation model introduced in the “Capturing Phase Behavior of Ternary Lipid Mixtures with a Refined Martini Coarse-Grained Force Field” [Journal of Chemical Theory and Computation 2018, 14, 11, 6050–6062]. This refined Martini model was reported to reproduce experimental phase diagrams for a ternary DOPC/DPPE/cholesterol mixture, including the coexistence of two liquid phases. However, we demonstrate that this coexistence only emerged due to an unfortunate choice of simulation parameters, which leads to poor energy conservation. Specifically, the constraints on the cholesterol model drained energy out from the membrane, resulting in two coexisting phases at drastically different temperatures. Using the simulation parameters recommended for the used cholesterol model, this artefact is eliminated, yet so is phase coexistence, *i.e.* experimental phase diagrams are no longer reproduced.

It is important to highlight that the present comment was submitted to Chemical Theory and Computation. However, it was rejected without peer-review by the Editor-in-Chief, who stated that the journal “rarely publishes such material”.

At room temperature and at certain ratios, the canonical mixture of dipalmitoylphosphatidylcholine (DPPC) with two saturated acyl chains, dioleoylphosphatidylcholine (DOPC) with two monounsaturated acyl chains, and cholesterol (CHOL) undergoes phase separation into liquid ordered (L_o) and liquid disordered (L_d) phases [1]. It is well-documented that the standard implementation of the coarse-grained (CG) Martini model [2] does not capture this behavior [3–5]. Therefore, phase separation and phase coexistence studies performed using the Martini model replace DOPC by dilinoleoylphosphatidylcholine (DLiPC) with two diunsaturated acyl chains as low transition temperature (T_m) lipid [6]. Unfortunately, no experimental phase diagrams for the DPPC/DLiPC/CHOL mixture exist to our knowledge, rendering the comparison between simulations and experiments qualitative at best.

Recently, Carpenter *et al.* tackled this outstanding issue by a careful refinement of the Martini parameters for DOPC and DPPC. They adjusted the bonded parameters of each bead separately to reproduce the bond length and angle distributions extracted from atomistic simulation data [3]. This approach diverts from the building block concept of the version 2 family of the Martini force field, where the number of bonded parameters is minimized. With this extended freedom to fine-tuning of the interactions, Carpenter *et al.* achieved almost quantitative agreement with the experimental phase diagrams for the mixture of DPPC, DOPC, and CHOL [1, 7]. Considering the known limitations of the CG models [8], this is an impressive milestone on the way to accurate modeling of complex lipid mixtures. In their work, Carpenter

et al. derived two different models; “Extensible parameters” where DPPC and DOPC head groups share identical interaction parameters, and “Optimal parameters”, where they are allowed to differ. The latter parameter set is of more interest, as it provides the best agreement with the experimental phase diagram [3].

Here, we demonstrate that the reported liquid–liquid coexistence in the model by Carpenter *et al.* (“Optimal parameters”) is an artifact caused by an unfortunate choice of simulation parameters for GROMACS. Namely, the used current cholesterol model by Melo *et al.* [9] was parametrized using a very conservative set of parameters for the LINCS constraint algorithm to properly model the ring structure of cholesterol. Unfortunately, these more conservative constraint options were not followed by Carpenter *et al.* [3], who instead followed the parameter set generally recommended for the Martini model. When the cholesterol model is simulated with these generally used parameters, *i.e.* 4th order expansion of the constraint coupling matrix (`lincs_order` equal to 4) and 1 iteration of constraints per integration time step (`lincs_iter` equal to 1), energy is not properly conserved due to the presence of virtual sites and corresponding coupled constraints in this model. This effect, which is also highlighted in the GROMACS manual [10], manifests itself especially at large integration time steps.

In the case of the study by Carpenter *et al.* [3], CHOL constraints drain out a substantial amount of energy from their neighborhood, which in the case of the studied ternary lipid mixtures consists preferentially of DPPC. Thus, this leads to the cooling down of CHOL and the surrounding DPPC lipids which together form a sort of ordered phase. In contrast — to maintain the target temperature of the thermostat — DOPC-rich region gets warmer than the target temperature of the thermostat, thus forming a very disordered phase. This Maxwell de-

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mon causes the temperatures of DPPC (and CHOL) and DOPC to diverge exponentially as a function of the integration time step from the target temperature of the thermostat, therefore resulting in the apparent L_o phase being dozens of K cooler than the L_d phase. Importantly, L_o/L_d coexistence regions reported by the authors and matching the experimental phase diagram in the DPPC/DOPC/CHOL mixtures are only recovered in simulations in the presence of this artifact.

We show that this build-up of temperature difference can be at least partially prevented by either 1) following the suggested LINCS parameters for CHOL simulations by Melo *et al.*, 2) coupling the different lipid types to separate thermostats, or 3) decreasing the simulation time step. All these options, that improve energy conservation, cause the L_o/L_d phase coexistence to vanish. Instead, an almost ideal mixing is instead observed.

Thus, when achieving a proper NPT ensemble, the model by Carpenter *et al.* does not present an improvement over the standard Martini v2.2 lipid model in capturing the phase coexistence in DOPC/DPPC/CHOL mixtures [3, 5, 6]. Therefore, further work is required before a direct comparison of experimental and Martini-based coarse grained simulations on phase-separating lipid mixtures is viable. Meanwhile, the DPPC/DLiPC/CHOL mixture in the standard Martini implementation will—despite its obvious limitations—serve as the main model of choice for studies on L_o/L_d phase coexistence.

I. RESULTS

To demonstrate the issues in the model by Carpenter *et al.*, we first followed the original simulation protocol reported in Ref. 3. We performed 15 μ s simulations of 3/3/2 and 2/1/1 compositions of the DPPC/DOPC/CHOL mixture (see Methods and Set 1 in Table I). Both of these compositions fall in the heart of the L_o/L_d coexistence region [3]. The simulations were performed at 298 K using time steps of 10, 15, 20, 25, 30, 35, and 40 fs. Importantly, 35 fs was used in the original work [3]. We characterized the degree of phase separation using the mean contact fraction [4] (f_{mix} , see Methods) extracted from the last 1 μ s simulation. For each value of the integration time step, we extracted f_{mix} and the temperature of the lipids. With blue markers in Fig. 1A), we demonstrate the obvious correlation of f_{mix} of the function of lipid temperature, both of which depend on the used integration time step. Notably, larger time steps lead to significantly cooler membranes (< 298 K) and smaller values of f_{mix} , *i.e.* higher degree of phase separation. With time steps that maintain the overall temperature of the lipids reasonably close to the target temperature of 298 K, both mixtures display nearly ideal mixing instead. The fact that the target temperature of the thermostat is not preserved even with small time steps is in line with a recent report on the imperfect en-

ergy conservation with the New-RF simulation settings used [11].

We also extracted the temperatures of the different lipid types. As this information not available in the original trajectories as no velocities were saved, we extended each simulation by 100 ns, during which we also saved the instantaneous velocities (see Methods). The temperatures of each lipid type are shown as a function of the integration time step in Fig. 1B). It is evident that the increase in time step causes the low- T_m lipid (DOPC) to heat up and the high- T_m (DPPC) lipid to cool down. The behaviors of DPPC and CHOL are similar. For time steps up to 20 fs, this effect is negligible, yet with the 35 fs time step used by Carpenter *et al.*, the temperature difference between the lipid components is already more than 20 K with DPPC being ~ 30 K below its T_m of 314 K. Thus, it is clear that decreasing the integration time step improves energy conservation, yet leads to a smaller level of phase separation.

To pinpoint the culprit of the poor energy conservation, we performed additional simulations with the input parameters slightly modified. First, we used the same integration time step of 35 fs as Carpenter *et al.* but with the LINCS parameters provided by Melo *et al.* [9]—namely two LINCS iterations per time step, and an 8th order expansion of the constraint coupling matrix (Set 3 in Table I). Data for these settings is shown in red in Fig. 1A). It is evident that this more accurate handling of the constraints leads to a much smaller degree of cooling of the lipids. With these settings, phase separation also essentially vanishes. The temperatures of each lipid type in the simulations with a time step of 35 fs and with various simulation settings are shown in Fig. 1C). The more accurate LINCS settings clearly lead to all the lipids being at almost the same temperature which also closely matches the target one. This indicates that energy conservation can be improved by at least these two ways—decreasing the time step or increasing the number of LINCS iterations and the order of the LINCS expansion. The fact that “LINCS” and “20” overlap in Fig. 1A) suggests that the effect on phase separation is independent on the means of improving energy conservation. However, both approaches lead to a disagreement between the simulation and the experimental phase diagram, contrary to the results reported in the paper by Carpenter *et al.*

We performed an additional simulation, in which we attempted to improve energy conservation by using a separate thermostat for each of the lipid types while still using an integration time step of 35 fs (Set 2 in Table I). As shown in Fig. 1A) by green dots, these settings lead to an almost as cool membrane as in the case when all lipids are coupled to the same thermostat. Still, the degree of phase separation is significantly decreased. Based on Fig. 1C), the used temperature coupling does not prevent CHOL and DPPC from cooling down, yet it does not allow DOPC to heat up. Therefore, the average lipid temperature decreases, whereas the temperature differ-

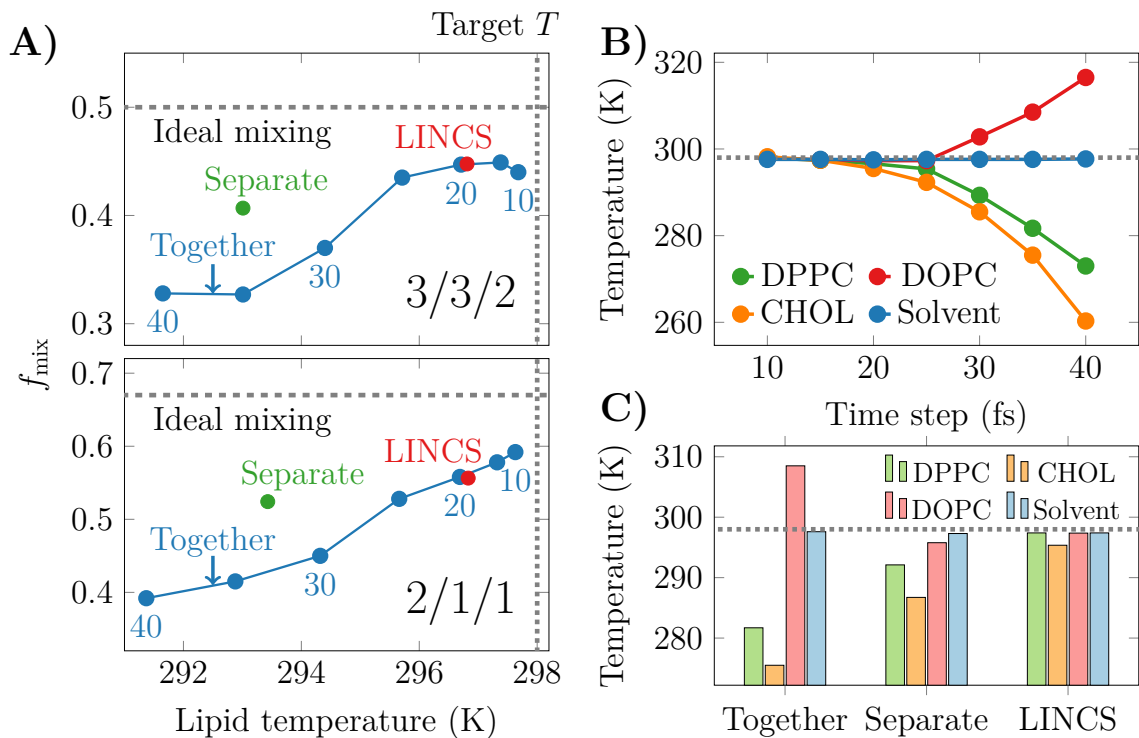


Figure 1. LINCS settings and temperature coupling affect the degree of phase separation. A) The degree of phase separation as a function of the simulation temperature of the lipids, both of which depend on the integration time step, indicated by point labels. Only the labels that are multiples of 10 are shown for clarity. The dotted gray line shows the target temperature of the thermostat. Data for the two studied mixtures are shown in the two panels, and the f_{mix} values corresponding to ideal mixing are highlighted by dashed gray lines. The blue markers show data for simulations in which all lipids are coupled **Together** to the thermostat (Set 1 in Table I). Green markers show data for simulations in which lipid types are coupled to **Separate** thermostats (Set 2 in Table I). Red markers show data for simulations in which more conservative **LINCSt** parameters are used (Set 3 in Table I). B) Temperatures of the lipid types and the solvent beads as a function of the integration time step. Data are only shown for the 3/3/2 mixture, yet the behavior in the 2/1/1 mixture is essentially identical. C) Temperatures of the lipid types and the solvent beads as a function of temperature coupling and LINCS options. Data are again shown for the 3/3/2 mixture only.

ence between DPPC and DOPC is minor resulting in no phase separation.

We also plot the final structures of selected simulations on the top row of Fig. 2. It is evident that proper phase separation takes place with a time step of 35 fs and following the simulation options of Carpenter *et al.* [3] (Set 1 in Table I), whereas decreasing the time step to 10 fs results in an almost ideal mixing of the lipid types. Similar behavior is observed with the conservative LINCS parameters (Set 3 in Table I). However, for the case in which all lipid types are coupled separately to a thermostat (Set 2 in Table I), some heterogeneity is still observed. The local temperature maps, calculated from the 100 ns simulation during which velocities were saved, are shown on the bottom row of Fig. 2. It is evident that the observed phase separation with the model by Carpenter *et al.* goes hand in hand with the uneven temperature distribution in the membrane, indicating that the systems is not sampling the proper equilibrium ensemble. With the simulation setup of Carpenter *et al.* [3], the temperatures of the

L_o and L_d phases can differ as much as 100 K between regions.

II. CONCLUSIONS

From the data presented above, we can draw the following conclusions: **1)** The use of LINCS parameters that are incompatible with the used CHOL model, combined with a large integration time step, results in poor energy conservation. In the case of DPPC/DOPC/CHOL mixture considered by Carpenter *et al.* [3], the virtual site model of CHOL [9] drains out a significant amount of energy from the system, mainly from DPPC which locates itself close to CHOL. **2)** When all lipids are coupled together to the thermostat, as was done by Carpenter *et al.* [3], the cooling of CHOL and DPPC leads to the heating up of DOPC. This temperature difference drives the separation of the lipids into hot (DOPC-rich) and cool (DPPC- and CHOL-rich) phases, whose tempera-

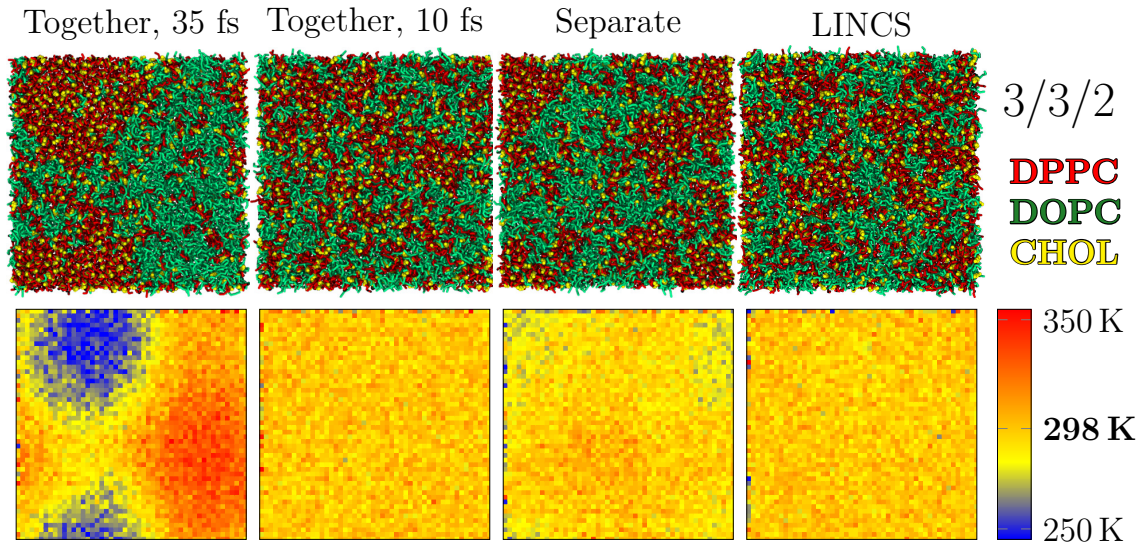


Figure 2. Top row: final structures of chosen simulations with the 3/3/2 DPPC/DOPC/CHOL mixture after 15 μ s. DPPC, DOPC, and CHOL are shown in red, green, and yellow, respectively. Only the system where all lipids are coupled to the same thermostat (“Together”) and a 35 fs integration time step is used undergoes phase separation. Some heterogeneity is also seen in other systems with DPPC and CHOL clustering together. Bottom row: the temperature maps calculated from the 100 ns simulations during which velocities are saved (see Methods). For the “Together, 35 fs” system, the two phases show almost 100 K difference in temperature. When the lipid types are coupled to separate thermostats (“Separate”), the DPPC-rich regions are slightly cooler than the remainder of the membrane. For the other systems, the temperature is close to the target on (298 K) in the entire membrane.

tures differ by significantly. This behavior is in obvious violation of the second law of thermodynamics. **3)** The poor energy conservation can be cured by either decreasing the integration time step or using the LINCS parameters that were used in the parametrization of the virtual site model of CHOL [9]. This better energy conservations leads to the loss of phase coexistence, and thus to poor agreement with experimental phase diagrams. Additionally, by coupling all lipid types to separate thermostats, the temperature difference between DPPC and DOPC is limited, and only a small degree of lipid demixing is observed.

It is also worth mentioning that the standard implementation of the current version 2.2 of the Martini model does not display phase separation of the DPPC/DOPC/CHOL mixture even when the simulation settings of Carpenter *et al.* are used (set 4 in Table I). This results from the fact that the standard Martini DOPC model mixes fairly well with cholesterol. Indeed, a hybrid DPPC/DOPC/CHOL mixture with the parameters of Carpenter *et al.* used for DPPC and the standard Martini parameters used for DOPC did not phase-separate. However, with DOPC parameters adapted from Carpenter *et al.* and DPPC parameters from the standard Martini implementation, phase separation was recovered. This indicates that the unfavorable mixing of DOPC and CHOL in the model by Carpenter *et al.* is central for the co-cooling of DPPC with CHOL, which is required for the formation of a cool L_o phase.

All in all, while the model of Carpenter *et al.* seemed to finally reproduce the experimental phase diagram of the well-studied DPPC/DOPC/CHOL lipid mixture, our results demonstrate that it does due to a simulation artifact. This artifact relates to a poor energy conservation, which results from an unfortunate combination of large integration time steps and not using the the LINCS parameters required by the CHOL model. These choices lead to a clear violation of target NPT ensemble. Unfortunately, this compromises the validity of any findings obtained with the model by Carpenter *et al.* [3], and suggests that further work is still needed until a Martini-like coarse-grained model reproduces the phase behavior of the canonical DPPC/DOPC/CHOL mixture.

III. METHODS

A. Simulations

In the first set of simulations, we strictly followed the protocol of Carpenter *et al.* [3] and built lipid membranes with dimensions of $30 \times 30 \times 15$ nm², which had a total of 3040 lipids and ~ 77000 solvent beads, out of which 10% were modeled as antifreeze particles. The systems were set up by the `insane` tool [12]. We considered two compositions in the heart of the L_o/L_d coexistence region, namely DPPC/DOPC/CHOL ratios of 3/3/2 and 2/1/1. We applied restraints with a force constant of

2 kJ/(mol×nm²) to the phosphate beads of the phospholipids in one leaflet. All in all, we used the same equilibration protocol and simulation parameters as Carpenter *et al.* in the commented paper [3]. Namely, the New-RF simulation parameters [13] were used, unless otherwise mentioned. To pinpoint the violation of the NPT ensemble to the used CHOL model, we performed the following simulations (see also Table I):

First, following Carpenter *et al.*, the lipids and the solvent were separately coupled to a thermostat with a target temperature of 298 K, the 15 μ s simulations with time steps of 10, 15, 20, 25, 30, 35, and 40 fs, saving the trajectories every 1 ns. Secondly, we performed 15 μ s simulations with a 35 fs time step but with all the lipid types (DPPC, DOPC, and CHOL) coupled separately to a thermostat. Thirdly, we performed 15 μ s simulations with two iterations of the LINCS algorithm (`lincs_iter` = 2) and using the 8th order expansion of the constraint coupling matrix (`lincs_order` = 8). Here, all lipids were coupled together to the thermostat.

We also run additional 100 ns simulations, starting from the final structures of the aforementioned simulations, and saved the velocities (.trr file) so that the temperatures of each lipid type could be extracted using `gmx traj`. As the used cholesterol model has constraints, we corrected for the missing degrees of freedom.

B. Analyses

The contact fraction f_{mix} , describing the level of lipid demixing, was adapted from Ref. 4, and is defined as

$$f_{\text{mix}} = \frac{c_{\text{US-S}}}{c_{\text{US-S}} + c_{\text{US-US}}}, \quad (1)$$

where for example $c_{\text{US-S}}$ refers to contacts between unsaturated (US, here DOPC) and saturated (S, here DPPC) lipids. Thus, the smaller the value of f_{mix} , the sharper the separation is. A contact is registered if the phosphate beads of the lipids were within 1.1 nm.

The temperatures of the lipids were extracted using `gmx energy`. When the lipid types were coupled separately, their temperatures were extracted separately and the averaging was performed over the degrees of freedom. For CHOL, this was less than $3N$ due to the constraints. The temperatures of each lipid type were extracted from the 100 ns trajectories containing velocities (see Simulations Methods above) using `gmx traj`. The values were corrected for the missing degrees of freedom of CHOL.

The heat maps of temperature distribution were calculated by projecting the lipid center of mass onto the macroscopic plane of the membrane. While performing the binning, each point was weighted by the instantaneous temperature of the corresponding molecule. For DPPC and DOPC, the number of degrees of freedom in each lipid was taken as $3N$, while in the case of CHOL the decrease of degrees of freedom in the presence of constraints was accounted for.

Table I. Simulated systems. Composition is given as DPPC/DOPC/CHOL. “ Δt ” is the integration time step in fs. “ T -coupl.” refers to the way the lipid temperatures are coupled; the lipids are being coupled either together to one thermostat or separately to three thermostats.

Composition	Δt	T -coupl.	<code>lincs_order</code>	<code>lincs_iter</code>
Set 1. Lipids coupled Together				
3/3/2	10	Together	4	1
3/3/2	15	Together	4	1
3/3/2	20	Together	4	1
3/3/2	25	Together	4	1
3/3/2	30	Together	4	1
3/3/2	35	Together	4	1
3/3/2	40	Together	4	1
<hr/>				
2/1/1	10	Together	4	1
2/1/1	15	Together	4	1
2/1/1	20	Together	4	1
2/1/1	25	Together	4	1
2/1/1	30	Together	4	1
2/1/1	35	Together	4	1
2/1/1	40	Together	4	1
<hr/>				
Set 2. Lipids coupled Separately				
3/2/2	35	Separately	4	1
2/1/1	35	Separately	4	1
<hr/>				
Set 3. Conservative LINCS settings				
3/2/2	35	Together	8	2
2/1/1	35	Together	8	2
<hr/>				
Set 4. Unmodified Martini v2.2				
3/2/2	35	Together	4	1

ACKNOWLEDGMENTS

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IV. DATA AVAILABILITY

The inputs and outputs for our simulations with the model by Carpenter *et al.* are available as follows:

1. Long simulations with different time steps:
 - DOI: 10.5281/zenodo.3956709 (3/3/2 mixture)
 - DOI: 10.5281/zenodo.3956797 (2/1/1 mixture)
2. Short simulations with different time steps and with velocities saved:
 - DOI: 10.5281/zenodo.3956761 (3/3/2 mixture)
 - DOI: 10.5281/zenodo.3956812 (2/1/1 mixture)
3. Additional simulations of the 3/3/2 mixture with different temperature coupling or LINCS settings:
 - DOI: 10.5281/zenodo.3956775 (3/3/2 mixture)
 - DOI: 10.5281/zenodo.3956814 (2/1/1 mixture)