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August 25, 2023

Dear Dr. Marrink,

Thank you for considering the original version of our manuscript, “Understanding the Free Energy Landscape of Phase Separation in Lipid Bilayers using Molecular Dynamics”. Based on the reviewers’ comments, we are now submitting a revised version. Below, excerpts from your decision letter and the reviewers are marked in red, while our responses are in black text and indented.

In the cover letter, you wrote:

As you will see, the referees find your work interesting, but are of mixed opinion on the overall significance of the work. Referee #3 does not support publication for that reason. However, in my opinion this recommendation is not well justified, and I would welcome a revised version of your manuscript. In particular, please pay attention to the following points: (i) try to provide a better rationale for why certain CVs work better than others, to what extent they are generalizable to other membrane systems, and how they can distinguish between local domain formation and macroscopic phase separation, (ii) try to connect your results better to the thermodynamics of phase separation, including improvement on the statistical quality of the analysis.

We will respond to Referee #3 below. We have extended the discussion of the first point (see the Discussion section). In particular, we think that the effective lengthscale measured by the individual CVs has something to do with their effectiveness, although determining this precisely is extremely challenging. Per your second point, we have added a second approach to extract the free energy change upon separation (see Fig 8) using a modified version of the coordinate (see Fig 6) and extended the discussion of the meaning of ΔΔGsep (see second subsection of Discussion). Figure S7 contains all of the free energy curves from individual replicas, but now we also discuss their convergence explicitly in the text.

We thank Reviewer #1 for their careful and thoughtful review:

1. Figures 5, 6, and 7, and accompanying discussion. The discussion below Figure 5 is confusing. The DPPC-DAPC-chol plot shows double well behaviour at lower temperatures, and (I think) single well behaviour at higher temperatures. (It is hard to say, because the curves are all piled on top of each other.) But the text says that "both basins correspond to relatively high FLC and appear to be phase separated based on visual inspection." It is also mentioned that at the higher temperature "60% of the lipids prefer to be in clusters." Is this system phase separated? Normally when a free energy curve like this moves from a double well to a single well as a function of some thermodynamic control parameter, one well corresponds to one phase, and the other well corresponds to the other phase. Is that the case here?

We largely agree with the reviewer’s assessment and have rewritten the discussion accordingly (particularly the second section of the discussion, titled “Interpreting ΔΔGsep”). We now argue that the lower-FLC basin represents a single phase with very non-ideal mixing. We now discuss two different free energy changes, both of which are valuable to think about: the free energy difference between 2 phases and 1, and between 2 phases and a relatively well-mixed single phase.

More broadly, the results are never clearly connected to the basic thermodynamics of phase separation. Some attempts are made in this direction in the section entitled "Computing the free energy of separation" (did the authors mean to write "phase separation here?"), but the presented analysis falls short of the mark, in the reviewer's opinion. The difficulty seems to be that it is not straightforward to transform the FLC collective variable into a useful order parameter (in the Landau sense, not the SCD sense), with clearly separated values that discriminate the two phases. Perhaps this is not surprising, since the thermodynamic variable that is varied is the temperature. As temperature is varied the composition of the two phases also changes; meanwhile, the FLC is a local composition variable. Thus one expects that the values of the FLC CV that defines the two phases will change with temperature. I expect that this approach would be cleaner if instead of temperature composition were varied, in a such a way that the system follows a tie-line through the coexistence region.

Again, we mostly agree with the reviewer’s comment: cleanly extracting the free energy change of phase separation, while the central goal of the entire project, was definitely the weakest part of the original manuscript. We’ve modified the title of the section to be “Computing the free energy of phase separation”, as suggested, and greatly expanded the discussion. We concede the problem is not entirely solved, but we think this paper is a good step forward.

As for the variation in the composition of the phases with temperature, we agree that is a large part of the challenge, so the revised version of the manuscript explicitly discusses it, including the ability of the modified version of FLC (called FLCopt), which uses a significantly longer cutoff and as a result does a far better job of distinguishing less-local structure. We also discuss the notion of varying overall bilayer composition along a tie-line; if we had a good way to know the tie-lines in advance, this would definitely make for simpler interpretations.

Alternatively, one might consider an analysis that is expected to give a clean signal as a function of temperature — the heat capacity. This has the advantage of obtaining an estimate of the transition temperature for each system (at least for the finite size simulation), at the location of the peak in the heat capacity, which ties the results back to the thermodynamics of phase separation. The POPC system is also useful here, as one expects no signature of a phase transition, based on the longer, standard CG simulations.

This is an excellent idea. Unfortunately, our testing revealed that heat capacities are simply too noisy to be interpretable, because the amplitude of fluctuations in the potential energy in a system this size is on the order of thousands of kcal/mol, perhaps 2 orders of magnitude larger than the signal we’d be hoping to extract.

2. The presented work falls short of delivering data of high statistical quality as promised in the introduction. The work is premised by stating correctly that it is challenging to obtain simulation results for lipid phase separation of good statistical quality. If one could obtain such data it would be of great value. Simulation models could be rigorously tested, and experimental results for more complex mixtures rationalized. However, no such demonstrations are presented. No phase boundaries are reported, no transition temperatures reported, the compositions of the two phases are nowhere obtained. The data points in Fig. 7 are presented without error bars. I agree with the authors that the goal is a worthy and impactful one, but as it stands the work seems to get only halfway to the finish line. A new sampling protocol is presented that looks promising, but the proof that it really works for calculating key observables is not provided.

We would argue that extracting the boundaries of phases (which in turn is necessary to extract their internal compositions) is a separate problem from what we are attempting to accomplish here. We discuss in the manuscript how to extract transition temperatures (the point where the free energy difference is 0), but given the uncertainties of getting the free energy difference there are large uncertainties in Tm.

Computing error bars for free energy curves is a challenging technical problem, because those curves are only determined up to a constant and the choice of how to align them can alter the apparent meaning of the errors. Instead, we presented the free energy curves for the individual replicates as Figure S7 (they rarely differ by more than 0.25-0.5 kcal/mol). We have added a sentence to this effect just below the first discussion of Figure 5. We considered putting the all the replicate curves in the figure but doing so would have required each temperature to be on its own panel, which in turn made comparison more complex.

We agree with the reviewer that we are not presenting a complete solution to the problem. However, we believe that it is a worthwhile step forward. Moreover, we’ve added discussion of some further directions we think will be fruitful (other coordinates that might lead to cleaner interpretation, and the effects of system-size on the results).

3. The introduction states that there is functional phase separation in the living cell membrane, but the evidence supports (at best) instead localized domains of different composition. It is also claimed that this phase separation exists across all domains of life. Both statements are an overreach, in the reviewer's opinion.

We respectfully disagree. However, we’ve added additional references that use this nomenclature, most notably recent work from Dr. Keller’s lab on the melting curves for phase separation in vacuolar membranes in live yeast.

In a few places nice sounding turns of phrase are used, but with unclear meanings: for example, "molecular grammar" and "curated ensemble." I'm not sure what either of these phrases mean.

We have rewritten both sentences.

The explanation of the CVs (Eqs 1,2,3) is not very clear.

We have expanded the description of the CVs.

Reviewer #2:

In this manuscript, Poruthoor and Grossfield describe a weighted ensemble (WE) approach to study the free energy landscape of phase separation in model membranes using molecular dynamics (MD) simulations. The proposed method and protocols provide a useful means by which to probe the thermodynamics of lipid phase separation using MD simulations; this is important but has been missing in the field. The work is rigorous and the manuscript is well written overall. However there are a few issues that I would like to encourage the authors to revisit. One is that they did not provided a sufficient rationale for why two of the three collective variables failed in their WE analysis while the third, which is related to the other two or at least not fundamental unique, works so well. At the minumum a hypothesis/hypotheses could be offered in order to complete the discussion, even if they may not test the hypothesis here.

We have expanded the discussion of the behavior of the different collective variables, including a new one introduced in the revision (FLCopt). While we cannot draw firm conclusions about what makes one more effective, we attempt to rationalize the behavior we observed.

Another limitation is the rather terse Discussion section. This could be expanded through, for example, a more thorough discussion of the possible physical basis and hence different performances of the three reaction coordinates, especially given the data in Fig 3.

We agree, and have expanded the discussion section accordingly. We now discuss the behavior of the different coordinates and the meaning of the computed free energy changes.

Fig 7 should also be interpreted more. For example, what is the basis for the different temperature dependence of deltadetaG for the two bilayer models?

This is now discussed in the Discussion section.

Overall, this is an important piece of work from a group with a strong track record in free energy methods, with a potentially broad application but can be strengthened a bit more, as noted above.

We thank you for you kind words and hope you agree the revised manuscript is stronger.

Reviewer #3:

The paper presents results of coarse-grained (CG) MARTINI simulations of three ternary mixtures, two of which are known to exhibit phase separation and one not. For the simulations, the authors use Weighted Ensemble (WE) techniques, which supposedly expedite the phase segregation of the mixtures when starting from a uniform distribution of lipids. The work is purely methodological and contains no new biophysics.

While we agree that the major import of the work is methodological, it is an attempt to treat a problem with significant biophysical implications. The present work sets the stage for a number of potential applications, and as such we think it is appropriate for Biophysical Journal.

This is not to suggest that papers presenting new methods or improvements of existing methods are not interesting and publishable, but it is far from obvious whether the WE implementation presented herein is truly a big step forward: WE methods are not new and the generalization of the results to simulations of other membranes is not clear. The most interesting result in the work is the demonstration that collective variables can serve as "reaction coordinates" for WE simulations, but (as shown in the work) some collective variables perform poorly, and it is not obvious which collective variable works efficiently for a given membrane under investigation.

We believe the results are clear: one coordinate (FLC) samples efficiently for both systems capable of phase separating, while the other two do not; we provide a clear demonstration of the sampling by measuring probability flux between states (Fig 4 panels C and D). One of the more significant findings is that the obvious choice of collective variable, local density (CEI), does not effectively enhance the sampling. In the revision, we include discussion as to why we think this occurs.

I find the choice to apply the WE methods to CG simulations problematic, for two reasons: First, the problem of slow phase separation in mainly a problem in atomistic simulations.

Depending on the coordinate used to track separation, it can take between 10s of nanoseconds to multiple microseconds, even in a coarse-grained simulation. More importantly, though, while observing a single phase-separation event is tractable, that non-equilibrium relaxation process doesn’t tell us about the thermodynamics. Second, there is nothing in our formalism or approach that is specific to coarse-grained models. Rather, our choice to use MARTINI was driven by computational convenience while we were refining the protocols. Indeed, each FLC calculation requires between 1 and 2 months of wall-clock time using a single GPU, which means that performing the process of identifying and evaluating coordinates using all-atom simulations requiring 100x more computer time would have been completely intractable.

Second, calculations of free energy differences from CG simulations is always a risky business because of the loss of degrees of freedom in the coarse-grained picture which causes the entropic component of the free energy to be replaced by enthalpy. Therefore, the thermodynamics of phase separation captured by MARTINI simulations is not necessarily correct.

We agree that there’s no reason to expect the MARTINI thermodynamics to be quantitatively correct, for the reasons given. However, a) the MARTINI model is extremely common in the field, so understanding its thermodynamic behavior is valuable, and b) this work sets the stage for calculations using atomistic models, now that the basic protocols and approaches have been worked out.

Some additional comments:  
  
1. The authors state, regarding computer simulations, that "as a rule they cannot quantify the underlying thermodynamics" (page 2). This is probably correct for atomistic and CG molecular simulations, but miss the contribution of lattice simulations to the field (see, e.g., the works of Almeida and others).

We agree; we’ve reworded the statement to make it clear that we intended to refer to MD-based approaches and have added references to lattice simulations.

2. I find it disappointing that the authors do not explain the essence of WE methods, and only give the technical details for running WE simulations in GROMACS. Readers who are not familiar with the concept of WE simulations will have hard time to follow the work.

Thank you for pointing out this oversight. We added a paragraph to the methods summarizing the method and explaining our rationale for picking it.

3. The collective variables proposed in the paper are quantities that are sensitive to local clustering of molecules with themselves. The logic behind this choice is clear, but it neglects the fact that ternary mixtures not exhibiting thermodynamic phase separation still show formation of domains (termed microscopic or local phase separation in some papers). This is clearly seen in the DPPC-POPC-Chol simulation results in Fig. 1. The authors should discuss how the collective variables distinguish between macroscopic phase separation and formation of local domains.

As discussed above, we have updated the discussion and interpretation significantly, and try to identify how coordinates might distinguish between non-ideal mixing in a single phase and the formation of distinct phases. We also mention alternative approaches one might use going forward that rely on identifying distinct populations of lipids.

It would be also nice to include a general discussion on the phase behavior of ternary mixtures.

We believe a full discussion of ternary mixtures would be beyond the scope of this work. However, we reference several reviews in the introduction.

To conclude, the paper presents an application of WE methods for simulations of ternary mixtures of saturated and unsaturated lipids with cholesterol. The results may be promising, but the generalization of the work to other mixtures is not clear, let alone to more complex biological membranes. The paper presents no new physics and its value is limited to readers who are experts in these kind of simulations. I think that the work fits better to a more computational-oriented journal and recommends the authors to elaborate on the concept of WE simulation methods and on the formation of liquid ordered domains in mixtures not showing phase separation.

As discussed above, we have substantially improved the manuscript to address some of these concerns.

As you can see, we have made significant modifications to the manuscript in order to address the reviewers’ concerns. Hopefully, you will agree these revisions make the paper acceptable for publication in Biophysical Journal.

Sincerely,



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