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DISCUSSIONS

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Structure and dynamics of asymmetric membranes: general discussion

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Milka Doktorova opened discussion of the paper by Markus Deserno: It has been shown that under some conditions asymmetric vesicles can exhibit more than two types of 'phases' or 'environments' as evidenced by the differential partitioning of fluorescent lipid probes in experiments (*e.g.* Fig. 2 in ref. 1). This is not captured in conventional phase diagrams robustly characterized with symmetric model membranes and used here as a basis for examining the effects of differential stress on asymmetric 3-component membranes. How can the potential asymmetry-driven formation of a third 'bilayer phase' be incorporated into this analysis and is this something that the coarse-grained simulations can capture and provide the means to investigate further?

1 T. A. Enoki and F. A. Heberle, *Proc. Natl. Acad. Sci. U. S. A.*, 2023, **120**, e2308723120.

Markus Deserno responded: In the paper you reference,[1] Enoki and Heberle examine asymmetric binary membranes comprising two types of phospholipids (DPPC/DOPC at 295 K in their case). Disregarding differential stress for a moment, this situation is characterized by a single variable in each leaflet – say, the DPPC mole fraction. This nevertheless results in an already fairly subtle two-dimensional compositional phase diagram with 3 pure phases (shown in Fig. 4 of their paper): (i) both sides DPPC rich and ordered, (ii) both sides DOPC rich and disordered, and (iii) one side DPPC rich and ordered while the other side is DOPC rich and disordered. Between these phases lie three coexistence regions with generally tilted tie-lines (*e.g.*, two coexisting disordered phases on one side meeting an ordered phase coexisting with a disordered one on the other, each in mutual registration) – meaning, we observe four different leaflet mixing proportions. Moreover, under certain conditions we even get a three-phase coexistence region, bordering the three two-phase regions, which then amounts to six different mixing proportions. Once we add differential stress to this scenario, we have a new variable and hence a three-dimensional phase diagram. I am not aware that this has ever been studied, either in experiment or in theory. Much of this clearly goes beyond the physics we have learned in symmetric systems, and so we obviously do not expect trivial modifications of the "old" phase diagrams. But we do expect that the physical phenomenology we have learned to be important must be part of any explanatory models we wish to develop.

In the asymmetric ternary case we discuss in our paper (**https://doi.org/10.1039/d4fd00196f**) things are evidently even more complicated. However, our goal is not simply to extrapolate from ternary symmetric systems to ternary asymmetric ones. Instead, we use very generic thermodynamic reasoning (equilibrium conditions, Gibbs phase rule, mechanical stability, *etc.*) to suss out how many degrees of freedom we even have. We argue that there are four (in addition to temperature and net tension), and only if we fix two of them (say, the saturation ratios in both leaflets) do we get something we can easily plot. But you will notice that in most of the examples I have discussed in some detail I have not yet included the possibility of phase separation in each leaflet, by picking the saturation ratios to be sufficiently large or small so that they fall outside the coexistence region (at least the coexistence region as we know it from the symmetrical case). Once they go inside, one (or both) leaflets can laterally phase separate. This will not change the abundance asymmetry α nor the cholesterol mole fraction φ, but the compositions of the coexisting leaflet phases will split – first tentative guess: along the tie lines known from symmetric phase diagrams. But once this happens, inter-leaflet domain coupling needs to be included, as well as curvature effects (since the two laterally coexisting phases need not have the same torque). All this will quantitatively (and likely also qualitatively) change the observed behavior compared to what we know from symmetric systems. I must admit that at this moment I have no idea how this will all play out, or what a good set of thermodynamic variables is, or what observables we could measure that would be particularly informative about the underlying state. We need to develop better theories to address these situations and hope that the structure of these theories gives us some idea of the right variables or observables.

Of course, computational models would in principle include all these effects, or at the very least we typically know what molecular parameters we would have to adjust in order to account for them. But seeing things happen in simulation is not yet the same as understanding *why* they happen. We still require models, and given that the present situation is indeed quite complex – both theoretically and experimentally – I suspect we will have a lot on our plate for the foreseeable future.

1 T. A. Enoki and F. A. Heberle, *Proc. Natl. Acad. Sci. U. S. A.*, 2023, **120**, e2308723120.

John M. Seddon asked: If you move the lipid composition closer to the critical point in the ternary diagram, will you observe large curvature fluctuations in the bilayers, and will there be any difference between symmetric and asymmetric bilayers?

Markus Deserno replied: By the submission deadline of our contribution to this *Faraday Discussions* meeting we had not yet looked for this effect, but since then we have started to examine this question. I still have no data, but my firm expectation is that we will see enhanced curvature fluctuations – by the well-known composition–curvature coupling argument first made by Stan Leibler.[1] One of its predictions is that non-ideal mixing enhances this effect, and in a naïve quadratic mean-field theory the effective shape fluctuations actually diverge as we approach the critical point (of course, they do not in reality – we just need a more careful theory).

While we have not yet seen this in simulations, it appears that at least two other groups have: Pöhnl *et al.*[2] have examined binary mixtures of phospholipid and cholesterol, at the MARTINI level, while Hossein *et al.*[3] have looked at phospholipid mixtures featuring phosphatidylcholine (PC) *versus* phosphatidylethanolamine (PE) headgroups, using both MARTINI 2 and 3 as well as an atomistic force field (CHARMM C36).

I am unsure whether asymmetry would entail any obvious qualitative changes. Clearly, the effect relies on leaflet-specific composition–curvature coupling, but it does not have to be "symmetric" in magnitude to "work". There are quite a number of interesting effects to probe with some detail, and we are starting to look at this. The biggest challenges I expect to face are sampling issues: getting good statistics on shape fluctuations in the precious long wavelength regime is already difficult. And compositional fluctuations come with an even slower relaxation time that you need to exceed in order to sample correctly. To measure the effect precisely enough that we do not merely see its correct "direction" but can also quantitatively compare it to its predicted magnitude will be difficult.

1 S. Leibler, *J. Phys.*, 1986, **47**, 507.
2 M. Pöhnl, *et al.*, *Nat. Commun.*, 2023, **14**, 8038.
3 A. Hossein, *et al.*, *J. Phys. Chem. B*, 2024, **128**, 6317.

Madan Rao enquired: What ensemble have you chosen to represent the phase diagrams you presented? Do you fix the tension in the two leaflets, or do you fix the overall bilayer tension?

Markus Deserno responded: The Gibbs phase diagrams shown in our paper (**https://doi.org/10.1039/d4fd00196f**) are meant to refer to the zero bilayer tension situation, but I have not yet thought carefully about how they would change under an applied tension. Similarly, the torque- and differential stress diagrams in Fig. 7 and 10 of our paper, respectively, were based on actual simulations that were run at zero bilayer tension.

We have argued previously that it is convenient to look at bilayer tension and differential stress as the obvious symmetrized and antisymmetrized linear combinations of the individual leaflet tensions. This is useful because the bilayer tension is often zero (or in some well-defined sense "small") so that only the differential stress is an observable we need to explicitly account for. But, of course, the value of its complementary variable, the total bilayer tension, still must be stated, and in virtually all cases we have looked at so far that value was zero.

Madan Rao queried: Do you explicitly include a transbilayer interaction between components across the bilayer, either at a microscopic scale or at a coarse grained scale (for instance, as an "interfacial" contribution)? This clearly has both entropic and enthalpic parts and is strictly an emergent statistical quantity.

Markus Deserno replied: We do not yet have a theoretical description of asymmetric ternary systems in terms of, say, a phenomenological free energy. But our coarse-grained simulations must of necessity "commit" to some form of transbilayer interaction. Specifically, since we use a solvent-free model, lipids from the two different leaflets must attract one another, for otherwise the leaflets would simply unbind. The dominant contribution of this attraction comes from the last tail beads (recall that our coarse-grained (CG) lipids consist of a single hydrophobic head bead and three consecutive tail beads, of which the last ones directly "touch" one another across the leaflets).

What your question is probably driving at is whether we choose this enthalpic contribution to the transbilayer interaction to be dependent on lipid type, and the answer is that we do not. After all, it is just methyl groups touching! Whether a lipid is of the "ordered" or "disordered" type (as determined by intra-lipid interactions that affect how much it can "wiggle") does not influence the strength of the enthalpic interaction.

I wish to emphasize that we could have easily enforced domain registration if we had chosen that interaction to be stronger between like-type lipids, but we felt that this would mostly just be putting in by hand the known experimental outcome, instead of exploring whether it would naturally arise for different reasons. And these different reasons exist, because effects such as thickness mismatches, or the specific form of line tensions, depend on the type of registration. Possibly even more interesting, there is an *entropic* contribution to registration, because a rigid phase leaflet loses undulation entropy, and if it lies opposite a soft phase leaflet, it forces that leaflet to suffer a similar loss because they must ultimately share the same overall bilayer shape. If, instead, rigid phases align with rigid phases and soft phases with soft ones, then the overall entropy is larger. Years ago I had offered this simple argument as an explanation why adhesion between two vesicles at the brink of phase separation might actually demix their membranes in the adhesion zone, as experimentally observed, even if all lipids have the same head groups.[1]

1 V. Gordon, *et al.*, *Europhys. Lett.*, 2008, **84**, 48003.

Madan Rao said: This is related to the question on transbilayer coupling. How would you discuss issues of bilayer registry, partial registry or anti-registry? What would be the tuning parameter and how would it appear in your phase diagram?

Markus Deserno responded: Assuming you now want me to comment on a suitable theoretical approach, I would consider, as a first step, a phenomenological Landau free energy that includes a coupling term between the compositions of the two leaflets.

For the binary (or effective binary) case, this has been done before.[1–4] These papers really set the stage for how to think about coupled phase separation in opposing leaflets. In fact, recently Enoki and Heberle[5] have used a simple version of such theories to explain the experimental phase diagram of asymmetric vesicles in which each side is a binary DPPC/DOPC mixture. The (single!) phenomenological coupling parameter between the local order parameter in the two leaflets can be tuned so that the morphology of the theoretically predicted phase diagram is compatible with what is experimentally observed.

In all these prior theories, there is effectively only one important order parameter in each leaflet, which results in just a single coupling constant. Of course, for ternary systems each side has three different concentrations, which upon symmetric quadratic coupling gives 6 possible coupling constants (*i.e.*, the number of independent entries of a symmetric 3×3 matrix). This number is too big, of course, because *principally* the concentrations are not independent, and because *practically* not all of these couplings might be equally important. We have not yet looked carefully into this, though, and – to the best of my knowledge – neither have others. So I cannot really answer your question more definitively.

1 A. J. Wagner, *et al.*, *Biophys. J.*, 2007, **93**, 4268, DOI: 10.1529/biophysj.107.115675.
2 G. Garbès Putzel and M. Schick, *Biophys. J.*, 2008, **94**, 869, DOI: 10.1529/biophysj.107.116251.
3 S. May, *Soft Matter*, 2009, **5**, 3148, DOI: 10.1039/b901647c.
4 J. J. Williamson and P. D. Olmsted, *Biophys. J.*, 2018, **115**, 1956, DOI: 10.1016/j.bpj.2018.10.003.
5 T. A. Enoki and F. A. Heberle, *Proc. Natl. Acad. Sci. U. S. A.*, 2023, **120**, e2308723120, DOI: 10.1073/pnas.2308723120.

Heiko Heerklotz requested: Can you please comment on the order of magnitude of the torque caused by cholesterol redistribution in a hypothetic plasma membrane containing the same number of phospholipids in the outer and inner leaflet? Would such a torque be likely to interfere with membrane structure and function?

Markus Deserno answered: Let's start by getting a feel for the orders of magnitude. Consider as an example the asymmetric membranes discussed in Elani *et al.*,[1] which were made from DOPC and POPC lipids. Computational results from Venable *et al.*[2] give spontaneous leaflet curvatures of $J_{0\mathrm{m}}(\mathrm{DOPC}) = -0.071\ \mathrm{nm}^{-1}$ and $J_{0\mathrm{m}}(\mathrm{POPC}) = -0.032\ \mathrm{nm}^{-1}$. If for a start we ignore any additional leaflet stresses, then the spontaneous bilayer curvature of an asymmetric DOPC/POPC membrane would be $J_0 \approx -0.02\ \mathrm{nm}^{-1}$, and the associated spontaneous curvature torque would be $|\kappa J_0| \approx 30 k_\mathrm{B}T \times 0.02\ \mathrm{nm}^{-1} \approx 2.5\ \mathrm{pN}$. How "big" is this?

Spherical vesicles made from such asymmetric DOPC/POPC leaflets would have a vanishing bending energy at a radius $R = 2/|J_0| = 100\ \mathrm{nm}$, with DOPC on the inside. But the actual giant unilamellar vesicles (GUVs) in the experiments of Elani *et al.*[1] had radii between 10 μm and 20 μm, at least 2 orders of magnitude larger. As a consequence, their bending energy would have to be around 4 orders of magnitude larger than that of a relaxed vesicle, and so they should have an extremely strong tendency to vesiculate or tubulate into shapes with a curvature closer to their actual preferred one. We have argued that this might not happen because these vesicles are actually under a differential stress that creates a counter-torque. In the present case this requires a differential stress of around $\Delta\Sigma = \kappa J_{0,\mathrm{b}}/z_0 \approx 2$ mN m^{-1}, and that order of magnitude ends up being typical. This shows that an unbalanced torque of just a few pN would give rise to dramatic shape changes.

Now, how much torque is created when cholesterol redistributes? In a previous paper[3] Dr Varma and I had examined this situation. As a simple example, consider an asymmetric membrane containing two species of phospholipids on its two sides, with vanishing differential stress but a difference $\delta\chi$ between the non-ideal mixing parameters of cholesterol and the respective phospholipids, such that one of the sides is preferred (by essentially $\delta\chi \times k_\mathrm{B}T$). Combining some of the results in the paper I just cited, one can show that in the simplified case where all lipids (including cholesterol) have the same area per lipid, a, the differential stress $\Delta\Sigma$ as a function of cholesterol concentration ϕ is given by

$$\Delta\Sigma \approx \frac{1}{2}K_\mathrm{A}\delta\chi \frac{\phi(1-\phi)}{1+\frac{1}{2}\frac{\phi}{1-\phi}K_\mathrm{A}a/k_\mathrm{B}T}$$

Fig. 1 here plots this as the blue curve for the special case where $\delta\chi = 1$, area modulus $K_\mathrm{A} = 240$ mN m^{-1}, and a specific lipid area $a = 0.6$ nm^2. In our previous paper,[3] we also discuss the more difficult case where cholesterol is smaller than the lipids, and then we get the slightly reduced red curve in Fig. 1 here. Qualitatively, the outcome is the same though: the asymmetric partitioning of added cholesterol ramps up a differential stress that reaches a maximum value at around 20 mol% cholesterol and a magnitude that depends on the partitioning preference, but is again roughly a few mN m^{-1}. This is of the same magnitude as the "Elani case"[1] I discussed above. Hence, the associated torque would also be comparable, so we should again expect a strong tendency towards tubulation.

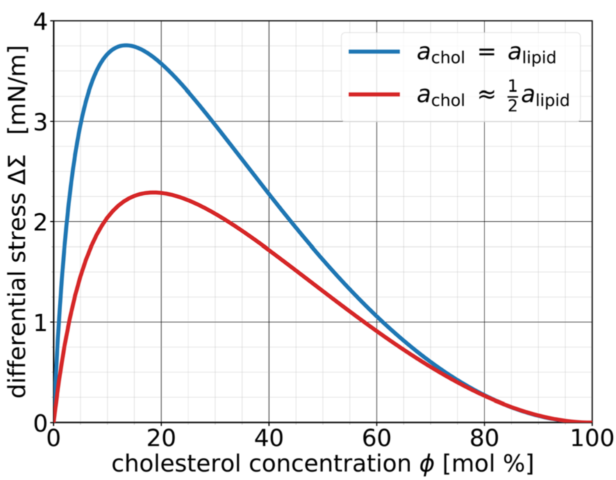

Fig. 1 Differential stress $\Delta\Sigma$ as a function of cholesterol concentration ϕ.

With all this in mind – yes, I believe that the torques arising from cholesterol redistribution can be large enough to drive very noticeable shape deformations, even though the details will always depend on exactly how much cholesterol we add or re-distribute. As a rule of thumb, which we justify in our paper, we expect cholesterol to nevertheless be a less potent driver of shape deformations than phospholipids, because it divides its impact between the two leaflets.

1 Y. Elani, *et al.*, *Chem. Commun.*, 2015, **51**, 6976–6979, DOI: 10.1039/c5cc00712g.
2 R. M. Venable, *et al.*, *Chem. Phys. Lipids*, 2015, **192**, 60–74, DOI: 10.1016/j.chemphyslip.2015.07.014.
3 M. Varma and M. Deserno, *Biophys. J.*, 2022, **121**, 4001–4018, DOI: 10.1016/j.bpj.2022.07.032.

Oded Farago asked: You consider changes in the thermodynamic variables that bring one of the leaflets into the two-phase region. This, in turn, should lead to changes in the mechanical properties of the bilayer. How are these changes handled in the calculations, and how do they show in the torque foliation curves?

Markus Deserno replied: I do not know yet, because we have not pushed our calculations that far. At this point our fairly generic thermodynamic reasoning avoids referring to a specific equation of state. Once phase coexistence occurs, some additional degrees-of-freedom counting arguments (basically the Gibbs phase rule) should enforce further constraints. All this might lead to important qualitative changes even before we consider mechanical aspects such as changes in rigidity, which will affect the torque, or that such asymmetric systems would also have a spontaneous curvature. For instance, if the spontaneous bilayer curvature is not the same in different regions of the bilayer, then what do we mean by a "zero torque condition" in the first place? Zero *average* torque, maybe?

How does all of this affect the torque foliations? Again, I cannot say for sure, because we have no theory yet for the specific details. I have thought a little bit about what would happen if the "cyan point" in the +-leaflet (Fig. 4 of our paper; https://doi.org/10.1039/d4fd00196f), while moving along its torque foliation curve, enters a two-phase region. My initial guess was that the torque foliation curve might become a straight line between the "entry" and "exit" points of the phase coexistence region. But since the system now is mechanically asymmetric, with the complications I just mentioned above, it is not clear to me whether this naïve linear expectation will hold, considering all the other effects that now matter and which have not been part of the currently proposed thermodynamic framework.

Oded Farago remarked: The nano-scale liquid ordered domains that you observe in the one-phase region of the phase space are local density fluctuations that always appear, especially near the binodal of the two-phase region (see, *e.g.*, ref. 1).

1 T. Sarkar and O. Farago, *Soft Matter*, 2023, **19**, 2417, DOI: 10.1039/d2sm01025a.

Markus Deserno responded: I agree that these systems should always show density fluctuations, or maybe more relevant here, *compositional* fluctuations, but I would expect them to be essentially molecular in size, just a few times as big as a single lipid's footprint. (Unfortunately I do not know how to make the vague term "a few" more precise!) Of course, near critical points these fluctuations can grow truly macroscopic, but that is because the respective susceptibilities diverge. This is not true as we merely approach a binodal sufficiently far away from the critical point. In fact, even slightly entering the coexistence region (sampling metastable states before nucleation sets in), the local free energy will give you no reason to expect enhanced fluctuations – not until you actually reach the spinodal. So I would disagree with the expectation that in the vicinity of the binodal compositional fluctuations are noticeably more pronounced than deeper into the pure phase.

That said, it is also possible that our unusually large "flakes" are small, ordered domains *inside* the two-phase region. That is possible because we do not know the location of the binodal very precisely. Measuring the composition of a phase requires partitioning all available molecules between the coexisting phases, which in our case is accomplished by a hidden Markov model. However, its output depends – among other things – on the training point, the chosen features, and the trajectory one actually trained on. The underlying systematic uncertainties are challenging to quantify, and we have not yet tried to do so.

Rumiana Dimova asked: How would an external mechanical stress (tension) couple with this thermodynamic framework of asymmetric membranes and do you expect a difference in the response of symmetric *vs.* asymmetric membranes?

Markus Deserno answered: I have not yet thought carefully about what would happen if we permit a nonzero net tension, but I expect several new phenomena to arise. At the very least, it is clear that tension can affect the bilayer *via* (i) any type of "area physics" and (ii) net curvature. I generally expect these to be coupled.

Let me illustrate this with a simple thought experiment: if the two leaflets differ in their (effective) area moduli, imposing an area strain will induce different leaflet stresses, which will then create a torque. Indeed, since the two leaflets need to stay together, any net tension can only ever change the areas in the same way, and so I expect a net tension to always create a torque proportional to the difference in leaflet area moduli. Incidentally, since this all makes sense within linear response, we should conversely expect torques to affect the net tension.

It gets more interesting if the two leaflets contain ordered domains that can melt under tension, especially if they cover different amounts of the area. This is closely related to the *Faraday Discussions* paper by Krompers *et al.* that was presented yesterday by Dr Heerklotz (**https://doi.org/10.1039/d5fd00003c**). He focused on temperature-driven melting or gelling, but tension would also be able to melt gel domains. Of course, in that case it is less likely that we can observe a curvature response; but if instead of fluid and gel phases we have L_o and L_d domains, then there is a different way in which this could manifest: melting an L_o domain in one leaflet would free up its cholesterol, some of which might be pushed into the other leaflet. Hence, a net tension could affect the trans-leaflet cholesterol distribution. We have briefly checked in our simulations whether tension could change the relative area fraction of coexisting L_o and L_d domains, but our domain identification algorithm was not precise enough to ascertain an effect.

Finally, should we be able to realize a scenario where one leaflet could phase separate but the other does not (because its composition is so far from the coexistence region that even the "imprinting" from the opposite side is not strong enough to trigger demixing), then we would have a system in which we have laterally coexisting asymmetric domains that almost certainly differ in their spontaneous curvature. As demixing progresses, *i.e.*, as domain coarsening sets in, this triggers ever larger deviations from a membrane's initial (say, flat) state. Hence, a net applied tension should at some point arrest coarsening and the system should end up with a compositionally and geometrically modulated phase, with a characteristic length scale set by the tension.

Andreas Heuer enquired: Does it help to include the information about the average area of the lipids for both leaflets in the general analysis, *e.g.*, in relation to the differential stress? Possibly as a reasonable approximation which is easy to access?

Markus Deserno responded: Yes, this information is both useful and important, because it is a basic factor in determining differential stress. In fact, in one of our recent papers[1] Dr Varma and I have accounted for specific lipid areas when it came to mixing between different lipid species. We examined a membrane that had two different types of phospholipids in its two leaflets (say, POPC and DOPC), plus additional cholesterol, rendering each side a binary mixture. Since the relative leaflet areas affect the stress, and cholesterol is significantly smaller than a typical phospholipid, we assigned different areas per lipid to each species. The equilibrium area of each leaflet was then postulated to be additive: $A_{\rm eq} = N_{\rm p}a_{\rm p} + N_{\rm c}a_{\rm c}$, where $N_{\rm p}$ and $N_{\rm c}$ are the number of phospholipids and cholesterol molecules in the leaflet, respectively, and $a_{\rm p}$ and $a_{\rm c}$ are their specific areas. We also included an empirical area-correction in the Flory–Huggins type mixing free energy, and the results we get for many observables reflect obvious expectations.

As plausible as this looks, it remains questionable how accurate this approach is when applied to real systems, because "area additivity" is a fickle assumption to make, especially when applied to cholesterol. The notion that a lipid even *has* an area – *i.e.*, a molecular property independent of the specific bilayer it is part of – is a major simplification. This is of course generally true when thinking about molecules (recall the well-known example that mixing 50 ml of water with 50 ml of ethanol does not yield 100 ml of vodka), but it is even more subtle in the case of lipids: unlike water or ethanol, lipids have a rich ensemble of molecular conformations, *and this ensemble depends on a lipid's neighbors*. If a disordered lipid has relatively rigid cholesterol molecules nearby, its tail fluctuations get suppressed and it takes up a smaller area than it would if it were surrounded by other lipids of its own type. Hence, if we were to replace 30% of all phospholipids in such a membrane by cholesterol, the area would not merely go down because cholesterol is intrinsically smaller than the phospholipid. It would go down *further* than that because cholesterol orders neighboring phospholipids, *i.e.*, it changes the molecular ensemble of a different neighboring lipid species. Obviously, such effects are not included when we rely on area additivity. A few years back this condensing effect has been discussed very nicely in a simulation study by Leeb and Maibaum.[2]

To make a quantitative theoretical improvement to the simple additivity ansatz, the only practical solution I see at this point is to replace it by an empirical equation of state, which presumably would have to be determined from experiment or, likely easier, from simulation.

1 M. Varma and M. Deserno, *Biophys. J.*, 2022, **121**, 4001.
2 F. Leeb and L. Maibaum, *Biophys. J.*, 2018, **115**, 2179.

Andreas Heuer asked: Is it possible to use the properties of the fluctuations of the membrane system during the MD simulations, to learn about the parameters, entering the analysis of the general model with the four degrees of freedom?

Markus Deserno replied: Yes, this is definitely possible. Of course, the challenge is to develop quantitative theories that permit us to interpret the experimentally measured fluctuations.

Let me give you an example, though, that suggests we can obtain a useful but difficult to measure parameter from the shape undulations of a mixed membrane. Picture a symmetric binary membrane in which the two phospholipids form a well-mixed phase at all mixing ratios. What is the bending rigidity of that membrane? One might expect some linear interpolation between the rigidities of the two pure phases, but one typically finds that it dips below that. A possible explanation is softening due to composition curvature coupling, as first discussed by Stan Leibler.[1] In that case, the extent of softening depends on the spontaneous curvature difference between the two lipid species. This parameter is very difficult to reliably measure in lamellar phases, so one typically resorts to experiments performed on inverted hexagonal phases, which curvature-wise are orders of magnitude removed from the local conditions in fluctuating lamellar phases. I have seen experimental data on this from my colleague Stephanie Tristram-Nagle, which beautifully align with theoretical predictions, and which will appear later this year in a special issue of the *Biophysical Journal* honoring the late Adrian Parsegian. This, in my view, is a vivid example where fluctuations in mixed systems allow you to measure an observable that is otherwise quite difficult to obtain.

Of course, in reality everything is more subtle. "Lipid spontaneous curvature" is yet another one of those mythical lipid properties, just like "area", which only make sense in the context of an entire ensemble, not as an immutable molecular property that Avanti or Sigma could print on their sample vials. This has been well documented by Sodt *et al.*,[2] and yet the theoretical analysis I have just outlined straight up assumes that a lipid bilayer's local spontaneous curvature can be obtained as an average of individual molecular properties weighted by the local lipid concentrations. It is very hard to make analytical progress without such approximations, and so many scientists (me included) make them as a first step; but one should remain alert to complications arising from such simple assumptions.

1 S. Leibler, *J. Phys.*, 1986, **47**, 507.
2 A. J. Sodt, *et al.*, *Phys. Rev. Lett.*, 2016, **117**, 138104.

Michael M. Kozlov enquired: Does the effective spontaneous (intrinsic) curvature of cholesterol in your computations depends on the surrounding lipid? What is its characteristic value?

Markus Deserno responded: When we developed the ternary model system in ref. 1, we focused on lipid interactions that affect mixing but left all lipid shapes as essentially "straight" (*i.e.*, we did not "by hand" impose any spontaneous curvatures). However, as you correctly remind us, cholesterol affects the conformational ensemble of its surrounding lipids, which is really how we should think of spontaneous curvature arising – see ref. 2. We have not yet measured what its value is, but we briefly mention some indirect findings in our paper. Eqn (20) in the paper (**https://doi.org/10.1039/d4fd00196f**) recalls that the difference between the torque coming from differential stress and the total torque must be the torque due to spontaneous curvature, $\kappa J_{0,\mathrm{b}}$. Since we have measured both the differential stress and the torque, we can deduce what $\kappa J_{0,\mathrm{b}}$ is, and we found it to vary relatively little even as we change cholesterol from 0 to 40 mol%. We take this to mean that "our" coarse-grained cholesterol affects the conformational ensemble of lipids only weakly – not surprising considering how simple our lipids are. That said, to get the spontaneous curvature from its associated torque, we need to divide out the bending rigidity κ, and we have not yet measured its value as a function of cholesterol content – something which all by itself is a surprisingly subtle question that especially for unsaturated lipids has given rise to a very lively debate over the past few years.

1 M. Varma, *et al.*, *J. Chem. Phys.*, 2024, **161**, 114103.
2 A. J. Sodt, *et al.*, *Phys. Rev. Lett.*, 2016, **117**, 138104.

Di Jin asked: How do you relate the simulation results to experimental observations? In particular, would there be a way to apply the insights from nanometric simulations to interpret experimental observations of micron-sized phase-separated domains by techniques such as AFM?

Markus Deserno answered: This is an excellent question, and one which I am frequently asked by new students: How do we relate our highly coarse-grained simulations to experiment? Our lipids do not look like actual lipids, our values for area, length, order parameters, moduli, relaxation rates, *etc.*, do not match an actual system, and sometimes (especially with dynamics) they might even be far off. How would we distinguish a saturated from an unsaturated lipid if all we have is four beads? These and other issues may make one legitimately wonder: what is the point? I suppose your question was not meant to be quite that far reaching, but please grant me a soap box moment.

You likely know the aphorism "All models are wrong. But some models are useful." It reminds us that the goal of modeling is not to capture reality in a 1-to-1 fashion. The famous short story "On Exactitude in Science" by Jorge Luis Borges really nails this sentiment. Models leave out detail, and some very good models leave out almost everything. To describe the motion of Earth around the Sun, we can model both celestial objects as point particles. To pull yet another authority: Einstein supposedly said, "Make things as simple as possible, but no simpler." Alas – who decides what is "as simple as possible", and when do we know we made it "too simple"?

These decisions are made by the modeler, and later again by the person who wants to learn something from the model. A good rule is: to make it into the model, a certain property must matter for what we wish to explain. Nothing gets in gratuitously. But there is a subtlety: say I want to describe the flow of water through a pipe. Do I need to model hydrogen bonds? Probably not. Do I need to account for density and viscosity? Almost surely yes. But wait – these are determined to no small part by water's hydrogen bonds! True, but we only need the macroscopic emergent properties of "density" and "viscosity" to describe flow, we do not need to understand how they emerge from a more fine-grained atomistic reality. Modeling hence very often means radically short-circuiting pathways of emergence.

Finally – back to membranes. Our coarse-grained model does not contain hydrogen bonds. It does not even contain atoms! (Or water, for that matter.) But it captures a few key (emergent!) properties of lipids that matter for its physics: a lipid's approximate shape, its amphipathic nature, the fact that tails have some conformational entropy. These, it turns out, suffice to make these lipids aggregate into two-dimensional fluid bilayer membranes that exhibit stretching- and curvature elastic properties with values of the moduli that are surprisingly meaningful. If we distinguish different lipid species by their size and tail "wiggliness", we can represent saturated and unsaturated lipids, and even some bravely simplified version of cholesterol. If we adjust their pair interactions, we can affect their mixing behavior and, for instance, make cholesterol melt gel phases or let ternary systems exhibit L_o/L_d phase separation. All these "successes" are examples where emergent membrane properties that are largely determined by generic lipid properties are reproduced, and that means I can reason about them using such simplified models. I can now go on to explore other aspects of the L_o/L_d physics, such as what happens when both sides of the bilayer are ternary mixtures but one of the components, cholesterol, can rapidly flip-flop. I can ask whether phase segregation or domain melting can be driven by differential stress, or membrane tension, or a change in a membrane's curvature state, or adsorption to a substrate.

Since you asked specifically about AFM imaging – what aspects of such experiments could we mimic/explain/predict? For starters, micron-sized domains will be difficult, simply because this would be a very large system. A 10 μm × 10 μm field of view would contain about 300 million lipids, which even in our highly coarse-grained model would mean more than a billion particles. But maybe we do not need micron domains? Maybe a domain with a size of 30 nm would be OK for what you want to understand? We could for instance ask whether adsorption to a substrate that flattens the membrane and reduces its thermal undulations would increase the temperature at which it phase-separates. We could ask whether preferential absorption of one phospholipid species *versus* another would change the leaflet compositions enough to see domains on one side, but maybe not on the other. We could try to pin some lipids on the substrate and ask whether somehow "shearing" the upper leaflet would get any possibly matching domains out of registration, and if so, by how much – thereby mimicking the beautiful experiments done some time ago by Blosser *et al.*[1] But wait, you say, in our model we usually see domain anti-registration! And here we come to a point where we reach a problem: maybe we reached the limits of the model? Or we have not yet simulated big enough systems? Or have not yet done some necessary tuning? At any rate, we need to step back and think before we blindly apply our model to this problem.

1 M. C. Blosser, *et al.*, *Biophys. J.*, 2015, **109**, 2317–2327.

Simon Connell said: This is more a comment than a question, Markus. In Fig. 11 and 12 in your paper (**https://doi.org/10.1039/d4fd00196f**), you look at a composition in the co-existence region near the L_d binodal, and the effect of changing the composition of one leaflet thus driving the opposing leaflet into (or further away from) the coexistence region (Section 4.3.2). You observed a stable L_o domain in the $\alpha = -10\%$ (16.7% Chol) as expected in the co-existence region, but on the boundary and in the single phase (α = 0%, 25.5% Chol and α = +10%, 32.5% Chol) you saw remnant transient "flakes" dynamically fluctuate in and out of existence. We work on the dynamics of lipid phases using high resolution and high speed AFM, along with fluorescence microscopy, and in a paper currently under review[1] we have observed these transient "flakes" near phase boundaries. This is with symmetric and not with asymmetric bilayers, so I understand the system is a little different. However, we observe these "transient flakes" a long way from the critical point in terms of composition, near phase boundaries, and if starting from the critical point and increasing temperature, they are seen up to 10 °C higher. This opens up the possibility of testing some of your ideas in real experiments, with observation of transient flakes down to 5 nm in size.

1 G. R. Heath, S. D. Evans and S. D. Connell, Critical fluctuations of lipid domains display raft-like behavior, under review.

Markus Deserno responded: Thank you for this very interesting comment. As Dr Farago has pointed out in an earlier question, we should always see transient compositional fluctuations, except I do not know what size scale I should expect (other than "molecular"). My personal sense is that the flakes we observe, and whose size and dynamics we really have not yet characterized more quantitatively, are bigger than what could be justified based on compositional fluctuations far away from a critical point.

That said, there have long been claims in the literature that complex lipid mixtures may have nanodomains, whose origin and thermodynamic justification I have not yet spent enough time to understand. To my knowledge, the first experimental evidence was reported by Heberle *et al.*,[1] who observed what they describe as (nano-) "domains" with an effective size in the 5–15 nm range in small unilamellar vesicles of ~60 nm diameter (albeit for quaternary mixtures). The domain sizes were determined by fitting a model of randomly dispersed circular patches to small angle neutron scattering data and were found to correlate with bilayer thickness mismatch of the coexisting L_o and L_d phases. At the risk of displaying too much of my own ignorance, but here is why I find this confusing: if these are true equilibrium domains, then what stops them from growing? And if they are transient fluctuations, why are they so big?

1 F. A. Heberle, *et al.*, *J. Am. Chem. Soc.*, 2013, **135**, 6853.

Thais Enoki communicated: I would like to include a minor comment to highlight the importance of ternary phase diagrams. The use of ternary phase diagrams in *in vitro* experiments does not aim to ignore more than 99% of the lipid species in the plasma membrane; instead, all lipids are represented by categories. The extensive repertoire of lipids is classified into three categories, each representing key characteristics that are of considerable significance. The two phospholipids in the phase diagram represent a group of lipids with high and low melting temperatures, and the third category is cholesterol. Indeed, as mentioned in the paper (**https://doi.org/10.1039/d4fd00196f**), the model can capture some emergent phase behavior, not an intricate lipidomic fingerprint.

Markus Deserno communicated in reply: I believe we agree on the physics, so this is almost surely just a language issue, regarding the precise meaning of "ignore". Of course we do not restrict to ternary systems out of ignorance about reality, or because we are too lazy to do "the real thing". We model ternary systems because we have the strong expectation that the classification into ordered lipids, disordered lipids, and cholesterol captures the essence of the effects we are presently curious about. My point is that we do not necessarily have an irrefutable prior reason to believe that this will actually work. The fact that it appears to do is a deep observation about the physics of the situation, about exactly what type of emergent variables matter, and how the process of emergence ends up identifying lipid order as the dominant variable.

But even if it does, we know that all the different lipid types in a real biomembrane present us with a whole spectrum of such melting temperatures, not just two. And it is not obvious to me that such a spectrum can always be described as if we only had two main categories of phospholipids, ordered and disordered. Clearly, as we tune the temperature, and as we cross several such main transition temperatures for some of our lipids in the mix, one would think we ought to notice that. Maybe if two representatives of the "saturated lipid class" in our mix have similar but not quite identical main transition temperatures, we would observe something strange if only we could examine the critical point very carefully.

I do not know what the answer is, and I suspect there will be more subtle observables that would be sensitive to such details, observables where an effective ternary model would fail. When we wrote that in our paper (**https://doi.org/10.1039/d4fd00196f**), we merely wanted to draw attention to the fact that ternary mixtures are well-motivated models of nature, but as with all models, it is always healthy to remember what we ignored.

Zhi Li opened discussion of the paper by Reinhard Lipowsky: Have you done some research about how ions affect the membrane tension? To what extent do you think the ions could change the membrane tension compared with mechanical bending tension?

Reinhard Lipowsky answered: Most ions are expelled from the hydrophobic core of the bilayer but can bind to or intercalate into the two headgroup layers. If the ions bind to one of the headgroup layers, they will increase or decrease the area per lipid in this leaflet. If the ions repel each other and increase the area per lipid, they increase the leaflet tension as well. On the other hand, if the lipids condense around the ions and attain a smaller area per lipid, the leaflet becomes compressed corresponding to a decreased or even negative leaflet tension.

The effect of ions and other small solutes on the bilayer asymmetry and the associated spontaneous curvature has been studied by coarse-grained molecular dynamics simulations of planar bilayers[1] and nanovesicles[2] as well as by experimental studies of giant unilamellar vesicles (GUVs).[3] In the simulations, the ions were modelled as small solutes and it was demonstrated that they generate a large transbilayer asymmetry and spontaneous curvature in planar bilayers.[1] The binding of small solutes to nanovesicle membranes leads to the formation of closed membrane necks, to recurrent shape fluctuations between open and closed necks, and to the fission of the closed membrane necks.[2] The experimental studies in ref. 3 used different concentrations of LiCl, NaCl, and KCl in the interior and exterior aqueous solution and measured the spontaneous curvature by pulling tubes from aspirated GUVs.

1 B. Różycki and R. Lipowsky, *J. Chem. Phys.*, 2015, **142**, 054101, DOI: **10.1063/1.4906149**.
2 R. Ghosh, *et al.*, *ACS Nano*, 2021, **15**, 7237, DOI: **10.1021/acsnano.1c00525**.
3 M. Karimi, *et al.*, *Nano Lett.*, 2018, **18**, 7816, DOI: **10.1021/acs.nanolett.8b03584**.

Georg Pabst added: Several experimental studies have reported that monovalent and divalent ions can modulate the intrinsic curvature of lipids. These ion-induced changes influence the spontaneous curvature of membrane leaflets, thereby altering the associated mean torque. In our recent work,[1] we demonstrated how such curvature modifications can affect the activity of an integral membrane enzyme in an asymmetric bilayer. This study also provides references to additional research exploring the impact of ions on intrinsic lipid curvature.

1 P. Piller, P. Reiterer, E. F. Semeraro and G. Pabst, *RSC Appl. Interfaces*, 2025, **2**, 69, DOI: **10.1039/d4lf00309h**.

Aileen Cooney asked: You discussed membrane–condensate interactions, have you looked how at other systems interact with membranes? Specifically hydrogel or colloidal systems?

Reinhard Lipowsky replied: For systems consisting of biomembranes interacting with condensate droplets, we use the theoretical framework of what we call “fluid elasticity”. This framework uses two basic assumptions to characterize the elastic and morphological responses of the vesicle–droplet systems.[1] The first assumption is that the condensate droplets are enclosed by a liquid–liquid interface and that the shape of this interface is governed by its interfacial tension. The second assumption is that the biomembranes are in a fluid state, which implies that their morphology is determined by a few curvature-elastic parameters such as bending rigidity and spontaneous (or preferred) curvature, in addition to the mechanical tensions within the membranes (**https://doi.org/10.1039/d4fd00184b**).

Hydrogels consist of networks of polymers (or peptides) in aqueous solutions. The macromolecules can be cross-linked by chemical bonds or by physical entanglement. Our theoretical framework can also be applied to hydrogel droplets interacting with biomembranes provided the shape of these droplets is governed, at least on long time scales, by a liquid–liquid interface with a well-defined interfacial tension. From an experimental point of view, this tension may be difficult to measure.

As far as rigid colloids are concerned, we also looked at the interactions of rigid nanoparticles with biomembranes. In this case, the interfacial tension of the condensate droplets is replaced by the adhesion free energy between membrane and particle surface. The membranes can again be described within the framework of curvature elasticity.[2]

1 R. Lipowsky, *Membranes*, 2023, **13**, 223, DOI: 10.3390/membranes13020223.
2 J. Agudo-Canalejo and R. Lipowsky, *ACS Nano*, 2015, **9**, 3704, DOI: 10.1021/acsnano.5b01285.

John M. Seddon said: The Gaussian curvature modulus κ_G is more difficult to measure experimentally than the bending modulus κ. In previous work, you showed that the profile of a vesicle phase-separated into two domains (for example, L_o and L_d) is determined by the difference in κ_G between the two bilayer domains. Could this provide a useful method for determining κ_G of a bilayer?

Reinhard Lipowsky responded: Consider a vesicle enclosed by a lipid bilayer that contains several molecular components and can undergo phase separation into two intramembrane domains, a and b. Let us denote the corresponding Gaussian curvature moduli by $\kappa_{G,a}$ and $\kappa_{G,b}$. Applying the Gauss–Bonnet theorem to such a vesicle, the domain boundary between the a and b domains generates a term that is proportional to the difference $\kappa_{G,a} - \kappa_{G,b}$ of the two Gaussian curvature moduli.[1] If the two Gaussian curvature moduli happen to be equal to each other, the domain boundary remains located within the membrane neck during the closure of this neck. On the other hand, if $\kappa_{G,a}$ is different from $\kappa_{G,b}$, the domain boundary is displaced from the closing neck. This displacement was originally obtained by numerical integration of the shape equations for axisymmetric vesicles,[2] has been observed experimentally,[3] and can be obtained in a simple and transparent manner from the matching condition for the mean curvature along the domain boundary.[4]

It seems possible to extend these theoretical considerations in such a way that one could determine the individual values of the Gaussian curvature moduli $\kappa_{G,a}$ and $\kappa_{G,b}$ rather than their difference but, at present, I am not aware of a feasible method to do so. It is interesting to note that different Gaussian curvature moduli will also displace the contact line between a vesicle membrane and a condensate droplet away from the closing membrane neck adjacent to the droplet.[5]

1 F. Jülicher and R. Lipowsky, *Phys. Rev. Lett.*, 1993, **70**, 2964, DOI: 10.1103/PhysRevLett.70.2964.
2 F. Jülicher and R. Lipowsky, *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top.*, 1996, **53**, 2670, DOI: 10.1103/PhysRevE.53.2670.
3 T. Baumgart, *et al.*, *Biophys. J.*, 2005, **89**, 1067, DOI: 10.1529/biophysj.104.049692.
4 R. Lipowsky, *Eur. Phys. J. E: Soft Matter Biol. Phys.*, 2024, **47**, 4, DOI: 10.1140/epje/s10189-023-00399-z.
5 R. Lipowsky, *Soft Matter*, under review.

Manpreet Kaur asked: How does the shape of the lipid (especially conical and inverted-conical shape lipids) affect the membrane tension? And what about the regions, where such non-bilayer lipids are clustered by strong lipid–lipid interactions?

Reinhard Lipowsky replied: To simplify the discussion, let us focus on the bilayer tension of a symmetric lipid bilayer, which exhibits the same lipid composition in both leaflets. This bilayer tension is a membrane-elastic parameter that describes the elastic response of the bilayer membrane to external constraints or forces that lead to the stretching (or compression) of the bilayer. Imagine, for instance, that we pull at the bilayer by external tangential forces in order to stretch it. As long as the bilayer does not rupture, these tangential forces must be balanced by the molecular forces between the different molecules within the membrane. In general, the membrane molecules experience a variety of molecular interaction forces such as van der Waals and electrostatic forces or forces arising from ions that adsorb onto the head groups of the lipids.

In order to determine the bilayer tension for a specific lipid bilayer with a certain lipid composition, one has to compute the local stress profile across the bilayer. By definition, the local stress within a lipid bilayer is equal to the negative local pressure. Therefore, stress profiles are equivalent to pressure profiles. From an intuitive point of view, the local stress is positive close to the interfaces between the lipid headgroups and the adjacent aqueous solutions, because these interfaces can reduce their interfacial free energy by reducing their areas, whereas the local stress is negative in the hydrophobic core, corresponding to a positive pressure arising from the confined hydrocarbon chains. More precisely, the stress profile is obtained from the anisotropic pressure tensor, which has a normal component P_N and a tangential component P_T, as recently reviewed in Appendix B of ref. 1. The bilayer tension is then obtained by integrating the stress profile over the coordinate perpendicular to the bilayer.

The local stress profile for a planar lipid bilayer in aqueous solution has been computed using three molecular models: binary Lennard-Jones fluids;[2] bead models for dissipative particle dynamics;[3–5] and the Martini force field for lipids.[6] Furthermore, in all of these molecular models, the hydrophobic chains of the lipids were treated as semiflexible polymers consisting of molecular groups that experience both a harmonic bond potential and a three-body bending potential, which favors a certain tilt angle between neighboring molecular groups along the hydrocarbon chains of the lipids. It is important to note that these interaction potentials between the hydrocarbon groups also affect the local stress profile across the bilayer.

Therefore, the bilayer tension of a planar lipid bilayer depends on all interaction potentials between the molecular groups within the membrane. The dependence of this tension on the shape of the lipids can be studied by using different architectures of the lipid molecules.[1–6] In ref. 5, we studied a binary mixture of two lipids with a small and a large headgroup. The lipid with the large headgroup may be regarded as a conical lipid. We focussed on the stress profiles for tensionless bilayers with zero bilayer tension, which are required for the computation of the bilayer's spontaneous curvature. Even for tensionless bilayers, the stress profiles vary significantly with the mole fraction of the large-headgroup lipids, see Fig. 2 in ref. 5.

1 R. Lipowsky, *et al.*, *Biomolecules*, 2023, **13**, 926, DOI: **10.3390/biom13060926**.
2 R. Goetz and R. Lipowsky, *J. Chem. Phys.*, 1998, **108**, 7397, DOI: **10.1063/1.476160**.
3 J. C. Shillcock and R. Lipowsky, *J. Chem. Phys.*, 2002, **117**, 5048, DOI: **10.1063/1.1498463**.
4 B. Różycki and R. Lipowsky, *J. Chem. Phys.*, 2015, **142**, 054101, DOI: **10.1063/1.4906149**.
5 A. Sreekumari and R. Lipowsky, *J. Chem. Phys.*, 2018, **149**, 084901, DOI: **10.1063/1.5038427**.
6 M. S. Miettinen and R. Lipowsky, *Nano Lett.*, 2019, **19**, 5011, DOI: **10.1021/acs.nanolett.9b01239**.

Sylvie Roke enquired: Most lipids have charges, when one does measurements of bending and tension, electrostatic interactions cannot be avoided, and these are strong, especially when compared to other interactions. How are such interactions incorporated/connected to membrane tension, bending computations that you presented?

Reinhard Lipowsky responded: The mechanical tension within a lipid bilayer, for which I use the short term "bilayer tension", is a membrane-elastic parameter that describes the elastic response of the bilayer membrane to external constraints or forces. Imagine, for instance, that we stretch a planar bilayer by tangential forces. As long as the bilayer does not rupture, these tangential forces must be balanced by the molecular forces between the different molecules within the membrane. In general, the membrane molecules experience a variety of molecular interaction forces such as van der Waals and electrostatic forces as well as forces arising from ions that adsorb onto the head groups of the lipids.

In order to compute the bilayer tension for a specific lipid bilayer with a certain lipid composition, one has to compute the local stress profile across the bilayer. By definition, the local stress is equal to the negative local pressure within the bilayer. From an intuitive point of view, the local stress is positive close to the two interfaces between the two lipid headgroup layers and the adjacent aqueous solutions, because this interface can reduce its interfacial free energy by reducing its area, whereas the local stress is negative in the hydrophobic core, corresponding to a positive pressure arising from the confinement of the hydrocarbon chains. More precisely, the stress profile is obtained from the anisotropic pressure tensor, which has a normal component P_{N} and a tangential component P_{T}, as reviewed in Appendix B of ref. 1. The bilayer tension is then obtained by integrating the stress profile over the coordinate perpendicular to the bilayer.

The local stress profile for planar lipid bilayers in aqueous solution has been computed using three different molecular models: binary Lennard-Jones fluids;[2] bead models for dissipative particle dynamics;[3–5] and the Martini force field.[6] Furthermore, in all of these molecular models, the hydrophobic chains of the lipids were treated as semiflexible polymers consisting of molecular groups that experience both a harmonic bond potential and a three-body bending potential, which favors a certain tilt angle between neighboring molecular groups along the hydrocarbon chains of the lipids. All of these interactions contribute to the stress profile as shown in Fig. 11 of ref. 2, which depicts the individual contributions from the different interactions. The total stress profile is obtained from the sum of the individual contributions as shown in Fig. 12a of ref. 2.

Stress profiles as obtained from dissipative particle dynamics of planar lipid bilayers are displayed in Fig. 4 and 10 of ref. 3, in Fig. 7 of ref. 1 based on data from ref. 4, and in Fig. 2 and 3 of ref. 5. Finally, a stress profile as obtained from the Martini force field is shown in Fig. 14b of ref. 1 based on data from ref. 6. The Martini force field includes Coulomb potentials for electrostatic interactions. Compared to the stress profiles as obtained from dissipative particle dynamics without Coulomb potentials, the stress profile as obtained from the Martini force field with Coulomb potentials exhibits additional fine structure, corresponding to small additional minima and maxima of the profile. However, when we compute the bilayer tension by integrating over the coordinate perpendicular to the bilayer, these additional minima and maxima will average out to a large extent.

1 R. Lipowsky, *et al.*, *Biomolecules*, 2023, **13**, 926, DOI: **10.3390/biom13060926**.

2 R. Goetz and R. Lipowsky, *J. Chem. Phys.*, 1998, **108**, 7397, DOI: 10.1063/1.476160.
3 J. C. Shillcock and R. Lipowsky, *J. Chem. Phys.*, 2002, **117**, 5048, DOI: 10.1063/1.1498463.
4 B. Różycki and R. Lipowsky, *J. Chem. Phys.*, 2015, **142**, 054101, DOI: 10.1063/1.4906149.
5 A. Sreekumari and R. Lipowsky, *J. Chem. Phys.*, 2018, **149**, 084901, DOI: 10.1063/1.5038427.
6 M. S. Miettinen and R. Lipowsky, *Nano Lett.*, 2019, **19**, 5011, DOI: 10.1021/acs.nanolett.9b01239.

Sylvie Roke asked: Has anybody tried to look at tension parameters and relate them to actual interactions?

Reinhard Lipowsky answered: As mentioned in my reply to the previous question, the contributions of the different molecular interactions to the stress profile have been analyzed in ref. 1 for a Lennard-Jones fluid, as displayed in Fig. 11 of ref. 1. In general, it is always possible to decompose the total stress profile into the individual contributions arising from the different molecular interactions. Thus, it is also possible to identify the individual contribution arising from the Coulomb potentials in the Martini force field.[2] However, as emphasized in my reply to the previous question, this Coulombic contribution is expected to make a relatively small contribution to the bilayer tension as obtained by integrating the total stress profile $s = s(z)$ over the coordinate z perpendicular to the planar bilayer.

1 R. Goetz and R. Lipowsky, *J. Chem. Phys.*, 1998, **108**, 7397, DOI: 10.1063/1.476160.
2 M. S. Miettinen and R. Lipowsky, *Nano Lett.*, 2019, **19**, 5011, DOI: 10.1021/acs.nanolett.9b01239.

Milka Doktorova requested: Can you elaborate in a little more detail about the origins of the fluctuation tension? In the abstract it is stated that it governs the excess membrane area stored in the vesicle shape fluctuations. In that sense, does fluctuation tension vary with lipid composition?

Reinhard Lipowsky responded: Membranes in aqueous solutions undergo thermally-excited shape fluctuations in the form of bending undulations in order to increase their configurational entropy. Thus, when we look at the shape of a membrane, we will, in general, observe shape fluctuations that represent normal displacements of the membrane from its average shape. The analysis of these shape fluctuations is particularly simple for a planar bilayer. In this case, the average shape is provided by a plane and the shape fluctuations lead to local and transient displacements from this planar shape, see the simulation snapshot in Fig. 2 of the paper (**https://doi.org/10.1039/d4fd00184b**). This snapshot was obtained for a planar bilayer with vanishing bilayer tension. The bilayer tension can be increased by changing the projected area of the planar bilayer, corresponding to the base area of the simulation box, without changing the number of lipid molecules, see Fig. S5 in the supplementary information of the paper (**https://doi.org/10.1039/d4fd00184b**). The bilayer tension is increased by increasing this base area as in Fig. S5c, which acts to flatten the bending undulations and to decrease the excess area stored in these shape fluctuations.

For planar bilayers with periodic boundary conditions, the shape fluctuations can be decomposed into a discrete set of Fourier modes. More precisely, such a decomposition is possible provided the lateral size of the bilayer exceeds a certain minimal size. The Fourier analysis in ref. 1 revealed that this minimal size is about 6 nm for a lipid bilayer with a thickness of about 4 nm. On smaller scales, the shape fluctuations represent molecular protrusions corresponding to the normal displacements of single lipids or small groups of neighboring lipids. Thus, one has to consider a sufficiently large segment of a planar bilayer with vanishing bilayer tension in order to observe the bending undulations of this bilayer. Furthermore, the number of discrete Fourier modes increases as one increases the lateral size of this bilayer.

As described in Section 3.5 of the paper (**https://doi.org/10.1039/d4fd00184b**), the fluctuation tension of planar bilayers is defined *via* the spectrum of these discrete Fourier modes, see eqn (16) of the paper. The fluctuation tension vanishes for vanishing bilayer tension as demonstrated for one-component planar bilayers in ref. 1 and further corroborated by the fluctuation spectra of planar bilayers with two lipid components.[2] One interesting and open question is whether or not these two tensions are also equal for non-zero tension values, see the references cited in Section 3.5 of the paper.

1 R. Goetz, *et al.*, *Phys. Rev. Lett.*, 1999, **82**, 221, DOI: **10.1103/PhysRevLett.82.221**.
2 A. Sreekumari and R. Lipowsky, *J. Chem. Phys.*, 2018, **149**, 084901, DOI: **10.1063/1.5038427**.

Gerald W. Feigenson opened discussion of the paper by Michael M. Kozlov: I am interested in the calculation and thoughts on mid plane energy where two phases are touching each other. This is simple chemically, with the main difference being density, so I wonder if you have any comments?

Michael M. Kozlov replied: Should be both. Computational models reveal such differential energy (see *e.g.* ref. 1 and references therein and in our paper (**https://doi.org/10.1039/d4fd00186a**)).

1 T. A. Enoki and F. A. Heberle, *Proc. Natl. Acad. Sci. U. S. A.*, 2023, **120**, e2308723120, DOI: **10.1073/pnas.2308723120**.

Oded Farago asked: Can you point to the differences between the membrane-mediated interactions between domains discussed in your work, and membrane-mediated interactions between cone-shaped proteins – a problem that has been investigated in prior studies.

Michael M. Kozlov answered: The difference is in the effects of the lipid tilt/splay deformations. They come on top of and interfere with the curvature deformations and modify the resulting interaction forces.

Zhibo Deng enquired: Would you consider incorporating species such as cholesterol into one of the monolayers to induce the formation of a lipid domain? If so, would you expect the resulting differential energy to initiate membrane curvature?

Michael M. Kozlov responded: We analyzed the effect of cholesterol and other lipid molecules with relatively large intrinsic curvatures. It boils down to a decrease in the effective splay (bending) modulus.

Ilya Levental asked: Would this set of differential transbilayer interactions be more relevant for bilayer domains or protein complexes?

Michael M. Kozlov answered: The interaction we analyzed might be relevant to the formation of bilayer domains out of the monolayer ones. Bilayer domains might interact as well if, *e.g.*, they are intrinsically curved. Yet I believe, based on the previous work, that similar bilayer domains would repel each other (I am talking about the membrane-mediated interaction only).

Concerning the protein complexes, the logic is the same. Similar protein complexes are expected to exercise an attractive membrane-mediated interaction (at least for large separations) if they are located in opposing leaflets, and a repulsive interaction in case they sit in the same leaflet.

Ilya Levental requested: About the attractive *vs.* repulsive energies, could you please expand on this?

Michael M. Kozlov responded: The model predicts that the interaction between domains located in opposing leaflets is attractive for large inter-domain distances. For small separations, the interaction can be either attractive or repulsive, depending on the ratio between the domain's rigidities and those of the surrounding lipid monolayers. The relevant rigidities are those for the monolayer bending (splay) and tilt (the average tilting of the lipid hydrocarbon chains with respect to the normal to the monolayer surface). For technical simplicity, we took this ratio to be the same for the bending rigidities and the tilt ones. If this rigidity ratio is larger than a certain "critical" value, *i.e.* the domains are sufficiently rigid, the interaction at small separations is predicted to be repulsive. Otherwise (for relatively soft domains), the interaction for small separations is attractive, which means that attraction persists for all separations. The critical rigidity ratio depends on the membrane tension and the domain's size. For example, for large tensions (flat membranes) and relevant domain sizes (say larger than a hundred nm), the predicted critical rigidity ratio is 3, as presented in the paper (**https://doi.org/10.1039/d4fd00186a**). We have the results for the critical ratio and the separation after which the interaction turns repulsive (if it does) for the whole range of tensions (not shown in this paper).

Gonen Golani enquired: What is the tension required to fulfill the requirement of a flat membrane compared to the rupture tension of the membrane?

Michael M. Kozlov replied: The required tension must be much larger than the tilt modulus, the latter constituting a few tens of mN m^{-1}. Therefore, the case of a completely flat membrane is interesting theoretically to start understanding the system, while the analysis of the experimentally feasible situations requires the accounting of the effects of small tensions. We have got these results but I did not have time to include them in the manuscript.

Damiano Andreghetti asked: The model you propose seems to share some features with the O(2) model (*XY* model) or theoretical frameworks used for liquid crystals. An interesting aspect of these theories is the presence of Goldstone modes (or gapless excitations) arising from continuous symmetry breaking. Do you expect your model to exhibit this behavior as well?

Michael M. Kozlov answered: I never thought about it.

Thais Enoki said: In your paper (**https://doi.org/10.1039/d4fd00186a**), you mentioned that you are not aware of any experimental data enabling the evaluation of the differential contact energy, $\Delta\varepsilon$. Although I do not have a model-free estimation of differential contact energy, we estimate this parameter by comparing an experimentally determined phase diagram of asymmetric bilayers of DPPC/DOPC[1] to the phase diagrams proposed by Wagner *et al.* (2007)[2] and later May (2009).[3] I want to share with you this paper for your evaluation and consideration to be cited along with the paper from May that you are already citing. It is important to note that our estimation agrees with the values in the range you mention in your paper.

If I understand it correctly, and as exploited in the 1D model, in the presence of one domain, there is a differential contact energy that is less favorable than the contact energy of the blue lipids (disordered lipids). Thus, this configuration tends to reduce the number of lipid molecules opposing the domains or make them tilt to decrease the contact area. These changes create deformations that propagate in the membrane (x-axis).

In the model's extension, there are two domains in opposite leaflets, and in this case, the model mimics a symmetric bilayer (I believe). The deformations mentioned above raise repulsive or attractive interactions that drive anti-registration or registration of domains in a symmetric-like bilayer. Can you add a line tension to this model, and how would that affect the results?

Could you add domains into the same leaflets, mimicking an asymmetric bilayer in lipid composition and phase behavior? In other words, one leaflet would have one phase, and the other would have two phases. In this case, would domains attract or repel each other? If domains repel each other, could this suggest that the interactions drive small domains, as they will never coalesce into larger domains? By adding a line tension to this model, how do you expect to change the deformations and interactions between domains? Would you expect the predictions from these models to be different in a spherical geometry compared to a plane geometry?

1 T. A. Enoki and F. A. Heberle, Experimentally determined leaflet–leaflet phase diagram of an asymmetric lipid bilayer, *Proc. Natl. Acad. Sci. U. S. A.*, 2023, **120**, e2308723120, DOI: **10.1073/pnas.2308723120**.

2 A. J. Wagner, S. Loew and S. May, Influence of monolayer–monolayer coupling on the phase behavior of a fluid lipid bilayer, *Biophys. J.*, 2007, **93**, 4268–4277, DOI: **10.1529/biophysj.107.115675**.

3 S. May, Trans-monolayer coupling of fluid domains in lipid bilayers, *Soft Matter*, 2009, **5**, 3148–3156, DOI: **10.1039/b901647c**.

Michael M. Kozlov responded: Many thanks for sending me your paper.[1] It is great to have an estimation deduced from experiments rather than MD simulations. I will cite your paper in my upcoming paper on domain interaction in membranes subject to finite tensions and undergoing therefore bending deformations in addition to the tilt/splay deformations.

"If I understand it correctly, and as exploited in the 1D model, in the presence of one domain, there is a differential contact energy that is less favorable than the contact energy of the blue lipids (disordered lipids). Thus, this configuration tends to reduce the number of lipid molecules opposing the domains or make them tilt to decrease the contact area. These changes create deformations that propagate in the membrane (x-axis)." Correct. In the manuscript submitted to the meeting (**https://doi.org/10.1039/d4fd00186a**), I considered only a flat membrane, implying that the membrane is subject to a large enough tension. In this case the deformations are tilt/splay of hydrocarbon chains. I already have results for a more general case of arbitrary tensions, including the experimentally relevant ones. In addition to tilt/splay there are also bending deformations, which change the picture but on a qualitative level the predictions are similar. I am going to write it down and include a reference to your work as one of the central ones.

"In the model's extension, there are two domains in opposite leaflets, and in this case, the model mimics a symmetric bilayer (I believe). The deformations mentioned above raise repulsive or attractive interactions that drive anti-registration or registration of domains in a symmetric-like bilayer. Can you add a line tension to this model, and how would that affect the results?" The interaction is predicted to be always attractive at large inter-domain distances. But at vanishing and small distances it can be either attractive or repulsive depending on the domain rigidity. The former case corresponds to a spontaneous registration. The latter case corresponds to some equilibrium non-vanishing distance between the domains. In order to register the domains have to overcome this distance by "jumping" over an energy barrier.

"Could you add domains into the same leaflets, mimicking an asymmetric bilayer in lipid composition and phase behavior? In other words, one leaflet would have one phase, and the other would have two phases. In this case, would domains attract or repel each other?" The domains in the same leaflet would repel each other. I did not explicitly calculate that since the answer is obvious based on previous works (including ours) on the bending-mediated interactions between cap-like membrane inclusions.

"If domains repel each other, could this suggest that the interactions drive small domains, as they will never coalesce into larger domains? This means that separately nucleated domains would have to overcome large energy barriers to coalesce. So coalescence would be kinetically hindered. By adding a line tension to this model, how do you expect to change the deformations and interactions between domains?" The long-range interaction considered by the model would be independent of the line tension. Yet, the "microscopic" origin of the line tension itself would need to be understood and modeled.

"Would you expect the predictions from these models to be different in a spherical geometry compared to a plane geometry?" Definitely, there will be additional features of the interaction such as a different distance dependence. But I hope that the qualitative essence will remain the same. My student is trying to compute the 3D model. It is technically more challenging.

1 T. A. Enoki and F. A. Heberle, Experimentally determined leaflet–leaflet phase diagram of an asymmetric lipid bilayer, *Proc. Natl. Acad. Sci. U. S. A.*, 2023, **120**, e2308723120, DOI: **10.1073/pnas.2308723120**.

Malavika Varma opened discussion of the paper by Daniel Huster: From an experimental technique perspective, how substantial is the difference in measurements between symmetric and asymmetric systems in Fig. 2 of the paper (**https://doi.org/10.1039/d4fd00192c**)?

Daniel Huster responded: The error bars for the determination of ^{2}H NMR order parameters are below symbol size in the plots. So the differences are significant.

Robert V. Law asked: In Fig. 1A & B, D & E of your paper (**https://doi.org/10.1039/d4fd00192c**), there is a comparison between the asymmetric and symmetrical bilayers. What is the origin of the isotropic line in the middle of the asymmetric (Fig. 1B and 1E) in the ^{2}H NMR spectra?

If these isotropic signals are from microvesicles or micelles, did you also confirm the presence of these microvesicles or micelles by static ^{31}P NMR spectroscopy by looking for any isotropic lines at 0 ppm?

Were there any issues *e.g.* Fourier artefacts in signal digitisation when you had a sharp isotropic signal combined with the broader ^{2}H NMR signals?

Daniel Huster replied: The isotropic line in the ^{2}H NMR spectra comes from highly curved lipid structures, possibly very small (*i.e.* budded) vesicles. Overall, this contribution amounts to 7% (for POPC in the outer layer) and 9% (for POPS in the outer layer) of the ^{2}H NMR spectra of deuterated lipids. We have also carried out ^{31}P NMR studies of the multilamellar vesicle (MLV), which detect *all* lipids (not just the outer leaflet). There was only a very small isotropic contribution in these spectra.

Robert V. Law questioned: If these signals are from microvesicles or micelles, can they possibly recombine with the membranes during ^{2}H NMR T_2 of the experiment? Would this affect the values of the order parameters and relaxation times? If these signals are from microvesicles or micelles, why do they occur only in the asymmetric system?

Daniel Huster answered: I guess budded vesicles cannot recombine with the membranes as this would increase the number asymmetry which is unfavorable. The order parameters and relaxation measurement come ONLY from the anisotropic part of the ^{2}H NMR spectrum, so the isotropic signal is not considered in either of these measurements. The symmetric system is prepared under no stress, lipid powder is hydrated in buffer and the membranes are in full equilibrium. Due to the exchange with cyclodextrin (CD), some (number) asymmetry could be introduced, which may lead to budding.

Edward Lyman asked: The differences in order parameter plots between the symmetric and asymmetric vesicles are intriguing. It would be extremely informative to be able to assign the S_{CD}'s to particular carbons. Is there some way to do this, short of carbon-by-carbon deuteration?

Daniel Huster replied: We are doing order parameter measurements with better site-specificity. We need ^{13}C labeled lipids to measure order parameters under magic-angle spinning conditions. We have done this for some symmetric systems and will soon provide these order parameters for asymmetric membranes. The key idea is to measure the motional averaging of the ^{1}H–^{13}C dipolar coupling, which probes the same C–D bond vector as measured in ^{2}H

NMR. Right now, we only have ^{13}C labeled PE but will get ^{13}C labeled PC, so we will do experiments on PC_{out}/PE_{in} large unilamellar vesicles (LUVs).

Edward Lyman enquired: Since the samples are multilamellar, only the outermost leaflet gets exchanged. Does that give enough of a signal (it apparently does), and is it feasible to get enough signal out of LUVs?

Daniel Huster answered: Yes, we can get sufficient signal for ^{2}H NMR and also for ^{13}C NMR. The latter will be done in LUVs. Due to the lateral diffusion of lipids in LUVs, the order parameters will also be lower as we have to consider averaging by diffusion over the curved surface. But comparing the lipids in the outer and in the inner leaflet will be straightforward in these preparations. We are working on it…

Manpreet Kaur said: A 2021 study,[1] with CHO cells, shows symmetric membranes have reduced tension relative to asymmetric membranes. Your work also shows that artificial asymmetric membranes are stiffer than symmetric ones. I would expect the artificial membrane with lipids you chose to have more mis-packing. Why is an artificial asymmetric membrane more stiff, is it because of multivesicle bodies affecting membrane properties?

1 A. Shiomi, *et al.*, Extreme deformability of insect cell membranes is governed by phospholipid scrambling, *Cell Rep.*, 2021, **35**, 109219, DOI: **10.1016/j.celrep.2021.109219**.

Daniel Huster responded: Well, a cell is of course a much more complicated system with many more lipids and other species in the membrane. In model membranes, it has been shown experimentally[1,2] and theoretically[3] that asymmetric membranes are stiffer. Also, the experimental systems so far did not contain cholesterol, which plays an important role in membrane stiffness.

1 Y. Elani, S. Purushothaman, P. J. Booth, J. M. Seddon, N. J. Brooks, R. V. Law and O. Ces, *Chem. Commun.*, 2015, **51**, 6976, DOI: **10.1039/c5cc00712g**.
2 K. Karamdad, R. V. Law, J. M. Seddon, N. J. Brooks and O. Ces, *Chem. Commun.*, 2016, **52**, 5277, DOI: **10.1039/c5cc10307j**.
3 A. Hossein and M. Deserno, *Biophys. J.*, 2020, **118**, 624, DOI: **10.1016/j.bpj.2019.11.3398**.

Heiko Heerklotz asked: Could a linewidth analysis of NMR (^{31}P?) be used as an alternative approach to AF4 to quantify the fraction of lipid in budding-induced daughter vesicles (<30 nm) as compared to mother vesicles (>80 nm?).

Daniel Huster replied: Yes, this could be possible, however, better than ^{31}P NMR where the anisotropies are almost completely averaged by lateral diffusion, ^{2}H NMR of DPPG-d_{62} should work better. Fig. 2 here is a NMR lineshape simulation considering averaging in 30 nm and 70 nm vesicles.

Erwin London enquired: Would it be worthwhile to repeat these experiments at a lower temperature at which the POPE enriched leaflet would be in the ordered gel state? It might be expected to result in a stronger effect on the lipids in the opposite leaflet than at higher temperatures.

Daniel Huster answered: Absolutely right, and we have already started these experiments. Will report more on this as soon as possible.

Markus Deserno asked: As has by now been pointed out numerous times at this meeting, the two leaflets of an asymmetric membrane do not have to be under the same tension. Looking at the order parameters in your NMR experiments, shown in Fig. 2 of your paper (https://doi.org/10.1039/d4fd00192c), I was wondering whether the curious *z*-dependent shifts might be caused by this differential stress? I know you are not a simulator, but have you discussed this with your simulation colleagues? If that were the reason for these shifts, it would basically amount to a (proxi-) measurement of differential stress *via* NMR, which would be amazingly useful.

Daniel Huster responded: All agreed. We collaborate with Robert Vacha on this issue. I am not sure how to measure leaflet-specific stress, though. I know Koenig and Gawrisch have measured the lateral compressibility by NMR/X-ray.[1]

1 B. W. Koenig, H. H. Strey and K. Gawrisch, Membrane lateral compressibility determined by NMR and X-ray diffraction: effect of acyl chain polyunsaturation, *Biophys. J.*, 1997, **73**, 1954–1966, DOI: 10.1016/S0006-3495(97)78226-2.

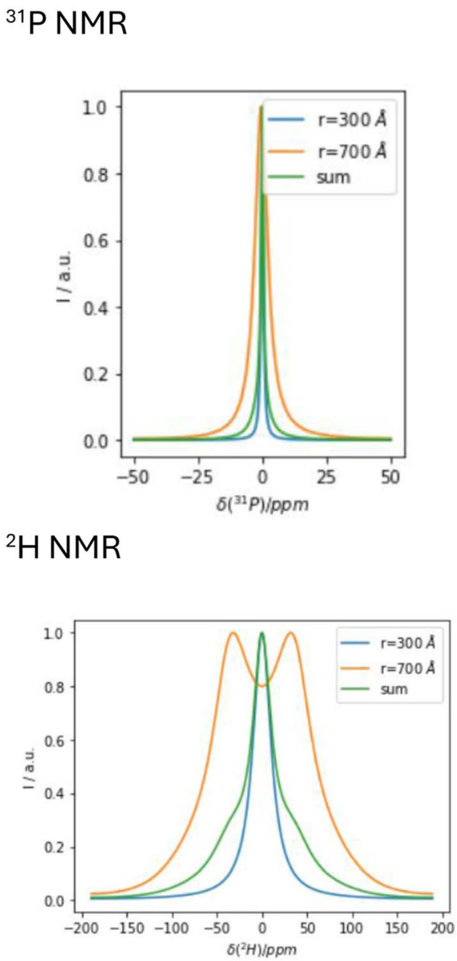

Fig. 2 NMR lineshape simulation considering averaging in 30 nm and 70 nm vesicles.

Robert Vacha remarked: We have calculated lipid order parameters from simulations with membranes at different tensions and we have not observed changes in order parameters similar to the ones obtained in NMR experiments so far.

Daniel Huster added: Yes, the interesting question to answer is: are the changes in order parameters we see in ^{2}H NMR related to cyclodextrin binding, a problem in the MD force field or periodic boundary conditions, or a new feature of asymmetry between the two leaflets? We will do experiments in POPC MLVs with the outer leaflet exchanged with POPC-d_{31}.

Milka Doktorova said: Obtaining structural data in the form of order parameters and relaxation rates for CH bond dynamics in asymmetric membranes with NMR provides a great opportunity to validate theoretical predictions and computational observations for asymmetric bilayers. In that respect, it is important to ensure that the experimentally measured parameters are not influenced by factors other than the asymmetric distribution of the bilayer lipids. Since the exchange in the NMR samples was performed with methyl-β-cyclodextrin (CD) and there is evidence that some of the CD is present in the sample after the exchange and washes, it is important to verify that the remaining CD is not affecting the results by *e.g.* binding to the bilayer and contributing to the apparent differential effect of asymmetry on the top and bottom parts of the chains. Have you compared the order parameter of POPC exchanged into POPC MLVs following the same protocol? This would serve as a direct control in this experimental setup and should reveal any potential artifactual effects from the protocol on the measured order parameters and relaxation rates.

Daniel Huster responded: This is indeed a very good suggestion. We have done this experiment and compared the ^{2}H NMR order parameters of symmetric POPC-d_{31} MLVs (in the absence of CD) with asymmetric MLVs POPC$_{\mathrm{in}}$/POPC-$d_{31}{}^{\mathrm{out}}$. Within experimental error, the order parameters are identical. This suggests that (i) CD does not influence POPC order parameters and (ii) the new features we see in the asymmetric membranes could indeed be caused by different tension in the individual leaflets of the asymmetric membranes.

Milka Doktorova asked: As mentioned in the text of the paper (**https://doi.org/10.1039/d4fd00192c**), one limitation of the current protocol is the limited accessibility only to the outer leaflet properties of the asymmetric outermost bilayer in the multilamellar vesicles. Can this type of NMR measurement be done on large or giant unilamellar vesicles instead where potentially the inner leaflet can also be labeled and examined? What are the minimal requirements for obtaining such structural data with regards to vesicle diameter and lipid concentration?

Daniel Huster answered: Yes, in principle, GUVs are possible. However, for good sensitivity, the lipid concentration should be really high, so GUVs should be not so diluted and concentrated. If they do not break while gently centrifuging them, I think this could work. Lipid concentration around 1 mg of ^{2}H-labeled lipid in the NMR volume (∼0.5 ml) should be fine. A diameter of 200 nm or bigger should also work, but extruded LUVs of a diameter larger than 100 nm already contain multilayers…

Edward Lyman commented: Since the papers of Hossein, Varma, and Deserno[1,2] have come out the simulation community has been grappling with the problem of initializing asymmetric sims from a "correct" (or at least reasonable) membrane stress state. It seems to me like these S_{CD} measurements may be an incredibly informative window into this problem. One could run a series of simulations that replicate (*e.g.*) the compositional asymmetry of your sample, but over a range of number imbalance conditions. Because the force fields do a quite good job at capturing S_{CD}'s at a quantitative level, one could use the NMR data to select the simulation model that best matches the data. If you can't talk Markus Miettinen (or someone else) into this let me know and we will do it for you!

1 A. Hossein and M. Deserno, *Biophys. J.*, 2020, **118**, 624, DOI: 10.1016/j.bpj.2019.11.3398.
2 M. Varma and M. Deserno, *Biophys. J.*, 2022, **121**, 4001, DOI: 10.1016/j.bpj.2022.07.032.

Daniel Huster responded: Yes, absolutely. Robert Vacha has done preliminary simulations on our system but could not recover the order parameters so far. We will keep working on this issue for sure. We will also do ^{1}H–^{13}C order parameters where the assignment is clear, which may also help.

Michael M. Kozlov enquired: To what extent does the order parameter depend on the tension? I am asking since the in-plane area of lipid molecules should be rather independent of the tension because of the high value of the stretching rigidity.

Daniel Huster replied: I think this has not been worked out theoretically. I would guess that order parameter profile and the lateral stress profile ($\sigma(z)$) and the lateral pressure profile ($p(z)$) are related but I am not aware that this has been studied.

Gonen Golani opened discussion of the paper by Milka Doktorova: Can cryo-EM *in vivo* be sensitive enough to detect density differences between the monolayers in a realistic biological setting? In *in vitro* systems, will we still observe a difference in the electron density if the membrane is rich with cholesterol that can flip-flop to 'fill the gaps' in the monolayer with a lower phospholipid density?

Milka Doktorova responded: While cryo-EM can reveal structural details of biological membranes (as done for example, in a recent study[1]), it is not presently clear whether that can be done at the resolution needed to analyze the intensity variation across the bilayer as done in liposomes, and measure parameters like ω that inform on the lipid abundance imbalance between the two leaflets. One of the main difficulties in this endeavor comes from the requirement for relatively thin samples, *i.e.* a few hundred nanometers in thickness, which allows for imaging the membranes without 'squishing' them and potentially distorting the projected bilayer profiles. Ongoing technical advances could make this easier in the future but for now, this remains a challenge.

Regarding the *in vitro* systems, since lipid headgroups are more electron dense than any part of cholesterol, we do not expect Chol to interfere with the phospholipid (PL) imbalance that can be detected in the intensity profiles of the cryo-EM bilayer images. In fact, our computational analysis directly shows how even when Chol interleaflet distribution changes dramatically, ω varies systematically with PL imbalance. However, while we do not expect trends with PL imbalance to be affected, it is possible that Chol can change the absolute value of ω in bilayers with different lipid compositions *via* its effects on bilayer properties (and consequently, changes in the shape of the intensity profiles).

1 D. Glushkova, S. Böhm and M. Beck, *bioRxiv*, 2025, preprint, DOI: 10.1101/2025.03.13.642912.

Fabio Lolicato remarked: It is worth noting a methodological point regarding the calculation of area per lipid. Specifically, in a symmetric bilayer, if the area is normalized by the total number of molecules (phospholipids + cholesterol), the average area per molecule decreases as cholesterol is added. This is because cholesterol is smaller than a typical phospholipid, and mixing molecules of different sizes reduces the average area. In this context, one might infer that the volume per molecule also decreases. However, if the area is instead normalized by the number of phospholipids only, the interpretation becomes more complex. M. Javanainen[1] has shown that when the area is divided by the number of phospholipids alone, the average area per phospholipid remains nearly constant up to 20 mol% cholesterol. This indicates that cholesterol contributes no excess area up to this concentration. Beyond 20 mol%, however, the area per phospholipid begins to increase.

1 M. Javanainen, P. Heftberger, J. J. Madsen, M. S. Miettinen, G. Pabst and O. H. S. Ollila, *J. Chem. Theory Comput.*, 2023 **19**, 6342, DOI: 10.1021/acs.jctc.3c00648.

Milka Doktorova responded: Analysis of leaflet packing can be done by either counting or disregarding Chol. One case underestimates, while the other overestimates, the actual area occupied by the phospholipids. Our analysis shows that, considering Chol, the trend in leaflet packing does not follow trends observed in symmetric bilayers. If we calculate the area per phospholipid, A_P, instead (without counting Chol), the area of DAPC for example increases with Chol concentration in the leaflets of both symmetric and asymmetric membranes as shown in Fig. 3 here. However, the smaller effect on A_P up to $\sim$20 mol% Chol observed in symmetric bilayers is not seen in the asymmetric ones. Instead, A_P increases steeply even at low Chol concentrations, demonstrating that trends in lipid packing in the leaflets of asymmetric membranes do not correspond to those of symmetric stress-free bilayers regardless of how the lipid packing is quantified.

Fabio Lolicato commented: Simulating highly asymmetric bilayers is still a developing area, and I don't yet know what the best practice is in this context. In particular, I'm concerned about the physical accuracy of such simulations. In a bilayer with lipid imbalance between the two leaflets, the (xy) dimensions of the simulation box are typically dictated by the leaflet with the higher area per lipid. This sets the overall box size and can lead to an artificially increased area per lipid in the opposing leaflet. Since lipid force fields are generally parameterized to

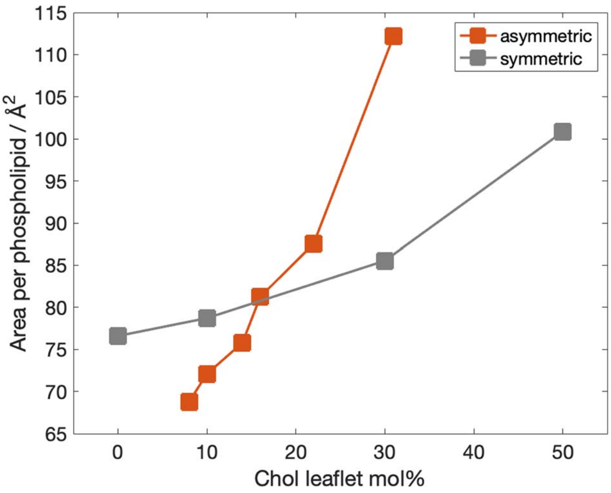

Fig. 3 Area per phospholipid, A_p, *versus* Chol concentration in a DAPC leaflet opposite of either DAPC leaflet (in symmetric bilayers, gray) or DPPC leaflet (in asymmetric bilayers, red).

reproduce a specific area per lipid, forcing lipids into a non-ideal packing environment may alter their physical properties and introduce artifacts.

Milka Doktorova responded: The bilayer area in typical *NPT* simulations of asymmetric bilayers is free to fluctuate and is governed not by the area per lipid of individual leaflets but by the differential stress in the membrane which arises from the mutual adaptation of the leaflets to the same shared total lateral area. Therefore, the simulation conditions represent a membrane state in which the two leaflets are constrained to have the same area. This is indeed the case for almost any existing model and biological membrane which are closed membrane surfaces.

Replacing one bilayer leaflet with a different one in simulations is analogous to replacing one protein binding partner with another one. If simulations can reproduce the behavior of the individual components, they present an ideal platform for studying the behavior of the assembly. It is true that lipid force fields have been calibrated against the bulk properties of symmetric bilayers for which there is extensive structural data from experiments (in contrast to asymmetric membranes). However, even though the parameterization relies on tension-free bilayers for example, simulations can reproduce the effects of net tension on bilayer area and dynamics, indicating that they are capable of capturing the properties of leaflets experiencing tension or compression such as those arising from differential stress.

It is important to note that in simulations of asymmetric bilayers there are important considerations that have to be taken into account. For instance, due to the presence of periodic boundary conditions a small bilayer patch is not able to relieve differential stress by *e.g.* curving or allowing lipids to flip between leaflets; there are no extra membrane pools and the number of lipids is fixed. This can occur for instance, in local regions of the plasma membrane, segregated from the bulk membrane *via* diffusion barriers. Analysis of the lateral pressure distribution in the simulated bilayer can reveal the amount of differential stress and propensity for curvature, and these can be related to the observed biophysical properties of the membrane. Until we are able to measure lipid number imbalance and fully characterize the asymmetry of membranes *in vitro*, we won't be able to directly validate the computational results with experiments. However, by specifically accounting for the constraints of the simulation conditions, we can use simulation results to improve our understanding of the effects of differential stress on bilayer structure and dynamics, and formulate testable hypotheses.

Fabio Lolicato queried: Do you think that a difference in area per lipid actually exists between the two leaflets in living cells? Or could it be that this imbalance is somehow compensated, either by the asymmetric shape of membrane proteins, differential protein crowding, or varying numbers of membrane-associated proteins on each side of the bilayer?

Milka Doktorova answered: Based on existing data, we believe there is a difference in the lipid packing (average area per lipid) in the two leaflets of living plasma membranes.[1] Some of the most direct evidence comes from experimentally measured differences in the readout of environment-sensitive probes confined to the exoplasmic or cytoplasmic leaflet (*e.g.* ref. 2–4). There are many advantages to a potential imbalance in the packing densities of the leaflets. Having a relatively compressed and impermeable exoplasmic leaflet, for instance can protect the cell while the ability to dynamically and locally change the relative packing of the two leaflets provides much greater functionality in terms of both the internal properties of the bilayer (*e.g.* lateral stress profile) and its ability to modulate differential interactions with peripheral and inserting proteins from the cytoplasm *versus* the extracellular medium. Therefore, considering the distinct lipid compositions of the two leaflets, equal areas per lipid would seem to be a very special limiting case with no clear advantages and thus unlikely to be actively maintained.

1 M. Doktorova, J. L. Symons, X. Zhang, H.-Y. Wang, J. Schlegel, J. H. Lorent, F. A. Heberle, E. Sezgin, E. Lyman, K. R. Levental and I. Levental, *Cell*, 2025, **188**, 2586, DOI: 10.1016/j.cell.2025.02.034.
2 C. Patra, M. Samad, T. van Zanten, M. Singh, R. A. Vishwakarma, P. P. Singh and S. Mayor, *bioRxiv*, 2024, preprint, DOI: 10.1101/2024.07.23.604763.
3 J. H. Lorent, K. R. Levental, L. Ganesan, G. Rivera-Longsworth, E. Sezgin, M. Doktorova, E. Lyman and I. Levental, *Nat. Chem. Biol.*, 2020, **16**, 644, DOI: 10.1038/s41589-020-0529-6.
4 A. Gupta, T. Korte, A. Herrmann and T. Wohland, *J. Lipid Res.*, 2020, **61**, 252, DOI: 10.1194/jlr.D119000364.

Giacomo Fiorin asked: Previously, when examining symmetric bilayers containing cholesterol you had collaborated with scientists who performed neutron spin echo (NSE), which is a powerful method to determine dynamic properties of a membrane, such as viscosity and interleaflet friction. For asymmetric bilayers like the plasma membrane, which are made of one ordered leaflet and a disordered leaflet, measuring such properties would be of great interest. Besides informing NMR experiments on asymmetric membranes, what are your thoughts about informing NSE experiments as well?

Milka Doktorova replied: Calculating representative NSE data from simulations is certainly feasible. This has been demonstrated for example, with coarse-grained simulations of flat bilayer patches and vesicles in ref. 1. Calculating the intermediate scattering functions from all-atom simulations should technically be doable as well, although the bilayer would have to be larger (*e.g.*, on the order of a few thousand lipids per leaflet). This is necessary to be able to obtain the decay of the dynamics at smaller wavevectors q (corresponding to larger real distances). For instance, a typical q range for analysis of bilayer dynamics with NSE is 0.037–0.12 $Å^{-1}$ (corresponding to length scales of ∼50–170 Å) and a bilayer with dimensions of 35 × 35 nm would have ∼2000 lipids per leaflet, assuming area per lipid of 60 $Å^2$. Since the dynamic range extends to ∼100 ns, the simulations should also be at least 1–2 μs long. The intermediate scattering functions would yield the decay rates which can then be related to various bilayer parameters such as viscosity and bending rigidity. Doing this analysis for asymmetric bilayers with all-atom simulations would allow for selective deuteration of various lipids/lipid components (as done in the generation of representative SANS data) and should reveal the sensitivity of the measurement to the dynamics of bilayers with various degrees and types of asymmetry. It may also be interesting just from the point of view of analyzing the dynamics (thickness fluctuations) of the simulated bilayers *via* the NSE-based framework of parsing the intermediate scattering functions.

1 J.-M. Y. Carrillo, J. Katsaras, B. G. Sumpter and R. Ashkar, *J. Chem. Theory Comput.*, 2017, **13**, 916, DOI: **10.1021/acs.jctc.6b00968**.

Alberto Giacomello enquired: It is interesting to see that the area per lipid goes up as the cholesterol content in the leaflet is increased in asymmetric membranes. I was wondering whether you expect this behaviour to have consequences on the phase behaviour of asymmetric membranes as opposed to that of symmetric membranes?

Milka Doktorova answered: What our results suggest is that the interleaflet distribution of Chol, driven by differential stress, can produce leaflet properties that do not follow trends observed in symmetric bilayers with these compositions. It is therefore difficult to make predictions for the phase behavior of the asymmetric leaflets based on our understanding of phase separation in symmetric bilayers. If we were to draw any parallels to symmetric systems, the closest analog would probably be a bilayer with the lipid composition of one of the asymmetric leaflets, which is subjected to different amounts of net bilayer tension. This analysis would ignore any possible effects of interleaflet coupling in the asymmetric environment, but would show the effects of tension on the lateral mixing of the lipids.

Alberto Giacomello asked: Have you tried simulating cholesterol contents above 30%?

Milka Doktorova responded: We have not tried simulating these particular membranes with higher cholesterol concentration. Generally, adding more Chol would be easy, *i.e.* the bilayers should be able to easily tolerate it, and we would not expect big differences in Chol interleaflet distribution. We did a similar study in a different set of coarse-grained simulations, presented in Fig. 2A in ref. 1.

There, the outer leaflet had again saturated lipids while the inner leaflet was enriched in PUFA lipids and the phospholipid (PL) imbalance was fixed with the inner leaflet having 35% more PLs than the outer leaflet. We varied the overall Chol concentration in the bilayers and saw similar Chol enrichment in the underpopulated saturated leaflet across all simulations (Fig. S2A in ref. 1); however the bilayer remained in a stable flat morphology only when Chol concentration was ≥30 mol% of all bilayer lipids.

1 M. Doktorova, J. L. Symons, X. Zhang, H.-Y. Wang, J. Schlegel, J. H. Lorent, F. A. Heberle, E. Sezgin, E. Lyman, K. R. Levental and I. Levental, *Cell*, 2025, **188**, 2586, DOI: 10.1016/j.cell.2025.02.034.

Georg Pabst said: (i) Achieving a phospholipid (PL) imbalance of up to 2 experimentally seems quite challenging. How do you envision realizing this in a controlled and reproducible manner?

(ii) I am confused by the approach used to calculate the scattering form factor in eqn (1) in the paper (https://doi.org/10.1039/d4fd00200h). Why not simply compute the scattering amplitude directly from the scattering length density of a flat bilayer – that is, skip the squaring step, followed by taking the square root? A more rigorous calculation would also yield the form factor (and hence the scattered intensity) on an absolute scale. Note: this also makes modelling more reliable (see ref. 1 and 2).

(iii) I do not believe that the center of mass (COM) for SAXS/SANS is a straightforward or reliable measure of PL imbalance, at least not as suggested. Several issues need to be considered:

(a) Membrane thickness fluctuations contribute to the scattering signal in the relevant *q*-range, as discussed in ref. 1 and 2.

(b) Experimental noise, including incoherent scattering in SANS at high H_2O content, needs to be properly accounted for and can introduce significant uncertainties.

(c) The choice of *q*-range for the analysis appears somewhat arbitrary and could easily bias or mislead the conclusions.

1 M. P. K. Frewein, M. Doktorova, F. A. Heberle, H. L. Scott, E. F. Semeraro, L. Porcar, and G. Pabst, *Symmetry*, 2021, **13**, 1441, DOI: 10.3390/sym13081441.
2 M. P. K. Frewein, P. Piller, E. F. Semeraro, K. C. Batchu, F. A. Heberle, H. L. Scott, Y. Gerelli, L. Porcar and G. Pabst, *J. Membr. Biol.*, 2022, **255**, 407, DOI: 10.1007/s00232-022-00234-0.

Milka Doktorova responded: The bilayer with imbalance of 2 is very stressed and while this is tolerated in the simulations, it is unlikely that such state can be realized in a synthetic model system *in vitro* without causing budding, rupture or other morphological responses, as we point out in the text (https://doi.org/10.1039/d4fd00200h). Still, in our opinion, it is informative to examine it as a limiting case in this simulation study to gain better understanding of the trends and extents to which differential stress can affect bilayer properties.

Regarding question (ii), we apologize if the explanation we provide in the text is confusing. In retrospect, it would have been more straightforward to give the equation for the form factor as a function of the scattering length density (SLD) profile and the intensity as a function of the form factor (rather than the other way around, as we did it), but the result is the same.

Regarding absolute scale, we first note that the analytical scattering models used in the mentioned references are not derived on an absolute scale, nor does either paper evaluate or discuss whether or how analyzing data on an absolute scale makes the modelling more reliable. In our opinion, the larger issue is that extrusion (the typical method for preparing LUVs for scattering) can change the sample concentration either by dilution from water/buffer in the dead volume of the extruder or by lipid adsorption to the filter, so even if scattering curves are put on an absolute scale, there is still sample-to-sample variability in concentration. In our experience, fitting always requires an arbitrary scale factor for each dataset to account for this (generally unknown) variability in lipid concentration. Therefore, we do not see advantages in analyzing the scattering data (both from experiments and simulations) on an absolute scale.

Regarding question (iii), we agree that many factors can complicate the interpretation of the scattering data. This is true whether one uses an analysis based on fitting to models (as in the mentioned references) or a model-free approach like we propose. These confounding factors include not only thickness fluctuations, but also lateral lipid clustering (including phase separation), especially in mixtures of deuterated and protiated lipids (see ref. 1). While neither of these factors is accounted for in the simulated form factors presented here, in principle it is possible to include them with a hybrid MD/Monte Carlo approach similar to that in ref. 1.

We fully agree that the proposed analysis would not be advisable for SANS data of vesicles in a high-H_2O medium due to the large increase in incoherent scattering.

As for the *q*-range, it is not arbitrary but was instead chosen to encompass the region of the scattering curves that is most reliable (*i.e.*, least affected by background scattering); of course, other ranges could be explored using the provided methodology. We note that this analysis is much cruder than modeling-based approaches which can provide detailed structural parameters of the bilayers. Yet, this simple representation is able to quantify differences between asymmetric bilayers directly from the scattering form factors, allowing for potential calibration of the underlying PL number imbalances with simulations.

1 F. A. Heberle, V. N. P. Anghel and J. Katsaras, *J. Appl. Crystallogr.*, 2015, **48**, 1391, DOI: 10.1107/S160057671501362X.

Daniel Huster queried: You observed an increase in area of lipid as a function of cholesterol; normally, highly unsaturated lipid does not mix well with cholesterol. So I could see that the area stays the same as adding more cholesterol due to de-mixing. But how can it increase? In the upper leaflet with saturated chains only, one would expect a decrease in area due to the condensation effect of cholesterol.

Milka Doktorova replied: Cholesterol has a clear condensing effect in symmetric bilayers: it not only has smaller area than a phospholipid which naturally decreases bilayer area when it replaces lipids, but in the case of saturated lipids it also effectively orders their chains further compressing the area. However, in asymmetric membranes, the Chol concentration in a leaflet is driven by differential stress (among other factors), and is not the same as simply adding or removing Chol in the leaflet, as in symmetric bilayers. Instead, in asymmetric membranes the area of the leaflets (which essentially determines the area per lipid) is determined by the area of the bilayer which is a direct consequence of the stored differential stress; it is not determined by Chol concentration alone. In other words, the Chol concentration in the leaflets is an after-effect of PL imbalance and differential stress, and that is why the resulting area per lipid that we observe does not have to (and does not, in our simulations) follow the condensing effect that Chol has in stress-free symmetric bilayers.

Gerald W. Feigenson enquired: I enjoyed the new MD simulations. Have you tried, or could you try, instead of DAPC, cytoplasmic-type lipids with 16 or 18 *sn*1 chain? Such lipids are very different in interactions with cholesterol, *e.g.* as measured by maximum cholesterol solubility. Such cytoplasmic lipids could very well change your MD results.

Milka Doktorova answered: We can certainly try using different lipids in the simulations. For example, in ref. 1 we ran a series of coarse-grained bilayer simulations in which the two leaflets had lipid compositions representative of those of red blood cell plasma membranes, *i.e.* with sphingomyelin and PC in the top leaflet and mixed chain lipids in the bottom leaflet. There, we kept the phospholipid imbalance between the leaflets fixed (with the cytoplasmic leaflet having 35% more lipids than the exoplasmic leaflet) and varied the total cholesterol concentration in the membrane. In the bilayer with 30 mol% Chol, we saw Chol enrichment in the top leaflet but it was noticeably smaller compared to bilayer A.1 (the closest analog in the present study). This illustrates that, as suggested, the presence of mixed chain lipids can change the interleaflet distribution of Chol. However, the relative apparent effects of Chol on lipid packing (*e.g.* the trends in Fig. 1B in the paper (**https://doi.org/10.1039/d4fd00200h**)) are driven mainly by differential stress and we do not expect that to change with variations in lipid composition, but we would have to run the simulations to confirm.

1 M. Doktorova, J. L. Symons, X. Zhang, H.-Y. Wang, J. Schlegel, J. H. Lorent, F. A. Heberle, E. Sezgin, E. Lyman, K. R. Levental and I. Levental, *Cell*, 2025, **188**, 2586, DOI: **10.1016/j.cell.2025.02.034**.

Robert V. Law asked: Do you always assume cholesterol is colinear with lipids (with its hydroxyl group pointing towards the water layer)? Some work with PUFA lipids indicates that cholesterol can be perpendicular to bilayer, between the two bilayer leaflets.[1]

1 N. Kučerka, *et al.*, *J. Am. Chem. Soc.*, 2009, **131**(45), 16358–16359, DOI: **10.1021/ja907659u**.

Milka Doktorova responded: Generally, Chol is rarely seen to partition to the bilayer midplane in all-atom simulations. To verify that, we calculated the distribution of Chol tilt angles in our trajectories (defined by the angle between the vector connecting atoms C3 and C17 in CHARMM36 notation, and the bilayer normal, *i.e.* the positive direction of the z axis of the simulation box). The majority of Chol was either in a tight upright orientation in the DPPC leaflet (tilt angles around 10°) or adopting a wider range of tilt angles in the opposite DAPC leaflet (tilt angles around 150°). This is illustrated in Fig. 4 here for the two bilayers with most extreme PL imbalances, A.1 and A.6. There is no density at 90° confirming the absence of molecules lying flat at the midplane. In coarse-grained simulations of these bilayers, we do see some density of tilt angles around 90°, however analysis of the time evolution of the tilts of individual Chol molecules shows that these are states that Chol adopts only transiently as it flips between the leaflets. Therefore, we do not have reasons to believe that Chol at the midplane is affecting our results.

Markus Deserno said: In Fig. 1B of your paper (https://doi.org/10.1039/d4fd00200h) you argue that the effect of cholesterol concentration in a single leaflet can be quite different in symmetric *versus* asymmetric bilayers – namely, the area per lipid of both the saturated DPPC and the unsaturated DAPC leaflet increases with increasing leaflet cholesterol concentration, as opposed to the observations in a symmetric system, where the trend quite naturally goes the other way. I am not sure I entirely follow the explanation given in the text, but I would like to suggest what I think might be a simpler rationalization of this observation, and I am curious whether you would agree with it. For ease of reference, I have included as Fig. 5 here a version of your Fig. 1B with a few extra annotations in it, and with the symmetric trends removed for clarity.

My argument relies on the fact that the driving force in your sequence of simulations is not cholesterol but differential stress (as created by abundance asymmetry; cholesterol then just goes along for the ride). The bilayer in which both cholesterol leaflet fractions are close to 30 mol% features a very large abundance asymmetry: the DPPC leaflet has about twice as many phospholipids as the DAPC leaflet, as we can see in your Fig. 1A of the same panel. Much cholesterol is hence squeezed out of its more "natural" DPPC environment, leading to its unusual 50 : 50 partitioning between the DPPC and the DAPC side.

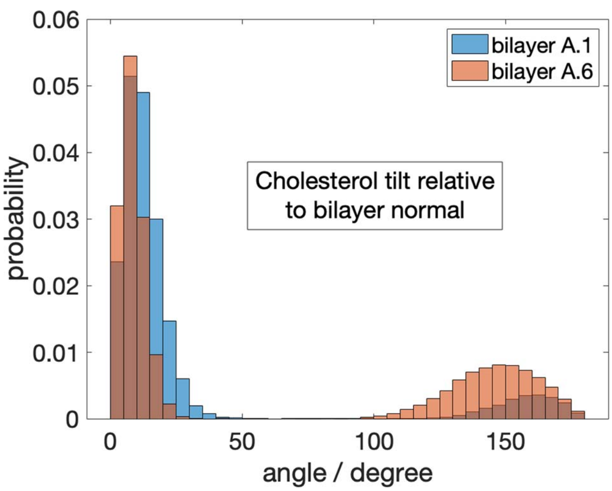

Fig. 4 Cholesterol tilt relative to bilayer normal (positive direction of the *z* axis of the simulation box) for the two simulator bilayers with most extreme PL imbalances, A.1 and A.6. Shown are the probability distributions of Chol tilt angles in the two bilayers.

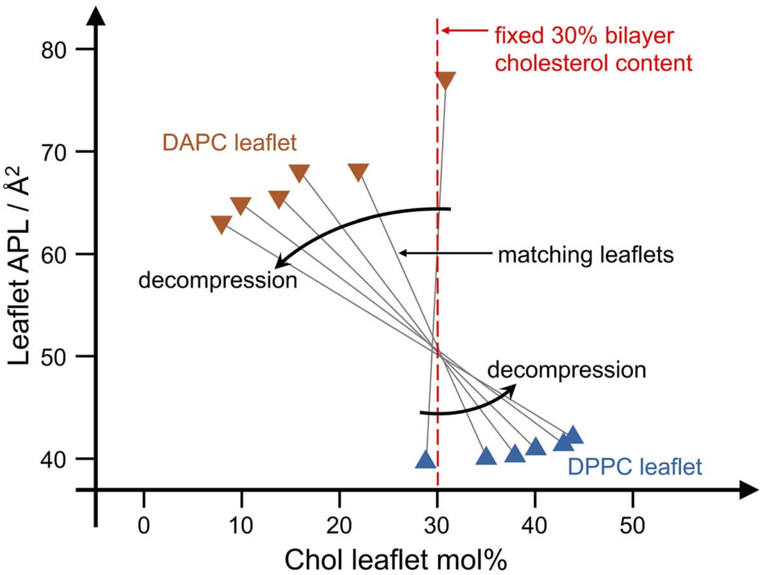

Fig. 5 Annotated version of Fig. 1B from https://doi.org/10.1039/d4fd00200h.

For the ease of discussion, an equivalent but easier to interpret realization of your abundance scan is to say that we run simulations at a fixed number of cholesterol lipids, which then also means a fixed number of phospholipids (say, 200 lipids overall, of which 60 (*i.e.*, 30%) are cholesterol and 140 are phospholipids, whose relative DPPC/DAPC abundance we now tune). The "decompression" that happens upon lowering the abundance asymmetry (indicated by the two black curved arrows in my Fig. 5 here) then effectively amounts to removing DPPC lipids from the DPPC leaflet, turning them into DAPC lipids, and inserting those into the DAPC leaflet. The number of DAPC lipids in the DAPC leaflet therefore *increases* upon decompression, while the area of the bilayer also shrinks (because the huge compressive stress in the DPPC leaflet is relieved). Both effects imply a reduction in the area per lipid in the DAPC leaflet, and the only confounding effect that could counter that is that you also lose cholesterol lipids from there into the DPPC leaflet. However, this is a smaller correction that cannot undo the bigger effect of differential stress relaxation. This means that plotting the effect against the emergent cholesterol leaflet fractions might lead one to the possibly misleading view that cholesterol "acts funny" when added to a DAPC leaflet in an asymmetric membrane. Of course, there is nothing wrong with your observed correlation, but it strikes me as a somewhat more indirect consequence of a bigger driver that is hiding in the background. Would you agree with this attempt at an alternative rationalization?

Milka Doktorova replied: We completely agree that differential stress is the main driving force here. Within the representation of fixed numbers of lipids and cholesterol, it is not entirely clear why decompression of the DPPC leaflet by turning DPPC into DAPC lipids and moving them to the DAPC leaflet would *shrink* the area of the bilayer since that would be associated with a much larger compressive force on the DAPC leaflet. We think this is more likely to slightly *increase* the bilayer area. However, the result is the same in that cholesterol movement to the DPPC leaflet is not able to counter the effect of differential stress relaxation and instead the area per lipid would increase in the DPPC leaflet and decrease in the DAPC leaflet in a way opposite of what we would expect from the corresponding changes in the leaflets' cholesterol concentrations. Plotting the area per lipid as a function of Chol leaflet mol% may be misleading if perceived as 'adding' Chol to a leaflet and seeing its response (as opposed to varying the relative numbers of lipids and analyzing the Chol distribution resulting from that). However, we believe the trends are still informative as they demonstrate that if we were able to measure the Chol concentration in one of the leaflets of an asymmetric membrane, for example, and see that it changes under different conditions or between membranes, we cannot make inferences about the relative packing of the leaflet based solely on Chol concentration. In the presence of differential stress, Chol does not behave as Chol in stress-free symmetric bilayers, and this is one of the main points that this analysis illustrates.

Francisco N. Barrera asked: In different simulations you can observe that the lower membrane leaflet gets thinner as the DAPC lipid chains bend more appreciably. Do you expect that this thinning affects the partition of cholesterol to this leaflet due to the changing local hydrophobic mismatch? This question is assuming that cholesterol will remain in a "vertical" state in all cases.

Milka Doktorova answered: The DAPC leaflet does indeed become thinner as the tension in the leaflet increases in the simulation series, and this is accompanied by an increase in the cholesterol concentration in that leaflet. This preferential partitioning of cholesterol to the DAPC leaflet is likely influenced by a number of different factors which affect its chemical potential. The changes in thickness are driven by the differential stress in the bilayer (compressing the top leaflet and expanding the bottom one). While cholesterol concentration is correlated with thickness in the DAPC leaflet, this is not the case in the DPPC leaflet where for bilayers A.4, A.5 and A.6 the leaflet thickness remains constant at 24.1 Å but Chol decreases from 38 to 29 mol%. Therefore, it would be hard to attribute the observed changes in Chol distribution to thickness in general, however it is possible that when it comes to the unsaturated DAPC leaflet, its thinning (driven by differential stress) contributes to making the environment more favorable for Chol relative to the stress-free state of the leaflet.

Michael M. Kozlov opened discussion of the paper by Oded Farago: By calculating the energies of the membrane leaflets, you are using the expression for the energy proportional to the square of the difference between the mean and spontaneous curvatures. This means that the reference state for the energy computations is always the state in which the leaflet's curvature is equal to the spontaneous curvature. This can be done if the spontaneous curvature of each leaflet is a constant. Yet, if there is a lipid exchange between the leaflets, the spontaneous curvatures of the leaflets are changing. In that case a direct comparison of energies computed by using the quadratic expression before and after the exchange appears problematic since these energies are related to different reference states. What would be your comment on that?

Oded Farago replied: Indeed, we assume that there is no exchange of lipids between the leaflets, which is applicable on times much smaller than the flip-flop times of lipids. The theory definitely needs to be extended when cholesterol, which is rapidly flip-flopping, is present. With that said, the key finding is that local phase separation may be accompanied by curvature instability because of the *lateral* inhomogeneities in the spontaneous curvature and bending rigidity. It is true that the variations in these parameters due to lipid monolayer exchange must be considered, but these will not necessarily change the outcome (*i.e.*, curvature instability) as long as the membrane is laterally inhomogeneous. In other words, the theory assumes quadratic curvatures energetics, and the instability arises from changes in the free energy parameters that are coupled to local phase transitions (and may involve also lipid exchanges).

Markus Deserno said: We had also considered the nature of the fluid–gel transition in a number-asymmetric membrane[1] and found a phase diagram that contains both continuous and discontinuous transitions. I cannot quite tell from the specific plots you display in your paper (**https://doi.org/10.1039/d4fd00182f**) whether the scenario you discover is similar or not, so I wanted to inquire. For ease of reference, I append a cleaned-up phase diagram we later published in ref. 2 (Fig. 6 here). As a function of relative lipid number asymmetry δn and (essentially) temperature T, we find that both the upper and the lower leaflet of the membrane can be in three different states: completely fluid ("f"), completely gel ("g"), or in a state where both fluid and gel phases coexist ("c"). We only observe a first order transition when, for not too large asymmetry, we heat or cool the system across the temperature where the "usual" (symmetric) gel transition used to be. In that case we see a "coexistence swap": a bilayer where, for instance, the upper leaflet is in a coexistence region and the lower leaflet is fluid would upon cooling go into a bilayer in which the upper leaflet fully turns into a gel and the lower leaflet enters fluid/gel coexistence. All other transitions – which

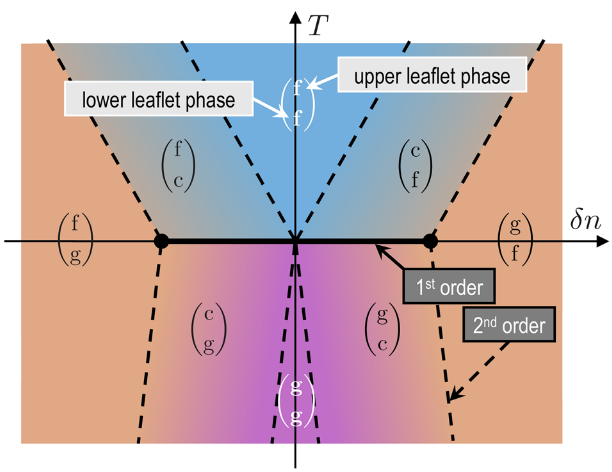

Fig. 6 Phase diagram as a function of temperature T and relative lipid number asymmetry δn.

basically amount to the emergence or dissolution of leaflets with coexistence – are continuous.

We have a theoretical model that is essentially equivalent to two coupled van der Waals gases, but this model assumes that the membrane always stays flat – there are no curvature contributions in our theory or any of the accompanying simulations. I understand that you have a slightly different situation where the bilayer has two oppositely curved regions, and this seems to matter in your case. Could you clarify how this pans out?

1 S. L. Foley, A. Hossein and M. Deserno, *Biophys. J.*, 2022, **121**(16), 2997–3009.
2 S. L. Foley, M. Varma, A. Hossein and M. Deserno, *Emerging Top. Life Sci.*, 2023, **7**(1), 95–110.

Oded Farago responded: Fig. 10 in the paper (**https://doi.org/10.1039/d4fd00182f**) suggests that both monolayers transform simultaneously into a state of coexistence: the upper one – coexistence between gel and dense fluid; and the lower one – between two dilute fluid phases with slightly distinct densities. Thus, the short answer to your question is that this kind of a phase transition deviates from the ones proposed in your work because your phase diagram does not show a state where both leaflets are at coexistence. A close look at our Fig. 10 reveals another interesting thing: the monolayer densities in one of the segments seem to follow a linear dependence on δn with respect to the monolayer densities prior to the transition, while the densities in the monolayer of the other segment exhibit discontinuities. These observations suggests that coupling between the lateral (density) and normal (curvature) degrees of freedom must be considered in order to understand the phase behavior of asymmetric membranes. Eqn (16)–(21) in the paper highlight some of the considerations that one must consider when attempting to minimize the bilayer free energy with coupling between area and curvature.

Rumiana Dimova asked: You find that near the gel transition, asymmetry can induce phase separation and curvature instabilities. What are the potential biological implications of this finding, particularly in the context of lipid rafts? Are there particular systems close to gel transitions to which the findings will apply directly?

Oded Farago answered: The observed curvature instability is not limited to the first order gel transition occurring in single-component lipid bilayers. It can happen also in multi-component mixtures where other phase transitions take place, *e.g.*, the second order phase separation in ternary mixtures. In general, the transition is driven by the inter-molecular interactions. When it happens, different regions in the membrane have different mechanical properties and the mechanical coupling between the different segments lead to curvature instability (*i.e.*, different segments exhibiting distinct mean curvatures subject to the global constraint on the average mean curvature). In the context of lipid rafts, it is known that many biological processes take place in these domains, involve changes in local molecular distributions as well as curvature extrusions. The work demonstrates that the local changes in composition, lead not only to domain formation and local phase separation, but also to curvature instability arising from the membrane asymmetry and the variations in the mechanical parameters characterizing the different phases. It may be relevant to several biological processes like endo- and exo-cytosis.

Giacomo Fiorin enquired: One of the main advantages of Monte Carlo simulations, as you have highlighted in your paper (**https://doi.org/10.1039/d4fd00182f**), is the ability to fine-tune the moves of individual particles to implement complex physical forces on them.

Do you think that your approach could be extended to engineer different curvature preferences in the lipid molecules of each leaflet, possibly without changing their respective areas per lipid molecule (APLs)?

This would be greatly advantageous compared to deterministic MD simulations, both coarse-grained or atomistic, which currently do not provide a method to describe an asymmetric membrane where the preferred curvature is the only parameter that changes between the leaflets.

Oded Farago replied: A model of cone-shaped lipids with preferred curvature can be easily generated by changing the sizes of the beads forming the coarse-grained lipids. This has, in fact, already been done in previous works. What Monte Carlo allows you to do is to: (i) preserve the lipid asymmetry in simulations runs longer than the flip-flop times of the lipids (by rejecting moves that cause flip-flops), and (ii) perform simulations in a restricted configuration phase space in order to measure the free energies of specific membrane structures. With Molecular Dynamics you must follow the dynamics of the system over the whole phase space, subject to periodic boundary conditions.

Sarabjeet Kaur opened discussion of the paper by Sylvie Roke: How does second harmonic imaging accurately capture signals at the water surface, given that water molecules are inherently unstable, especially considering that water is transparent and its molecules are free-floating, making signal detection seemingly challenging?

Sylvie Roke responded: The second harmonic (SH) photons are created by tiny charge oscillations within molecules that occur at the second harmonic frequency. These oscillations are very weak but present in all water molecules, and if one detects very carefully, indeed SH scattering can be recorded from every substance, including water. This process was discovered in 1965,[1] and was termed hyper Rayleigh scattering. The emission can be enhanced when molecules are correlated in their orientational distribution, which creates a so-called coherent enhancement. On a surface, there is such a correlation, since the plane of the surface together with the separation between molecular species leads to a non-zero orientational distribution of molecules. This coherent emission is what is detected in SH imaging of water, and it is $\sim 10^7$ times weaker than resonant two-photon emission, under comparable imaging conditions.[2]

1 R. W. Terhune, P. D. Maker and C. M. Savage, Measurements of nonlinear light scattering, *Phys. Rev. Lett.*, 1965, **14**, 681–684, DOI: **10.1103/PhysRevLett.14.681**.
2 M. Eremchev, D. Roesel, P.-M. Dansette, A. Michailovas and S. Roke, High throughput wide field SH imaging of giant unilamellar vesicles, *Biointerphases*, 2023, **18**, 031202, DOI: **10.1116/6.0002640**.

Sarabjeet Kaur asked: Referring to the plot Fig. 6c in your paper (**https://doi.org/10.1039/d4fd00197d**) that shows second harmonic (SH) intensity *versus* time, is the observed SH signal decay a reliable and direct proxy for proton translocation across the membrane, or could it be influenced by other interfacial dynamics that affect the measured timescale?

Sylvie Roke replied: It is unlikely that other interactions/processes are changing the SH response, besides proton translocation. SH imaging probes changes in the orientational distribution of water in the narrow volume adjacent to the interface that is ~1 nm thick. Protons will change this water structure and neutralize/interact with PS head groups. These interactions initially reduce the amount of charges on one leaflet, which change the orientational distribution of water molecules on that leaflet, creating a bigger contrast (at t_2). Once the protons reach the other leaflet the orientational distribution of water will be impacted in the same way as the opposing leaflet, which creates a more symmetric orientational distribution of water molecules on the bilayer as a whole.

Felix M. Goni questioned: You mentioned that the ionic concentration near the membrane surface is 1 M. In my view, units of concentration are only meaningful when applied to bulk volumes. Can you really apply concentration units to a two-dimensional nanovolume?

Sylvie Roke responded: Indeed, the unit of concentration is in content per volume. The hydrated region of the bilayer has a thickness of several water layers and so we can consider this region as a volume.

Rumiana Dimova asked: Have you considered whether some of the second harmonics hotspots observed in GUVs or cells might originate from highly curved membrane regions – such as nanotubes or invaginations or even submicroscopic membrane undulations – rather than from charge or hydration asymmetry? Note that membrane nanotubes are not detected with transmission-light microscopy such as in phase contrast observations. It would be great if you are able to compare a tense *vs.* flaccid GUV held by a micropipette (tubulation and fluctuations are suppressed in tense GUVs) rather than under osmotic shocks, which can lead to membrane poration and subsequent tension relaxation.

Sylvie Roke answered: Yes, we have observed tubules growing out of the free-standing lipid membranes (FLMs) and they do look rather differently than the surface potential fluctuations. We have performed both osmotic shock experiments as well as aspiration experiments on GUVs, but the results are not published yet.

Rumiana Dimova said: In one of your slides, you showed a movie of the signal from the giant vesicle membrane, but there also appeared to be non-negligible signal inside and outside the vesicle, even though the surrounding medium shouldn't contribute. This may be due to signal enhancement. Could you comment on the potential risks of over-enhancement leading to artifacts or over-interpretation of the data?

Sylvie Roke replied: There are a few aspects to consider here, namely (i) the origin of the SH emission and (ii) how the images/videos were created. (i) SH photons are created by tiny charge oscillations within molecules that occur at the second harmonic frequency. These oscillations are very weak but present in all water molecules. SH scattering can be recorded from every molecularly anisotropic substance, including neat water. The emission is enhanced when (water) molecules are correlated in their orientational distribution, which creates a so-called 'coherent enhancement'. On a surface, there is such a correlation, since the interface creates a non-zero orientational distribution of molecules. These two effects, 'incoherent' and 'coherent' emission, both take place (see *e.g.* ref. 1 for an explanation) with the latter being bigger than the former. When GUVs are SH imaged, both are detected, albeit with different intensities. It is therefore possible that the high intensity tail of the bulk water SH emission distribution gets to be on the same order of magnitude of the low intensity tail of the interfacial SH emission. (ii) The imaging of GUVs is done with linearly polarized light, and only the component that is normal to the surface contributes to the emission. This is why the raw data shows a 'ring' of vanishing intensity. Dividing the intensity image by the normal component of the electrostatic field squared provides the true surface contribution. This is shown in detail in Fig. S4 of ref. 2.

1 M. Flór, D. M. Wilkins, M. de la Puente, D. Laage, G. Cassone, A. Hassanali and S. Roke, Dissecting the hydrogen bond network of water: charge transfer and nuclear quantum effects, *Science*, 2024, **386**(110), eads4369, DOI: **10.1126/science.ads4369**.
2 D. Roesel, *et al.*, Ion-induced transient potential fluctuations facilitate pore formation and cation transport through lipid membranes, *J. Am. Chem. Soc.*, 2022, **144**(51), 23352–23357.

Nasim Mirzajani asked: In relating the SH intensity to the transmembrane potential difference, $\chi^{(2)}$ contributions in eqn (3) of your paper (**https://doi.org/10.1039/d4fd00197d**) have been neglected on the basis that the value of the difference in $\chi^{(2)}$ across the membrane is smaller than the value of $\chi^{(3)}$. However, to make a direct comparison, $\chi^{(3)}$ would have to be multiplied with the potential difference, as it appears in the equation. The potential difference values (<200 mV) would seem to bring the values of $\chi^{(2)}$ and $\chi^{(3)}$ contributions closer to each other. How would taking $\chi^{(2)}$ contributions into account change the subsequent interpretation of the data? Are there further studies that can be done to evaluate the role of surface chemical interactions in this system, without the field-driven interactions?

Sylvie Roke responded: The unit of the surface $\chi^{(2)}$ tensor is $m^2\ V^{-1}$ while that of the $\chi^{(3)}$ tensor is $m^2\ V^{-2}$. For an asymmetric lipid membrane, the first one has been determined to be on the order of $\sim 10^{-24}\ m^2\ V^{-1}$, while the second one is $\sim 10^{-21}\ m^2\ V^{-2}$.[1] Multiplying this by 0.2 V (we need to consider the unit V, not mV), gives $0.2 \times 10^{-21}\ m^2\ V^{-1}$. For a symmetric lipid bilayer the $\chi^{(2)}$ contribution is even smaller than the value for an asymmetric bilayer. So, for water imaging in all cases the third-order contribution is bigger than the second-order one, which results in a parabolic curve when the image-integrated SH intensity is measured as a function of applied bias (see Fig. 2B and 3D in the paper (**https://doi.org/10.1039/d4fd00197d**)). For a resonant process *e.g.* when applying SH resonant fluorophores, the resonant enhancement results in a situation where the $\chi^{(2)}$ contribution is larger than the $\chi^{(3)}$ contribution. This should result in different (linear) dependence when the integrated intensity is plotted as a function of applied bias.[2]

1 O. Tarun, C. Hannesschläger, P. Pohl and S. Roke, Label-free and charge-sensitive dynamic imaging of lipid membrane hydration on millisecond time scales, *Proc. Natl. Acad. Sci. U. S. A.*, 2018, **115**, 4081–4086.
2 B. A. Jemet, V. Nikolenko and R. Yuste, Second harmonic imaging of membrane potential of neurons with retinal, *J. Biomed. Opt.*, 2004, **9**(5), 873–881.

Edward Lyman enquired: I am really interested in new measurements of lipid flip-flop rates catalyzed by scramblases, especially with better time resolution. So, is it possible to reconstitute a scramblase (like TMEM16F) into an asymmetric black lipid membrane prep? Then activate it with Ca^{2+}, and observe how lipid asymmetry is lost?

Sylvie Roke answered: If the scramblase can be embedded in the free-standing lipid bilayer then this experiment should be possible. From the optical side there are no reasons why this could not be done.

Di Jin asked: When you have symmetric ion concentrations across a membrane containing PA lipids, you have shown you observed localised transmembrane potential. In the absence of PA lipids, would you still expect to observe the signal?

Sylvie Roke responded: The intensity is there because the PA head groups are charged, and these charges create an orientational distribution of water molecules, which has a non-zero component along the surface normal, by means of charge–water interactions. If the PA groups were not present, and the membrane had only PC groups, it would be on average charge neutral on each leaflet. There might still be some other interactions which provide an interfacial non-isotropic distribution, but since these interactions are much weaker, they are currently not easily detectable with SH imaging. As an example, see Fig. 1B and 2D in ref. 1.

1 O. Tarun, C. Hannesschläger, P. Pohl and S. Roke, Label-free and charge-sensitive dynamic imaging of lipid membrane hydration on millisecond time scales, *Proc. Natl. Acad. Sci. U. S. A.*, 2018, **115**, 4081–4086.

Di Jin enquired: Can you explain how the localised transmembrane potential is related to fusion?

Sylvie Roke replied: Membrane potential fluctuations are accompanied by transient electrostatic field fluctuations that might aid in the creation of membrane nanopores, or defects that can aid the fusion process. There is no investigation at this time available but I can imagine this as a potential scenario.

Michael Kaltenegger questioned: I was wondering how comparable FLMs and GUVs are as systems for inferring proton translocation rates from SH-intensity measurements. Specifically, I'm considering the impact of the larger volume of solvent on the other side of a FLM, compared to the smaller volume inside a GUV, on the time required for the pH difference to diminish. Wouldn't it inherently take more time for the pH contrast to disappear in the FLM system compared to GUVs due to the differences in solvent volumes?

Sylvie Roke replied: The contrast that appears in the SH images is determined by the water molecules adjacent to the membrane, in a layer that is typically 2–3 water molecules thick. When adding H^+ to the outside (GUV)/upper leaflet (FLM), the SH intensity increases on the same time scale (Fig. 6C in the paper (**https://doi.org/10.1039/d4fd00197d**)) in both systems. This means that protons have started interacting with the interfacial water/lipid head groups of the leaflet nearest to where they were injected. The SH intensity decreases again because the opposing leaflet begins to undergo a similar structural transformation, which restores the proton induced asymmetry that was maximum at t_2. Protons leaving/not in the ~1 nm thick interfacial region do not impact the SH intensity. This means that the diffusion into the bulk is unimportant for the experiment.

Iwona Swiderska opened discussion of the paper by Alexander P. Fellows: (i) You were comparing PC to PS lipids in terms of their domain formation. Do you expect this domain behavior difference to be purely electrostatic, *i.e.* related to the charge of the headgroup, and can it be therefore generalized to other lipid types with the same charge?

(ii) Do you expect the domain behavior to be different between a monolayer and a bilayer system and if so, how?

Alexander P. Fellows replied: I certainly think that electrostatics plays an important role in the domain formation with charged lipids. Clearly bringing together charged head-groups in such close proximity creates substantial electrostatic interactions that will impact the packing structure. However, the fact that these charged lipids overcome these repulsive interactions to form condensed domains shows that other factors are at play, particularly the lateral van der Waals interactions between the tail-groups. In this sense, I would generally expect other lipids with the same head-group charge and tail functionality to behave in a similar fashion (*i.e.* not depending that strongly on the specific chemical structure of the head-group), but just having the same charge on the head-group with tails of different chain length or degree of saturation would likely result in a different phase structure in the membrane. These are, however, definitely important questions that need to be addressed going forward.

In regards to the differences between mono- and bi-layer systems, it is definitely the case that the trans-leaflet interactions will play a role in the domain behaviour. We have even heard quite a few talks emphasising the importance of these interactions, with them acting to slightly perturb the domain structures and, importantly, lead to correlated packing structures in the two leaflets. Nevertheless, despite these differences, monolayer studies give significant insight into the fundamental lateral interactions unpinning domain formation, which can then be extended to bilayer studies to independently assess the impact of the inter-leaflet interactions. While the studies we present here are not currently feasible in bilayer systems, this is an area we are actively pursuing.

Edward Lyman addressed Alexander P. Fellows, Sylvie Roke and John C. Conboy: Is it possible to detect any of these spectroscopic observables in the near field in the liquid state? Maybe with a tip enhanced detection, or with some kind of patterned substrate? The goal is to obtain better spatial resolution. I know that Markus Raschke at University of Colorado Boulder was saying that he could do this in liquid state at some point, but I didn't follow this so closely.

Alexander P. Fellows responded: I completely agree that gaining better spatial resolution is an important factor for the future characterisation of these sorts of systems. It is definitely the case the tip-enhanced measurements have started to become a research focus,[1] and we have even applied such a method to model membrane systems in the past.[2] The complication with these methods, however, is primarily associated with the impossibly small signals being generated from the nanoscopic region, not to mention the uncertainty in polarisation generated from the tip. I believe that other super-resolution methods, such as those implemented in other nonlinear optical techniques,[3,4] might be more promising for improving resolution, but will still fall far away from the resolution that is possible in near-field approaches.

1 A. Sakurai, S. Takahashi, T. Mochizuki and T. Sugimoto, Tip-enhanced sum frequency generation for molecular vibrational nanospectroscopy, *Nano Lett.*, 2025, **25**(16), 6390–6398.
2 A. P. Fellows, Using Vibrational Laser Spectroscopy to Study Biologically Relevant Interfaces, PhD thesis, University of Cambridge, 2022.
3 J. H. Park, S.-W. Lee, E. S. Lee and J. Y. Lee, A method for super-resolved CARS microscopy with structured illumination in two dimensions, *Opt. Express*, 2014, **22**, 9854–9870.
4 A. Zhitnitsky, E. Benjamin and O. Bitton, *et al.*, Super-resolved coherent anti-Stokes Raman scattering microscopy by coherent image scanning, *Nat. Commun.*, 2024, **15**, 10073.

Sylvie Roke replied: Near-field and tip-enhanced approaches are possible under very well-defined situations, and the need for the tip or the patterned surface is not easily compatible with also having a freely suspended membrane in the aqueous phase. Another complication is the separation of the response of the tip and the sample, and the way in which the tip modifies the sample.

John C. Conboy replied: Measurement of liquid state flip-flop could not be measured due to rapid mixing of the lipids, gel phase lipids were therefore examined. However, for sum-frequency vibrational spectroscopy (SFVS) of hybrid bilayer systems which contain only one monolayer of lipid, there is theoretically no impediment to characterization of Ca^{2+} and Mg^{2+} binding and interfacial water structure for LC phase lipids. Markus Raschke uses infrared scattering scanning nearfield optical microscopy (IR-sSNOM) for nanoscale characterization of surface structures, which may be applicable toward spectroscopy of hybrid bilayers.[1–3] Flip-flop measurement requires the ability to track the movement of lipids between leaflets, which in the case of sum-frequency is derived from changes in asymmetry with time. Unlike sum-frequency, IR would be unable to distinguish between the vibrational resonances of lipids contained within proximal or distal leaflets and would not be applicable to flip-flop measurement. However, there have been recent reported cases of tip enhanced sum-frequency which would be applicable.[4,5]

1 Y. Wang, S. C. Johnson, N. Nookala, J. F. Klem, S. R. Turner, R. L. Puro, M. Hu, I. Brener, E. A. Muller, A. Belyanin, M. A. Belkin and M. B. Raschke, *Laser Photonics Rev.*, 2024, **18**, 2301148.

2 R. L. Puro, T. P. Gray, T. A. Kapfunde, G. B. Richter-Addo and M. B. Raschke, *Nano Lett.*, 2024, **24**, 1909–1915.
3 R. Wilcken, B. L. Esses, R. S. Nithyananda Kumar, L. A. Hurley, S. E. Shaheen and M. B. Raschke, *Sci. Adv.*, 2025, **11**, eads3706.
4 A. Sakurai, S. Takahashi, T. Mochizuki and T. Sugimoto, *Nano Lett.*, 2025, **25**, 6390–6398.
5 P. Roelli, I. Pascual, I. Niehues, J. Aizpurua and R. Hillenbrand, *arXiv*, 2025, preprint, arXiv:2501.01784, DOI: 10.48550/arXiv.2501.01784.

Syma Khalid said: Regarding the distinct patches of lipids you observe, in a plasma membrane, by mass ~50% is composed of proteins – these membrane proteins often have distinct lipid fingerprints. It is unlikely a patch of lipid of the size that you report from *in vitro* studies would be present within a plasma membrane as there would always be a native membrane protein nearby. How would this alter the image?

Alexander P. Fellows responded: You are certainly correct that the lipid domains formed in these pure lipid systems are substantially larger than what would be formed in physiological settings and that proteins play an important role in the macroscopic structure of the membrane. Nevertheless, the underlying aim of such work on model lipid systems is to understand the fundamental lipid–lipid interactions governing domain formation that will still be prevalent in the presence of other membrane components. In this sense, observing differences in the domain line tension, compositional enrichment, *etc.* gives crucial insights into the favourability of certain structural motifs that help understand the wider impact on membrane structure due to processes such as PS exposure.

Ilya Levental addressed Alexander P. Fellows and Sylvie Roke: What is the applicability to biological systems? Would you see the asymmetries this way or would the proteins get in the way?

Alexander P. Fellows replied: The question of applicability to biological systems really comes down to what can be learned. In relatively simple model systems such as the two-component monolayers that we have studied here (**https://doi.org/10.1039/d4fd00187g**), we can really isolate the signals from the individual components and learn a lot about their specific 3D orientational distributions and intermolecular environments. As you add more and more complexity to the system, you generally sacrifice some of the information content. For ‘real’ biological systems, these kinds of second-order techniques can certainly tell you a lot about the amount and distribution of anisotropic molecular structure, but it would be very difficult to get this kind of information on the individual component level.

Sylvie Roke answered: The SH imaging method as presented here enables the time- and spatially-resolved recording of membrane potentials of living cells as demonstrated in ref. 1, in which membrane potential videos were made of living mouse brain neurons that were undergoing de- and repolarization events. It can be thought of as a future all-optical electrode-free, non-invasive version of electrophysiological recordings. The spatial resolution is determined by the optical resolution, which offers a drastic improvement compared to electrodes, which typically only record one data point per cell.

1 M. E. P. Didier, O. Tarun, P. Jourdain, P. Magistretti and S. Roke, *Nat. Commun.*, 2018, **9**, 5287.

Martín E. Villanueva opened discussion of the paper by John C. Conboy: Have you calculated the compressibility modulus for the 10% DPPS/DPPC mixture based on the obtained Langmuir monolayer isotherms? Additionally, how do you correlate the interfacial behavior observed in the monolayer with the molecular translocation (flip-flop) events detected in the corresponding lipid bilayers? This would be particularly relevant in the context of establishing monolayer–bilayer correspondence.

John C. Conboy replied: The compressibility moduli for monolayers of DPPC, DPPS, and 10% DPPS in DPPC were all calculated (Fig. 3) in the manuscript (**https://doi.org/10.1039/d4fd00206g**). The DPPC monolayer was unaffected by Ca^{2+}. Conversely, the pure DPPS monolayer displayed a decrease in compressibility upon exposure to Ca^{2+}. The change in compressibility of the 10% DPPS mixture was too small to quantify with statistical certainty; however, from the behaviors of the single component monolayers, a 10% DPPS in 90% DPPC monolayer would be expected to have a slight decrease in compressibility, roughly 1/10th the response of the 100% DPPS system if a linear response is assumed. As flip-flop motions require a local deformation of the bilayer to accommodate headgroup transfer through the hydrophobic core, the flip-flop rate is expected to decrease as the rigidity of the monolayer increases.[1] The slowed flip-flop kinetics of the 10% DPPS in DPPC is therefore consistent with an overall rigidification of the bilayer in response to Ca^{2+} ions.

1 T. C. Anglin and J. C. Conboy, *Biochemistry*, 2009, **48**, 10220–10234.

Ben John asked: Given that you used a PEG spacer, does it interact or mix with the lipids upon casting onto the substrate? If so, could it contribute to the signal you're detecting? And have you examined the system without PEG to compare?

John C. Conboy answered: The PEG forms a highly hydrated "mushroom" phase spacer between the bilayer and underlying support.[1–4] The PEG–PE lipid is fully protonated and will contribute to the sum-frequency generated in an asymmetric bilayer but will not flip-flop due to the inability to move the large PEG polymer through the membrane. The sum-frequency generated by the PEG is therefore constant over the course of the experiment and simply factored into the baseline. The PEG is not expected to influence the behavior of surrounding lipids, as the polymer is within the aqueous phase adjacent the membrane rather than intercalated within the membrane itself. The non-perturbing nature of the spacer was verified with DPPC controls where the flip-flop rate of DPPC was measured in the presence and absence of added PEG–PE lipid. No statistically significant difference was noted.

The notable way in which the PEG spacer influenced lipid behavior was the abolishment of Ca^{2+} salt bridging between the PS headgroups and silica support. 10% DPPS in DPPC deposited without the PEG spacer resulted in a biexponential decay in the presence of Ca^{2+} (see Fig. 7 here). Comparison of the flip-flop behavior of DPPS deposited in the proximal *versus* distal leaflets confirms the biexponential decay is due to Ca^{2+} salt bridging of the PS headgroups with the support. DPPS starting in the proximal leaflet near the silica was found to translocate far more slowly than distal DPPS and would not mix to completion, as indicated by significant residual asymmetry. Conversely, DPPS starting in the distal leaflet would mix quickly to homogeneity, with the generated sum-frequency appearing to reach a minimum, but would then re-establish asymmetry as evident from a subsequent increase in sum-frequency intensity. The observed behaviors imply DPPS can flip freely from the distal leaflet, whereas flops out of the proximal leaflet are hampered. PS lipids which salt bridge to the surface can effectively be captured and accumulate within the proximal leaflet. Consistent with the hypothesis is the observation of both single exponential behavior as well as complete mixing of the DPPS lipids with identical rates of PS flip-flop for both proximal and distal leaflets upon incorporation of the PEG spacer.

1 A. J. Diaz, F. Albertorio, S. Daniel and P. S. Cremer, *Langmuir*, 2008, **24**, 6820–6826.
2 M. L. Wagner and L. K. Tamm, *Biophys. J.*, 2000, **79**, 1400–1414.
3 V. Kiessling and L. K. Tamm, *Biophys. J.*, 2003, **84**, 408–418.
4 V. Kiessling, C. Wan and L. K. Tamm, *Biochim. Biophys. Acta*, 2009, **1788**, 64–71.

Manpreet Kaur enquired: In the artificial membranes, how is Ca^{2+} causing the PS flip-flop? How do you propose the hydrophilic head group flip-flops across the membrane?

John C. Conboy replied: There are two possible mechanisms for how Ca-exposed PS lipid flip-flop could proceed. Firstly, complexed PS could simply flip

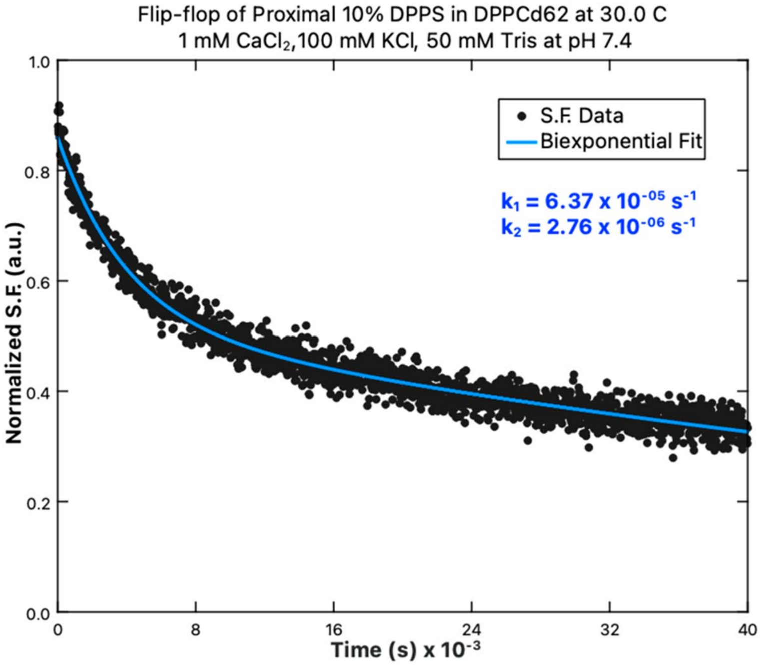

Fig. 7 Flip-flop behavior of proximal 10% DPPS in DPPC-d_{62} in the presence of Ca^{2+}.

as a $[PS]_2Ca$ unit and the slowed PS flip-flop rates observed in the presence of Ca^{2+} would therefore be attributed to the higher energetic cost of moving a larger complex through the bilayer. More likely however, is that flip-flop is preceded by a dissociation of the complex and the slowed rate of Ca-exposed PS flip-flop is influenced by the rate of the prerequisite dissociation step. Some preliminary data from induction of PS asymmetry in tethered vesicles may support this hypothesis. Asymmetry of PS in vesicles extruded without any internal Ca^{2+} can be induced by exposure to external 1 mM Ca^{2+}, presumably due to accumulation of PS toward the Ca-exposed leaflet. Preliminary data has shown that induced asymmetry can be cycled *via* removal of the external Ca^{2+} through complexation with EGTA. Such would theoretically not be the case if $[PS]_2Ca$ complexes could flip as a unit, as one would expect the internal and external Ca^{2+} concentrations to eventually equilibrate due to shuttling of Ca^{2+} ions over the bilayer. If Ca^{2+} were to transport into the vesicle, removal of the external Ca^{2+} would then result in a Ca^{2+} concentration differential and PS lipids would be expected to accumulate on the interior rather than return to baseline.

Robert V. Law said: Many of the phospholipids (*e.g.* DMPC) you use in your flip-flop measurements are in the gel phase as your measurements are done at 21 °C. From the literature, there is a well-established understanding that in the gel phase, lateral diffusion is very slow.[1,2]

Is it possible that due to the method that you use in the preparation of your supported bilayers that they are unusually defective (*e.g.* many pores *etc.*) or slightly dehydrated? Might the possibility of these contribute to the unusually fast flip-flop rates that you find?

1 D. Marquardt, *et al.*, *Langmuir*, 2017, **33**(15), 3731–3741.
2 J. Sot, *et al.*, *J. Mol. Liq.*, 2022, **363**, 119874.

John C. Conboy responded: Defect-mediated enhancement of lipid flip-flop rates in planar supported lipid bilayers (PSLBs), as postulated by Marquardt *et al.*,[1] cannot be ruled out entirely without high fidelity, sub-micron resolution AFM to characterize surface defects. However, flip-flop measurements of PSLBs from Langmuir–Blodgett/Langmuir–Schaffer (LB/LS) deposition are highly reproducible in a way which seems inconsistent with defect-mediated flip-flop. From the calculations of Marquardt, fluctuations in surface defects of less than a percent of surface coverage should have pronounced effects on the measured flip-flop rates. A reduction in surface coverage from 99.9% to 99.5% is predicted to speed flip-flop by an order of magnitude. Highly reproducible flip-flop measurements would imply highly reproducible defects, which, while not impossible, does seem unlikely. Further, flip-flop measurements performed by Kornberg and McConnell in vesicles are far faster than those reported for aLUVs, a discrepancy which cannot be attributed to surface defects.[2]

1 D. Marquardt, F. A. Heberle, T. Miti, B. Eicher, E. London, J. Katsaras and G. Pabst, *Langmuir*, 2017, **33**, 3731–3741.
2 R. D. Kornberg and H. M. McConnell, *Biochemistry*, 1971, **10**, 1111–1120.

Georg Pabst asked: (i) Based on your results, is it correct to conclude that, in the absence of flippases, phosphatidylserine (PS) would predominantly reside in the exoplasmic leaflet? If so, can you estimate the energetic barrier that the enzyme must overcome to facilitate its translocation to the cytoplasmic leaflet?

(ii) How would you expect the flip-flop rates you measured in the gel phase to extrapolate to the fluid phase? Would you anticipate any changes in the dynamics of Mg^{2+} and Ca^{2+} interactions with the lipids that might significantly affect the flip-flop behavior?

John C. Conboy replied: Yes, in the absence of flippases and other cellular control mechanisms the native lipid flip-flop dynamics measured here would predict an enrichment of PS toward the Ca-exposed exoplasmic leaflet. Without specific action by the cell to counteract the differential rates of flip and flop, a slow rate of inward flip combined with a fast rate of outward flop should result in an equilibration such that $N_{\text{external}}/N_{\text{internal}} = k_{\text{flop}}/k_{\text{flip}}$. The theorized enrichment of PS toward the Ca-exposed leaflet is in agreement with observations of both the Hampton and Bratton groups where the degree of PS externalization was dependent on the Ca^{2+} concentration differential over the bilayer.[1,2] The barrier to flip-flop can be estimated through extrapolation from the best line fit of the Ca-exposed 10% DPPS flip-flop rates in Fig. 10 in the paper (**https://doi.org/10.1039/d4fd00206g**) to physiologic temperature (37 °C) to then calculate $\Delta G^{\ddagger}$ using the Eyring relation (eqn (20) in the paper). The predicted $\Delta G^{\ddagger}$ in this case would be ~95 kJ mol^{-1}. However, this value is particular to the binary DPPS/DPPC mixture as tested, which is simplified and non-physiologic. A more holistic matrix would include a blend of saturated and unsaturated phospholipids which would be in the liquid crystalline state at physiologic temperature.[3,4] If the tested mixture is extrapolated out to the phase transition of DPPS, 54 °C, the LC phase flip-flop barrier would then be ~88 kJ mol^{-1}. However, there is difficulty in predicting the thermodynamic and kinetic properties of the LC phase using only data collected within the gel phase. Saturated, gel phase lipids had to be used here in order to slow the flip-flop process sufficiently to be measured. As a general assessment, higher temperatures and fluid lipids are expected to result in faster rates of flip-flop for both the Ca-exposed and Ca-free PS lipids. The specific rates of translocation within each environment are unknown, however, the relative kinetics of Ca-exposed and Ca-free PS lipid flip-flop $[k_{\text{PS}}/k_{(\text{Ca:PS})}]$ are hypothesized to be similar even in the LC phase. Phase segregation of PS from PC in the presence of Ca^{2+} has been noted in both gel and LC phases,[5,6] which indicates PS–Ca interactions are not phase-specific.

1 M. B. Hampton, D. M. Vanags, M. I. Pörn-Ares and S. Orrenius, *FEBS Lett.*, 1996, **399**, 277–282.
2 D. L. Bratton, V. A. Fadok, D. A. Richter, J. M. Kailey, L. A. Guthrie and P. M. Henson, *J. Biol. Chem.*, 1997, **272**, 26159–26165.
3 A. J. Verkleij, R. F. Zwaal, B. Roelofsen, P. Comfurius, D. Kastelijn and L. L. van Deenen, *Biochim. Biophys. Acta*, 1973, **323**, 178–193.
4 J. H. Lorent, K. R. Levental, L. Ganesan, G. Rivera-Longsworth, E. Sezgin, M. Doktorova, E. Lyman and I. Levental, *Nat. Chem. Biol.*, 2020, **16**, 644–652.
5 C. P. Tilcock, P. R. Cullis and S. M. Gruner, *Biochemistry*, 1988, **27**, 1415–1420.
6 M. Ross, C. Steinem, H.-J. Galla and A. Janshoff, *Langmuir*, 2001, **17**, 2437–2445.

Rumiana Dimova addressed John C. Conboy and Sylvie Roke: Calcium and magnesium differ not only in their binding modes to PS, but also in their position within the Hofmeister series, with Ca^{2+} being more chaotropic than Mg^{2+}. To what extent do you think the differences you observe in lipid packing, headgroup ordering, and flip-flop dynamics might arise from broader Hofmeister effects on interfacial water structure or membrane hydration, beyond specific ion–lipid interactions? And do you think techniques like those developed by S. Roke could help disentangle these contributions?

John C. Conboy responded: The more chaotropic nature of Ca^{2+} and its ability to more effectively displace water from the lipid headgroups is very likely a large factor in the pronounced effect Ca^{2+} ions have on PS behavior. Papahadjopoulos noted that Mg-complexed PS lost significant weight when vacuum dried, whereas Ca-complexed PS did not, with this difference in weight being attributed to water content.[1] Papahadjopoulos therefore attributed the fusogenic activity of Ca^{2+} and the ability of Ca^{2+} ions to phase segregate PS from PC to the displacement of water and more effective binding compared to Mg^{2+}, which did not display the same effects.[1–7] The reduced ability of Mg^{2+} to effectively coordinate the PS headgroups, as calculated by Martin-Molina *et al.*[8] and inferred from our observed reduced amine peak ordering compared to Ca^{2+} in the SFVS data, could very well be due to Mg^{2+} being insufficiently chaotropic to disrupt and displace waters associated with the PS headgroup. The capacity of each ion to coordinate the lipid headgroup appears tightly linked with the relative chaotropic nature of each species and the two effects may not be entirely distinguishable from one another. Second harmonic generation could be utilized as a complementary technique for tracking net water structure at the lipid/water interface, however in our current study sum-frequency provides useful structural information (ice- and water-like bonding characteristics) beyond just a simple water ordering parameter achievable with SHG. A comparative analysis of the ice- and water-like peaks, combined with the amine peak derived Ca–PS binding isotherm data, also allowed for the separation of molecular reorientation and third-order coupling contributions to the $\chi^{(3)}$ effect. Knowledge of the net water structure alone would have been insufficient to the derivation of the Ca–PS and Mg–PS binding isotherms key to this assessment due to the tendency of water order to maximize at high field strengths near highly charged surfaces.[9,10] The ability to detect and track the reorientation of head-group amines during Ca^{2+} and Mg^{2+} complexation was crucial for characterizing the charge state of the PS lipids at low concentrations of Ca^{2+} and Mg^{2+} where the water ordering was beyond saturation and insensitive to changing surface charge.

1 C. Newton, W. Pangborn, S. Nir and D. Papahadjopoulos, *Biochim. Biophys. Acta*, 1978, **506**, 281–287.
2 D. Papahadjopoulos, G. Poste, B. E. Schaeffer and W. J. Vail, *Biochim. Biophys. Acta*, 1974, **352**, 10–28.
3 K. Jacobson and D. Papahadjopoulos, *Biochemistry*, 1975, **14**, 152–161.
4 D. Papahadjopoulos, W. J. Vail, C. Newton, S. Nir, K. Jacobson, G. Poste and R. Lazo, *Biochim. Biophys. Acta*, 1977, **465**, 579–598.
5 D. Papahadjopoulos and A. Portis, *Ann. N. Y. Acad. Sci.*, 1978, **05467**, 50–66.
6 A. Portis, C. Newton, W. Pangborn and D. Papahadjopoulos, *Biochemistry*, 1979, **18**, 780–790.
7 N. Duzgunes, J. Wilschut, R. Fraley and D. Papahadjopoulos, *Biochim. Biophys. Acta*, 1981, **642**, 182–195.
8 A. Martin-Molina, C. Rodriguez-Beas and J. Faraudo, *Biophys. J.*, 2012, **102**, 2095–2103.
9 D. E. Gragson and G. L. Richmond, *J. Am. Chem. Soc.*, 1998, **120**, 366–375.
10 S. Kielich, *IEEE J. Quantum Electron.*, 1969, **5**, 562–568.

Sylvie Roke replied: The observation that Ca^{2+}, Mg^{2+}, and Ba^{2+} when interacting with partially charged phospholipid membranes all create different membrane potential fluctuations[1,2] is a manifestation of ion specific interactions. For example, Mg^{2+} interacts with lipid head groups through an ion pair complex which still involves a hydration shell (solvent separated ion pair), whereas Ca^{2+} directly binds to the lipid head group (contact ion pair). The first results in weaker interactions that have a smaller impact on the membrane potential distribution/interfacial water orientation, while the latter leads to larger interactions that have a larger impact on the membrane potential distribution/interfacial water orientation.

1 O. B. Tarun, *et al.*, Transient domains of ordered water induced by divalent ions lead to lipid membrane curvature fluctuations, *Commun. Chem.*, 2020, **3**(1), 17.
2 D. Roesel, *et al.*, Ion-induced transient potential fluctuations facilitate pore formation and cation transport through lipid membranes, *J. Am. Chem. Soc.*, 2022, **144**(51), 23352–23357.

Markus Deserno said: You made a remark in your presentation that cells meticulously regulate the intracellular calcium concentration, but that the same would not be true for magnesium. I found that puzzling, because in view of the $\sim$70 mV electrostatic potential difference across the plasma membrane, I would then expect the magnesium concentration – purely by the electrostatic contribution to their chemical potential – to be about 200 times as concentrated inside cells relative to the extracellular fluid. Is this really true, or am I missing something here?

John C. Conboy responded: The predominant contribution to the electrostatic potential of animal cells is the regulation of K^+ and Na^+ ions within the cytosol.[1] Na^+ ions are removed from the interior and replaced with K^+ ions in a $3Na^+ : 2K^+$ ratio, which results in a deficit of positive internal charges. Additionally, K^+ ions are free to diffuse through ion channels. As the internal concentration of K^+ is far higher than the external K^+ concentration due to active ion shuttling, diffusion favors outward efflux. The final equilibrium concentration is a balance between the negative potential which attracts K^+ toward the interior and the concentration gradient driven efflux. Theoretically, it is true that Mg^{2+} ions would accumulate toward the interior in response to the electrical driving force, however the cell membrane is not permeable to Mg^{2+} ions.[2] For eukaryotes, a large variety of transporters exist which serve to shuttle magnesium between the plasma and cytosol, as well as the cytosol and organelles (see page 17 of Vink and Nechifor).[3] However, the end result is simply that the cytosolic and exoplasmic concentrations of Mg^{2+} ions are roughly equal.

1 B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts and J. Watson, *Molecular Biology of the Cell*, Garland Publishing, New York, 3rd edn, 1994.
2 E. Murphy, *Circ. Res.*, 2000, **86**, 245–248.
3 R. Vink and M. Nechifor, *Magnesium in the Central Nervous System*, University of Adelaide Press, South Australia, 2011.

Tyler Reagle asked: Have you investigated (i) whether the rate of DPPS flip-flop is linear with respect to the mole fraction of DPPS in the distal leaflet (denoted by X_x where the subscript "x" refers to either the "distal" or "proximal" leaflet) and (ii) whether the 10% DPPS used for the flip-flop measurements falls within such a linear-regime? Significant deviation from linearity might reflect non-ideal behavior arising from Coulombic-repulsion and/or accumulation of mechanical-tensions within the leaflets (which would be incurred if there is net transport of DPPS into the distal leaflet that is not balanced by a counter-flow of DPPC lipids);[1] the condensing effect of Ca^{2+} upon the molecular area of DPPS could incur an additional contribution to a mechanical-tension in the distal leaflet. Elementary rate-laws for the flip-flop of DPPS could be expressed in terms of the chemical activity of this lipid in a given leaflet (denoted by Λ_x, where the subscript "x" refers to the "distal" or "proximal" leaflet),[2] given by $R_{\text{flip}} = k_{\text{flip}}\Lambda_{\text{distal}}$ and $R_{\text{flop}} = k_{\text{flop}}\Lambda_{\text{proximal}}$, where R_{flip} and R_{flop} are the rates of DPPS flip and flop, respectively. Following the Debye–Hückel limiting law, Λ_x and X_x are related by $\Lambda_x = \gamma_x X_x$, where γ_x is the activity coefficient of DPPS in a given leaflet (which presumably depends on X_x, the density of adsorbed cation, and the mechanical conditions experienced by the lipid). Accounting for non-ideality in the rate-laws leads to $R_{\text{flip}} = k_{\text{flip}}\gamma_{\text{distal}}X_{\text{distal}}$ and $R_{\text{flop}} = k_{\text{flop}}\gamma_{\text{proximal}}X_{\text{proximal}}$, where γ_{distal} and γ_{proximal} might not be equal. If DPPS does behave non-ideally under a given set of conditions but ideal behavior is assumed, then the products $k_{\text{flip}}\gamma_{\text{distal}}$ and $k_{\text{flop}}\gamma_{\text{proximal}}$ are the "measured" or "perceived" rate-constants for DPPS flip and flop, respectively, which would be obtained from fitting of kinetic data. If DPPS does not deviate significantly from ideal behavior (*i.e.* $\gamma_x \rightarrow 1$), then these products yield unbiased estimates of k_{flip} and k_{flop}. If (a) DPPS does deviate significantly from ideal behavior under certain conditions and (b) the fitting equation for the time-dependent spectroscopic data (eqn (7) in the paper (**https://doi.org/10.1039/d4fd00206g**)) could be reformulated to account for this in some way, it would be very interesting to know whether the Ca^{2+}-dependence of DPPS flipping could be explained by changes in the activity coefficient(s) of DPPS or if this effect comes from changes to the activation-energy barrier for flip-flop (conveyed by changes in k_{flip} after non-ideality has been accounted for).

1 T. Reagle, *et al.*, *Soft Matter*, 2024, **20**, 4291, DOI: **10.1039/d3sm01772a**.
2 A. Radhakrishnan and H. M. McConnell, *Biochemistry*, 2000, **39**, 8119, DOI: **10.1021/bi0005097**.

John C. Conboy responded: The present study did not examine different mole fractions of DPPS, however a previous publication by Brown and Conboy determined flip-flop rates for DSPC mixtures containing varying fractions of DSPG, which, like DPPS, is also net negative at neutral pH.[1] It was determined the transition state free energy, $\Delta G^{\ddagger}$, is linear with respect to the mole fraction of PG. The rate of flip-flop, however, responds exponentially [rate = $\exp(-\Delta G^{\ddagger}/RT \ln((k_BT)/h))$]. As altering the mole fraction of negative lipid within the membrane modulates the average distance (x) between the charged headgroups, and potential (ϕ) scales exponentially with distance [$\phi/\phi_0 = e^{-\kappa x}$], the exponential scaling in the rate of flip-flop with respect to PG mole fraction was attributed to coulombic interactions between the lipids. The influence of charge repulsion on rates of flip-flop was further confirmed through modulation of the inverse Debye length (κ) such that 25% PG in low ionic strength PBS would experience the same coulombic forces as 75% PG in high ionic strength PBS. When compared, these two systems had statistically identical rates of flip-flop. While chemical activities were not calculated, these data demonstrate charged lipid flip-flop is indeed dependent on the local ionic environment, in other words the charge screening (activity) of the PG lipids does modulate the rate of translocation. However, changes in PS activity (charge screening) does not explain the data obtained in the present study, as all 10% DPPS flip-flop measurements were performed using a fixed ionic strength in the presence and absence of Ca^{2+}. In addition, as all 10% DPPS bilayers contain one leaflet of 10% DPPS in DPPC-d_{62} and one leaflet of 10% DPPS-d_{62} in DPPC-d_{62}, all coulombic effects are symmetric over the bilayer and no disruptive mechanical tension should occur between the two leaflets. The slowing effect of Ca^{2+} on DPPS flip-flop must therefore be attributed to a direct interaction rather than effects due to altered PS activity. These effects would however need to be addressed in cases where the starting coulombic forces are unequal, in which case the rate equation could be rederived with non-ideality and unequal rates of apparent k_{flip} and k_{flop} (R_{flip} and R_{flop} in the above notation) in mind.

1 K. L. Brown and J. C. Conboy, *J. Phys. Chem. B*, 2015, **119**, 10252–10260.

Christian Schwieger questioned: Looking at the water spectra, why are the two water components, *i.e.* free and bound water, increasing in the same way?

John C. Conboy replied: The two water resonances centered at 3200 and 3400 cm^{-1} represent the ice- and liquid-like O–H symmetric stretches, respectively.[1–4] These represent all water which is ordered near the surface. While both result from the symmetric O–H stretch of water, the ice-like resonance is indicative of a strong, tetrahedral H-bonding structure like that seen in ice. Conversely, the liquid-like peak results from a weaker H-bonding structure, namely bifurcated H-bonding whereby hydrogens are shared unevenly between neighboring oxygens. The sum-frequency intensity of the water resonances are expected to scale with interfacial charge density due to a third-order, $\chi^{(3)}$, effect (eqn (10) in the paper (**https://doi.org/10.1039/d4fd00206g**)).[5–12] The $\chi^{(3)}$ effect represents two phenomena: the direct coupling of the three electric fields, dependent on the third-order hyperpolarizability of the material (γ), and rearrangement of dipolar molecules in response to changing surface charge, which is merely dependent on the second-order hyperpolarizability of the material (β) (eqn (11) in the paper). While both water peaks respond the "same" in the sense that both gain in intensity as surface charge density increases, the response of the ice-like peak is actually greater due to the aforementioned reorganization term. In short, an increase in surface charge will induce some number of disordered waters to migrate to the semi-ordered 3400 cm^{-1} population, while some number of the pre-existing semi-ordered waters will migrate to the tetrahedrally ordered 3200 cm^{-1} population. As the tetrahedrally ordered population represents a terminal state, where further population conversion is not possible, the number of waters contained within the tetrahedrally ordered state is expected to grow more rapidly than as with the semi-ordered state, which will gain waters from the disordered population, but also lose waters to the maximally ordered state. A fit of the transition probabilities of the 3200 and 3400 cm^{-1} resonances *versus* the interfacial electric field strength (Fig. 7 in the paper) found that the response of the ice-like peak is roughly double that of the liquid-like.

1 D. E. Gragson and G. L. Richmond, *J. Am. Chem. Soc.*, 1998, **120**, 366–375.
2 G. L. Richmond, *Chem. Rev.*, 2002, **102**, 2693–2724.

3 A. G. de Beer and S. Roke, *J. Chem. Phys.*, 2016, **145**, 044705.
4 V. Ostroverkhov, G. A. Waychunas and Y. R. Shen, *Chem. Phys. Lett.*, 2004, **386**, 144–148.
5 P. E. Ohno, H. F. Wang and F. M. Geiger, *Nat. Commun.*, 2017, **8**, 1032.
6 P. E. Ohno, H. F. Wang, F. Paesani, J. L. Skinner and F. M. Geiger, *J. Phys. Chem. A*, 2018, **122**, 4457–4464.
7 S. Ong, X. Zhao and K. B. Eisenthal, *Chem. Phys. Lett.*, 1992, **191**, 327–335.
8 X. Zhao, S. Ong and K. B. Eisenthal, *Chem. Phys. Lett.*, 1993, **202**, 513–520.
9 B. F. Levine and C. G. Bethea, *J. Chem. Phys.*, 1976, **65**, 2429–2438.
10 P. D. Maker and R. W. Terhune, *Phys. Rev.*, 1965, **137**, A801–A818.
11 R. W. Terhune, P. D. Maker and C. M. Savage, *Phys. Rev. Lett.*, 1962, **8**, 404–406.
12 S. Kielich, *IEEE J. Quantum Electron.*, 1969, **5**, 562–568.

Walter Nickel opened discussion of the paper by Gonen Golani: In calculations regarding energy barriers for membrane fusion, was this considering protein-dependent membrane fusion, *e.g.* catalysed by SNARE proteins? Or considered in context of membrane fusion *in vitro* that can also be triggered in a protein-independent manner? The principal question here would be as to whether your conclusions can be applied to cellular systems in which membrane fusion occurs by dedicated protein machineries such as SNARE proteins or viral fusion proteins.

Gonen Golani answered: The calculation is general and applies to both protein-dependent and protein-independent fusion. Specifically, we compute the energy barrier for fusion and how it is affected by membrane asymmetry. The driving force required to overcome this barrier is beyond the scope of this work, as it is not directly determined by lipid asymmetry. In other words, we focus on one side of the process, the energy barrier to fusion, without addressing the mechanism by which this barrier is overcome (*e.g.*, SNARE). The implicit assumption is that most biologically relevant fusion events proceed through a similar pathway, which is similarly influenced by induced asymmetry.

John M. Seddon remarked: I was surprised that the saddle-splay term doesn't vary with $\Delta\zeta$ (Fig. 4 in the paper; **https://doi.org/10.1039/d4fd00189c**). Is this to be expected?

Gonen Golani responded: The meaning of $\Delta\zeta$ is the difference between the spontaneous curvatures of the two monolayers. Since the elastic model is quadratic in tilt and splay, the first-order effect of $\Delta\zeta$ enters through the splay term in the energy density (which is the analog of mean curvature in the absence of tilt). This is why the splay energy is most strongly affected by $\Delta\zeta$. Saddle-splay energy is only indirectly influenced: changing $\Delta\zeta$ alters the minimal energy configuration of the hemifusion intermediates compared to a symmetric membrane, and as a result, the saddle-splay energy also changes. However, this effect is relatively small.

John M. Seddon asked: Will your work help to design lipid nanoparticles with more efficient endosomal escape, for delivery of nucleic acids or other therapeutics into cells?

Gonen Golani replied: Possibly. Our work showed that inducing a certain type of asymmetry can reduce the energy barriers for fusion. If fusion is indeed the rate-limiting step in delivery, this could offer a way to optimize the process.

Ilya Levental enquired: Two types of asymmetry, compositional and density. Which is most relevant? Splaying lipids promoting fusion, *e.g.* PE accumulating on cytosolic leaflet?

Gonen Golani answered: To my understanding, the most important form of asymmetry affecting the energy barriers for fusion is the level of lipid unsaturation. A higher concentration of unsaturated lipids in the cytoplasmic monolayer is expected to raise the energy barrier for fusion from the outside while lowering it from the inside. Other types of asymmetry play a lesser role. For example, we calculated the effect of a density difference, which manifests as differential stress. This had a minimal impact on the energy barrier for lipid mixing and a somewhat greater effect on content mixing, though still less significant than the influence of intrinsic lipid curvature.

Patricia Losada Pérez said: You mentioned that lipid splay is the dominant contribution. Excluding the action of lipids that induce non-zero spontaneous curvature, what is the driving mechanism of lipid splay? Can one predict or quantify lipid splay in some way?

Gonen Golani responded: The energetic contribution of splay arises from the difference between the actual geometrical splay of lipids near the fusion site and their intrinsic splay (curvature), which is an inherent property of the lipids. Lipids with bulkier head groups and narrower tails typically exhibit positive intrinsic splay (curvature), while lipids with smaller heads and broader tail regions have negative intrinsic splay. The intrinsic splay of lipids can be measured experimentally (*e.g.*, ref. 1), and the actual splay adopted by the lipids can be calculated numerically, as done in this work (**https://doi.org/10.1039/d4fd00189c**). However, to the best of my knowledge, there is currently no experimental method to directly measure the difference between the actual and intrinsic splay near the fusion site.

1 M. K. Dymond, *Chem. Phys. Lipids*, 2021, **239**, 105117.

Michael M. Kozlov asked: How does the overall tension affect the energy of the fusion intermediate?

Gonen Golani answered: Membrane tension has opposing effects on fusion intermediates. The energy required for stalk formation increases, as forming a stalk demands additional membrane area compared to the pre-fusion configuration. This was addressed both experimentally and theoretically in previous work,[1] where we found that the energy barrier increased by a few kT. In contrast, expansion into a hemifusion diaphragm is favored because it involves lipid flow into the surrounding membranes, releasing energy. Therefore, we expect the hemifusion diaphragm radius to increase with tension. Finally, the expansion of the fusion pore is also favored, as membrane tension lowers the energy barrier for pore formation.

1 P. Shendrik, *et al.*, *ACS Nano*, 2023, **17**, 18942.

Michael M. Kozlov questioned: Does the tension change the inter-membrane distance?

Gonen Golani replied: Yes, in general, tension reduces membrane undulations and, as a result, decreases the repulsive interactions between membranes. This effect was considered in recent work by the same authors.[1] However, in the current study (**https://doi.org/10.1039/d4fd00189c**), the membrane tension was not changed – only the monolayer tension was varied. Specifically, to avoid addressing this effect, we explicitly assumed that the sum of the monolayer tensions, which defines the membrane tension, vanishes. In other words, the two monolayers have tensions of equal magnitude but opposite sign. Under these conditions, the membrane's undulation spectrum remains unchanged, and the inter-membrane distance stays the same.

1 P. Shendrik, *et al.*, *ACS Nano*, 2023, **17**, 18942.

Rumiana Dimova enquired: In your experiments, membrane fusion involves two bilayers of different composition, whereas in the simulations, the membranes have identical composition. How do you think accounting for this compositional asymmetry in the simulation model would affect the predicted energy barriers and potentially influence the preferred directionality of fusion?

Gonen Golani responded: We have tested fusion between symmetric and asymmetric membranes in simulations, although these results were not included in the paper to maintain focus on the principal idea. In these simulations, the qualitative behavior remained the same, although the magnitude of the effect was reduced. We note that the goal of the simulations was not to achieve an exact fit to the experimental system – several factors are not known with sufficient accuracy – but rather to demonstrate qualitative agreement, which is unchanged if one membrane is symmetric.

Heiko Heerklotz asked: A membrane asymmetry that favors fusion with vesicles from the interior and opposes fusion from the outside seems advantageous in most but not all cases. How do you envisage the cell is dealing with the few cases where fusion events from the outside are favorable?

Gonen Golani answered: The hypothesis is that fusion from the extracellular side can be induced by lipid scrambling. Indeed, several reports indicate that plasma membrane scrambling – mediated, for example, by TMEM16 – promotes cell–cell fusion, a process involved in tissue development (*e.g.*, ref. 1).

1 Y. Zhang, *et al.*, *Sci. Adv.*, 2020, **6**, eaba0310, DOI: **10.1126/sciadv.aba0310**.

Georg Pabst requested: Could you speculate on the role of long-range interactions – such as screened electrostatics, van der Waals forces, and membrane undulations – in promoting the preferential fusion of asymmetric vesicles? For instance, some asymmetric membranes have been found to be significantly more rigid than their symmetric counterparts.

Gonen Golani responded: Membrane interaction and rigidity play important roles in fusion. Membranes are usually repulsive at long range and one of the roles of the work of fusion proteins is to overcome this repulsive force and bring the membranes to close enough distance so they could fuse. This process, and the possible effect of asymmetry in this context, was out of the scope of the present work but could definitely be important. For example, scrambling of charged lipids could dramatically change the interaction profile between the membranes. Membrane rigidity is another important factor that was not considered here and plays an important role – a more rigid membrane is harder to fuse. However, I would speculate that if one monolayer becomes stiffer and the other softer there might be more complex effects. These should be quantified using numerical calculations.

Oded Farago opened discussion of the paper by Giacomo Fiorin: Technical questions: How do you measure the bending modulus in the simulations? How do you impose curvature on the simulated membranes (is it done using the buckling method for measuring the bending rigidity – see ref. 1)?

1 M. Hu, P. Diggins IV and M. Deserno, *J. Chem. Phys.*, 2013, **138**, 214110.

Giacomo Fiorin answered: Firstly, free-energy landscapes were computed by applying biasing potentials on the membrane curvature and unbiasing the resulting probability distributions using established MD simulation techniques. Secondly, each computed free-energy landscape was fitted against the mathematical expression of the same landscape as predicted from continuum theory. For very large bilayers, this theory would be the Helfrich–Canham model, and its only parameter would be the bending modulus, k_c. However, given the very small bilayer sizes it is also necessary to account for lipid orientation, as originally proposed by Hamm and Kozlov[1] and confirmed by MD simulations and X-ray diffraction measurements.[2,3] The additional parameter, k_t, was derived by fitting the resulting maps of lipid orientations against the theoretical prediction, as previously done in Fiorin *et al.*[4]

Rather than the buckling method used by Deserno, our computational approach[5] is more similar to the one by den Otter and Briels,[6] but with the following two differences:

(i) We used atomistic simulations and constant-pressure conditions, which allow the bilayer's projected area to adapt to increased local curvature without adding membrane tension.

(ii) Sinusoidal deformations were used in this work purely for mathematical convenience, but the same scheme supports arbitrary types of curvature,[5,7] as well as changes in other structural parameters such as thickness (work to be published).

1 M. Hamm and M. M. Kozlov, *Eur. Phys. J. E: Soft Matter Biol. Phys.*, 2000, **3**, 323–335.
2 M. C. Watson, *et al.*, *Phys. Rev. Lett.*, 2012, **109**, 028102.
3 M. S. Jablin, *et al.*, *Phys. Rev. Lett.*, 2014, **113**, 248102.
4 G. Fiorin, *et al.*, *PNAS Nexus*, 2023, **2**(8), pgad269.
5 G. Fiorin, *et al.*, *J. Comput. Chem.*, 2020, **41**, 449–459.
6 W. K. den Otter and W. J. Briels, *J. Chem. Phys.*, 2003, **118**(10), 4712–4720.
7 W. Zhou, *et al.*, *eLife*, 2019, **8**, e50576.

Malavika Varma asked: What are your thoughts on extending this work to mixtures with and without cholesterol?

Giacomo Fiorin replied: Quantification of the bending mechanics of asymmetric membranes containing cholesterol is critical to understand the dynamics of the plasma membrane, and will likely be the subject of future work. To that end, some challenges remain to be addressed: for example, our atomistic computations of simple asymmetric bilayers are so far consistent with proposed theoretical models, but not yet in quantitative agreement with experimental data. This apparent discrepancy needs to be addressed.

Furthermore, in multi-component bilayers the densities of each lipid species may also vary with the local curvature. This effect did not appear to be significant in symmetric POPC or DOPC bilayers with cholesterol,[1] but this behavior may not necessarily apply to other lipid compositions. For asymmetric bilayers, this is probably even more true due to differential stress, which would dramatically affect the lipid packing of each leaflet and thus the propensity of cholesterol to flip-flop. Although these effects would be accounted for in our atomistic simulations, theoretical modeling would also be required to correlate simulations with experiments.

1 G. Fiorin, *et al.*, *PNAS Nexus*, 2023, **2**(8), pgad269.

Walter Nickel queried: Are your conclusions valid in the context of native cellular membranes? There is quite a complexity observed for cellular membranes with a number of relevant parameters. Plasma membranes are among the stiffest bilayers, but they are not only characterised by lipid asymmetry but also high levels of cholesterol. By contrast, the endoplasmic reticulum is less asymmetric but also contains low levels of cholesterol. In addition, what are the relative contributions of membrane proteins to membrane stiffness? In particular, membranes from different organelles contain vastly different protein to lipid ratios. So what do you think is the contribution of the reported effects on membrane stiffness in native membranes with different lipid compositions and different protein to lipid ratios?

Giacomo Fiorin replied: The conclusions of this study are directly applicable to asymmetric lipid bilayers with homogeneous leaflets. These bilayers are, in turn, synthetic models of the plasma membrane used to infer its mechanical properties from *in vitro* experiments. Quantification of bending mechanics of symmetric bilayers with cholesterol using this method was done previously in ref. 1. Estimation of the effect of embedded membrane proteins would probably require computations on larger systems, and with varying protein to lipid ratios.

1 G. Fiorin, *et al.*, *PNAS Nexus*, 2023, **2**(8), pgad269.

Milka Doktorova said: The zero-tension state in the bilayers is estimated from the defined area asymmetry parameter. As stated in the text (**https://doi.org/10.1039/d5fd00006h**), the calculation of lateral pressure profiles from the simulation trajectories has caveats but standard protocols exist including within NAMD which was the software used for the simulations in this study. Have you tried to calculate the leaflet tension in the bilayers with some of these protocols to see how it corresponds to the area asymmetry, or do you have some specific observations indicating that it is unreliable for estimating the differential stress in the bilayers?

Giacomo Fiorin replied: We thank Dr Doktorova for bringing up calculations of lateral stress profiles as an approach to quantify the tension of each leaflet. As acknowledged in our paper (**https://doi.org/10.1039/d5fd00006h**), this would be an alternative to considering each leaflet individually and using the changes in area per lipid molecule to infer changes in tension. We have not used the former approach in our work, neither using the NAMD protocol, nor any others, because our focus on bilayers with homogeneous leaflet compositions allowed us to avoid the associated caveats.

To provide some context, the direct computation of local stress and tension from MD simulations may be done using a variety of schemes, most of which have not yet been implemented in mainstream software packages such as NAMD. It should be noted that these schemes can differ in how they generalize continuum-mechanics equations to the atomic scale. As a consequence, the numerical results of these methods may differ significantly from each other. A few examples can be found in ref. 1–4.

The method implemented in NAMD and raised in this question by Dr Doktorova is based on the theoretical framework used by Lindahl and Edholm,[5] but with modifications in the way that σ_{ij}, *i.e.* the virial stress contribution due to a force between two atoms i and j, is apportioned in space. Specifically, when recording σ_{ij} onto geometric "slabs" to compute a stress profile, NAMD makes an additional approximation: it assigns σ_{ij} only to the slabs containing atoms i and j, omitting any slabs between them. This discretization scheme may impact the calculation of lateral pressure in individual leaflets, particularly for atoms near the bilayer midplane. That is, if atoms i and j are in the same leaflet, but j extends into the opposing leaflet due to interdigitation, NAMD always attributes half of the value of σ_{ij} to the opposing leaflet, irrespective of how deeply atom j penetrates into it. (Another technical limitation to the NAMD implementation is that reciprocal-space contributions to the virial stress are estimated using a different algorithm than the one used to run the simulations, *i.e.* Ewald *vs.* Particle-Mesh-Ewald.)

On the other hand, direct computation of local stress can be applied to heterogeneous leaflets, unlike the strategy used in our work. We note that the approximations mentioned above do not necessarily render the calculated pressure profiles "unreliable" *per se*. For example, the NAMD discretization strategy mostly affects the region near the midplane, where the two leaflets interact, but the majority of the atoms can be neatly attributed to either leaflet and thus their contribution is dominant. This assumption is similar to the one adopted in this work, where the area asymmetry of individual leaflets was used. For future work, we are in fact considering the direct computation of local stress, but with methods designed to reduce or eliminate discretization errors.

1 O. H. S. Ollila, *et al.*, *Phys. Rev. Lett.*, 2009, **102**, 078101.
2 T. Nakamura, *et al.*, *Comput. Phys. Commun.*, 2015, **190**, 120–128.
3 J. M. Vanegas, *et al.*, *J. Chem. Theory Comput.*, 2014, **10**(2), 691–702.
4 R. M. Elder, *et al.*, *Chem. Phys. Lett.*, 2019, **731**, 136580.
5 E. Lindahl and O. Edholm, *J. Chem. Phys.*, 2000, **113**, 3882–3893.

Alberto Giacomello questioned: When fitting the free energy profile as a function of the curvature one obtains the thermodynamic variable conjugate to it. So what is the thermodynamic observable that you are obtaining by fitting the free energy profiles? To my understanding, it should be a combination of the bending modulus and the non-local bending modulus, which is in turn connected to the area compressibility of two leaflets.

Giacomo Fiorin responded: I assume that you are referring to the non-local curvature energy term as defined in recent work by the Deserno lab (eqn (16a–d) in ref. 1). This represents the combined penalties associated with the compression or expansion of the two leaflets. In that work, the non-local curvature energy term is expressed as a function of $\langle c \rangle$, the mean over the membrane's surface of the local curvature c. However, in our MD simulations the bilayers are subject to periodic boundary conditions, which constrain $\langle c \rangle$ to be zero; furthermore, the numbers of lipids in the two leaflets are fixed during all simulations of the same bilayer. As a result, all parameters influencing the non-local curvature energy are constant, and this term does not contribute to the computed free-energy profile in a manner that is accounted for by the theory.

It is important to note that the values of the non-local curvature energy may vary significantly between the bilayers considered in this work, but these bilayers were all simulated independently and free-energy differences between them were not computed. Performing such comparisons would likely require simulations without periodic boundary conditions, which in turn would necessitate significantly larger system sizes.

1 A. Hossein and M. Deserno, *J. Chem. Phys.*, 2021, **154**, 014704.

Oded Farago asked: What is the origin of the increasing bending rigidity at moderate asymmetry, and why does it decrease at larger asymmetry. Is the stiffening of the membrane related to a phase transition or partial gelation of compressed layers?

Giacomo Fiorin replied: Although the increase in bending modulus is correlated with asymmetry, we do not currently have direct evidence of a phase transition from a quantifiable order parameter. Note that this fact does not necessarily reflect the absence of a phase transition, especially considering that we have so far only examined variations in lipid density but not yet in other measures of lipid packing. Furthermore, attributing the observed stiffening to gelation would arguably also require a clearer relationship between the proposed cause and effect, *i.e.* an explanation to why compression of POPC leaflets appears to cause first a stiffening and then a softening.

Edward Lyman said: Fig. 1A and 5A in your paper (**https://doi.org/10.1039/d5fd00006h**) report the free energy when deforming the membrane. If I understand the method correctly, the deformation is applied by imposing a long wavelength undulation *via* a collective variable (a sinusoidal deflection of the membrane), and the free energy obtained by standard collective variable methods.

In these panels there are an awful lot of data points. Is each one a separate simulation? There also are no error bars on any of the points. You have indicated that this is because the errors are smaller than the points. Is this correct, and if so, how were the errors analyzed? If indeed the errors are this small I find it quite surprising, because these are very small amplitudes, and I would expect that a lot of sampling is needed to overcome the thermal noise.

Giacomo Fiorin responded: Thank you for pointing out that the data points in the free-energy plots could be interpreted as individual umbrella-sampling simulations: in fact, those points represent the discretized bins of the free-energy profile computed from the simulated histograms using the WHAM method.

There are specific reasons why the estimated sampling error is very small. For each bilayer, the umbrella-sampling windows were initialized by extracting random snapshots of a 3 μs conventional MD simulation, and the aggregated simulation time between all windows amounts to an additional 7 μs. At the wavelength corresponding to the bilayers' lateral sizes (*i.e.* below 10 nm) the variations in bilayer curvature are tightly coupled to the variations in lipid orientation. The correlation times of the latter are of the order of nanoseconds for homogeneous phospholipid bilayers (see *e.g.* ref. 1). Therefore, even within a single block of simulated data (five blocks for each bilayer were used to estimate errors), the total simulated time exceeds the relevant correlation times by at least two orders of magnitude. Lastly, due to the stratification strategy of umbrella sampling, each simulated window was restrained to fluctuate near a given curvature, resulting in near-uniform sampling across the free-energy landscape.

1 R. M. Venable, *et al.*, *Chem. Phys. Lipids*, 2015, **192**, 60–74.

Markus Deserno said: Let me preface this question with just a little bit of "historical context". When a few years back we found out that abundance asymmetry alone, and hence differential stress, could increase a membrane's bending modulus, we got the idea that this might be related to some type of ordering in the compressed leaflet. We then looked for instance at the hexatic order parameter and found this to be true. This led to a prediction: it should be easier to order the compressed leaflet if the membrane itself is already closer to the gel transition! We tested this in a follow-up publication[1] and indeed could confirm it – at least within the limited data we could obtain back then.

But here is the sad part of this story: when we looked at published experimental results from groups who observed asymmetric membranes to stiffen, this correlation is not very apparent. For instance, Lu *et al.*[2] see only about 40% stiffening in systems where the high melting lipid (DMPC) is essentially at its gel transition, while Elani *et al.*[3] see 150% stiffening at a temperature (295 K)[4] around 24 K above that of the high melting lipid (POPC). This is not at all what we expected, and this made us worry whether our idea even holds water.

So here is my question: with your more detailed membrane model (compared to our highly coarse-grained one), have you investigated this correlation between increased ordering and magnitude of the stiffening? Do you maybe plan to do this at different temperatures, *i.e.*, distances from the main phase transition? I am asking because I want to assure you that what we had claimed back then is by no means the final word on this question. I have a sense it might be more complicated than that.

1 A. Hossein and M. Deserno, *J. Chem. Phys.*, 2021, **154**, 014704, DOI: **10.1063/5.0028255**.
2 L. Lu, *et al.*, *Soft Matter*, 2016, **12**, 7521–7528, DOI: **10.1039/c6sm01349j**.
3 Y. Elani, *et al.*, *Chem. Commun.*, 2015, **51**, 6976–6979, DOI: **10.1039/c5cc00712g**.
4 J. M. Seddon, personal communication.

Giacomo Fiorin answered: Thank you for this valuable historical note, which is both informative and helpful in framing future investigations. Coarse-grained (CG) models in general, and the MARTINI force field in particular, have known limitations in describing the transition between the liquid and gel phases. For example, the work by Salerno *et al.*[1] on long-chain hydrocarbons above their melting point has shown that MARTINI potentials differ from atomistic ones in ways that go beyond a simple reduction in the number of degrees of freedom. While developing a CG force field for lipids with a high melting point,[2] we also found that a single set of CG parameters could not simultaneously reproduce experimental properties of both the liquid and gel phases: eventually, we settled on simulating only the former. It appears that current CG models can represent the key characteristics of either phase reasonably well, but the predicted transition mechanism may still require further validation.

Regrettably, our answer to whether we correlated stiffening with curvature-induced ordering is no: due to time constraints we did not include a computation of the hexatic order parameter. We will likely use these computations in future work. However, we did examine local lipid density, another quantity influenced by molecular order, and found no correlation with curvature (see Fig. S4 and S5 in the supplementary information of the paper; **https://doi.org/10.1039/d5fd00006h**). That said, we cannot rule out the possibility that gel-phase domains may emerge over timescales or length scales beyond those explored in this study (several microseconds and ~8 nm). Given the computational cost of atomistic simulations, particularly when conducted across a range of temperatures, further work in this direction will require careful planning.

1 K. M. Salerno, *et al.*, *Phys. Rev. Lett.*, 2016, **116**, 058302.
2 C. M. MacDermaid, *et al.*, *Biophys. J.*, 2020, **118**(7), 1588–1601.

Tiemei Lu asked: Is it possible to simulate a full spherical (GUV) membrane instead of a flat membrane? (Not just patches of full vesicles.)

Giacomo Fiorin answered: In principle, MD simulations can tackle systems of any size. In practice, though, the longest simulation times attainable by typical MD simulations are limited (currently, 10–100 μs), and this limit also decreases slightly with increasing system sizes. Furthermore, the relevant correlation times of curvature deformations increase very rapidly with the bilayer size (see *e.g.* ref. 1 and related work). Therefore, attaining the necessary sampling would be very challenging for atomistic simulations of even relatively small vesicles (~100 nm), and barely approachable with coarse-grained simulations. For micrometer-sized giant vesicles, MD simulations of any kind are not possible for the foreseeable future. Instead, MD simulations can be used to study GUVs indirectly, *e.g.* by providing the parameters used in continuum-mechanics equations from first-principles computations.

1 A. G. Zilman and R. Granek, *Phys. Rev. Lett.*, 1996, **77**(23), 4788–4791.

Rumiana Dimova addressed Giacomo Fiorin and Markus Deserno: Your studies suggest that compositional asymmetry alone does not significantly alter bending rigidity when the two leaflets experience equal stress. However, introducing stress or packing asymmetry appears to increase the bending rigidity. In contrast, Elani *et al.* (2015)[1] reported a 2–3-fold increase in bending rigidity for asymmetric DOPC/POPC membranes, which is considerably higher than the modest changes observed in your work. How do you interpret this discrepancy?

I would also like to offer a speculative comment: the emulsion transfer method used for GUV preparation in Elani's study[1] may result in a very different stress asymmetry between the leaflets, which is lipid specific. Since the two monolayers are formed separately (each at its own interface), differences in interfacial tension and lipid packing may lead to unequal stresses when the bilayer is finally assembled. In addition, the emulsion-transfer method may result in some lipids intended for one leaflet migrating to the "wrong" one, as we seem to observe in our own work.[2] Could such differences in (i) the final leaflet stress and (ii) composition account for the more pronounced effect seen in their measurements compared to your simulations?

1 Y. Elani, *et al.*, *Chem. Commun.*, 2015, **51**, 6976–6979, DOI: 10.1039/c5cc00712g.
2 D. Drabik, *et al.*, *Biophys. J.*, 2024, **123**, 2406–2421, DOI: 10.1016/j.bpj.2024.05.031.

Giacomo Fiorin replied: These are valuable considerations when comparing experimental and simulated data. In the case of asymmetric POPC/DOPC vesicles, it is noteworthy that the same laboratory has used fluctuation analysis to estimate different bending moduli for asymmetric bilayers: one study reported a value 2.2 times higher than that of the symmetric mixture,[1] while another found a factor of 1.5.[2] It is noteworthy that the latter result also has a higher experimental uncertainty, and even agrees with our own estimate of 1.25. However, as we acknowledge in our paper (https://doi.org/10.1039/d5fd00006h), our estimate is not consistent with the one of Elani *et al.*[1] Given the relatively small size of the bilayers in our simulations, our initial approach to reconcile this discrepancy has been to explore the potential influence of length scale.

Your first point suggests that, in the study by Elani *et al.*,[1] the densities of the two leaflets may be influenced not only by the intrinsic biophysical properties of the lipids but also by the specifics of the preparation method – implying that the vesicles could be subject to significant curvature strain. This appears to be a plausible interpretation, although we lack sufficient technical insight into the transfer method used to speculate further. It is important to note, however, that MD simulations can be biased in a similar way due to periodic boundary conditions, which impose a zero mean curvature. If the asymmetric bilayer configuration observed by Elani *et al.*[1] could be reproduced in a future simulation, it might require either a higher degree of leaflet asymmetry than we have considered or the introduction of non-zero membrane tension.

We appreciate your observation regarding potential spurious lipid transfer between leaflets and the insertion of oils, as suggested in your work.[3] While it is difficult to predict the precise effect of a partial loss of asymmetry, it could plausibly soften the bilayer, thus increasing the discrepancy between experimental measurements and simulations and reinforcing the importance of understanding its cause. Moreover, the increased bilayer thickness and reduced interdigitation due to oil insertion present additional questions that are worth investigating.

Finally, although we have not yet examined bilayers with charge asymmetry, your findings on POPC/POPA systems offer a potentially valuable benchmark – while recognizing the limited correlation between area modulus (K_A) and bending modulus (k_c).

1 Y. Elani, *et al.*, *Chem. Commun.*, 2015, **51**, 6976–6979, DOI: 10.1039/c5cc00712g.
2 K. Karamdad, *et al.*, *Chem. Commun.*, 2016, **52**, 5277–5280, DOI: 10.1039/c5cc10307j.
3 D. Drabik, *et al.*, *Biophys. J.*, 2024, **123**, 2406–2421, DOI: 10.1016/j.bpj.2024.05.031

Markus Deserno answered: Let me start with the Elani work.[1] It is true that we never saw a stiffening driven by compositional asymmetry alone, *i.e.*, in the absence of differential stress. But we have no proof either that Elani *et al.*[1] ever did. Recall that we simply do not know the differential stress in their experiments; but as we had explained in our original paper,[2] and as I have revisited in my earlier answer to a question of Dr Heerklotz, the very fact that Elani *et al.*[1] actually could observe stable fluctuating GUVs in their experiments suggests that their bilayers are under a differential stress of a few mN m^{-1} in order to cancel the spontaneous curvature torque.

I agree that the extent of stiffening observed by them is larger than what we see in our simulations, and there is an easy and a more complicated take on this. The easy answer is that I am not surprised that we see less stiffening, considering that we attribute this effect to a change in lipid order, and our highly coarse-grained lipids have much less entropy to lose than an actual lipid. It is not uncommon that order-affiliated phenomenology is underestimated in coarse-grained models, just as the differences in diffusion constants between liquid and gel phases, or liquid ordered and disordered phases, is much less pronounced at that level. The more complicated answer is that our original hypothesis ("ordering of the compressed leaflet drives stiffening") makes a clear prediction ("stiffening should be more visible if the high melting lipid is closer to its main phase transition") which does *not* appear to be borne out by the available experimental studies. It is therefore quite possible that we have not yet figured out the main reason why such membranes stiffen.

Regarding the comment that the specific protocol for making the asymmetric vesicles might create additional lipid-specific sources of differential stress: yes, this is definitely possible, and we had in fact thought about this for a while. In the end, my take is that the details of the creation process are likely very messy, and trying to theoretically understand at a quantitatively predictive level what kind of additional stresses might sneak in during the complex non-equilibrium emulsification process might be too difficult for us to explain with our current state of understanding. In contrast, it is much easier to summarily reason about the final outcome, and this leads to something I facetiously call the "anthropic principle" of asymmetric vesicle creation. Recall that in philosophy and cosmology the anthropic principle typically goes as follows: we might ask why our universe is "set up" in such a miraculously "fine-tuned" way that it supports the evolution of intelligent life. And one disarmingly disappointing answer to this is: "Well, if it weren't, nobody would be around to ask that question." So in our GUV creation example we could ask: how is it that the very messy production process [insert your favorite protocol] gives rise to stable gently fluctuating asymmetric vesicles which therefore have to be under close to vanishing torque? And a possible answer is: "If it didn't, we wouldn't be talking about this production process, because it wouldn't create objects of interest." In short, I believe whatever complicated processes are going on that preferentially partition lipids or affect tension, torque, and differential stress, if at the end of the day we get stable GUVs, then these GUVs must satisfy the conditions conducive to their own existence (such as, I submit, close to vanishing torque).

1 Y. Elani, *et al.*, *Chem. Commun.*, 2015, **51**, 6976–6979, DOI: **10.1039/c5cc00712g**.
2 A. Hossein and M. Deserno, *Biophys. J.*, 2020, **118**, 624, DOI: **10.1016/j.bpj.2019.11.3398**.

Michael M. Kozlov opened discussion of the paper by Axel Voigt: How can one estimate the values of the elastic coefficients of the higher order contributions to the energy?

Axel Voigt replied: To the best of my knowledge the bending rigidities of the higher order terms have not been measured. An estimate has been considered in ref. 1, where the term related to $\mathscr{K}^2$ is used to model periodic structures. This requires $k_{4,2}$ to be negative, which is stabilized by an even higher order term related to $\mathscr{K}^4$ with a positive coefficient. The same approach is used in ref. 2 to model neck formation. In our context we assume $k_{4,2}$ to be positive and to have a stabilizing effect. Instead of favouring the formation of saddles, we favour developable surfaces, surfaces with zero Gaussian curvature.

1 R. Goetz and W. Helfrich, *J. Phys. II*, 1996, **6**, 215–223.
2 T. Shemesh, *et al.*, *Biophys. J.*, 2003, **85**, 3813–3827.

Michael M. Kozlov said: If one compares your expression for that energy with that presented in Marin Mitov's 1978 paper,[1] one wonders why in the expression you are using the coefficients of the higher order terms depend on the spontaneous curvature.

1 M. D. Mitov, *C. R. Acad. Bulg. Sci.*, 1978, **31**, 513–515.

Axel Voigt responded: In ref. 1 an elastic energy as a function of director derivatives is considered and expanded leading to the higher order terms. A spontaneous curvature is not explicitly considered in this setting. We take a functional form which depends on the mean and the Gaussian curvature and consider a Taylor expansion at the spontaneous curvature. While setting the spontaneous curvature to zero leads to the same terms also considered in M. Mitov,[1] the methodology is different. To some extend M. Mitov considers a "physical correction" and we a "geometric correction" to the small curvature assumption in the classical Canham/Helfrich model.

1 M. D. Mitov, *C. R. Acad. Bulg. Sci.*, 1978, **31**, 513–515.

Markus Deserno said: Dr Kozlov expressed worries about using some spontaneous mean curvature as the expansion point also for higher order terms. I am not so terribly worried about that – of course you could decide to write these terms in this form, and it would amount to certain choices for some of the expansion coefficients, or how some cubic terms show up. As long as all terms contributing to any given order are present, this just amounts to "renaming" the various moduli. But in your expression all terms are *not* present, which then amounts to nontrivial statements about the underlying physics, and I was wondering what your thought process was for leaving out certain terms. For instance, you do not have a cubic term of the form $\mathscr{H}^3$, and you also do not have a term of the form $\mathscr{K}(\mathscr{H}-\mathscr{H}_0)^2$, which is quartic. I presume you made some conscious choices here – can you help me understand what considerations went into this?

Axel Voigt responded: We agree that there are additional terms with the same order. A complete list of third and fourth order terms can be found in ref. 1 or 2. We derive the higher order terms by a Taylor expansion of the bending energy density as a function of the mean and the Gaussian curvature at the spontaneous curvature. We only keep terms which are bounded from below. This excludes all odd geometric orders, *e.g.* $\mathscr{H}^3$ but also the quartic term $\mathscr{K}(\mathscr{H}-\mathscr{H}_0)^2$, as the mean and the Gaussian curvature also can be negative. While in different context such terms are considered to initiate instabilities, we are interested in stabilizing effects of the higher order terms. Therefore all higher order bending rigidities are nonnegative and only nonnegative terms in the expansion are considered. Including the missing terms would require to specify certain relations between the bending rigidities in order to guarantee boundedness from below.

1 M. D. Mitov, *C. R. Acad. Bulg. Sci.*, 1978, **31**, 513–515.
2 M. Deserno, *Chem. Phys. Lipids*, 2015, **185**, 11–45.

Gonen Golani enquired: Lower-order terms such as membrane tension, which can arise from pressure differences, appear to be absent from the energy considerations at equilibrium. These terms could stabilize cylindrical shapes, as observed in tether-pulling experiments. How would the introduction of membrane tension or a pressure difference affect the results?

Axel Voigt replied: As the membrane is considered to be inextensible (locally area preserving) an additional membrane tension does not have any effect. The pressure already serves this role, here it is the Lagrange multiplier to enforce the inextensibility constraint.

John M. Seddon asked: I assume that the modulus $k_{4,2}$ is positive in sign, and that this favours parabolic surfaces in general, rather than specifically cylindrical tubes?

Axel Voigt answered: Yes, in our investigation (**https://doi.org/10.1039/d4fd00202d**) we only consider terms which guarantee boundedness from below. Therefore $k_{4,2} \geq 0$. Without any constraints this would lead to smooth surfaces whose Gaussian curvature vanishes everywhere, so-called developable or ruled surfaces. Cylindrical tubes belong to this class. However, due to the constraints on inextensibility and enclosed volume such surfaces cannot be realised for any closed surface.

Markus Deserno questioned: You mentioned that you are not just interested in energy-minimized shapes (*i.e.*, solution to the shape equation) but also the time evolution of shape transformations. Your dynamical equations indeed contain an in-plane membrane viscosity, but there are other sources of dissipation as well. The viscosity of the embedding solvent of course comes to mind, but the friction between the two leaflets – which might have to slide past one another as the vesicle deforms – is another. Do you have any plans on incorporating them, or alternatively a good reason why you can ignore them?

Axel Voigt responded: Indeed there are other dissipation mechanisms. Our goal was to consider a minimal model, which puts the effect of surface viscosity and higher order curvature terms in the bending energy into the focus. Any coupling with the surrounding bulk phases (the embedding solvent) is neglected. This is justified in the limit of a large Saffman–Delbrück number. As the viscosity of the membrane is typically larger than the viscosity of the bulk phases, this is typically the case. To neglect interleaflet friction is probably less justified. An "effective" membrane viscosity, expressed by the Reynolds number Re, or the general friction term with the friction coefficient γ probably provide zeroth order approximations. A better approximation requires to model both leaflets, approaches in this direction can be found in ref. 1 building on ref. 2.

1 A. Torres-Sanchez, *et al.*, *J. Fluid Mech.*, 2018, **871**, 218–271.
2 U. Seifert, *et al.*, *Europhys. Lett.*, 1993, **23**, 71–76.

Markus Deserno commented: It would seem that inter-leaflet friction would require the explicit modeling of two leaflets, but I believe there is a way around that: the parallel surface theorem allows us to calculate the local area difference between the two leaflets as a function of local curvatures, and I presume a little bit of differential geometry will then show that the local inter-leaflet strain rate scales like the time evolution of curvature (or curvature gradients). I suspect this could be put into your model without too much extra effort. It is conceivable, though, that this does not greatly contribute at the large scales you appear to be interested in.

Axel Voigt replied: This sounds like a meaningful and numerically feasible approach worth exploring.

Di Jin asked: How would you interpret Reynolds number in terms of membrane properties and what is the typical range of Reynolds number, and what does it imply?

Axel Voigt answered: By interpreting membranes as fluid deformable surfaces, essentially two-dimensional incompressible fluids with bending properties, the Reynolds number follows the same definition as in classical fluid dynamics. It relates to the membrane viscosity which has been measured for various bilayer systems, see ref. 1, *e.g.* for DPPC : DOPC : Chol (1 : 1 : 1) the reported membrane viscosity is 17.7 ± 3.06 nPa s m. Together with low fluid velocities in the membrane and small length scales this leads to low Reynolds numbers. All numerical simulations are therefore considered in a regime which also would allow for a Stokes approximation.

1 H. A. Faizi, R. Dimova and P. M. Vlahovska, A vesicle microrheometer for high-throughput viscosity measurements of lipid and polymer membranes, *Biophys. J.*, 2022, **121**, 910–918.

Michael M. Kozlov opened a general discussion: A differential stress of a few mN m^{-1} should be able to drive formation of membrane pores and relaxation of the stress difference due to the trans-monolayer lipid exchange through these pores. Do you think this may happen?

Rumiana Dimova answered: In principle, I would expect that a differential stress of a few mN m^{-1} could indeed be sufficient to drive pore formation and thereby allow for trans-monolayer lipid exchange to relax the stress difference. However, I am not aware of direct experimental evidence confirming this mechanism.

I suppose that some of the most widely used experimental methods for creating asymmetric membranes, such as leaflet exchange *via* hemifusion or individual lipid exchange assisted by cyclodextrin, likely proceed without introducing large differential stresses. In contrast, the phase-transfer method, where two monolayers formed at oil–water interfaces are combined into a bilayer, could intuitively result in significant differential stress due to differences in the initial interfacial tensions of these monolayers.

Our own experience with this method, especially when using charged lipids, suggests that the resulting asymmetric GUVs are indeed less stable compared to their symmetric counterparts.[1,2] Unlike symmetric GUVs, these asymmetric vesicles tend to rupture spontaneously over time. Furthermore, we have observed that the pore edge tension of these asymmetric membranes is lower,[2] and they are prone to collapse when subjected to external stresses such as electric fields.

These observations are consistent with the idea that differential stress may indeed promote pore formation, but to my knowledge, a direct, real-time observation of stress relaxation *via* pores in this context is still lacking.

1 D. Drabik, *et al.*, *Biophys. J.*, 2024, **123**, 2406–2421, DOI: 10.1016/j.bpj.2024.05.031.
2 F. S. C. Leomil, *et al.*, *Langmuir*, 2024, **40**, 4719–4731, DOI: 10.1021/acs.langmuir.3c03370.

Conflicts of interest

There are no conflicts to declare.