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DISCUSSIONS

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Behaviour and interactions of proteins and peptides with and within membranes; from simple models to cellular membranes: general discussion

Mibel Aguilar, Kareem Al Nahas, Francisco Barrera, Patricia Bassereau, Margarida Bastos, Paul Beales, Burkhard Bechinger, Boyan Bonev, Izabella Brand, Amitabha Chattopadhyay, William DeGrado, Patrick Fuchs, Ana J. Garcia Saez, Bart Hoogenboom, Shobhna Kapoor, Paula Milán Rodríguez, Justin Molloy, Paul O'Shea, Georg Pabst, Sreetama Pal, Amy Rice, Aurelien Roux, John Sanderson, John Seddon, Lukas K. Tamm and Aishwarya Vijayakumar

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Aishwarya Vijayakumar opened the discussion of the paper by Shobna Kapoor: Does the charge of the lipids on the membrane influence its interaction with the cytoskeleton?

Shobhna Kapoor responded: Yes, the charge of the lipids on the membrane does influence its interaction with the cytoskeleton. In our work (DOI: 10.1039/d0fd00051e), some mycobacterial lipids had no net charge at physiological conditions and these lipids did not affect the membrane adhesion energy (implying no changes to the membrane–cytoskeletal interactions). However, these did influence the actin morphology re-distribution. Most likely, the electrostatic interactions between bacterial lipids and the host cell membrane/actin network underneath it play a role in the selective recruitment of host proteins and lipids, impacting the membrane–cytoskeletal interactions.

Paula Milán Rodríguez asked: What is the lipid transfer mechanism from the bacterial membrane to the host membrane?

Shobhna Kapoor replied: There are two possible mechanisms of lipid transfer from the bacterial surface to the host cell membrane. First is *via* the transfer of exogenous lipids within extracellular vesicles (EVs) released by the bacteria. The second is *via* direct lipid transfer upon direct contact of the bacteria with the host cell. At the moment, which of these is at play for the transfer of mycobacterial lipids to the host cell is not known. One way to approach this issue is by the use of double labeled bacteria, wherein the bacteria are labeled with a fluorophore and

the lipids are also labelled with an orthogonal fluorophore. The absence of the lipid signal in the bystander cells (which have just the bacteria signal) would argue in favor of the EV mediated transfer of lipids from the bacterial surface to the host cell.

Patricia Bassereau commented: Which type of actin-related process is triggered eventually?

Shobhna Kapoor answered: Migration and phagocytosis/endocytosis are among the most applicable actin-related host processes impacted eventually by exogenous bacterial lipids. This has indeed been shown with some mycobacterial lipids. Other processes that could be implicated are autophagosome maturation and formation,[1] wherein actin has been recently shown to play a critical role. Thus, modulation of these processes would work in the favor of the pathogen to either foster its host uptake or enhance its survival by blocking the autophagosome maturation.

1 K. Zientara-Rytter and S. Subramani, *Autophagy*, 2016, **12**, 2512–2515.

Patricia Bassereau remarked: Could it trigger some activation of CDC42?

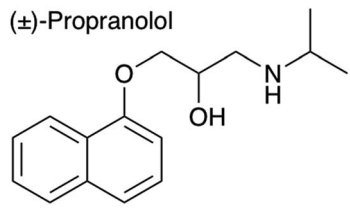
Shobhna Kapoor replied: There is a possibility for the activation/modulation of the activity of CDC42 GTPase in mycobacterial host cross talk, as CDC42 is implicated in actin nucleation *via* the ARP3–WASP pathway. However, direct proof for the same by mycobacterial lipids has not been shown. We think that global host cell proteomics in the presence of mycobacterial lipids might shed some light on these issues.

Shobhna Kapoor commented: The changes in the cellular actin cytoskeleton are associated with tether force in general and we see the same with our exogenously added mycobacterial lipids. Specifically, we have observed the host actin to be re-distributed between patches, filaments and punta in presence of mycobacterial lipids, impacting tether force distribution. However, the contribution of each actin morphology on the tether force is not known. For this, we would have to move to model systems.

William DeGrado opened a general discussion of the paper by John Sanderson: Have you looked at many histidine containing peptides? Can histidine residues in peptides serve as catalysts of the reaction by forming acyl imidazoles that then transfer to Lys?

John Sanderson answered: We haven't looked at many, but we have some circumstantial evidence that His is acylated so it is likely that there is some transfer onwards. It will be very structure dependent though. When we look at the lipidation of propranolol, which has both a secondary alcohol and a secondary amine which are well placed for intramolecular transfer (the structure is presented below) we find that lipidation on the oxygen forms an ester, but the process stops at that point as long as the O-lipidated propranolol is embedded in the membrane. If we chemically synthesise an authentic sample of *O*-palmitoyl or

O-oleoyl propranolol, if we are not careful the acyl group migrates to the nitrogen before we get the material into the NMR spectrometer – it is very facile, as you would imagine. As the *N*-acyl product is an amide, it is effectively irreversible in these conditions. It therefore appears that propranolol is O-acylated in the membrane by virtue of its penetration depth and orientation favouring this nucleophilic site. The *O*-acyl product is isolated by virtue of being embedded in the membrane, which prevents the migration from occurring, presumably for steric reasons. It is reasonable to expect that similar arguments would apply for a lipidated peptide, with the caveat that there is more conformational space to explore for a potential acyl group acceptor, so there may be some scenarios where acyl group transfer is possible.



William DeGrado asked: Do you think it is possible that the fatty acid acyl group might transfer from the acylated imidazole to the hydroxyl of cholesterol?

John Sanderson replied: Yes, that is certainly possible in theory. We have seen lipidated products that we think are lipidated on His, but these have been difficult to characterise. They have the retention time properties expected for a lipidated peptide, but fragment in-source very easily in the mass spectrometer. However, when we look at all the potential lipidation sites, we can rule out many of them apart from His because we have seen them elsewhere (identified by tandem MS approaches). So we have circumstantial evidence that His is acylated, but not concrete proof. As for the transfer to cholesterol, I have looked through old data sets to try to see whether there is any evidence of cholesteryl esters. I have not been able to find any, but then neither the chromatography nor the MS conditions were optimised for cholesteryl esters (lipidated peptides elute earlier than lipids, and cholesteryl esters are liable to in-source fragmentation). It is something we will look for at some point, because we also want to rule out (or in) transfer from a cholesteryl ester to a peptide, which may be something that happens in lipid droplets or lipoproteins. There is some evidence that amyloid peptides can associate with lipoproteins, and many real (*ex vivo*) amyloid deposits contain cholesterol and cholesteryl esters, so this might be a relevant route for them to be lipidated.

Amy Rice asked: Is there evidence of the back transfer of acyl chains from the lipidated peptides to lysolipids or is this transfer mostly irreversible?

John Sanderson responded: It is certainly irreversible for the transfer to amino groups (Lys, N-terminus) as the products are amides. Transfer to Ser is reversible in principle, but we have not examined it to date. I have looked over some of our

old data to see whether there is any evidence of *e.g.* acyl group scrambling or formation of cholesterol esters, but I have not found any evidence – although those experiments were not optimised for detecting those products.

Mibel Aguilar remarked: Melittin acts very quickly – and you observed the lipidation over 24 hours. Could this be a reversible reaction which occurs in cells transiently?

John Sanderson responded: The rate is indeed slow relative to the membrane lytic activity. Lipidation could be transient, especially if there is an acylase in the cell that can reverse lipidation. This kind of acylase activity is something that we are actively looking for at the moment. Transient lipidation might also feature in enabling some peptides to cross membrane boundaries more easily.

Francisco Barrera queried: Is it known if the acylation process that you describe occurs in cellular membranes? If that was the case, it would maybe imply that membrane proteins get slowly acylated, in a process that could constitute a sort of "membrane protein aging". However, maybe cellular enzymes (deacylases) could selectively revert this process?

John Sanderson answered: We have not looked at peptides – there are some challenges to doing that – but we have looked at small organic molecules, including the drug propanolol, a beta blocker.[1] Propranolol has two potentially reactive sites – a secondary alcohol and a secondary amine. We conducted initial experiments in simple single-component liposomes and established that there was indeed lipidation of this drug. We synthesised four authentic lipidated propranolol analogues (palmitoyl and oleoyl derivatives at each of the alcohol and the amine groups) and used these to find the best conditions for extracting the lipidated propranolol from the membrane mixture. We then incubated propranolol with liposomes made from commercial liver extracts and verified that we saw a series of lipidated products, and verified that we could extract them. Finally we administered propranolol to liver cells and extracted the cells after 24 h and saw a similar series of lipidated products. The challenges with doing this with peptides like melittin include the potential for degradation by peptidases, but also the problem with handling a large number of controls. We know that melittin lipidation occurs on at least 5 locations, so to repeat the process that we used for propranolol, we would need 10 different synthetic analogues as controls. There are also challenges to proving where the acyl group comes from *in vivo* because you have other good sources of acyl groups, most notably coenzymes (thioesters). We were on the point of doing experiments to try to resolve this when the first lockdown happened. We had loaded cells with isotopically labelled oleate and planned to used CoA synthetase inhibitors to do isotope chasing experiments.

We have also looked at proteins. There are some proteins in locations where you might expect this kind of acyl modification to accumulate because the proteins are not turned over. An example of this is aquaporin 0 (AQP0) in lens fiber cells. Because of the role they perform, these post-mitotic cells have many of their organelles removed (because they scatter light) and proteins such as AQP0 are not turned over – the AQP0 in the nucleus of your lens has been there since birth. When we look at the lipidation of AQP0,[2] we find all the hallmarks of this

lipidation process: lipidation occurs at two sites that are proximal to the membrane interface and not predicted as lipidation sites by known consensus sequences. Lipidation is also complete, and there is a series of lipidated products found, with a relative abundance in line with the cell membrane fatty acid composition. So, we think that AQP0 is lipidated by this process, but the issue raised above – potential lipidation by CoA derivatives – has not been ruled out yet. There are other proteins that have caught our attention, including surfactant protein C in lung surfactant – again a location where there may not be means to recycle or correct lipidated proteins. SP-C is lipidated on Cys residues, again with a pattern that is unusual. In this case, the product is mostly palmitoyl – which is expected as DPPC is a major component of lung surfactant.

The typical half-life for a protein is about 100 h, so there is time for acyl modifications to accumulate. So, in principle, the cell may correct for these by recycling the protein, or by reversing the acylation. There are some enzymes, such as some sirtuins for example, that have very broad substrate specificity and may fulfil this role. This hypothesis is developed more fully in the *BioEssays* article.[3] In that article, I have also hypothesised that the lipidation of amyloid peptides is a mechanism to drive these peptides into conformations that are on-pathway for fibril formation. There are some arguments in favour of this: in parallel to our work with melittin, many amyloid peptides adopt amphipathic helices in the membrane; experiments with amyloid peptides are typically done with similar peptide to lipid ratios to ours; the lag phase has kinetics similar to our lipidation kinetics; and lipidation would be expected to be sensitive to changes in the lipid profile and/or peptide sequence, which can account for the spatial and temporal difference in amyloid nucleation.

1 H. M. Britt, C. A. García-Herrero, P. W. Denny, J. A. Mosely and J. M. Sanderson, *Chem. Sci.*, 2019, **10**, 674–680.
2 V. S. Ismail, J. A. Mosely, A. Tapodi, R. A. Quinlan and J. M. Sanderson, *Biochim. Biophys. Acta Biomembr.*, 2016, **1858**, 2763–2768.
3 J. M. Sanderson, *BioEssays*, 2020, **42**, 1900147.

Sreetama Pal asked: Do you expect the melittin lipidation events to mature or coalesce into the peptide acting as a lipid-solubilizing detergent (as reported earlier[1,2]), especially if you keep decreasing the lipid-to-peptide molar ratio?

1 A. Therrien, A. Fournier and M. Lafleur, *J. Phys. Chem. B*, 2016, **120**, 3993–4002.
2 A. Therrien and M. Lfleur, *Biophys. J.*, 2016, **110**, 400–410.

John Sanderson responded: There are two points here. First, we can measure an apparent CMC for the peptide, so there is every reason to believe that it will have detergent-like properties. But at some point you will fall below the CMC if you decrease the concentration, and at that point I would expect the peptide to remain as a peptide anchored in the membrane by the acyl group. It is also worth remembering that the lipidation by-product is a lysolipid, so the situation with regard to detergent activity is a little more complex. Second: our peptide to lipid ratios are quite high for pragmatic reasons. If we use a lower P : L, it becomes harder to characterise the peptide in the excess of lipid. We have, however, run experiments at 1 : 100, below the critical concentration for toroidal formation, and seen a similar lipidation process. I should add that we generally load our

sample onto the LC column without any treatments so that we know we are not losing any lipidated material during chemical extraction.

Paul Beales remarked: Could you take a peptide and predict where lipidation might occur or not? Or is this still an empirical observation on a case by case basis?

John Sanderson responded: We found this process because we doing some work with Alison Rodger on the kinetics of melittin binding to liposomes and found that equilibrium was hard to attain.[1] This lipidation emerged from trying to understand that. Prediction is currently challenging, but I have just started a project to examine this. At the moment, the location at the hydrophobic/hydrophilic boundary seems to be critical. This has not generally been picked up for a couple of reasons: first, no-one has been looking for it, and second, you cannot predict the process on the basis of a consensus sequence – it depends on how something sits in the membrane. Another thing we know is that when you lipidate Lys21 or Lys23, the peptide no longer digests with trypsin – so if you were doing a trypsin digest of a membrane protein, you have a transmembrane sequence that is lipidated, making it more hydrophobic, and that will either form a large insoluble fragment, or elute off the end of your LC gradient.

1 A. Damianoglou, A. Rodger, C. Pridmore, T. R. Dafforn, J. A. Mosely, J. M. Sanderson and M. R. Hicks, *Protein Pept. Lett.*, 2010, **17**, 1351–1362.

Sreetama Pal asked: Do you observe any lipid dependence in the lipidation-mediated peptide folding? Since peptide folding during or after membrane adsorption would include favorable hydrogen-bonding interactions of the peptide, membrane, and surrounding water molecules, it might be interesting to specifically explore the role of lipids that are prone to participating in hydrogen bonds (such as PE) or influencing hydration dynamics (such as PG or other negatively charged lipids).

John Sanderson replied: In terms of the folding, that is not something that we have looked at. Our work (DOI: 10.1039/d1fd00030f) used a synthetic peptide obtained commercially with fatty acyl chains at either the N-terminus or the side chain of Lys23. We looked at the folding of this in the absence of membranes, but it would be interesting to know whether the folding changes if the peptide is anchored in the membrane by the fatty acyl group. It is notable, though, that we do see transfer of a second and sometimes a third acyl group to the peptide in some circumstances, and these transfers occur preferentially at the same locations (N-terminus, Lys23), so my guess is that the membrane-associated singly lipidated peptide is still helical. We have looked at the effects of components like PE on lipidation, and generally we see transfer from both PE and PC in the ratio you would expect based on the composition, but with an enhanced rate. In some cases, such as DOPC/DPPS, we have only observed transfer from one component. In PC/PG mixtures, we see transfer from both components.[1]

1 R. H. Dods, J. A. Mosely and J. M. Sanderson, *Org. Biomol. Chem.*, 2012, **10**, 5371–5378.

Paul Beales said: Could there be specific nearest neighbours that would increase the likelihood of an amine being lipidated? Normally, the amine would be in a hydrophilic residue that often sits away from the membrane. For it to sit at the hydrophilic–hydrophobic interface, are you looking for a lysine to be close to a few hydrophobic amino acids? While it is not possible to fully predict, might local residues be used to score a likelihood that a lysine (or other amine residue) might get lipidated? This could then be used in bioinformatics approaches to seek other peptides of potential interest.

John Sanderson replied: It is possible that some neighbouring residues could transfer on the acyl group. So, for example, initial transfer from the lipid to a His or Ser may then lead to subsequent intramolecular transfer to a second residue. As this would be through space you would need to know both the sequence relationship between the two groups, and the structure type (helix *vs.* sheet). It should be possible to predict those, however. Propranolol is an interesting case here, as if we make *O*-acyl propranolol *in vitro*, intramolecular O to N acyl migration occurs before we can analyse it – it is very fast. In membranes, transfer from the lipid to the alcohol occurs, but there is not subsequent transfer to the nitrogen – the intramolecular transfer is inhibited, presumably for steric reasons within the membrane. This highlights that local structural effects, including how the lipidated molecule partitions, are likely to be crucial.

Izabella Brand enquired: May lipopolysaccharides undergo diacylation and trigger acylation of a peptide? Does the acylation of the peptide depend on the pH? Are any amino acids/amino acid sequences particularly sensitive to acylation?

John Sanderson answered: The acyl group transfer process occurs from ester groups on the lipid. So, to use lipid A as an example, in principle there are four ester groups that could serve as the source of an acyl group. It is also possible – again in principle – that an alcoholic group of lipid A is transiently lipidated before passing the acyl group on to a suitable acceptor. Transfer from the amides of lipid A would not be expected to occur. We haven't addressed the pH dependence of the process – we've generally used physiological pH for the bulk medium. Changes in pH could have complex effects on the process. First, the charged ammonium forms of the Lys side chain and the N-terminal amino group are not reactive, so you might expect the rate of transfer to these sites to slow down at a low pH, particularly for the N-terminal amino group, which has a pK_a in bulk solution closer to the physiological pH. However, the effective pH in the membrane interface is not the same as that of the bulk solution, so it is not trivial to understand the effects of changes in the bulk pH on the ionisation state of the peptide and its net orientation in the membrane interface. You might also expect there to be changes in the rate determining step of the process at low and high pH (based on studies of aminolysis and transesterification in bulk solution, mostly by Jencks and Bruice[1,2] in the 1960s to 1980s), so it may well be the case that the overall rate of transfer to all sites (including the serine side chain) changes fundamentally at extremes of pH. The effects of pH are on our list of things to look at, specifically with regard to the reactivity in endosomes, as it looks like some drugs get trapped in these organelles, and lipidation may be a cause, or outcome, of that.

So far, most of the lipidation that we have seen has been to amino groups (N-terminus, Lys side chain) and hydroxyl groups (Ser). We also think that lipidation of the His imidazole occurs, as well as possibly also the guanidinium of Arg. In the latter two cases, the evidence comes from the retention times of the products, combined with a high tendency to fragment in-source in MS analyses, alongside other sequence considerations (*i.e.* we have identified all the other possible products already). We also anticipate that the thiol of Cys will be lipidated, but we have not been able to see that yet.

1 A. C. Satterthwait and W. P. Jencks, *J. Am. Chem. Soc.*, 1974, **96**, 7018–7031.
2 T. C. Bruice and S. M. Felton, *J. Am. Chem. Soc.*, 1969, **91**, 2799–2800.

Paul Beales queried: To follow on from the sensitivity to the lipid composition, does that mean melittin will get lipidated in some cell membranes but not others? Could this have a role in selectivity for different cell types?

John Sanderson replied: For interfacial helices such as melittin, it may be possible to develop methods to predict likelihood based on neighbouring residues and predictions of amphipilicity. These will need to be nuanced though if they are to predict the difference in reactivity according to membrane composition. We have seen variations in melittin reactivity. The reactivity is higher in PC membranes containing cholesterol. In mixtures of PC with PS, PG or PE, reactivity is generally higher than that in PC alone, but there are some quirks. So, for example, in a membrane composed of DOPC and DMPG (4 : 1), we see transfer from both lipids. In DOPC/DPPS (4 : 1), we only see transfer from the oleoyl component.[1] This could lead to selectivity in different cell types according to differences in the lipid composition, or even in one cell type under different stress conditions. We have proposed this as one method by which amyloid peptides may be lipidated under specific circumstances if the membrane composition permits it.

1 R. H. Dods, J. A. Mosely and J. M. Sanderson, *Org. Biomol. Chem.*, 2012, **10**, 5371–5378.

Georg Pabst asked: Can you comment on the role of Mel partitioning in different membrane systems? Your experiments (DOI: 10.1039/d1fd00030f) used lipid concentrations (50 μM) where this should be important. In other word, have you repeated the experiments at different lipid concentrations? For example, you could go to high lipid concentrations ($\sim >$ a few mM) and repeat the experiments at the same peptide to lipid ratio. Under such conditions, effects from differential partitioning between your lipid systems should be insignificant (all Mel should be bound). Possibly, this could even speed up peptide lipidation in the time range, and maybe then this effect becomes relevant for the biological activity.

John Sanderson answered: We have looked at the effect of different peptide to lipid ratios, but not the effect of absolute concentrations. However, the data we have for cholesterol are very interesting.[1] Melittin binds to POPC/cholesterol with a lower affinity than POPC, yet the rate of lipidation increases, alongside a change in selectivity away from N-terminal lipidation towards lipidation on the side chain of Lys23. This is ultimately because the "on" rate is not rate-limiting in the process, even when the binding is weak. I do agree though that increasing the

absolute concentration will lead to a high percentage of the peptide being bound, which will simplify analysis of the kinetics.

1 H. M. Britt, J. A. Mosely and J. M. Sanderson, *Phys. Chem. Chem. Phys.*, 2019, **21**, 631–640.

Boyan Bonev enquired: Does the lipidation of melittin benefit from PLA release from lipids?

John Sanderson responded: Probably. It is certainly the case that any formation of a lysolipid by PLA in an otherwise perfect membrane will promote initial lipidation. We looked at bee venom melittin, which is contaminated with PLA2, and found that there was significant reactivity with all components of the membrane. The lysolipid formed by PLA2 dominated though, as the rate of the phospholipase reaction is much faster than that of the lipidation reaction.

Lukas K. Tamm said: It is well known that peptides such as melittin change orientation in the membrane depending on their concentration or the degree of hydration of the lipid bilayer. Could these effects also influence the degree of acylation as a result of the position and orientation of the peptide in the membrane?

John Sanderson responded: Yes, indeed that may be the case. To date, we have not systematically studied the effects of hydration and concentration. Our concentrations tend to be defined by what we can study analytically, so if our peptide to lipid ratio drops much below 1 : 100 the analysis becomes more challenging because the peptide signals are swamped by the lipid. We do not perform any extraction on the peptide–lipid mixtures to avoid components (*i.e.* lipidated peptides) being lost during the process. The untreated mixture is loaded onto the LC column. However, you make a good point and we will look at the effects of absolute concentration in the future, which will affect the bound melittin ratios. There is some empirical evidence that hydration levels alter lipidation, as samples prepared at low hydration between glass slides for solid state NMR appear to be stable when refrigerated for long periods, whereas preparations with liposomes in excess water are less stable.

Sreetama Pal commented: Regarding the influence of cholesterol in the membrane interaction of melittin behavior, there have been some studies showing that although melittin binding to membranes decreases in the presence of cholesterol, cholesterol preferentially interacts with the tryptophan residue of melittin.[1,2] This could be one reason behind your observations that melittin lipidation in the presence of PC/Chol membranes is higher than that in PC membranes.

Having said that, I would expect any effect of cholesterol on melittin lipidation to not be dependent solely on the binding/affinity parameters, since cholesterol is also known to influence a range of local and global membrane physical properties and there could be a combinatorial effect of these factors.

1 H. Raghuraman and A. Chattopadhyay, *Biophys. J.*, 2004, **87**, 2419–2432.
2 P. Wessman, A. A. Strömstedt, M. Malmsten and K. Edwards, *Biophys. J.*, 2008, **95**, 4324–4336.

John Sanderson responded: Indeed, it is known that cholesterol decreases the affinity of the peptide for the membrane and changes the penetration depth of the peptide – there are red edge excitation shift data that demonstrate this, which we cited in our paper.[1] The penetration depth, and probably also orientation, are responsible for the shift in selectivity away from the N-terminus towards Lys23. The enhanced rate is most likely a consequence of increased water penetration to the carbonyl region where the reaction occurs. Ultimately, several things are contributing to the process – some are mechanistic (physical organic chemistry) relating to water involvement in the rate determining step; others relate to the biophysics of peptide absorption into the membrane interface – the preferred depth and orientation *etc.* Binding affinity is not really a factor, exemplified by the finding that even low molecular weight organic molecules with little membrane affinity can be lipidated.[2]

1 H. M. Britt, J. A. Mosely and J. M. Sanderson, *Phys. Chem. Chem. Phys.*, 2019, **21**, 631–640.
2 H. M. Britt, A. S. Prakash, S. Appleby, J. A. Mosely and J. M. Sanderson, *Sci. Adv.*, 2020, **6**, eaaz8598.

Boyan Bonev asked: Perhaps the proton chemical shift may turn in something from carbon 2.

John Sanderson replied: Yes, that is not something that we have considered, but there should be a significant difference between ester *vs.* amide *vs.* acid. It is known that the ^{13}C shift of the free fatty acid varies with pH, which is a tool we could use to examine the pH close to the interface. I am unsure whether the shift at the 2-position also has pH dependent shifts.

Paul Beales commented: Some venoms have phospholipases and membrane active peptides. Perhaps they work in synergy in some cases?

John Sanderson replied: This may be the case. Most of our melittin work has been done with synthetic melittin for this reason, but in this paper (DOI: 10.1039/d1fd00030f) we did look at bee venom melittin. We saw the lipidation we expected, but also significantly raised quantities of lysolipid, mostly because the hydrolysis by phospholipase A is much faster than the lipidation process.

Boyan Bonev commented: Yes, this is an interesting conclusion and tangent from your study.

John Sanderson said: Magainin II and PGLa also have this lipidation activity,[1] but there is no evidence that it is related to their antimicrobial activity.

1 R. H. Dods, B. Bechinger, J. A. Mosely and J. M. Sanderson, *J. Mol. Biol.*, 2013, **425**, 4379–4387.

Margarida Bastos commented: Thanks a lot, for the answer and reference, which I already downloaded and read. The lipidation is a interesting aspect.

Bart Hoogenboom opened a general discussion of the papers by Boyan Bonev: I am not sure if I have fully understood this – do you have vesicles with PC on the

inner leaflet and LPS on the outer leaflet? Could you elaborate a bit more on how you prepare these and on how you ensure the asymmetry? Apologies if I have misunderstood this.

Boyan Bonev responded: No, the LUVs are symmetric but LPS is presented as a polymyxin target on the outer leaflet only.

Patricia Bassereau asked: Do resistant strains have different LPS? Would it be possible to target them with other drugs that use other LPS?

Boyan Bonev answered: Resistance is a very complex and multifaceted process. LPS adaptations can contribute to resistance, specifically *via* the alkylation or Etn-ation of LPS phosphates or pyrophosphates. An antibiotic combination approach or the use of adjuvants is certainly a very appropriate way forward.

Burkhard Bechinger commented: The spectra shown in Fig. 3 of the paper (DOI: 10.1039/d1fd00036e) show the ^{31}P powder patterns of DMPC lipids. In the presence of polymyxin, the discontinuities/'edges' at about 30 ppm and −15 ppm are less steep and thereby less well defined; is there heterogeneity in the lipid population, problems with ^{1}H decoupling or what else could explain the difference in the ^{31}P NMR spectra in the absence of the peptide?

Boyan Bonev replied: Wideline ^{31}P NMR spectra from fluid lipid membranes are inhomogeneous statistical sums of contributions from the effective CSA (CSAeff) of lipid phosphates, offset by the statistically weighted contribution from molecular populations with different orientations with respect to the external magnetic field. In the fluid phase, the magnitude of this CSAeff is determined by the residual anisotropy after the full ^{31}P CSA (*ca.* 200 ppm) is averaged by the fast (GHz) axial rotation of the lipid molecules to approximately 40–45 ppm, as seen for DMPC. Clean spectral outliers are observed, as the lateral molecular librations within the bilayer are slow compared to the NMR timescale (100s of MHz).

In the presence of a large molar fraction of membrane-perturbing compounds, such as LPS or polymyxin, and particularly in the presence of both, lipid packing can be disrupted, which increases the librational freedom, frequency and magnitude. As a result of this, the overall CSAeff is reduced significantly. In addition, the individual contributions to the inhomogeneous distribution are broadened homogeneously due to angular excursions caused by molecular librations (rocking) that introduce an additional partial averaging mechanism onto the axial lipid rotation. The superposition of such significantly broader homogeneous lines leads to rounding of the 0 and 90° "edges" of the powder distribution. This enhanced mobility on the NMR timescale can also partially interfere with proton decoupling. This latter effect, however, is not significant for phosphates, in which proton coupling is weak as there are no protons directly bonded to the phosphorus atom, but all proton bonding is at least two bonds away and mediated by oxygen atoms.

Kareem Al Nahas enquired: Would the use of LPS free anionic membranes (PC : PG, 3 : 1) instead of zwitterionic (only PC) yield membranolytic activity similar to those of polymyxin B and LPS membranes? In other words, is the

observed membrane activity of polymyxin B specific to the presence of LPS or is the enhanced activity a result of general electrostatic interactions?

Boyan Bonev responded: There is no difference between polymyxin B-induced leakage in PG vesicles with or without LPS. The presence of the PG charge overwhelms the system and sequesters most of the polymyxin in non-specific interactions, which masks the LPS-mediated ones. This, however, is not the composition of the outer leaflet of bacterial outer membranes, which consists almost entirely of LPS, and for this reason the PG/LPS model is inappropriate. The use of a zwitterionic lipid allows the teasing out of the LPS-specific effects.

Bart Hoogenboom asked: Can you relate your findings to recent evidence[1] that *E. coli* may develop resistance to polymyxin B by modification of the inner membrane, pretty much ignoring what happens at the outer membrane (and hence to LPS)?

1 G. Benn, I. V. Mikheyeva, P. G. Inns, J. C. Forster, N. Ojkic, C. Bortolini, M. G. Ryadnov, C. Kleanthous, T. J. Silhavy and B. W. Hoogenboom, *Proc. Natl. Acad. Sci. U. S. A.*, 2021, **118**(44), e2112237118, DOI:10.1073/pnas.2112237118.

Boyan Bonev replied: There are two obstacles to breaching the Gram-negative envelope, the OM and the IM. Each contributes independently to the MIC for a particular antibiotic. While we focus on the difference between the OM and IM, stress-related adaptations in the IM contribute to an increase in the MIC. That also depends on the growth phase point.

Bart Hoogenboom remarked: To clarify my previous question about resistance: clearly polymyxin disrupts the outer membrane, but I wonder if you can say something about the differences by which it disrupts the outer and inner membranes, with possible ramifications for resistance.

Boyan Bonev responded: In our study (DOI: 10.1039/d1fd00036e), we hypothesise that the presence of rLPS in lipid membranes facilitates polymyxin-mediated membrane disruption. In other words, we compare non-specific membrane breaches (no rLPS) to LPS-mediated membrane disruption. So, yes, this is our model of the rLPS-aided differential disruption of outer *vs.* inner membranes. LPS P-Etn-ation interferes with CAP-mediated OM disruption and leads to resistance.

Aishwarya Vijayakumar opened the discussion of the paper by Sreetama Pal: If lysine has a role in hydrophobic mismatching, have the roles of arginine and histidine been studied? Which type of NMR experiment is used to study the hydrophobic mismatching?

Sreetama Pal responded: The incorporation of arginine and histidine into the WALP scaffold has led to the identification of some interesting peptide behavior as a function of both hydrophobic mismatching and pH. For example, arginine–tryptophan interactions have been shown to control helical fraying at the edges of the WALP helix, with important consequences for the stability of transmembrane

peptides and proteins.[1] In peptides with a single arginine acting as a membrane interfacial anchor, the introduction of a glutamate near the arginine has been shown to induce multiplicity in peptide conformational states.[2] Similarly, the introduction of histidine residues has been shown to modulate the degeneracy in peptide conformational and orientational states, depending on hydrophobic mismatching and pH.[3] More importantly, the pH responsiveness of WALP analogs has been found to depend on the precise position of histidine residues.[4] It is worth mentioning here that, in spite of decades of research into peptide–membrane interactions, these aspects of peptide behavior remain enigmatic and are therefore worth investigating from the perspective of tryptophan fluorescence (on account of the central role of tryptophans in membrane protein organization, evolution and biology[5,6] and the substantial overlap of fluorescence timescales with a range of membrane-associated phenomena[7]). However, a robust analysis and meaningful interpretation of tryptophan dynamics in the context of such nuanced behavior would require 'basis sets' of tryptophan (fluorescence) signatures from simpler WALP variants, which remain limited.[8] These considerations informed our choice of KWALP and GWALP as suitably minimalistic systems for exploring the consequences of near-neighbor interactions between tryptophan and nonaromatic interfacial amino acids, such as lysine and glycine. The type of NMR experiments used to study hydrophobic mismatch would depend, among other things, on whether protein/peptide or lipid responses to mismatch conditions are being investigated. For example, since hydrophobic mismatching could induce the formation of non-bilayer (such as cubic or reversed hexagonal) lipid phases, lipid responses to mismatching can be tracked using ^{31}P-NMR (based on the fact that the environment of the phosphate in the phospholipid headgroup would be distinct in different membrane phases[9]). More subtle lipid responses, such as changes in the lipid acyl chain order in positive or negative mismatching conditions, could also be identified based on order parameters calculated using ^{2}H-NMR. On the other hand, peptide responses to mismatching range from localized changes in the conformational dynamics of the peptide backbone and side chains to more global changes in the peptide orientation (transmembrane or surface-adsorbed) and even aggregation. These aspects have been explored using a combination of ^{2}H-NMR and other specialized NMR-based methodologies, such as GALA (geometric analysis of labeled alanines[10]) and PISEMA (polarization inversion with spin exchange at magic angle[11]).

1 S. J. Sustich, F. Afrose, D. V. Greathouse and R. E. Koeppe II, *Biochim. Biophys. Acta, Biomembr.*, 2020, **1862**, 183134.
2 J. R. Price, F. Afrose, D. V. Greathouse and R. E. Koeppe II, *ACS Omega*, 2021, **6**, 20611–20618.
3 F. Afrose, A. N. Martfeld, D. V. Greathouse and R. E. Koeppe II, *Biochim. Biophys. Acta, Biomembr.* 2021, **1863**, 183501.
4 F. Afrose and R. E. Koeppe II, *Biomolecules*, 2020, **10**, 273.
5 D. A. Kelkar and A. Chattopadhyay, *J. Biosci.*, 2006, **31**, 297–302.
6 R. E. Koeppe II, *J. Gen. Physiol.*, 2007, **130**, 223–224.
7 S. Pal and A. Chattopadhyay, in *Membrane Organization and Dynamics*, ed. A. Chattopadhyay, Springer, Heidelberg, 2017, pp. 1–9.
8 S. Pal, R. E. Koeppe II and A. Chattopadhyay, *J. Fluoresc.*, 2018, **28**, 1317–1323.
9 G. Gröbner and P. Williamson, in *Solid-State NMR: Applications in Biomembrane Structure*, ed. F. Separovic and M. A. Sani, IOP Publishing, 2020, pp. 1–30.
10 P. C. A. van der Wel, E. Strandberg, J. A. Killian and R. E. Koeppe II, *Biophys. J.*, 2002, **83**, 1479–1488.

11 V. V. Vostrikov, C. V. Grant, A. E. Daily, S. J. Opella and R. E. Koeppe II, *J. Am. Chem. Soc.*, 2008, **130**, 12584–12585.

Paul O'Shea enquired: Regarding your use of TCSPC to probe indole lifetimes, have you also looked at the polarisation of Trp, as the anisotropies can also be very revealing about the microenvironments? In an earlier paper, for example ref. 1, we were able to show that the Trp environment changed significantly within the interior of a protein using time-resolved anisotropy measurements, I think in your system it would work well.

1 N. Chadborn, J. Briant, A. J. Bain and P. O'Shea, *Biophys. J.*, 1999, **76**, 2198–2207.

Sreetama Pal answered: That's a great point! We did, in fact, measure trends in the fluorescence anisotropy of tryptophan residues in WALP analogs with excitation (Fig. 2c of the paper, DOI: 10.1039/d0fd00065e) and emission (Fig. 3c of the paper) wavelengths. The magnitude of change in the tryptophan fluorescence anisotropy with excitation (Fig. 2d of the paper) or emission (Fig. 3d of the paper) wavelengths did not show any significant differences across the three peptide analogs or in the presence/absence of negatively charged lipids. However, the absolute values of the fluorescence anisotropy for WALP tryptophans (the blue traces in Fig. 2c and 3c of the paper) were markedly lower than those for KWALP/GWALP (the green/maroon traces in Fig. 2c and 3c of the paper). This is possibly due to homo-fluorescence resonance energy transfer (homo-FRET) among the tryptophan pairs present at each end of WALP, but not KWALP/GWALP. Of course, this does not rule out contributions from specific near-neighbor interactions (for example, cation–π interactions among lysine and tryptophan residues in KWALP) in dictating the fluorescence anisotropy values. Time-resolved anisotropy measurements (as suggested) could be expected to provide more spatiotemporally resolved information about these aspects of tryptophan dynamics in WALP analogs.

Burkhard Bechinger asked: In the paper you proposed to use Trp fluorescence for tilt angle determination. However, in other membrane proteins there may be many other factors that influence the local environment of the Trp, including *e.g.* oligomerisation or membrane deformations. Therefore, the comparison with WALP may not be straightforward. In solid-state NMR, we have taken many years to elaborate the technique to determine tilt angles quite accurately, also including wobbling and rocking motions of the peptides (*e.g.* reviewed in ref. 1). In your opinion, where are the limitations of the fluorescence approach?

1 B. Bechinger, J. M. Resende and C. Aisenbrey, *Biophys. Chem.*, 2011, **153**, 115–125.

Sreetama Pal replied: I agree that the analysis of fluorescence readouts, such as the emission maximum, fluorescence anisotropy or fluorescence lifetime, would suffer from ambiguity of interpretation due to the multiplicity of factors that could influence these values. This is especially true for the spectral analysis of tryptophan fluorescence. However, since the red edge excitation shift approach employed here reports specifically on the solvation environment of the fluorophore (and not the fluorophore itself), the magnitude of REES (defined as the red shift in the fluorescence emission maximum on increasing the excitation wavelength) is known to be a very faithful marker of the membrane penetration depth of a fluorophore. Previous work from our group has established this

working relationship for a range of fluorophores known to preferentially partition into specific membrane depths.[1,2] These considerations, along with reports on the scaling of tilt angles with positions of tryptophan residues along the helix,[3] led us to propose the use of REES signatures for interfacial tryptophans as an indirect measurement of the WALP tilt angles. This is validated by our observations of a higher magnitude of REES in KWALP/GWALP tryptophans, relative to that in WALP (Fig. 2b of the paper, DOI: 10.1039/d0fd00065e), since the tilt angles of KWALP/GWALP are known to be higher than those in WALP.[4,5] However, your point is well-taken in that the proportionality between the REES signatures of tryptophans and peptide tilt angles must be tested and validated for a wide range of peptides and proteins (including more complex WALP variants) before this could be treated as a universal working rule applicable for all membrane proteins. Regarding the comparative usefulness of NMR and fluorescence methodologies for tilt angle determination, NMR approaches have definitely emerged as one of the most effective. However, to the best of my understanding, resolving the differences in tilt angles estimated by NMR and fluorescence remains a challenge. As suggested elsewhere,[6,7] this could be due to the differential overlap of NMR and fluorescence timescales with that of peptide motion, which could lead to underestimation of the tilt angles by NMR on account of motional averaging. Yet another source contributing to this discrepancy could be the differences in experimental conditions required for NMR and fluorescence, translating to different lipid-to-peptide ratios, which could influence peptide dynamics beyond a threshold value. On the other hand, fluorescence-based approaches could introduce complexities arising from changes in peptide organization and dynamics due to incorporation of extrinsic fluorescent labels (in the absence of tryptophans). In my opinion, one way forward could be to use a judicious combination of NMR and fluorescence approaches to define the lower and upper bounds of the peptide tilt angles, followed by the use of directed analytical and simulation approaches for further refinement.

1 A. Chattopadhyay and S. Mukherjee, *Langmuir*, 1999, **15**, 2142–2148.
2 H. Raghuraman, S. Shrivastava and A. Chattopadhyay, *Biochim. Biophys. Acta, Biomembr.*, 2007, **1768**, 1258–1267.
3 V. V. Vostrikov and R. E. Koeppe, *Biochemistry*, 2011, **50**, 7522–7535.
4 A. E. Daily, D. V. Greathouse, P. C. A. van der Wel and R. E. Koeppe, *Biophys. J.*, 2008, **94**, 480–491.
5 V. V. Vostrikov, C. V. Grant, A. E. Daily, S. J. Opella and R. E. Koeppe, *J. Am. Chem. Soc.*, 2008, **130**, 12584–12585.
6 S. Özdirekcan, C. Etchebest, J. A. Killian and P. F. Fuchs, *J. Am. Chem. Soc.*, 2007, **129**, 15174–15181.
7 A. Holt, R. B. M. Koehorst, T. Rutters-Meijneke, M. H. Gelb, D. T. S. Rijkers, M. A. Hemminga and J. A. Killian, *Biophys. J.*, 2009, **97**, 2258–2266.

John Sanderson remarked: I noted that the intensities for KWALP are lower than WALP. Is there any effect of the neighbouring lysine on the tryptophan excitation or emission?

Sreetama Pal replied: That would have been neat, but we did not find any obvious spectral shape changes in fluorescence excitation spectra for KWALP relative to WALP, although there was a small decrease in fluorescence intensity at the excitation maximum (the left panel in the Fig. 1) and a blue shift in the emission maxima (the right panel in Fig. 1), as also seen in Fig. 2a of the paper

(DOI: 10.1039/d0fd00065e). Interestingly, the fluorescence intensity for both WALP and KWALP tryptophans showed a modest increase in negatively charged POPC/POPG (dashed lines in Fig. 1) membranes relative to that in zwitterionic POPC membranes (solid lines). For GWALP, a reverse trend of decreased fluorescence intensity in negatively charged membranes was observed. The reason for the opposing trend for KWALP and GWALP is not apparent at this point in time, but could indicate that the modulation of the tryptophan microenvironment by interfacial lysines (in KWALP) occurs in a fundamentally different manner than that by interfacial glycines (in GWALP). In addition, it is worth mentioning here that the absorption spectroscopy based protein charge transfer spectra (ProCharTS) method could be a good alternative for specifically probing the interaction of charged and aromatic amino acids in peptides, since proximity to tryptophan residues has been reported to reduce ProCharTS absorbance in soluble peptides.[1] Of course, successful adaptation of ProCharTS for membrane-interacting peptides and proteins would first require the (rather non-trivial) development of analytical tools to identify and correct for membrane-induced scattering artifacts in absorption spectra.

1 M. Z. Ansari, A. Kumar, D. Ahari, A. Priyadarshi, P. Lolla, R. Bhandari and R. Swaminathan, *Faraday Discuss.*, 2018, **207**, 91–113.

Patrick Fuchs addressed Sreetama Pal and Burkhard Bechinger: Adding to the conversation of Sreetama and Burkhard: one difficulty is also to have a good model of the motion of the peptide over the time scale of the measurement.

Sreetama Pal replied: Yes, absolutely, and this is where computational approaches can provide some robust insights!

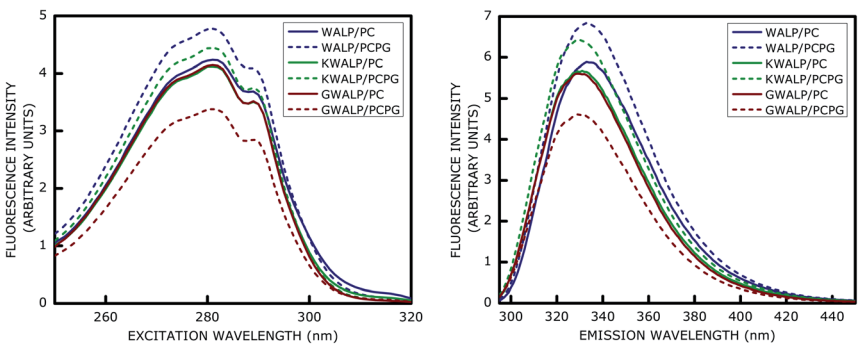


Fig. 1 Fluorescence excitation (left) and emission (right) spectra of WALP (blue), KWALP (green), and GWALP (maroon) tryptophan residues in zwitterionic POPC (solid lines, PC) and negatively charged POPC/POPG (dashed lines, PCPG) membranes. Excitation spectra were acquired with the emission wavelength set to the emission maximum of each peptide observed upon excitation at 280 nm (*i.e.*, 334 nm for WALP and 331 nm for KWALP/GWALP), while emission spectra were collected at an excitation wavelength of 280 nm. The spectra represent averaged traces from at least three independent measurements, with each spectrum recorded in the corrected spectrum mode. All experimental conditions are the same as in Fig. 2a of the paper (DOI: 10.1039/d0fd00065e).

Burkhard Bechinger answered: Indeed, extended sets of complementary solid-state NMR measurements were needed to develop models which describe the tilt and pitch angles as well as the wobbling and rocking motions of helical peptides (*e.g.* for surface oriented helices see ref. 1; for transmembrane helices see ref. 2).

1 M. Michalek, E. S. Salnikov and B. Bechinger, *Biophys. J.*, 2013, **105**, 699–710.
2 E. S. Salnikov, C. Aisenbrey, B. Pokrandt, B. Brügger and B. Bechinger, *Front. Mol. Biosci.*, 2019, **6**, 83.

Burkhard Bechinger said: Apart from motions of the whole peptide and/or changes in the bilayer properties and morphology, the Trp side chain can wobble/rotate around the Cβ–Cγ bond. How does this affect your analysis?

Sreetama Pal replied: That is a good point. These localized tryptophan motions could reflect subtle changes in the fluorophore microenviroment. However, the red edge excitation shift analysis employs steady state fluorescence anisotropy (Fig. 2c of the paper, DOI: 10.1039/d0fd00065e) and therefore, we have no direct handle on the tryptophan side chain dynamics. However, the apparent rotational correlation times calculated for tryptophan (Fig. 4 of the paper, DOI: 10.1039/d0fd00065e) could be more sensitive to tryptophan side chain motions, although deconvoluting those trends to identify the contribution of tryptophan side chain dynamics would require data acquisition in a time-resolved setup.

Patrick Fuchs remarked: Do you use also tilt/azimuthal rotation fluctuations to determine peptide orientation by fluorescence?

Sreetama Pal answered: Great question! Different types of fluorescence spectroscopy and microscopy have been used to estimate the tilt and azimuthal angles of membrane-interacting peptides and proteins. In the case of WALP, incorporation of the BADAN fluorophore at different positions along the helix was employed to construct a calibration plot for emission maximum as a function of the BADAN position, which was then used to calculate the tilt and rotation angles.[1] The fluorescence quenching based parallax approach[2] has been employed to calculate the tilt angle of the bee venom peptide melittin in its membrane-bound form, based on simple geometric considerations of the membrane penetration depth of two distinct fluorescent moieties in the peptide.[3] In addition, FRET efficiencies of membrane proteins have been correlated to changes in azimuthal angles.[4]

1 A. Holt, R. B. M. Koehorst, T. Rutters-Meijenke, M. H. Gelbe, D. T. S. Rijkers, M. A. Hemminga and J. A. Killian, *Biophys. J.*, 2009, **97**, 2258–2266.
2 A. Chattopadhyay and E. London, *Biochemistry*, 1987, **26**, 39–45.
3 S. Haldar, H. Raghuraman and A. Chattopadhyay, *J. Phys. Chem. B*, 2008, **112**, 14075–14082.
4 S. E. D. Webb, D. J. Rolfe, S. R. Needham, S. K. Roberts, D. T. Clarke, C. I. McLachlan, M. P. Hobson and M. L. Martin-Fernandez, *Opt. Express*, 2008, **16**, 20258–20265.

Patricia Bassereau opened a general discussion of the paper by Aurelien Roux: In the case of tissue bending, there are many internal forces involved. It is an active process. The bending of membranes due to lipid shapes that you mentioned occurs at equilibrium. Can you comment and is the analogy justified?

Aurelien Roux answered: I would say that at a short timescale, epithelial cells are responding elastically, which may indicate that they are in a sort of mechanical equilibrium. So in that short timescale, the analogy would be arguable, I think. However, on long time scales, above ten minutes, this may be less relevant, as cells would actively change their shape on that time scale.

John Seddon said: I like your idea of comparing lipid membranes with the monolayers of cells, in terms of their surface properties. It is clear that a layer of epithelial cells can develop a local negative Gaussian curvature. Could this curvature be extended through space to form a lattice of saddle-like structures, similar to an ordered sponge, that could play a role in tissue formation or tissue engineering?

Aurelien Roux answered: This is certainly a good idea. I can surely see that the local lattice of cells can create negative Gaussian curvature, and certainly, the negative Gaussian curvature could then extend, as in the lipid sponge phase, to form a foam like material.

Ana J. Garcia Saez enquired: How do cell/cell adhesion forces between neighbouring cells in the monolayer affect the bulging process? Can this be compared to lipid packing?

Aurelien Roux responded: Dear Ana, thank you for the excellent question. First, adhesion energy is associated with contractility, so cells with higher adhesion energy are also more contractile along the lateral parts of the cells. Thus, they usually are taller and thinner. This can be compared to the lipids with long, saturated acyl chains, which will have a higher density and will make thicker bilayers. The big difference is the dynamics, as cells can change their adhesion/contractility, allowing them to change the overall curvature of the surface (as proposed in Fig. 2 of my article, DOI: 10.1039/d1fd00040c), whereas lipids cannot change shape directly.

Justin Molloy asked: I am interested in whether the direction of the membrane deformation caused by the spiral protein assemblies can occur either way, *e.g.* positive or negative curvature relative to the leaflet to which the protein binds?

More specifically, do you think that the lipid composition of the inner and outer leaflets might affect the probability of the buckling direction or is it totally dominated by the face to which the protein binds?

Aurelien Roux replied: This is an excellent question. The buckling mechanism, at least theoretically, does not predict direction. The probability of breaking symmetry in one direction or another is 50% for both directions. But anything that would make the system asymmetric, including the fact that the protein is binding only one side, or that the lipid composition is different in the two leaflets of the membrane, could bias this 50/50 probability. More work needs to be done on this though.

Paula Milán Rodríguez queried: So your initial information is the shape and then from the shape you try to see which mechanism could provoke it?

Aurelien Roux responded: Exactly, but we need another information, which is force. And the exact mechanism of shape generation can be established from the quantitative relation between the force and the shape.

Paula Milán Rodríguez enquired: About the buckling of surface through growth under confinement: is this mechanism present in living organisms?

Aurelien Roux responded: This is an essential, important question that developmental biologist have tackled for about a century. Rhumbler, in the early 1900s, had developed a purely mechanical model (made of springs, ropes, *etc.*) that reproduced the shape taken by simple embryos, such as the sea anemone, during gastrulation. The theory about buckling by growth pressure arose at that time, and many experimental works associated with computational work showed that shapes of embryos can be reproduced by buckling-based algorithms. The main problem of measuring the force associated with the deformation remained, which is practically impossible *in vivo*. We used an *in vitro* approach to quantitatively link the shape of the epithelium and compressive stress to show that growth under confinement can generate sufficient compressive stresses to buckle the epithelium (see ref. 1). I should mention the experimental tour-de-force of Guillaume Charras, who buckled the epithelium using micro-levers, showing that the buckling can be compensated by cell contractility to flatten the epithelium after buckling for compressive rates below 35%.[2]

1 A. Trushko, I. Di Meglio, A. Merzouki, C. Blanch-Mercader, S. Abuhattum, J. Guck, K. Alessandri, P. Nassoy, K. Kruse, B. Chopard and A. Roux, *Dev. Cell*, 2020, **54**, 655–668.
2 T. P. J. Wyatt, J. Fouchard, A. Lisica, N. Khalilgharibi, B. Baum, P. Recho, A. J. Kabla and G. T. Charras, *et al.*, *Nat. Mater.*, 2020, **19**, 109–117.

Paul Beales commented: One difference with lipid bilayers is that they are fluid, whereas cell monolayers usually aren't (excepting metastatic effects!). Could you comment on how this lack of fluidity may give rise to differences between lipid and cell layers in your packing parameter model analogy? Instead, the cells might be better approximated by colloidal packing theories. Does the physics of topological defects in liquid crystals or colloidal monolayers have a role to play in understanding curvature effects in cell monolayers? I am particularly thinking of the work of the likes of David Nelson[2] on packing and defects on curved surfaces and the interactions that occur between the topological defects that must arise in these geometries. Have you considered these models in the context of cell packing and the curvature of cell layers?

Aurelien Roux replied: This is an excellent question. Indeed, it is essential for the material not to be completely fluid in order to have propagation of long range forces in cellular tissues. And yes, many people have used the Nelson description for studying how order and constraints propagates in active matter. We actually show that topologies with charge +1 (spirals, asters, vortices) can concentrate cellular forces and deform cell monolayers into protrusions, resulting in cellular tornadoes (see ref. 1).

1 P. Guillamat, C. Blanch-Mercader, K. Kruse and A. Roux, *bioRxiv*, 2020, 2020.06.02.129262, https://www.biorxiv.org/content/10.1101/2020.06.02.129262v1.
2 M. J. Bowick, D. R. Nelson and A. Travesset, *Phys. Rev. B*, 2000, **62**, 8738.

Paul Beales remarked: Regarding your model of ESCRT filaments as a spring, you predict that on an unsupported membrane they will remodel the membrane by pushing out towards the membrane, but if ESCRT spirals grow on a solid-supported membrane (as in your AFM studies), why do they not buckle away from the membrane due to elastic stress in this case since the solid support prevents them from buckling in the "usual" direction?

Aurelien Roux answered: Again, an excellent question Paul. First, the spring model does not define any particular direction for buckling. But any asymmetry, such as different lipid compositions in the two leaflets, or just the fact that ESCRT-III is binding only on one side of the membrane, may force the system to choose one direction over the other. But in both cases, when the membrane is attached to a solid substrate, the adhesion energy is so high (in particular on mica for AFM, and in clean glass experiments) that it prevents the membrane from any deformation, whatever direction it is. Thus, the spirals stay flat. Recent work from our colleagues (Simon Scheuring's group) imaging the spirals on soft substrates show spontaneous and reversible buckling in the center of the spirals (soon to be published). Also, buckling is aided by the addition of Vps2/Vps24 to the spirals, which increases the rigidity and twisting of filaments (see ref. 1 and 2).

1 J. Moser von Filseck, L. Barberi, N. Talledge, I. E. Johnson, A. Frost, M. Lenz and A. Roux, *Nat. Commun.*, 2020, **11**, 1516.
2 A.-K. Pfitzner, V. Mercier, X. Jiang, J. Moser von Filsek, B. Baum, A. Šarić and A. Roux, *Cell*, 2021, **182**, 1140–1155.

Patricia Bassereau opened a general discussion of the paper by Justin Molloy: You use your tracking system to probe the local viscosity. But, as you know, the probe mobility can be influenced by interactions with the cytoskeleton. There are many membrane components that the receptor could interact with, not just the surrounding lipids. Thus, I was surprised that you claimed to measure primarily the viscosity of the membrane with such experiments.

Justin Molloy responded: We make that claim because the M_1 and M_2 muscarinic acetylcholine receptors that we selected for this study exhibit MSD *vs.* dT plots which are linear and show no evidence of sub-diffusion or super-diffusion. However, other membrane proteins that we[1] and other groups have studied show distinctly non-linear MSD *vs.* dT plots. The experimental work of Kusumi, Jacobson and many others and the theory of Saxton (see the references in our article, DOI: 10.1039/d1fd00035g) indicate that these more complex diffusive behaviors might arise from interactions with cytoskeletal networks or other obstructions to free-diffusion.

1 G. I. Mashanov, M. Nobles, S. C. Harmer, J. E. Molloy and A. Tinker, *J. Biol. Chem.*, 2010, **285**, 3664–3675.

Patricia Bassereau said: People have been using single particle tracking for some time to reveal the organization of cell membranes, but it remains a very challenging task considering their complexity and the interaction with the cytoskeleton. What I find quite exciting in your results is the difference in probe

mobility in the plasma membrane of the same cell types, depending if they are primary cells, cells in culture or in a tissue.

Justin Molloy replied: Yes, we find that cell-lines and primary cell-cultures show little cell-to-cell variation within a cell type; however, the differences between a stable cardiac cell-line (HL1) and to primary cardiomyocytes are significant and the differences are greater still between cells embedded in tissue slices compared to those studied in a primary cell-culture. It is curious that even when we see a significant difference between adjacent cells within a tissue slice, our quadrat sampling method indicates that the plasma membrane of a given cell is homogeneous and the variation we see can not be explained by sampling statistics.

John Seddon asked: Is it clear that Monte Carlo is the best approach to simulating membrane protein self-diffusion?

Justin Molloy replied: One great advantage of Monte Carlo (*i.e.* single molecule stochastic models) simulations is that we can generate mock single molecule video datasets and test our analytical tools (*i.e.* test our image analysis methods against a known truth). Another advantage is that we can make the model system as complex or simple as we like. Sophisticated, closed analytical solutions require a lot of brain-power, whereas Monte Carlo simulations just require computing time (usually enough for a quick cup of coffee). Numerical simulations (*e.g.* networks or sets of ODEs) are not so easily adapted for 2D or 3D geometrical systems. So, we prefer Monte Carlo stochastic models, because each simulated molecule can take on its own unique properties, and we can build a physical 2D or 3D framework (or system) of the "cell" and "plasma membrane" and then give the system different viscosities, include cytoskeletal networks or protein binding sites *etc.* Then, we let the simulated molecules meander under thermal force with motional probabilities determined by the local physical environment.

Paul O'Shea enquired: The approach is very interesting, but could you clarify that although you indicate that although you rely on random sampling for this to behave, do you bias the initial starting conditions/point? Monte Carlo assumes a random sample/seed but if it is not random you can get spurious results. Are you able to confirm a random start? The problem is loosely analogous to that of the atomistic modeling of membrane lipids in which a starting condition of 'equilibrium' is desirable.

Justin Molloy responded: Yes, we get your point. The single fluorophore simulations are initialized by randomly seeding "virtual" molecules over the "virtual" membrane. So, they have random starting positions and any state variables are also randomly assigned. We then let the model run for a hundred (or a thousand) cycles so that the "molecules" and any state variables relax towards equilibrium. We then start to output simulation data and either simply watch the system fluctuate about the steady-state or, if we wish, perturb the system and observe it relax to a new steady-state.

Amitabha Chattopadhyay commented: I want to share some general remarks on the measurement of membrane diffusion. One needs to keep in mind that, in membranes, diffusion follows the Saffman–Delbruck model, which means that diffusion is proportional to the mass of the diffusing body in a logarithmic fashion (weak function of mass). Therefore, questions such as receptor dimerization in membranes are difficult to address using membrane diffusion measurements. Having said that, we were able to monitor the activation of G protein-coupled receptors by measuring the diffusion of the receptor using FRAP (since G-proteins are dissociated upon signaling, there is a mass difference).[1] We have also carried out the measurement of GPCR diffusion in membranes using FRAP, z-FCS, and SPT. Although we used the same construct and cell type in these experiments, the diffusion coefficients exhibited some variation due to differences in the sampling time in these measurements. Interestingly, in z-FCS measurements, it is possible to dissect out diffusion modes (such as random diffusion, anomalous diffusion, coralled diffusion) by the application of diffusion laws.[2] In recent times, these measurements have gained spatial resolution by using diffraction-limited microscopy. By using SPT measurements, we could gain further insights on relative distributions of receptors with various modes of diffusion.[3]

For a recent Perspective, see ref. 4.

1 T. J. Pucadyil, S. Kalipatnapu,K. G. Harikumar, N. Rangaraj, S. S. Karnik and A. Chattopadyay, *et al.*, *Biochemistry*, 2004, **43**, 15852–15862.
2 S. Ganguly and A. Chattopadhyay, *Biophys. J.*, 2010, **99**, 1397–1407.
3 S. Shrivastava, P. Sarker, P. Preira, L. Salomé and A. Chattopadhyay, *Biophys. J.*, 2020, **118**, 944–956.
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Justin Molloy answered: Thank you for raising this Amit and I apologize that we did not do justice to your previous work nor indeed the work of many others in the field in our short manuscript. This is a good opportunity to refer the general reader to your papers and the references therein.

Patricia Bassereau said: We did *in vitro* experiments to show there are limitations to Saffman–Delbrück (SD). We were using single particle tracking to measure the diffusion of voltage-gate potassium channels (KvAP) and of aquaporin 0 in giant liposomes. According to SD, these proteins should have similar diffusion coefficients since they have the same size. We showed that if you change the liposome tension, the diffusion of aquaporin was almost not changed but we observed a reduction by 50% for the lowest tension with KvAP, which is not predicted by SD. We propose that if a protein deforms a membrane (like KvAP), it affects its mobility in the membrane. This might also occur when membrane proteins change conformation, and shape due to some activity. But the question is whether or not the timescale of this change allows us to capture this effect in the diffusion. Experiments have been done *in vitro* to investigate this question.

Justin Molloy commented: If a protein causes local membrane deformation then, in terms of its mobility within the lipid bilayer, can you consider the effect as a (perhaps, tension-dependent) change in the effective radius of the protein *i.e.* otherwise consistent with the Saffman–Delbruck analysis?

Patricia Bassereau responded: This was our first guess that what is effectively diffusing is a "bump" with a size that depends on the membrane tension. But if you evaluate the effect, in particular the intrinsic curvature of the protein, you find that you would need a much higher curvature to explain our results, than the value that we had measured with separate experiments.[1]

1 F. Quemeneur, J. K. Sigurdsson, M. Renner, P. J. Atzberger, P. Bassereau and D. Lacoste, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **111**, 5083–5087.

Paul O'Shea said: Justin, in order to disentangle the various structures that may underlie the different diffusion processes of large membrane components (receptors), if you look at purely membrane surface indicators that can float *versus* a transmembrane receptor protein, the 2D diffusion coefficients of something that is just on the surface would be different in form *vs.* something embedded in the membrane and may offer a way of discriminating between the various diffusion/structural models. Personally, I think all of these structures probably exist in membranes at the same time but with different dynamics.

Justin Molloy responded: Yes, I agree Paul, the lateral diffusion of transmembrane proteins is likely to be very different from that of monotopic membrane proteins. We chose acetylcholine receptors as a model system because we are able to label them in different ways, express them in different cell types and because their molecular structures have been solved. In our earlier work with muscarinic receptors (M_1 and M_2 classes), we found they showed near-perfect Brownian motion in the lipid bilayer (linear MSD *vs.* dT plots). In the current study (DOI: 10.1039/d1fd00035g), we used these proteins as surrogate probes of the membrane bilayer structure; specifically testing if they show the same lateral diffusion constant across different cells types (primaries *vs.* cell lines, *vs.* tissue culture); between neighboring cells and different regions of membrane across an individual cell. Membrane heterogeneity might well exist on length and time scales that are outside our measurement window (30 ms to 2 seconds on membrane regions with a length scale of $\sim$1 μm^2) and indeed our findings would be strengthened if we had an additional, independent measure of membrane structure that we could cross-correlate with our single molecule tracking data. I think Patricia makes a similar point and we agree with both of you, but it is sadly beyond the scope of our current study.

Paul O'Shea said: Justin – you are suggesting that all the anomalous diffusion is driven by molecular motors located in the cytoskeleton? Or are there other mechanisms, perhaps a bulk lipid flow?

Justin Molloy replied: Anomalous diffusion can manifest either as an upward or downward curvature of mean squared displacement *vs.* time interval plots (MSD *vs.* dT) for super-diffusion and sub-diffusion, respectively. Anomalous sub-diffusion usually arises from passive effects, such as obstacles (molecular crowding), cages (cytoskeletal or extracellular matrix networks) or transient confinement (stochastic binding/unbinding) which all tend to impede the path of a free Brownian walk (which would show a perfectly linear MSD *vs.* dT plot).

Conversely, anomalous super-diffusion requires energy input, arising from *e.g.* convective flow, systematic drift or the action of molecular motors. To some extent, one can distinguish super-diffusion mechanisms by looking at the

autocorrelation and cross-correlation of molecular trajectories: convective flow and systematic drift both tend to show strong cross-correlation between different molecules (*i.e.* all (or many) molecules will "drift" in the same direction), whereas the direction of super-diffusion that is driven by individual molecular motors is usually uncorrelated between molecular paths; unless cytoskeletal tracks are aligned (*e.g.* cytoplasmic streaming in plant and amoeboid cells). Matters become more complicated when short-lived single molecule trajectories are studied, because then one finds that only a minority of molecules show perfectly straight-line MSD *vs.* dT plots because of simple statistical variation and this is easy to show by Monte Carlo simulations.

We have tried to address these sampling (perhaps better termed "under-sampling") problems in our paper (DOI: 10.1039/d1fd00035g).

Conflicts of interest

William DeGrado is an advisor to Innovation Pharmaceuticals, and there are no other conflicts to declare.

