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Theoretical and experimental studies of complex peptide–membrane systems: general discussion

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Mibel Aguilar opened a general discussion of the paper by Ronald J. Clarke: Did you try other negatively charged lipids to determine if the electrostatic interaction was specific for PS? Also, what was the P : L ratio for the QCM and CD experiments? Would you have expected the helical content to be higher than you observed?

Ronald J. Clarke replied: Yes, in fact we did investigate other negatively charged lipids. In the course of our studies we found that a simple but very effective method to screen for electrostatic interactions is to measure the static light scattering of suspensions of lipid vesicles or membrane fragments containing the Na^+,K^+-ATPase. If an interaction occurs, aggregation and flocculation of the colloidal system occurs, resulting initially in a large increase in light scattering.[1] At relatively high concentrations of polyamino acids, the light scattering increase is followed by a slower decay in scattering, probably due to permeabilisation and disruption of the lipid membrane. For these measurements we repurposed a fluorimeter in our laboratory and measured the scattering at 90° to the incident light at 826 nm, corresponding to a high intensity line of the instrument's xenon arc lamp. To determine whether the peptide interaction with the membrane is specific to phosphatidylserine, we carried out light scattering measurements using vesicles composed of 85 mol% dioleoylphosphatidylcholine (DOPC) and 15 mol% of either dioleoylphosphatidylserine (DOPS), dioleoylphosphatidylglycerol (DOPG) or dioleoylphosphatidic acid (DOPA). Vesicles containing DOPS, DOPG or DOPA all showed similar large increases in light scattering after the addition of poly-L-lysine, indicating that the electrostatic interaction of lysine with the membrane is not specific to PS.[2] Lysine also

interacts with PG and PA. In contrast, no increase in light scattering was observed when poly-L-lysine was added to uncharged vesicles composed of 100% DOPC or 85% DOPC and 15% dioleoylphosphatidylethanolamine (DOPE). Using the same technique we also found that the addition of poly-L-arginine to Na^+,K^+-ATPase-containing membrane fragments caused slightly larger increases in light scattering than those observed with poly-L-lysine, indicating that membrane interaction is not specific to lysine. Experiments using a negatively charged polyamino acid, poly-L-glutamic acid, showed no increase in light scattering. The interaction, therefore, appears to be nonspecific, simply requiring a negatively charged membrane surface and a positively charged amino acid side chain.

In the case of the QCM-D experiments, a precise peptide : lipid molar ratio (P : L) is difficult to estimate because the lipid is bound to the quartz crystal surface, whereas the peptide is delivered to the surface of the crystal within a continuous flow system. Thus, the lipid is situated on a two-dimensional surface, while the peptide is in a three-dimensional solution, so that they are strictly speaking, not comparable. Nevertheless, the concentration of lipid used in forming the bilayer was 3 mM and the concentrations of peptide used to study their interaction with the membrane were 10 μM for the Na^+,K^+-ATPase peptide and 9 μM for the H^+,K^+-ATPase peptide. Of the 3 mM lipid used in the flow system, a significant fraction of the lipid molecules would have passed through the system without binding to the crystal surface. When the peptides were added to the flow system, a very slow rate of 50 μl min^{-1} was used to allow sufficient time for the peptides to bind to the membrane.

In the case of the CD measurements the lipid concentration was 500 μM and the peptide concentrations were 100 μM for the Na^+,K^+-ATPase peptide and 90 μM for the H^+,K^+-ATPase peptide. However, prior to recording the CD spectrum extravesicular peptide was removed from the system by centrifugation, so that only intravesicular peptide was measured. The reason for this was to maximise the probability of membrane interaction and to avoid a large background of non-membrane-bound extravesicular peptide. Nevertheless, there is still probably a significant amount of unbound intravesicular peptide present, which could account for the fact that the measured helicities of the peptides do not reach 100%. It is worth bearing in mind that in the native protein systems, the peptides would be permanently anchored close to the membrane surface by the transmembrane domains of both ATPases. Therefore, the likelihood of membrane interaction would be much higher than in the studies we have reported here using free peptides.

1 A. Gorman, K. R. Hossain, F. Cornelius and R. J. Clarke, Penetration of phospholipid membranes by poly-L-lysine depends on cholesterol and phospholipid composition, *Biochim. Biophys. Acta, Biomembr.*, 2020, **1862**, 183128.
2 K. Nguyen, A. Garcia, M.-A. Sani, D. Diaz, V. Dubey, D. Clayton, G. Dal Poggetto, F. Cornelius, R. J. Payne, F. Separovic, H. Khandelia and R. J. Clarke, Interaction of N-terminal peptide analogues of the Na^+,K^+-ATPase with membranes, *Biochim. Biophys. Acta, Biomembr.*, 2018, **1860**, 1282–1291.

Leonie van 't Hag asked: Why do you think there is a small interaction with DOPC/cholesterol bilayers in the QCM experiment whereas there was none with pure DOPC? Did you also try DOPC/DOPS without cholesterol? This would be a nice addition to see if there was also a difference in that case.

Ronald J. Clarke replied: The small interaction with DOPC/cholesterol bilayers could perhaps be explained by the cholesterol-induced increase in membrane dipole potential.[1] Because the polarity of the dipole potential is positive in the membrane interior and negative on the membrane surface, cholesterol would be expected to make the membrane surface slightly more negatively charged, which could attract more peptide. However, the effect of cholesterol on peptide binding is quite small and much smaller than the effect observed after including phosphatidylserine, with a net negative charge. We haven't looked at peptide binding to DOPC/DOPS membranes in the absence of cholesterol. I agree that this could be useful to see what effect the omission of cholesterol makes. The native membranes with which the peptide would interact, however, contain a high mole percentage of cholesterol of 40%. Therefore, we have included cholesterol at the same level so as to reproduce the native situation as closely as possible.

1 T. Starke-Peterkovic, N. Turner, M. F. Vitha, M. P. Waller, D. E. Hibbs and R. J. Clarke, Cholesterol effect on the dipole potential of lipid membranes, *Biophys. J.*, 2006, **90**, 4060–4070.

Leonie van 't Hag commented further: I was wondering if you tried circular dichroism spectroscopy with vesicles in the presence of salt, and if that showed enhanced peptide folding? I guess the experiment was done in the absence of salt due to the light absorption of chloride in the far-UV region. This experiment works very well, however, when one uses fluoride salts. We have for example done this for CD measurements with peptides in cubosomes.[1]

1 L. van't Hag, X. Li, T. G. Meikle, S. V. Hoffmann, N. C. Jones, J. S. Pedersen, A. M. Hawley, S. L. Gras, C. E. Conn and C. J. Drummond, How peptide molecular structure and charge influence the nanostructure of lipid bicontinuous cubic mesophases: model synthetic WALP peptides provide insights, *Langmuir*, 2016, **32**, 6882–6894.

Ronald J. Clarke replied: Thank you for your good suggestion. At this stage we have only carried out measurements in 10 mM Tris buffer, because of its low absorbance above 190 nm[1] and in the absence of salt in order to maximise the strength of attraction of the peptides for the membrane. A problem we have is that the CD signals are quite small, because we are only measuring peptide encapsulated within the vesicles. Free non-encapsulated peptide was removed by centrifugation and resuspension of the pellet in order to maximise the probability of detecting membrane-bound peptide. In the native system the N-terminal peptide is attached to the rest of the protein, *i.e.*, the Na^+,K^+- or H^+,K^+-ATPase, and can, therefore, never escape the vicinity of the membrane. In the future we intend to synthesise peptides with hydrophobic anchors at the C-termini to more closely approximate the native situation. This should allow us to measure circular dichroism at higher peptide concentrations and obtain an improved signal-to-noise ratio. Once we've achieved this we could then more easily study the effect of the addition of salts, such as NaF.

1 S. M. Kelly and N. C. Price, The use of circular dichroism in the investigation of protein structure and function, *Curr. Protein Pept. Sci.*, 2000, **1**, 349–384.

Larisa Zoranić remarked: In the article, there is a reference: "the proteins of hyperthermophilic organisms preferentially contain lysine residues (*i.e.*, over

arginine), because of their greater number of rotameric conformations, which leads to an enhanced thermostability *via* an entropic stabilization mechanism". Could you say more about the preferences of lysine over arginine (residues of the N-termini) in the case of ion pumps? Is this preference also connected to the proposed electrostatically based detection mechanism for the changes in lipid membrane composition?

Ronald J. Clarke answered: This is a very interesting question. As in the case of hyperthermophilic organisms, the lysine residues of the N-terminal peptide of the Na^+,K^+- and H^+,K^+-ATPases could provide an entropic stabilisation when the peptide is not bound to the membrane. Berezovsky *et al.*[1] attributed the preference of hyperthermophilic bacteria for lysine over arginine to a higher number of accessible rotamers in lysine. This could partly be due to the fact that the lysine side chain has one more tetrahedral carbon than the arginine side chain. To estimate the possible order of magnitude of the entropic stabilisation due to the N-terminal peptide of the ATPases, we performed a prediction of the *Homo sapiens* peptides' secondary structures using the QUARK server[2] and inputted the resulting pdb files into the PLOPS server[3] to obtain a value of the entropy change for the complete unfolding of the peptides. The calculations yielded values of $-T\Delta S$ of -234 kJ mol^{-1} for the Na^+,K^+-ATPase and -322 kJ mol^{-1} for the H^+,K^+-ATPase.[4] These values are much greater than the free energy of ATP hydrolysis under physiological conditions of -55 kJ mol^{-1}.[5] Although it is unlikely that the N-termini undergo complete unfolding, and the entropy changes could be partially compensated by changes in the entropy of solvating water molecules, the calculations indicate that entropic stabilisation of the N-terminal peptides could make a significant contribution to the proteins' energetics and kinetics, *i.e.*, by modifying the activation energy barriers of partial reactions of the ion pumping cycles.

When lysines of the N-termini interact electrostatically with the surrounding membrane, they produce an enthalpic stabilisation, which could also modify activation energy barriers for ion pumping. In this case lysine may perhaps be preferred to arginine, because, according to molecular dynamics simulations,[6] arginine penetrates further into the membrane than lysine and, therefore, may bind so strongly that conformational transitions of the ATPases involving the release of the N-terminal peptide from the membrane, and which may be required for ion pumping, might be inhibited. The deeper penetration of an arginine residue into a lipid membrane than a lysine residue, can also be rationalised simply by considering the structures of the two amino acid residues at neutral pH. Both have positive charges, but in the case of lysine the charge is localized on an ammonium group, whereas in the case of arginine the charge is delocalized across a guanidinium group. Thus, the charge density and hence the Born energy within a membrane would be lower in the case of arginine than lysine, allowing greater penetration by arginine.

1 I. N. Berezovsky, W. W. Chen, P. J. Choi and E. I. Shakhnovich, Entropic stabilization of proteins and its proteomic consequences, *PLoS Comput. Biol.*, 2005, **1**, e47.
2 D. Xu and Y. Zhang, *Ab initio* protein structure assembly using continuous structure fragments and optimized knowledge-based force field, *Proteins*, 2012, **80**, 1715–1735.

3 M. C. Baxa, E. J. Haddadian, J. M. Jumper, K. F. Freed and T. R. Sosnick, Loss of conformational entropy in protein folding calculated using realistic ensembles and its implications for NMR-based calculations, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **11**, 15396–15401.
4 K. R. Hossain, X. Li, T. Zhang, S. Paula, F. Cornelius and R. J. Clarke, Polarity of the ATP binding site of the Na^+,K^+-ATPase, gastric H^+,K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase, *Biochim. Biophys. Acta, Biomembr.*, 2020, **1862**, 183138.
5 R. J. Clarke, M. Catauro, H. H. Rasmussen, and H.-J. Apell, Quantitative calculation of the role of the Na^+,K^+-ATPase in thermogenesis, *Biochim. Biophys. Acta, Bioenerg.*, 2013, **1827**, 1205–1212.
6 L. B. Li, I. Vorobyov and T. W. Allen, The different interactions of lysine and arginine side chains with lipid membranes, *J. Phys. Chem. B*, 2013, **117**, 11906–11920.

Paul O'Shea commented: Regarding your use of 150 mM KCl to subdue the electrostatic interactions; if Cl^- gets in the way of CD measurements and the other suggestion of using fluoride doesn't work (*i.e.* exchanging chloride for something else), I am sure you know that 150 mM monovalent K^+ chloride is about the same as a few mM divalent cations (*e.g.* Ca^{2+}) in electrostatic effects on membranes, so perhaps this could be used in CD?

Ronald J. Clarke answered: Yes, it is true that using $CaCl_2$ instead of NaCl or KCl one could achieve a higher ionic strength with a lower concentration of salt. However, in the case of Ca^{2+}, any effects seen could not simply be attributed to a nonspecific charge screening effect. Ca^{2+} reduces the membrane dipole potential of phosphatidylcholine membranes, probably by binding to its phosphate group.[1] It has also long been known that Ca^{2+} and Mg^{2+} bind to the headgroup of phosphatidylserine.[2,3] Therefore, assuming we do see changes in membrane binding of our peptides after adding $CaCl_2$, it's likely that these changes would be due to Ca^{2+} binding to the lipid headgroups rather than simply charge screening. This said, it would actually be very interesting to study the effect of Ca^{2+} ions because of their known role in cell signalling.

1 R. J. Clarke and C. Luepfert, Influence of anions and cations on the dipole potential of phosphatidylcholine vesicles: a basis for the Hofmeister effect, *Biophys. J.*, 1999, **76**, 2614–2624.
2 H. Hauser, D. Chapman and R. M. C. Dawson, Physical studies of phospholipids: XI Ca^{2+} binding to monolayers of phosphatidylserine and phosphatidylinositol, *Biochim. Biophys. Acta, Biomembr.*, 1969, **183**, 320–333.
3 C. Newton, W. Pangborn, S. Nir and D. Papahadjopoulos, Specificity of Ca^{2+} and Mg^{2+} binding to phosphatidylserine vesicles and resultant phase changes of bilayer membrane structure, *Biochim. Biophys. Acta, Biomembr.*, 1978, **506**, 281–287.

Paul O'Shea continued: Ron, the satellite peptide on the ATPase is very interesting, particularly the involvement of phosphorylation. Such a dramatic localised change of the charge density could lead to lateral movement of the ATPase (see *e.g.* ref. 1) as well as changes of the activity. I wonder therefore, does it have any bearing on the sorting of the ATPase in the membrane? There is precedence for electrostatic localisation of ATPases, as many years ago Jim Barber at Imperial College promoted the idea of chloroplast ATPase sorting and thylakoid stacking due to changes of the surface electrostatics.[2] I was intrigued by your story of the peptide flipping in or out based on phosphorylation and electrostatics. Does this have any basis on the sorting of the ATPases?

1 J. L. Richens, J. S. Lane, J. P. Bramble and P. O'Shea, The electrical interplay between proteins and lipids in membranes, *Biochim. Biophys. Acta, Biomembr.*, 2015, **1848**, 1828–1836.
2 J. Barber, Influence of surface charges on thylakoid structure and function, *Annu. Rev. Plant Physiol.*, 1982, **33**, 261–295.

Ronald J. Clarke responded: The question of the quaternary structure of the Na^+,K^+-ATPase is a very interesting one, which has been the topic of debate for many years. It is known that the minimal functional unit of the Na^+,K^+-ATPase consists of two subunits; the catalytic alpha subunit with ten transmembrane helices and the beta subunit with a single transmembrane helix, which is necessary for its trafficking to the plasma membrane and is probably involved in the pump's regulation. In addition, the Na^+,K^+-ATPase can be further modified by another single transmembrane FXYD protein, termed the gamma subunit in the kidney enzyme.[1] In 2007, with the publication of the first crystal structure of the Na^+,K^+-ATPase, it became clear that the ion transport pathway of the enzyme is located within the alpha subunit.[2] Therefore, further oligomerisation is not necessary to create the ion pathway. Nevertheless, there is a substantial amount of data indicating that the Na^+,K^+-ATPase is able to aggregate within the membrane to form $\alpha_2\beta_2$ dimers or $\alpha_4\beta_4$ tetramers.[3] The exact role of this aggregation of the protein is still uncertain, but kinetic evidence obtained *via* the stopped-flow technique indicates that the degree of aggregation depends on the concentration of the substrate ATP and the ionic strength of the surrounding solution, and that the aggregation into a dimer or higher oligomer decreases the rate of ion pumping but increases the ATP binding affinity.[4–7] The dependence of the state of aggregation of the Na^+,K^+-ATPase on the ionic strength of the surrounding solution is a strong indication that the protein dimers or oligomers are stabilized by an electrostatic interaction. Considering the data which we have shown in our paper, as well as in previously published work,[8] indicating an electrostatic interaction between the alpha subunit's N-terminus with the surrounding membrane, it appears likely that the N-terminus could be involved in determining the state of aggregation of the protein within the membrane, as you suggest.

1 M. V. Clausen, F. Hilbers and H. Poulsen, The structure and function of the Na^+,K^+-ATPase isoforms in health and disease, *Front. Physiol.*, 2017, **8**, 371.
2 J. P. Morth, B. P. Pedersen, M. S. Toustrup-Jensen, T. L. M. Sørensen, J. Petersen, J. P. Andersen, B. Vilsen and P. Nissen, Crystal structure of the sodium-potassium pump, *Nature*, 2007, **459**, 446–450.
3 R. J. Clarke and X. Fan, Pumping ions, *Clin. Exp. Pharmacol. Physiol.*, 2011, **38**, 726–33.
4 C. Lüpfert, E. Grell, V. Pintschovius, H.-J. Apell, F. Cornelius and R. J. Clarke, Rate limitation of the Na^+,K^+-ATPase pump cycle, *Biophys. J.*, 2001, **81**, 2069–2081.
5 R. J. Clarke and D. J. Kane, Two gears of pumping, *Biophys. J.*, 2007, **93**, 4187–4196.
6 R. J. Clarke, Mechanism of allosteric effects of ATP on the kinetics of P-type ATPases, *Eur. Biophys. J.*, 2009, **39**, 3–17.
7 Q. Jiang, A. Garcia, M. Han, F. Cornelius, H.-J. Apell, H. Khandelia and R. J. Clarke, Electrostatic stabilization plays a central role in autoinhibitory regulation of the Na^+,K^+-ATPase, *Biophys. J.*, 2017, **112**, 288–299.
8 K. Nguyen, A. Garcia, M.-A. Sani, D. Diaz, V. Dubey, D. Clayton, G. Dal Poggetto, F. Cornelius, R. J. Payne, F. Separovic, H. Khandelia and R. J. Clarke, Interaction of N-terminal peptide analogues of the Na^+,K^+-ATPase with membranes, *Biochim. Biophys. Acta, Biomembr.*, 2018, **1860**, 1282–1291.

Paul O'Shea continued: Does the peptide perhaps talk to cholesterol rich domains when it becomes incorporated in the membrane, and so may have a role

in targeting or localisation of the ATPase? Have you seen any domain formation as a result of the peptide interaction or localisation of the ATPases or even 'polymerisation' of the ATPases?

Ronald J. Clarke answered: Cholesterol certainly does have a strong effect on Na^+,K^+-ATPase activity, which increases significantly with increasing cholesterol content of the membrane up to the physiological level of around 40 mol% of the total lipid.[1] Cholesterol favours conversion of the enzyme into its E1 conformational state, the state to which Na^+ binds. The conformational transition into the E1 state is also necessary to allow phosphorylation of the Na^+,K^+-ATPase by ATP and transfer of Na^+ across the membrane. There is clear evidence from studies looking at the pattern of proteolytic digestion of the protein in its E1 and E2 (K^+ binding) conformations that the N-terminus does undergo significant movement during the E2-to-E1 transition.[2] Therefore, it is indeed possible that there could be some interplay between cholesterol and the protein's N-terminus involved in localising the Na^+,K^+-ATPase to cholesterol-rich domains. It would be interesting to perform some experiments imaging the distribution of the Na^+,K^+-ATPase to see if one could detect any localisation in lipid rafts or liquid-ordered domains.

1 A. Garcia, B. Lev, K. R. Hossain, A. Gorman, D. Diaz, T. H. N. Pham, F. Cornelius, T. W. Allen and R. J. Clarke, Cholesterol depletion inhibits Na^+,K^+-ATPase activity in a near-native membrane environment, *J. Biol. Chem.*, 2019, **194**, 5966–5969.

2 P. L. Jorgensen and J. H. Collins, Tryptic and chymotryptic cleavage sites in sequence of α-subunit of (Na^+ + K^+)-ATPase from outer medulla of mammalian kidney, *Biochim. Biophys. Acta*, 1986, **860**, 570–576.

Patricia Bassereau asked: If the pumping activity of the ATP-ase depends on peptide binding, how are the protein activity and the phosphorylation synchronized?

Ronald J. Clarke responded: The proteins' ion pumping activity is not synchronized with the phosphorylation of the conserved serine on the extramembranous N-terminus. P-type ATPase activity is driven by the hydrolysis of ATP, which causes an autophosphorylation (*i.e.*, not catalysed by another enzyme) of a conserved aspartic acid residue on the proteins' α-subunit. Although the entire α-subunit is of course a single polypeptide chain, this aspartic acid residue is not located within the proteins' N-terminal tail which we are investigating. Autophosphorylation of the aspartic acid residue is a catalytic phoshorylation required for ion pumping activity, whereas phosphorylation of the serine of the N-terminal tail by protein kinase C is a regulatory phosphorylation, which alters the ion pumping rate but isn't necessary to allow pumping to occur.

Margarida Bastos said: I am not sure about the relevance of deepness of insertion between Lys and Arg you referred to when answering our colleague, Larisa Zoranić , as although Arg might go deeper into membranes, the CPPs (cell penetrating peptides), rich in Arg, enter the cells, even with cargo, without destroying the membranes as AMPs do. This might be one of the reasons why Arg is the most common positively charged amino acid in CPPs.

Ronald J. Clarke replied: Yes, I agree that arginine is preferable to lysine in cell penetrating peptides to promote transport across the membrane. In the case of the lysine-rich N-terminal peptide of the Na^+,K^+-ATPase which I spoke about, we believe that the peptide moves on and off the membrane during the protein's ion pumping cycle. If the peptide was arginine-rich instead of lysine-rich, the peptide may be so strongly bound to the membrane that it acts as a permanent anchor rather than switching between on and off states. This interpretation is supported by molecular dynamics simulations comparing the arginine and lysine side-chains,[1] which showed enhanced binding of arginine, disrupting and permeabilizing the membrane.

1 L. Li, I. Vorobyov and T. W. Allen, The different interactions of lysine and arginine side chains with lipid membranes, *J. Phys. Chem. B*, 2013, **117**, 11906–11920.

Burkhard Bechinger added: Previously H-bonding interactions between Arg and lipid phosphates have been suggested that pull the lipid head groups into the membrane interior.[1] A difference between lysine and arginine is that the positive charge of the latter is 'smeared out' over a larger part of the molecule when compared to lysine, making the energy of placing an ion in a hydrophobic environment less unfavourable.[2] Furthermore, the arginine with three H-bond acceptors on the side chain should be able to associate with a larger hydration sphere which eases membrane insertion of the charge.

1 M. Tang, A. J. Waring and M. Hong, Phosphate-mediated arginine insertion into lipid membranes and pore formation by a cationic membrane peptide from solid-state NMR, *J. Appl. Chem. Sci.*, 2007, **129**, 11438–11446.
2 J. N. Israelachvili, S. Marcelja and R. G. Horn, Physical principles of membrane organization, *Q. Rev. Biophys.*, 1980, **13**, 121–200.

Sreetama Pal opened a general discussion of the paper by Paul Beales: It might be interesting to explore the role of calcium in these systems. Since you have phosphatidylserine in the system, the presence of calcium could induce isothermal phase transitions. The resulting spatial heterogeneity might provide additional control on the action of ESCRT.

Paul Beales responded: In these experiments we start with phase separated vesicles using a lipid composition close to the middle of the liquid–liquid coexistence region of the phase diagram. We already have divalent Mg^{2+} ions in solution for the ATP hydrolysis.

In future work, an interesting next step could be to make single phase systems close to the phase coexistence boundary and investigate how environment responsive phase separation couples with ESCRT activity. This wouldn't necessarily need calcium ions. ESCRT-II has been shown previously to trigger phase separation in supported lipid bilayers in the work by the group of James Hurley.[1] It is likely that this is a multivalent electrostatic clustering effect as many multivalent lipid clustering interactions have been shown to be able to trigger phase separation in the literature.

1 E. Boura, V. Ivanov, L.-A. Carlson, K. Mizuuchi and J. H. Hurley, *J. Biol. Chem.*, 2012, **287**, 28144–28151.

Patricia Bassereau asked: The L_o phase is more difficult to bend than L_d. So why would the L_o domain be recruited? The Snf7 assembly alone induces a local flat membrane shape but, bends it as soon as other ESCRT-III proteins are recruited, which should in principle recruit the more flexible L_d membrane.

Paul Beales responded: The curvature of the buds is still relatively small as their radius of curvature is still in the μm range. Therefore the energy cost of bending is not likely to be severe. The energy cost of bending will also be compensated by the reduction in line tension at the domain edge. The high curvature region of the buds is in the neck, where we still see evidence of the importance of the L_d domains in these regions.

Patricia Bassereau remarked: How would your mechanism work on asymmetric membranes, such as the plasma membrane?

Paul Beales replied: Great question. This would be a really interesting experiment to do but isn't something we have looked at. Asymmetric GUVs are not a standard system and so present challenges. If I was to speculate, bilayer domains are still likely to occur in the plasma membrane since this has been observed in asymmetric model membranes. Therefore the mechanisms we observe in these model membranes may well be relevant *in vivo* too.

Patricia Bassereau asked: You propose that the L_o domain is nucleated or recruited under the ESCRTs filaments. But, when the bud forms, the ESCRTs are expected to be at the neck where you also find the L_d membrane if I understand, and the L_o domain is more underneath but in the bud. Can you explain more about this?

Paul Beales responded: The model we present to explain our observations proposes that the L_o phase may be recruited beneath the growing ESCRT complex due to an entropic effect where suppressed membrane thermal undulations would favour the more rigid phase (analogous to a similar effect reported previously by Gordon and Deserno *et al.*[1]). Once the membrane begins to bud, either the L_o domain is kinetically trapped in this position, or the reduction in line tension by constriction of the neck becomes the energetic factor that favours the L_o domain localisation in the bud. This is currently our best hypothesis using plausible biophysical mechanisms. However, we do not present this as a proven mechanism and we are open to alternative models.

1 V. D. Gordon, M. Deserno, C. M. J. Andrew, S. U. Egelhaaf and W. C. K. Poon, Adhesion promotes phase separation in mixed-lipid membranes, *EPL*, 2008, **84**, 48003.

Durba Sengupta asked: Could the ESCRT cause L_o/L_d local "remixing" by modulating the line tension?

Paul Beales responded: On the contrary, ESCRT-II has been shown to induce phase separation on surface-supported membranes.[1] Furthermore, we do not see any evidence for phase remixing in our experiments – the timescale for which would be expected to be slow enough to be observable. As these membranes are in

a L_o–L_d region of the phase diagram, the bulk free energy of each phase is favourable in equilibrium. It would need a very large line tension to disfavour the phase separation and based on other reports, where there is a high line tension between membrane domains, membrane bending and vesicle fission has been reported. Interestingly, ESCRTs do drive membrane fission, so I would not discount that modulation of line tension could play a role in this function.

1 E. Boura, V. Ivanov, L.-A. Carlson, K. Mizuuchi and J. H. Hurley, Endosomal sorting complex required for transport (ESCRT) complexes induce phase-separated microdomains in supported lipid bilayers, *J. Biol. Chem.*, 2012, **287**, 28144–28151.

Amitabha Chattopadhyay said: Although asymmetric vesicles (liposomes) are difficult to prepare, Erwin London (Stony Brook University) has been able to generate asymmetric liposomes of different sizes using cyclodextrin-mediated exchange.

Paul Beales responded: I agree that asymmetric vesicles have been prepared but I feel this is a long way from a robust and standardised approach for the field. Knowing/controlling the precise compositions of the two leaflets of the membrane and characterising the lifetime of the asymmetry, which will dissipate over time due to lipid flip-flop, are examples of challenges in working with asymmetric vesicles. I agree that this is an important future direction for membrane biophysics research, but not something we have investigated at this stage.

Patricia Bassereau commented: A comment on phase-separation induced by ESCRT: it is a well-known effect that many multi-valent proteins that bind to lipids change the phase diagram and facilitate phase-separation (entropic effect). This is the case for toxins that bind to glycolipids, such as the Shiga toxin (15 Gb3) or Cholera toxin (5 GM1), or actin filament binding to PiP2 lipids.

Paul Beales answered: I agree. This has been seen in many systems as a general biophysical phenomenon where extraneous matter binds to the membrane and clusters specific lipids.

Patricia Bassereau also asked: If I understand you correctly, having L_d lipids in the "neck" can reduce the overall bending energy, but it will be higher that a uniform L_d bud? Maybe this is not an equilibrium process?

David P. Siegel answered: Let k_o and k_d be the bending elastic moduli of bilayers in the ordered and disordered phase, respectively. Let κ_o and κ_d be the Gaussian curvature elastic moduli of bilayers in those two phases, respectively. The Gaussian curvature of a separate GUV is 4π. In the limit of large vesicle size, introducing a "neck" connecting the GUV to the flat parent membrane makes a Gaussian curvature contribution of -4π. Let's call the GUV connected to the flat membrane a "bud." The curvature energy of a disordered phase GUV relative to the original flat bilayer $= 8\pi k_d + 4\pi\kappa_d$; that of a non-fissioned L_d-phase bud with an L_d phase "neck" is $8\pi k_d$, and that of a bud mostly in the L_o phase but with an L_d phase "neck" is $= 8\pi k_o + 4\pi(\kappa_o - \kappa_d)$. The difference in curvature energy between

the ordered phase bud with the L_d phase "neck" and the bud completely in the L_d phase is $8\pi(k_o - k_d) + 4\pi(\kappa_o - \kappa_d)$. In theory, for $(\kappa_d - \kappa_o) > 2(k_o - k_d)$, the curvature energy of the bud with the L_d neck and L_o phase body is lower than the bud completely in the L_d phase. However, as far as I am aware, the values of the Gaussian curvature moduli of bilayers in these phases are unknown.

Paul Beales added: This effect can be seen in the classic 2003 *Nature* paper from Baumgart,[1] Hess and Webb where they see the phase boundary is not in the neck of budding GUVs in L_o–L_d (which would minimise line tension) but the neck is L_d and the phase boundary sits just outside this due to the contribution of bending energy. Furthermore, by having the phase boundary near the neck of the bud, this lowers the overall line tension between coexisting phases. The reduction in line tension also needs to be considered alongside the curvature energies of the bulk phases.

1 T. Baumgart, S. Hess and W. Webb, Imaging coexisting fluid domains in biomembrane models coupling curvature and line tension, *Nature*, 2003, **425**, 821–824, DOI: 10.1038/nature02013.

David P. Siegel addressed Paul Beales and Patricia Bassereau: The curvature energy of a vesicle produced by budding is determined by the values of both the bilayer bending modulus and the bilayer Gaussian curvature modulus. This is also true of the curvature energy of the intermediate in budding, in which the vesicle is still connected to the parent membrane by a "neck." The Gaussian curvature elastic energy contribution of the "neck" is considerable, and different in sign than that of the final budded vesicle. The values of the Gaussian curvature modulus for the L_d and L_o phases of this composition are unknown, and they are difficult to measure. Depending on the values of these Gaussian curvature moduli with respect to the bending moduli, it is conceivable that a vesicle which is primarily in the L_o phase, but which has a neck region in the L_d phase, could have a total curvature energy which is lower than a bud completely in the L_o phase, and conceivably comparable to a bud totally in the L_d phase. Therefore Professor Beales' finding is not necessarily inconsistent with a simple membrane curvature energy model, even before we consider the effects of line tension.

Paul Beales responded: It would be great to be able to give full consideration of the Gaussian curvature moduli but these are elusive and have only been measured experimentally in a limited number of special cases.

Patricia Bassereau asked: The ESCRT complexes do not remain flat on the GUV if partners of Snf7 are present, and also Vps4. It is expected to induce the neck formation. Are the L_o domains moving away from the ESCRT filaments, but remain trapped in the forming bud?

Paul Beales replied: What we can say from these experiments is that L_o domains preferentially located in the ESCRT-induced buds and that PS is required in the L_d phase, which recruits the ESCRT complex. It also appears that the L_d phase locates to the necks of budding vesicles. We hypothesise in the paper that the suppression of entropic thermal undulations under the assembling ESCRT

complex may energetically favour the recruitment of the L_o phase. The budding and neck formation also minimises the line tension between phases. However, I think you'd agree that this is an interesting and unexpected observation, where further work will be required to fully pin down the precise mechanistic explanation.

Paul Beales added, in response to a comment from Aurélien Roux regarding VPS4: There still appears to be debate on this topic in the literature and we do not perform specific experiments that probe the role of Vps4. What is your opinion on the role of Vps4 given your experience with experiments in this area and the possible difference between *in vivo* and *in vitro*?

Aurélien Roux replied: I know that people are debating about Vps4 being a sole depolymerization factor. In my opinion, there is numerous evidence that Vps4 does not solely promote disassembly. First, overexpression of the dominant negative ATPase dead mutant also blocks the fission reaction *in vivo*. It is true for HIV viruses, cytokinetic abscission, and ILV formation. These is quite strong evidence that Vps4 is needed for fission. And we found the same in our *in vitro* reconstituted assay.

Paul Beales responded: Certainly we have seen that without the Vps4, we can get some ILV formation activity. We don't have the resolution in confocal microscopy studies to see what is happening with individual ESCRT components on the membrane. But we do then see a second round of ILV formation events with the addition of Vps4 and ATP.[1] So there appears to be some recycling of the ESCRT activity.

1 A. Booth, C. J. Marklew, B. Ciani and P. A. Beales, *In vitro* membrane remodeling by ESCRT is regulated by negative feedback from membrane tension, *iScience*, 2019, **15**, 173-184.

Paula Milán Rodríguez opened a general discussion of the paper by Reidar Lund: To what extent are the results (degree of insertion in the membrane) sensitive to the membrane lipid composition and P/L ratio? Have you tried other compositions and other ratios?

Reidar Lund replied: The experiments were performed on a variety of P : L ratios (reported in the paper) and we generally find that the interaction is peptide-dependent. For most peptides there is mostly an increase in amount accumulated, but for LL-37 we see a clear concentration dependent interaction pattern where the peptide seems to presumably bundle and insert deeper into the membrane with higher concentrations. We have previously reported data on DMPC/DMPG vesicles containing 2.5 mol% pegylated vesicles. We also varied the fraction of PG, and thereby the overall charge of the membrane, in the case of indolicidin. Surprisingly, we did not see significant changes in peptide insertion in this case.[1] We plan to study the effect of varying the amount of negatively charged lipids on the membrane interaction of other peptides besides indolicidin in future work.

1 J. E. Nielsen, V. A. Bjørnestad and R. Lund, Resolving the structural interactions between antimicrobial peptides and lipid membranes using small-angle scattering methods: the case of indolicidin, *Soft Matter*, 2018, **14**, 8750–8763.

Paula Milán Rodríguez asked: What are the other polar heads and aliphatic tails present in the *E. coli* membranes?

Reidar Lund answered: The lipid composition of *E. coli* has been widely studied, and found to depend on, amongst other things, the growth temperature of the bacteria. With growth at 37 °C the phospholipid headgroup composition of the inner membrane of *E. coli* is reported to be 75±1% phosphatidylethanolamine (PE), 19±1% phosphatidylglycerol (PG) and 6±2% diphosphatidylglycerol (DPG). While the acyl chain composition is 3.7% 14 : 0, 0.3% 14 : 1c7, 0.4% 15 : 0, 42.8% 16 : 0, 31.9% 16 : 1c9, 1.6% 18 : 0, 14.7% of 18 : 1c11, and 4.6% unidentified. This equals 46.9% unsaturated and 50.3% saturated acyl chains (not including the unidentified).[1]

1 M. Sven, A. Ann-Sofie, R. Leif and L. Göran, Wild-type *Escherichia coli* cells regulate the membrane lipid composition in a "window" between gel and non-lamellar structures, *J. Biol. Chem.*, 1996, **271**, 6801–6809.

Georg Pabst said: Your contribution seems to really rely on your specific need to have the unilamellar vesicles. This led you to the use of PEGylated lipids and studies of DMPE/DMPG in the gel phase. As a consequence, you compare gel phase data of DMPE/DMPG to fluid phase data of DMPC/DMPG (*i.e.* completely different states of membrane structure). How can you make conclusions on the activity of your studied peptides in the physiologically relevant state of membranes (which is fluid)? Instead, you could use other and physiologically more relevant lipid mixtures of *e.g.* POPE/POPG (possibly even add cardiolipin) and dose the peptide concentration in a way that does not lead to morphological or topological changes of your vesicles. This is fully compatible with scattering experiments on vesicles, including SANS, to enhance contrast (see *e.g.* reports from our group, *e. g.* ref. 1). Alternatively, you could use the peptide induced topological/morphological change as the readout for peptide activity.

1 M. Pachler, I. Kabelka, M.-S. Appavou, K. Lohner, R. Vácha and G. Pabst, Magainin 2 and PGLa in bacterial membrane mimics I: Peptide-peptide and lipid-peptide interactions, *Biophys. J.*, 2019, **117**, 1858–1869, DOI: 10.1016/j.bpj.2019.10.022.

Reidar Lund responded: First of all it should be noted that accurate determination of the peptide position within the membrane, unilamellar vesicles are needed. Having said that, we agree with the comment that fluid lipid membranes are desirable and we plan more experiments using unsaturated lipids in the future. Our intention in the TR-SANS experiment was to increase the temperature to above the melting point of the lipids. However, the stability of the current system we were working with did not allow us to do this. We do have experiments using other lipids, including cardiolipin, planned, but these have so far been postponed due to Covid-19 and neutron facility shut-downs.

Enrico Federico Semeraro commented: The scattering data analysis of the mixed lipid/AMP systems presents some gaps that the authors should address.

Specifically, (i) the presence of AMPs only on the outer bilayer leaflet, and (ii) the lack of critical comments on the uniqueness of the solution and on the confidence degree of the adjustable parameters.

(i) The presented results show that indolicidin, LL-37 and lacticin Q reside exclusively in the outer membrane leaflet, and aurein 1.2 penetrates deeper into the hydrophobic core. However, samples were measured waiting for equilibration (an aspect that should be specified), and it is unlikely that the spatial distribution of peptides is not equilibrated (at least partially) among both leaflets. Firstly, as examples, Ulmschneider reported the translocation of PGLa;[1] the laboratory of Vácha and colleagues reported extensively on the free energy barrier to cross lipid bilayers [see, *e.g.*, ref. 2 and 3]. Secondly, in order to justify the lack of effects induced by colistin, the authors write: "intracellular targets like peptide binding to ribosomes indicating passage through the outer and inner membrane has been presented." In this respect, there is evidence for intracellular targets for indolicin, LL-37 and an analog of aurein (to make the list short, see the review[4]). Thus, according to the authors themselves, peptide translocation and redistribution on both membrane leaflet is a required physical condition. Thirdly, the authors observed a detergent-like effect that cause micellization. Given the degree of perturbation of this process (membrane remodeling), it is physically extremely unlikely for the peptides to have an absolutely inaccessible inner leaflet.

(ii) The analysis of SAXS data that aims to retrieve the volume probability distribution of each component of a lipid membrane is not trivial and requires several constrains even in simple systems.[5] Often, contrast variation SANS measurements are used to reduce the degree of variability of the adjustable parameters. The addition of peptides, modeled as Gaussian volume distribution regardless of their size, can easily lead to overparameterization issues. These bias the uniqueness of the results, which then strongly depend on the initial assumptions and constrains of the applied model. Firstly, in the presence of peptides, the observed lift-off of the low-q minimum of the scattering patterns was addressed to peptide-induced transbilayer asymmetry. Unfortunately, asymmetry is not the only cause of such an empirical observation. Also changes in contrast profile, or volume of the unit cell (which depends on the ratio of partitioned peptides per lipids) can affect the scattering pattern in that region (just to name a few). As an example, the scattering analysis of the coexisting quasi-interdigitated phase has been reported,[6] where high-q data were fitted properly. Secondly, the precision of the structural details (in the nanometre length-scale) has to be reflected in the quality of the fitting in the high-q range (including $q > 0.1$ A A^{-1}, Fig. 3 in ref. 6), because it is the q-range that contains most of the information about such details. It appears, instead, that the authors only focused on the low-q region. Thirdly, the degree of confidence of the adjustable parameters should be included given the significant number of parameters. Without that, the interpretation of the results remains speculative. To conclude, considering all these points, I would appreciate from the authors critical comments on both data analysis and interpretation of results.

1 J. P. Ulmschneider, Charged antimicrobial peptides can translocate across membranes without forming channel-like pores, *Biophys. J.*, 2017, **113**, 73–81.
2 I. Kabelka and R. Vácha, Optimal Hydrophobicity and Reorientation of Amphiphilic Peptides Translocating through Membrane, *Biophys. J.*, 2018, **115**, 1045–1054.

3 I. Kabelka, R. Brožek and R. Vácha, Selecting collective variables and free-energy methods for peptide translocation across membranes, *J. Chem. Inf. Model.*, 2021, **61**, 819–830.
4 C.-F. Le, C.-M. Fang and S. D. Sekaran, Intracellular targeting mechanisms by antimicrobial peptides, *Antimicrob. Agents Chemother.*, 2017, **61**, e02340-16.
5 N. Kučerka, J. F. Nagle, J. N. Sachs, S. E. Feller, J. Pencer, A. Jackson and J. Katsaras, Lipid bilayer structure determined by the simultaneous analysis of neutron and X-ray scattering data, *Biophys. J.*, 2008, **95**, 2356–2367.
6 E. Sevcsik, G. Pabst, A. Jilek and K. Lohner, How lipids influence the mode of action of membrane-active peptides, *Biochim. Biophys. Acta, Biomembr.*, 2007, **1768**, 2586–2595.

Reidar Lund responded: This is an interesting question. However, we do not quite see why symmetry must be imposed *a priori*. First of all, difference in curvature of the inner/outer leaflet may change the interaction and lead to different lipid distribution, and consequently charge distribution. Secondly, the peptides were added to the outside of the vesicle (trying to crudely mimic the situation when applied to bacteria). There might thus be significant barriers for translocation of the peptide. Since we measured the mixtures over time in hours, and no change was detected, this means that we are in a stable, although possibly, metastable, quasi- equilibrium situation. We measured the time evolution and we did not detect any change at all. In fact, trial experiments using stopped-flow SAXS showed that, for example, indolicidin inserted on time scales down to a few milliseconds without detectable changes in the bilayer structure or peptide distribution. In ref. 1 we show how differences in asymmetric *versus* symmetric insertion of peptides can be resolved from SAXS data. Regarding the solubilization we do agree that resolving the peptide location in the remaining liposomes imposes a challenge, as the number of free parameters increases significantly. Furthermore, the scattering contribution from the micelles interferes with the first minimum which we show is essential to resolve the peptide insertion. This provides less confidence for more potent peptides at large P : L values which is why we aimed to start at the smallest P : L values that are detectable. We have also seen that even for rather weakly interacting peptides, such as indolicidin, solubilization indeed also occurs, but at very high P : L values not directly relevant biologically. However interestingly, such high P : L values of 1 : 1 are typical values for surface techniques such as QCM and reflectometry.

We are well aware that the fit analysis using the detailed model is complicated and needs constraints. However, for the neat bilayers there are well established data both from experiments and simulations published by, among others, Kučerka *et al.* We have applied all possible constraints in our analysis to minimize the free parameters. In fact, only the thickness is varied within soft constrains based on literature values by Kučerka *et al.*[4] For the peptide we introduce only two more – the width and main position within the bilayer. We try first to only vary the peptide-related parameters which usually works fine at low P : L ratios. At higher values, depending on the peptide, the thickness of the bilayer might change; and at even higher rations we observe some solubilization. We agree that at significant amounts of solubilization the conclusions become less clear. Concerning asymmetry, we are fully aware that there are several factors that influence not only the position but also the shape of the first minimum. However, we use low P : L values, below the solubilization limit, and vary the fraction of peptides to detect such changes. As discussed, the model allows us to explicitly let the density distribution of the peptide vary freely while properly calculating the electron density distribution throughout the bilayer. See *e.g.* ref. 2 for detailed discussions,

and ref. 3 for comparison with neutron reflectometry results. Previously reported work sets to be under the constraint of either needing to restrict the peptide to the head or tail, and symmetric distribution on both leaflets simultaneously. Concerning the negative remark about the fit quality at high q, we do not see that there are large deviations considering that we use a complete fit model for the whole q range. Please note that electron density distribution should give consistent results both at high and intermediate q. We do not believe in cutting the SAXS q range to improve the fit, we rather use data over as large q-range as possible doing our experiments using high resolution synchrotron SAXS instruments, and attempting to analyse complete data sets.

1 J. E. Nielsen, V. A. Bjørnestad, V. Pipich, H. Jenssen and R. Lund, Beyond structural models for the mode of action: How natural antimicrobial peptides affect lipid transport, *J. Colloid Interface Sci.*, 2021, **582**, 793–802.
2 J. E. Nielsen, V. A. Bjørnestad and R. Lund, Resolving the structural interactions between antimicrobial peptides and lipid membranes using small-angle scattering methods: the case of indolicidin, *Soft Matter*, 2018, **14**, 8750–8763.
3 J. E. Nielsen, T. K. Lind, A. Lone, Y. Gerelli, P. R. Hansen, H. Jenssen, M. Cárdenas, R. Lund, A biophysical study of the interactions between the antimicrobial peptide indolicidin and lipid model systems, *Biochim. Biophys. Acta, Biomembr.*, **1861**(7), 2019, 1355–1364.
4. N. Kučerka, J. F. Nagle, J. N. Sachs, S. E. Feller, J. Pencer, A. Jackson and J. Katsaras, Lipid Bilayer Structure Determined by the Simultaneous Analysis of Neutron and X-Ray Scattering Data, *Biophys. J.*, 2008, **95**, 2356–2367.

Margarida Bastos stated: You state in the abstract that "However, model systems based on PE-lipids (phosphatidylethanolamine) are more prone to destabilization upon addition of peptides, with formation of multilamellar structures and morphological changes. These properties of PE-vesicles lead to less conclusive results regarding peptide effect on structure and dynamics of the membrane." Nevertheless, this lipid is present in most bacterial membranes, mainly in the inner leaflet, so it is very important to use it, as has been discussed in the literature and as you also comment. I believe that this 'instability' is very important, and in fact is responsible for many AMP effects – AMPs are known to destroy membranes in various ways, not only by pore formation, they can form cubic structures, stacked bilayers, *etc.* So, lipids that have a tendency to form inverted structures, and that 'induce curvature' are very important for AMP action.

Aurélien Roux said: This is a very fair statement, and I guess the different modes of membrane destabilization will depend on the exact structure of the inverted lipid, and of its density. I guess one can draw phase diagrams of destabilization. I would be happy to see those diagrams.

Reidar Lund added: This is indeed a very good point. We specifically wanted to avoid multilamellar and non-lamellar phases in order to investigate the peptide organization at/in the membrane- and specifically, whether well-defined pores or channels can be detected. Also, we aimed at measuring the lipid dynamics in as "clean conditions as possible". We will look more into other mechanisms in the near future.

Boyan Bonev responded: Yes, but largely in model systems. With few exceptions, cytoskeleton/peptidoglycan-stabilised lipid membranes, as found in the bacterial envelope, do not buckle or undergo phase conversion to non-bilayer phases.

Kareem Al Nahas asked: Would the observed differences between PC and PE remain for other phospholipids with hydrophobic tails longer than DMPC/DMPE (*e.g.* DOPE/DOPC or DPPC/DPPE)?

Reidar Lund replied: Good question, we have not studied this yet. We plan to use POPE rather than DMPE in the future, due to the lower phase transition temperature. Through these experiments we will be able to also compare with previous collected data on POPC liposomes.

John Sanderson queried: What are the typical errors in measurement when you model the thickness? For example you state "The estimated overall thickness of the DMPE/DMPG membrane based on model fits was found to be 45 Å".

Reidar Lund replied: The thickness of the bilayer is constrained to literature values by Lee *et al.*,[1] Pan *et al.*[2] and Kučerka *et al.*[3]

1 T.-H. Lee, C. Heng, M. J. Swann, J. D. Gehman, F. Separovic and M.-I. Aguilar, Real-time quantitative analysis of lipid disordering by aurein 1.2 during membrane adsorption, destabilisation and lysis, *Biochim. Biophys. Acta, Biomembr.*, 2010, **1798**, 1977–1986.

2 J. Pan, F. A. Heberle, S. Tristram-Nagle, M. Szymanski ,M. Koepfinger, J. Katsaras, N. Kučerka, Molecular structures of fluid phase phosphatidylglycerol bilayers as determined by small angle neutron and X-ray scattering, *Biochim. Biophys. Acta, Biomembr.*, 2012, **1818**, 2135–2148.

3 N. Kučerka, B. van Oosten, J. Pan, F. A. Heberle, T. A. Harroun and J. Katsaras, Molecular structures of fluid phosphatidylethanolamine bilayers obtained from simulation-to-experiment comparisons and experimental scattering density profiles, *J. Phys. Chem. B*, 2015, **119**, 1947–1956.

John Sanderson asked: What is the timescale for the detergent effects of aurein 1.2 at high P : L?

Reidar Lund replied: We freshly mixed the solution just before the synchrotron SAXS experiment, so the solubilization we observe happens within a few minutes. To resolve the actual time scale of the solubilization we hope to utilize stopped-flow SAXS in future experiments.

Mibel Aguilar said: Aurein is a potent membrane lytic peptide at quite low concentrations. What concentrations did you use and did you see any evidence of bilayer disruption?

Reidar Lund responded: We think that the most relevant parameter in this case is the peptide–lipid (P : L) ratio rather than the concentration of peptide, as concentration can be misleading when comparing, for example, MIC values which are at a given bacterial cell amount and therefore not an independent concentration. In this work, we varied the P : L ratios 1 : 20, 1 : 50 and 1 : 100 which correspond to approximately 0.27–0.6 mg ml^{-1} aurein 1.2. At 1 : 20 we did see

significant solubilisation (~48% micelles) of the liposomes in the case of aurein 1.2 (significantly more than for the other peptides included in this study as seen from Table S1 of the ESI in our paper). We also observe some solubilisation at 1 : 50 P : L ratio (~5% micelles). This indeed supports aurein 1.2 being a potent membrane lytic peptide.

Evelyne Deplazes remarked: Do you have any insights into why colistin does not work?

Reidar Lund answered: We need to investigate this in more detail before concluding. Please also see my answer to the question from Georg Pabst below, addressed to Evelyne Deplazes and I.

Georg Pabst commented: Your time-resolved SANS data is a convolution of several processes (lipid transfer through the aqueous phase with and without peptide, peptide-mediated lipid flip-flop,…). How can you conclude on lipid flip-flop only? A speeding up of SANS contrast changes might be also due to peptide-mediated transfer between vesicles (*e.g. via* micellar-like aggregates). Have you tried writing down rate equations and seeing if they give reasonable results?

Reidar Lund responded: Indeed, we probe both exchange between vesicles and flip-flop. Both may be relevant to the AMP mechanism. This gives rise to two rate constants as reported by Nakano *et al.*[1] (and several follow-up papers), and also by us in a similar system. In the present work, we also observe two rate constants which become faster with the addition of peptides. In ref. 2 we show similar data for DMPC/DMPG liposomes, and is in this case we were able to extract quantitative values for the rate constants, as well as activation energies. In the present case, temperature variation was not straightforward since the structural stability was impaired at temperatures higher than about 37 °C. However, for exchange processes that occur in parallel either freely or *via* peptide complexation, we would expect the rate laws to give effective rate constants (~ sum of the two rate constants). Different exchange rates would thus be very challenging to detect using the current contrast design and set-up.

1 M. Nakano, M. Fukuda, T. Kudo, H. Endo and T. Handa, Determination of interbilayer and transbilayer lipid transfers by time-resolved small-angle neutron scattering, *Phys. Rev. Lett.*, 2007, **98**, 238101.
2 J. E. Nielsen, V. A. Bjørnestad, V. Pipich, H. Jenssen and R. Lund, Beyond structural models for the mode of action: How natural antimicrobial peptides affect lipid transport, *J. Colloid Interface Sci.*, 2021, **582**, 793–802.

Izabella Brand remarked: In the introduction you wrote: "… AMPs in some way or another mainly target the cytoplasmic membrane of the microorganisms". Do you know something about the transfer of these AMPs through the OM? Do they disrupt the structure of the OM?

Reidar Lund answered: This is an important point, We have not investigated this in detail and are not attempted to mimic the OM. However these are interesting questions that might be answered *e.g.* by floating bilayer mimics of OM. See *e.g.*, ref. 1.

1 L. A. Clifton, S. A. Holt, A. V. Hughes, E. L. Daulton, W. Arunmanee, F. Heinrich, S. Khalid, D. Jefferies, T. R. Charlton, J. R. P. Webster, C. J. Kinane and J. H. Lakey, An accurate *in vitro* model of the *E. coli* envelope, *Angew. Chem.*, 2015, **127**, 12120–12123.

Georg Pabst addressed Reidar Lund and Evelyne Deplazes: I am reflecting on the question above from Evelyne Deplazes to Reidar Lund on colistin:

Thermodynamic considerations of partitioning of amphiphilic compounds (*e.g.* AMPs) in membranes show that at the high lipid concentrations (> few mM) typically used for SAXS experiments all peptides can be considered to be bound (*i.e.*, the unbound fraction is negligibly small) – see the work by Steve White[1] or Heiko Heerklotz.[2] This means all colistin can be considered membrane bound (no if's and but's). The fact that no changes on the SAXS patterns are observed then strongly implies that the technique is simply not sensitive to the presence of colistin in the studied q-range (maybe because there is no change in overall membrane thickness). Possibly, some signatures are observed in the WAXS range (where you should have a signal because of measuring in the gel phase). Note also that raw inspection of SAXS data can be misleading if one looks only for changes such as shifts or lift-offs of the form factor minima. Compositional modelling may tell. In any case, the bottom-line of my comment is: not seeing changes in SAXS does not necessarily imply that there are no interactions.

1 S. H. White and W. C. Wimley, *Biochim. Biophys. Acta*, 1998, **1376**, 339–352.
2 H. Heerklotz and J. Seelig, *Eur. Biophys. J.*, 2007, **36**, 305–314.

Reidar Lund answered: Please first note that the conclusion of an apparent lack of interaction is not only relying on results from SAXS. We have also done TR-SANS (no change in lipid dynamics) and DSC (no significant change in lipid packing).[1] We are fully aware that a raw inspection of the SAXS curve is misleading. For example, there is a common misconception that changes in the minimum at intermediate Q towards higher Q, automatically can be interpreted as "membrane thinning". We showed in previous work that this is not necessarily the case as even subtle changes in the electron density distribution/contrast due to peptide insertion will affect this minimum. For SAXS we are in fact very sensitive to any change in electron density as the lipid component (head and tail) have a large difference and opposite sign of the contrast towards water. This means that the scattering cross-term between tail and head region is negative and the resulting structure is almost contrast matched on average. Technically speaking:

$$V_{(\text{tails})}\Delta\rho_{(\text{tails})} \approx V_{(\text{head})}\Delta\rho_{(\text{head})}$$

In fact, for certain lipid vesicles such as POPC, the vesicles are almost perfectly matched at low Q. Our system is thus very sensitive to insertion as well as the position within the membrane because the peptide will affect the electron density of the tails and head region quite differently. In summary, since the contrast for colistin is very similar to other AMPs, and the fact that other techniques do not show any effects of colistin, we conclude that for this lipid mixture, as well as DMPC/DMPG previously measured, colistin sulfate does not seem to insert at all. This might be different for other lipid mixtures. However, why colistin does not insert in a similar manner as the other peptides needs to be further investigated.

We also remark that colistin is in fact a mixture of compounds, and in this study we used commercially available colistin from Sigma-Aldrich.

1 J. E. Nielsen, V. A. Bjørnestad, V. Pipich, H. Jenssen and R. Lund, Beyond structural models for the mode of action: How natural antimicrobial peptides affect lipid transport, *J. Colloid Interface Sci.*, 2021, **582**, 793–802.

Evelyne Deplazes added: Thanks Dr Lund. It is very helpful information to know that colistin can be considered bound (no if's and but's) and that the effect might not be changes in thickness but changes in other properties not sensitive to SAXS measurements.

Izabella Brand said: You mentioned in the answer to Dr Pabst that you want to increase temperature to 44 °C, to observe the phase transition in DMPE vesicles. At temperature above 40 °C some proteins/peptides become denatured. Will such temperature increase affect the structure of your peptides?

Reidar Lund replied: Good point, we will validate this in the case of all of the helical peptides studied before doing experiments above 40 °C. While for indolicidin we have previously shown that the structure does not change at temperatures above 40 °C.[1]

1 J. E. Nielsen, V. A. Bjørnestad and R. Lund, Resolving the structural interactions between antimicrobial peptides and lipid membranes using small-angle scattering methods: the case of indolicidin, *Soft Matter*, 2018, **14**, 8750–8763.

Burkhard Bechinger remarked: In the paper you state that aurein can penetrate deeply into the membrane because of the low net charge. However there are many charges on this 13-residue peptide: 2K, E, D and the potentially charged N-terminus. The energies to insert all these charges into the hydrophobic interior are expected to be very unfavourable. How do you think this happens? Do you assume the p*K*s shift due to salt bridges or that dipoles formation is enough to neutralize the charges?

Reidar Lund replied: This is true, there are several charged side chains in aurein 1.2. Insertion of these charged units into the membrane could be viewed as rather unfavorable. However, it is well known that most AMPs are unstructured in buffered environments, and transform into more stable confined secondary structures upon interaction with lipid membranes. The folded structure of aurein 1.2 when interacting with micelles is described as a short well defined helix (PDB.file 1VM5).[1] In this graphic illustration it is rather clear that the charge residues are positioned in such a way that one has to assume that salt bridges are formed, thus neutralizing the overall charge and enabling integration into the lipid bilayer.

1 Wang, Guangshun, Y. Li and X. Li, Correlation of three-dimensional structures with the antibacterial activity of a group of peptides designed based on a nontoxic bacterial membrane Anchor, *J. Biol. Chem.*, 2005, **280**, 5803–5811.

Burkhard Bechinger commented: Could you explain in more detail what you mean by 'partial charge of indole ring'.

Reidar Lund replied: Tryptophan bears an indole ring which is well known for its ability to dock into the interfacial regions of different biological membranes and is well described, and their depth of interaction is stabilized by strong NOEs between the NH proton in the indole and the choline methyl and alkyl chain proton in a DPC system,[1] enabled as a result of mild charge distribution in the indole.[2] Specifically, in relation to peptide membrane interactions, the indole ring has been described to align on the membrane surface, stabilizing the peptide in the membrane.[3] Furthermore, it is not only the number of indoles, but also their sequential positioning in the peptide structure, which determines the local hydrophobic face, which eventually determines the peptide internalization effect.[4]

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3 W. Hu, K. C. Lee and T. A. Cross, Tryptophans in membrane proteins: indole ring orientations and functional implications in the gramicidin channel, *Biochemistry*, 1993, **32**, 7035–7047.
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David P. Siegel opened the discussion of the paper by Durba Sengupta: If I understand you correctly, you define leaflet curvature as a deformation towards or away from the bilayer midplane. Did you measure a displacement of the monolayer as a whole, or the membrane as a whole? Or is the displacement merely of the lipid–water interface? The radii of curvature are quite large (order 60 nm) compared to the thickness of the bilayer (Fig. 6 in your article). Moreover, the curvature fluctuations occur over an area roughly 5–10 nm wide; making the fluctuations rather local. Therefore, it seems to me that the variations in curvature around cav-1 could actually correspond to local variations in leaflet thickness. For

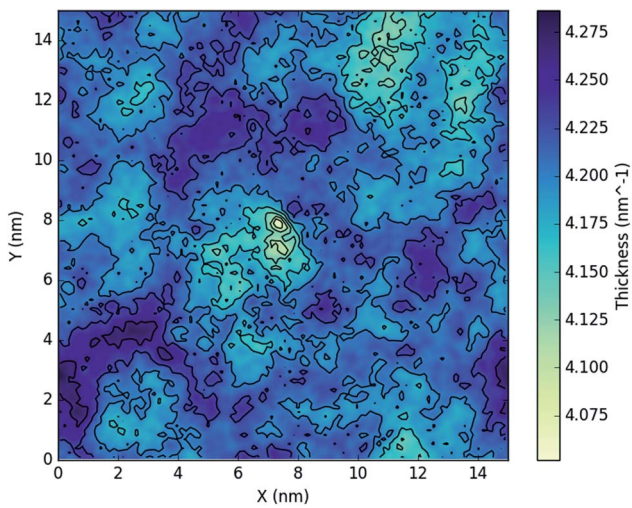

Fig. 1 The membrane thickness profile calculated from the head-group (*P*) distance between the two leaflets binding over the membrane plane.

purposes of comparing the curvature energy of different regions of a monolayer, as in caveolae and the surrounding planar membrane, there is an important distinction between variation in monolayer thickness and variation in the principal radii of curvature of a leaflet. At least according to continuum elastic theory, the curvature energy is determined by values of the bending modulus, the spontaneous curvature and the local curvature. In contrast, the energy required to vary the thickness of the leaflet depends on a different, chain stretching elastic constant. You seem to propose that the deformations which cav-1 induces in your simulations may play a role in stabilizing the 50 nm-scale membrane curvature observed in caveolae. I do not immediately see how variations in local leaflet thickness rationalize that. Obviously, a much higher concentration of the protein in the membrane may have that effect, but I am unsure that the present observations indicate that. I apologize if I have simply misunderstood your definitions.

Durba Sengupta replied: The sign of the curvature (positive or negative) is defined based on the mid-plane. The membrane thickness doesn't appear to change substantially (see Fig. 1 in this discussion).

Paul O'Shea asked: Can you comment on what your model would need to reconcile it with what we would see in a biological system?

Durba Sengupta replied: The model predictions such as cholesterol clustering, PS lipid interactions have already been validated by experimental approaches.[1] However, full-length protein structural models, protein oligomerization, and further post-translational modifications would be important steps in the future.

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Paul O'Shea remarked: Durba: your simulations imply the radii of the invaginations is a few nm, but in cells the radii is of the order of 80–100 nm – do you think you are looking at the same mechanism, *i.e.* is there perhaps a temporal feature that is missing or a time-domain not covered by your model?

Or in other words: What would your model need to reconcile with what we would see in a real biological system? This may help inform your model.

Durba Sengupta responded: Indeed fluctuations of these large bilayer models and instantaneous local curvature events need to be accounted for. We have averaged over multiple simulation sets and over time and don't observe further changes in the time evolution of curvature or lipid clustering. A single caveolin peptide construct would perhaps not stabilize the radii of 80–100 nm, but the models report on the local membrane remodeling induced by a single caveolin monomer.

Paul O'Shea further asked: Durba: This is interesting but I wonder, can you run the model for longer to perhaps see something that is stabilising; you seem to be out of equilibrium still?

Durba Sengupta replied: We did test the convergence of lipid clustering and curvature in time-blocks and don't observe large effects. However, we will take these comments on board and check multi microsecond time evolution as well.

Patricia Bassereau said: Caveolin monomers do not really exist. They are generally found as oligomers only. Since you show there is an interplay between curvature and the lipid composition, if the curvature created by a larger assembly of caveolins is not so strong, what would be your conclusions?

Durba Sengupta answered: Indeed caveolin oligomerization is a very critical aspect of caveolae. As of now, the model doesn't include the terminal domains required for oligomerization. The monomer simulations provide insight into the molecular mechanism of specific lipid–protein interactions and the intrinsic curvature induced. I hope that in the future we will be able to include oligomerization in our models.

Izabella Brand asked: Could you comment on the effect of the palmitoyl chain insertion in caveolin-1 into the membrane? Does this insertion have an effect on the membrane curvature?

Durba Sengupta replied: The lipid chain insertion appears to occur after the initial protein–membrane interaction. We have previously compared the protein-only construct and the lipidated-protein and don't discern differences in membrane curvature. However, further work is required for these complex membranes.

Aishwarya Vijayakumar queried: Does caveolin undergo post-translational modifications? How would it affect membrane curvature?

Durba Sengupta responded: The most significant post-translational modification is the addition of palmitoyl lipid tails. The construct we have considered has a single lipid tail modification (the full length protein has 3). In previous work, we compared the effects of the peptide with and without the lipid tail in simple model membranes. Although the membrane binding is modulated, we were unable to discern any curvature effects.

Aishwarya Vijayakumar asked: If we consider proteins that are instrinsically disordered, they do undergo post translational modifications that impact oligomerisation. In the case of caveolin, how would the oligomerisation be impacted?

Durba Sengupta answered: I would like to clarify that we have considered a protein construct that comprises of the main membrane interacting domains. The intrinsically disordered terminal domains have not been considered. Indeed specific phosphorylation sites have been identified in these domains, and careful work is needed to analyze their role in oligomerization.

Mibel Aguilar opened a general discussion of the paper by Bart Hoogenboom: I understand that cholesterol is important for some perforins. Does cholesterol

play a role in TMH1-PRF function, and have you looked at the effect of different cholesterol concentrations?

Bart Hoogenboom replied: Yes, we have looked at the role of cholesterol. We find that increased cholesterol content makes the membrane more ordered and therefore more resistant to perforin. Cholesterol is not required for perforin pore formation. Perforin pore forms absolutely fine in PC-only membranes. These observations are consistent between WT and TMH1-mutant perforin.

Cholesterol is required, however, for bacterial cholesterol dependent cytolysins (CDC), which are pore forming proteins of the same superfamily as perforin. Many of these CDCs use cholesterol as a binding target on the membrane.

Mibel Aguilar asked: Could you elaborate on the rationale for using cholesterol sulfate?

Bart Hoogenboom responded: We had found that PS scavenged perforin in a dysfunctional state and wanted to verify if that was due to the negative charge in the PS head-group. To test this, we investigated other negatively charged membranes. We used PG as well as cholesterol sulfate, as these result in a negative membrane surface charge, yet are chemically different from PS. The effects on perforin assembly were consistent between PS, PG, and cholesterol sulfate containing membranes, allowing us to attribute the "scavenging effect" to the negative surface charge.

Mibel Aguilar added: Have you done any QNM analysis on these materials to see if there are different properties in terms of the modulus and the adhesion?

Bart Hoogenboom responded: We have not, mainly because the mechanical properties of such supported lipid bilayers have been well documented in the literature, with ordered lipid domains showing enhanced stiffness.

Mibel Aguilar further commented: When you form these beautiful pores, where do you think the lipid that formed the initial membrane bilayers has gone? Is there lipid associated with these protein pores? Are you using a solution that causes the lipid to be washed away?

Bart Hoogenboom replied: There are different possible mechanisms by which the lipid may be removed. In earlier work on related cholesterol-dependent cytolysis,[1] we showed that lipids are essentially ejected, once protein assemblies cut into the membrane like cookie cutters. This can be explained as the inner walls of the pore-forming beta-barrel are hydrophilic, such that lipid patches inside the pore become unstable. This also emerged as a potential mechanism in more recent MD simulations.[2]

For perforin however, this is more subtle, since perforin's pore forming mechanism is more like a can-opener sliding into the membrane around the pore edge. We have not observed any signature of lipid-ejection for perforin, and speculate that there is a receding lipid edge as the pore size increases.

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Paul O'Shea said: Bart: This is a tricky problem; the need for Ca^{2+} at 1 mM as well as an ambient electrolyte concentration to alter the electrostatic interactions. Does it imply one set of interactions is specific *i.e.* for Ca^{2+}?

Bart Hoogenboom responded: Perforin specifically needs Ca^{2+} to bind to the membrane. This is not a purely electrostatic effect, as *e.g.* Mg^{2+} cannot assume this role.

Ana J. Garcia Saez asked: Did you see an effect of line tension at the domain borders?

Bart Hoogenboom responded: We did not observe significantly enhanced pore formation at boundaries between lipid domains. We did observe enhanced pore formation at the edges of lipid patches when we had incomplete lipid coverage of our substrates, but that may be of little physiological relevance.

Patricia Bassereau commented: You propose that a gel-phase forms in the lymphocyte membrane to resist the insertion of perforin in the contact zone with the target cell. How is the gel-phase triggered by the lymphocyte? How can the cell locally switch its membrane to an ordered organization? This should perturb its capacity for exocytosis in the contact zone. Is this a problem?

Bart Hoogenboom answered: That is a really intriguing question, but needs a bit of nuance.

Firstly, the lipid phases we discuss are not in the gel-phase, but in a liquid-ordered phase. In our experience, gel-phase membranes are generally quite robust against membrane-targeting peptides and proteins, but are of little physiological relevance.

Secondly, we find that lymphocytes are overall protected against perforin, also in experiments where they are exposed to recombinant perforin (so without synapse formation). This overall protection is reduced when we reduce the overall lipid order of the plasma membrane, using keta-cholesterol.

But then we would indeed expect this protection mechanism to be further enhanced at the immune synapse, as there is extensive experimental evidence that the lymphocyte membrane shows (further) enhanced order at the synapse. It coincides with the clearance in the actin cortex at the synapse, and *in vitro* work suggests that interactions between actin and the membrane can aid in pinning particular membrane domains.

Yet I don't think we fully understand how this local enhancement of lipid order might work.

Francisco Barrera asked: Some cancer cells have increased plasma membrane fluidity. Would this circumstance protect them from perforin's attack? Could they be more sensitive? This could be a nuanced situation because cancer cells can also expose PS, which might be expected to have the opposite effect.

Bart Hoogenboom replied: Since we observe that increased membrane packing/order (and thus reduced fluidity, I presume) protects against perforin, I would expect increased membrane fluidity to make such cells more sensitive to perforin attack. Indeed the exposure of PS could protect them, resulting in the opposite effect. Without actual experiments on such cells, we can speculate about the directions this may take, but it is hard to predict which effects will dominate.

Bart Hoogenboom opened a general discussion of the paper by Mibel Aguilar: How quickly do bacteria adapt their membrane lipid composition in response to changes in the environment?

Mibel Aguilar answered: Lipid production in bacteria is very finely tuned by the environment. Bacteria respond to various environmental stressors and conditions and can alter lipid synthesis at the genetic level. With the emergence of lipidomics, the complex changes are now becoming more evident, at least for bacteria, and the metabolic and genetic changes in response to environmental stresses is very quick.

The changes occur relatively quickly. For example, as indicated by Dr Bonev above, the conversion of acyl chain double bonds to cyclopropyl lipids is almost complete during the mid-phase of growth. With on-going improvements in lipidomic analysis, we will be able to track this more easily in future.

Amitabha Chattopadhyay addressed Bart Hoogenboom and Mibel Aguilar: How will bacterial growth rate affect your observations?

Mibel Aguilar replied: As discussed later in responses to Paula Milán Rodríguez, Paul O'Shea, Patricia Bassereau and Burkhard Bechinger during Session 4 (DOI: 10.1039/d1fd90068d), there are many cell properties that we need to monitor given that the lipid composition changed so significantly with the growth phase. We harvest the cells at a specific OD, so at least in the stationary phase, the culture is in a somewhat stable growth phase.

Conflicts of interest

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