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DISCUSSIONS

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Theoretical and experimental comparisons of simple peptide–membrane systems; towards defining the reaction space: general discussion

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Burkhard Bechinger opened discussion of the introductory lecture by William F. DeGrado: For the Influenza M2 transmembrane domain you mention the importance of the lipid composition, environmental and membrane details. Because X-ray (XR) investigations and structures in micelles have also been published, including in high impact journals, I would be interested what kind of details you still consider valid from such latter experiments? Which other details absolutely require a lipid bilayer and which peptide/lipid ratio do you consider acceptable?

William F. DeGrado responded: One really does need to look at whether a structure makes sense when all the biological and biophysical data are considered. My view is that crystal structures will provide energetically reasonable conformations, sometimes showing multiple states, even in a single unit cell. Often these states represent intermediates that are observed during function (*e.g.*, conductance in the case of M2 (see accompanying manuscript). For M2, we had companion data from Mei Hong, using SSNMR, which gave a very similar structure to our C-closed crystal structure. And it explained a large body of electrophysiological and pharmacological data. Another method we apply frequently now are unrestrained all-atom microsecond simulations of the protein embedded in a relevant lipid composition. Structures not compatible with this environment will drift away from the starting configuration over this time period. Overall, the

more data we can gather the better. Ultimately, we need to connect high-resolution data from cryo-EM and crystallography to the functionally relevant ensemble of structures in membranes with compositions closely matching those of the cellular membrane. In the end, however, we always need to compare all structures to experiments conducted in cellular and organismic context.

Burkhard Bechinger enquired: The LS-peptides adopt amphipathic helical structures when membrane-associated and should be happy to sit on the membrane surface. What driving forces make them go transmembrane and what is the proportion of the transmembrane *versus* in-planar populations?

William F. DeGrado responded: Yes indeed! They are surface absorbed, but a small fraction is inserted and stabilized by a transmembrane voltage. The inserted state can then associate as reviewed in ref. 1.

1 K. S. Åkerfeldt, J. D. Lear, Z. R. Waserman, L. A. Chung and W. F. DeGrado, *Acc. Chem. Res.*, 1993, **26**, 191–197.

Patricia Bassereau queried: Would the dual faces of the β3 integrin allowing interaction with mixed alpha partners contribute to drive integrins clustering at adhesion sites? For example, engaging two binding partners simultaneously as one driving force may result in clusters of β3 integrins (maybe mixed with alpha partners).

William F. DeGrado replied: I love the model and what you suggest is intriguing, but we do not have data to support it (or refute it). It would be interesting to find out.

Sreetama Pal asked: Based on the present understanding of lipid–protein interactions, what factors play a decisive role in determining the optimum number of helices in a transmembrane protein?

William F. DeGrado responded: Good question, I expect that the number of helices would be more dependent on the function, rather than folding. A protein would need to be large enough to create, for example, a binding site for small molecules, and in many cases also signal.

David P. Siegel continued: Professor DeGrado made the interesting speculation that some synuclein peptides could stabilize fusion/fission pores by binding with high affinity to lipid membranes with negative Gaussian curvature. I think one could determine whether peptides bound more readily to bilayers that already have negative Gaussian curvature. Such an activity might be a way certain peptide sequences are recruited to membrane fission or fusion sites *in vivo*. One could also determine if certain sequences, bound to such surfaces, decreased Gaussian curvature even more (*i.e.*, made the structures smaller). That could be done by seeing if the bound peptides reduce the lattice constant of those phases. One might simultaneously monitor both the extent of binding of the peptide and effects on the lattice constant using SAXS, on the same samples. For example, it might be possible to make "plugs" of cubic phase in X-ray capillaries, by temperature cycling host lipids across the bilayer/non-bilayer phase transition

temperature. One could add the peptides of interest by changing the column of fluid overlying the plugs. The extent of diffusion of the peptides into the cubic phase plug could be assayed by tracing the inherent fluorescence or Trp absorbance of the peptides under a light microscope. However, it is not clear how large a peptide can diffuse into cubic phase aggregates, either through the water channels in the bicontinuous structure, or along the bilayer–water interfaces. (The water channels are several nm in diameter, and the diameter can be changed as a function of temperature and lipid composition.) Obviously, this would have to be tested for the peptides of interest.

Durba Sengupta enquired: You mentioned lipid effects in M2 – but not in the helix dimers – where you mentioned structural motifs and polar residues. Wouldn't lipid effects be important there – especially with the polar residues embedded in the membranes? How could we probe it experimentally?

William F. DeGrado answered: M2 forms a tetramer, and it is important that the lipid chain length matches the length of the tetramer. In general, oligomerization also relies on matching of the hydrophobic thickness of the transmembrane domain (TMD) with that of the surrounding lipid. If the lipid bilayer is too thin or too thick, oligomerization can be reduced in model membranes. We have shown the effects of membrane on thermodynamic stability and functional dynamics in M2, and Dirk Schneider's group on glycophorin.[1–3]

1 A. L. Cristian, J. D. Lear and W. F. DeGrado, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 14772–14777.
2 C. W. Lin, B. Mensa, M. Barniol-Xicota, W. F. DeGrado and F. Gai, Activation pH and gating dynamics of influenza A M2 proton channel revealed by single-molecule spectroscopy, *Angew. Chem., Int. Ed.*, 2017, **56**(19), 5283–5287.
3 V. Anbazhagan and D. Schneider, *Biochim. Biophys. Acta*, 2010, **1798**, 1899–1907.

Durba Sengupta commented: The oncogenic mutations you mentioned in the growth factor receptors are at the head group region (or just below) so perhaps there are more lipid effects (than the simple hydrogen bonds between polar residues) that should be considered?

William F. DeGrado indicated that they have nothing to add.

Ana J. Garcia-Saez queried: Do you have an explanation of why up-regulation of chaperones is associated with membrane disruption? Is this happening in bacteria?

William F. DeGrado answered: Bacteria have two-component His kinases that sense and relay information about the environment to induce a transcriptional response of regulons beneficial to bacteria. Systems like Bae and Cox in *E. coli* sense capsule stress.

Paul O'Shea said: When you are looking at interactions of the lipids, you treat the lipid (such as cholesterol) that interacts as a single molecule with the proteinaceous species. I am thinking about dipole potentials and micro-viscosities which are more ensemble properties of the lipid and are there more specific

interactions. So the question is are you treating the lipid as a homogeneous matrix together with very specific lipid molecule interactions or can you include the lipid environmental heterogeneity as a factor in the interactions?

William F. DeGrado replied: Good question Paul. In analysis of natural proteins, we and others look specifically at lipid effects, particularly cholesterol in M2. Of course, there is a massive literature on this for antimicrobial peptides (AMPs). For designed systems, we don't explicitly consider lipid effects in the design process (other than hydrophobic length and positions of Tyr, Trp, Arg, Lys…) until we run microsecond molecular dynamics (MD), once we have a potential sequence designed. At that point we need to choose a lipid composition.

Patricia Bassereau opened discussion of the paper by Gregory A. Voth: Does the place in the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) where the virus is budding, possibly related to a local membrane composition or geometry, influence the M protein conformation, and thus budding?

Gregory A. Voth answered: It's pure speculation, but I would think so. We need to do coarse-grained simulations with many proteins to address this better. It would be wonderful to see you do some of your beautiful experiments on this protein binding to membrane tubules pulled from micro-pipettes like you have done for N-BARs (endophilin).[1]

1 M. Simunovic, E. Evergren, I. Golushko, C. Prévost, H.-F. Renard, L. Johannes, H. McMahon, V. Lorman, G. A. Voth and P. Bassereau, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, 11226–11231.

Patricia Bassereau enquired: Since you show that the local lipid composition influences the conformation, what triggers one conformation *vs.* the other in the cell?

Gregory A. Voth responded: We do not yet know. Perhaps its lipid domain dynamics that triggers the conformational change, or perhaps it is a response to larger scale membrane morphological changes during budding.

Patricia Bassereau asked: How do changes in cholesterol level in the cell affect virus assembly?

Gregory A. Voth responded: I am not sure anyone knows the answer to this. There has really been relatively little work done on the virus assembly and budding. It's a critical need.

John M. Seddon queried: Are the trans-membrane segments of the proteins sensitive in terms of conformation and/or dynamics to the lateral pressure profile within the lipid bilayer, and do you calculate this?

Gregory A. Voth answered: We don't yet know an answer to that question. However, I have a feeling that the M protein conformational states we see in the

simulations could be influenced by the lateral pressure profile. Yes, it should be possible to calculate it in the future.

Durba Sengupta asked: You showed high membrane deformation around the E channel – the viral envelope/membrane would be highly curved – would the high curvature alter the perturbation – and perhaps aid function?

Gregory A. Voth replied: It is possible. The pore in our model is pretty narrow so anything that increases its radius could affect ion conduction. On the other hand, the E channel appears to be non-selective.

Burkhard Bechinger enquired: From your simulations, can you decipher molecular details what kind of interactions make individual lipids (*e.g.* cholesterol) accumulate or deplete around the proteins? H-Bonds, van der Waals interactions, hydrophobic mismatch *etc.*?

Gregory A. Voth answered: Yes we can but we have not yet done so. One has to be a little careful about using local measures such as H-bonds though. N-BAR proteins are seen to aggregate in linear "strings" which are a result of membrane fluctuation-mediated forces between the proteins (like Casimir forces) and not local interactions.

Bart W. Hoogenboom asked: Could you speculate on how your findings with different lipids translate to real cell membranes?

Gregory A. Voth responded: Boy, that is a hard question. "Real" membranes are so complex yet many properties of membrane proteins have been elaborated pretty well in model membranes. We used a model for the ERGIC membrane.

Amitabha Chattopadhyay remarked: For HIV1, host cholesterol is known to be necessary for the virus to bud. It is not known whether this is true for SARS-CoV-2 virus.

Gregory A. Voth replied: I fully agree with you. However, the M protein which is believed to be important for SARS-CoV-2 budding shows a tendency in our simulations to sequester some cholesterol.

Izabella Brand asked: E protein is a channel protein. What triggers the opening and closure of the channel? Does the electric potential drop across the membrane affect the channel properties?

Gregory A. Voth responded: I'm afraid that no one really knows yet. Sorry! I don't think there is evidence the E channel is voltage gated though.

Georg Pabst opened discussion of the paper by Izabella Brand: The fabrication of lipid asymmetry on solid supports by the technique you are using has been shown to be challenging (see *e.g.* work by the groups of Lukas Tamm[1–3] and John Conboy[4]). How do you measure lipid asymmetry to make sure that your system is asymmetric to begin with?

1 J. M. Crane, V. Kiessling and L. K. Tamm, *Langmuir*, 2005, **21**(4), 1377–1388.
2 V. Kiessling, J. M. Crane and L. K. Tamm, *Biophys. J.*, 2006, **91**(9), 3313–3326.
3 V. Kiessling, C. Wan and L. K. Tamm, *Biochim. Biophys. Acta*, 2009, **1788**(1), 64–71.
4 T. C. Anglin, K. L. Brown and J. C. Conboy, *J. Struct. Biol.*, 2009, **168**, 37–52.

Izabella Brand replied: In general, fabrication of asymmetric lipid bilayers belongs to an experimental challenge. A freshly prepared bilayer is asymmetric, which is confirmed by electrochemical studies. The capacitance of a freshly prepared bilayer is $\sim$3 μF cm^{-2}. If a KLA molecule with its large polar head group faced the electrode surface, due to presence of water and sugar residues on the Au surface, an increase in the capacitance, due to a higher value of the dielectric constant of the water/sugar system, would be observed. However, potential cycling for several times, did not lead to any increase in the membrane capacitance. This result suggests that the asymmetry, at least to a large extent is preserved. Possibly, application of more negative potentials (as tested in simulation studies[1]) would cause some irreversible changes in the bilayer structure. Indeed, in our previous studies of asymmetric lipid bilayers containing glycolipids (GM1 and Gd1a) a gradual increase in the membrane capacitance in following potential scans was observed.[2] In parallel, the intensities of IR spectra of the sugar residues in the asymmetric POPE–KLA bilayer are attenuated, indicating a uniform orientation of the polar head groups in KLA. In contrast in the previous studies the intensities of the IR absorption modes in sugar residues adapted a random orientation, indicating structural rearrangements in the bilayer. A flip–flop was suggested. Note that KLA molecules bind divalent ions. It is responsible for the formation of a rigid leaflet, which might be responsible for the stability of the asymmetric bilayer.

1 T. J. Piggot, D. A. Holdbrook and S. Khalid, *J. Phys. Chem. B*, 2011, **115**, 13381–13388, DOI: 10.1021/jp207013v.
2 M. Nullmeier, H. Koliwer-Brandl, S. Kelm, P. Zägel, K.-W. Koch and I. Brand, *ChemPhysChem*, 2011, **12**, 1066–1079, DOI: 10.1002/cphc.201100036.

Georg Pabst asked: As a follow up of the previous question, lipid flip–flop was reported to be fast in solid supported bilayers by the Conboy group, see, *e.g.*, ref. 1. We suggested in Marquardt *et al.*[2] that this is due to unavoidable defects in solid supported bilayers. Do you think that lipopolysaccharides (LPS) will increase the stability of your asymmetric systems?

1 T. C. Anglin, M. P. Cooper, H. Li, K. Chandler and J. C. Conboy, *J. Phys. Chem. B*, 2010, **114**, 1903–1914.
2 D. Marquardt, F. A. Heberle, T. Miti, B. Eicher, E. London, J. Katsaras and G. Pabst, *Langmuir*, 2017, **33**(15), 3731–3741.

Izabella Brand answered: Yes, I think that the LPS increase the stability of the asymmetric bilayer. I have discussed this problem in the answer to the previous question.

Paul O'Shea enquired: Are capacitance changes due to structural rearrangements of the lipid? Or is there a contribution from the dielectric properties if the peptide attached to the membrane?

Izabella Brand responded: Both aspects are very important. The electric potentials cause some changes to the lipid bilayer alone (Fig. 2, curve 1, in the paper; DOI: 10.1039/d0fd00039f). These changes are due to the electroporation and adsorption–desorption of the lipid bilayer. In the presence of a peptide attached to the membrane the dielectric constant of the outer membrane (OM) changes (increases), agreeing with the observed increase in the membrane capacitance in the presence of melittin (Fig. 2, curves 2, 3, in the paper; DOI: 10.1039/d0fd00039f). The dielectric properties of melittin may not only lead to an increase in the capacitance, but may affect the membrane electroporation potential too. Melittin insertion into the bilayer will also affect the potential of zero charge of the OM, a phenomenon which was experimentally observed. Both lipids and peptides respond to electric potentials and contribute to experimentally measured capacitance values.

Paul O'Shea asked: Can you correlate the kinetics of these changes? Kinetics of binding and insertion of peptides including melittin has been done by other groups, including my own early reports (see for example ref. 1), and with melittin particularly (ref. 2). Do any of these rate changes correlate?

1 C. Golding, S. Senior, M. T. Wilson and P. O'Shea, *Biochemistry*, 1996, **35**, 10931–10937.
2 J. Wall, C. A. Golding, M. V. Veen and P. O'Shea, *Mol. Membr. Biol.*, 1995, **12**, 183–192.

Izabella Brand replied: Kinetics of melittin interaction with lipid bilayers was intensively studied over the past decades. However, large differences in kinetic data were reported. Many aspects affect the kinetics of melittin interaction with lipid bilayers. Among the critical factors are: the lipid : peptide ratio, the lipid composition of the bilayer, the shape of the supramolecular aggregate (*e.g.* planar lipid bilayer, vesicle), the presence of cholesterol in the bilayer or temperature. Despite differences in kinetics (rate constants, association constants), some similarities could be observed between different reports. There is a general consensus that disordered melittin adsorbs on the membrane surface. After adsorption on the membrane surface a transition to α-helix structure and membrane incorporation take place (see for example ref. 1). Saturation of the membrane surface with a melittin layer depends on the peptide concentration. However, after *ca.* 60 minutes of the interaction, saturation was observed. In our experiments, high concentrations of melittin were used (1 and 10 μM). Our results showed that after 15 minutes of the interaction the peptide still had a disordered structure. A change to α-helical structure was observed after 1 h of the interaction. This result is in good agreement with literature. Next steps of interaction include peptide insertion into the membrane and its disruption. This process is very specific to the lipid composition of the membrane. Since phospholipids were usually used in kinetic studies, a direct comparison to a bilayer containing lipopolysaccharide cannot be done.

1 M. G. Burton, Q. M. Huang, M. A. Hossain, J. D. Wade, A. H. A. Clayton and M. L. Gee, *Langmuir*, 2013, **29**, 14613–14621, DOI: 10.1021/la403083m.

Sreetama Pal questioned: Is it possible to identify (from the IR-based readouts you mention) whether any specific residues of melittin interact with the bacterial membrane mimic in use?

Izabella Brand responded: IR is sensitive to the conformation of a peptide/protein. However, the measured IR absorption modes are not specific for any particular amino acids. Thus, the measured signal is an average answer of all amino acids in the studied molecule. Thus, we are not able to detect specific amino acids in melittin interacting with KLA and POPE lipids.

Sreetama Pal asked: What is the orientation of the peptide after it adsorbs onto the membrane? In the abstract (DOI: 10.1039/d0fd00039f) you mention that "the N-terminus enters into the hydrophobic region of the membrane and forms a channel to the hydrophilic head groups in POPE". If this implies a pseudo-transmembrane orientation of melittin, how are the lipid headgroups positioned with respect to the peptide?

Izabella Brand answered: In this paper the orientation of the lipid molecules was studied. We observed that the carboxylate groups in the inner core of the KLA lipid interact directly with the peptide. This interaction weakens the binding of Mg^{2+} ions, which may be responsible for a loss of the outer leaflet compactness. In the hydrocarbon chain region an increase in the order of their packing was observed. This result agrees with previous studies and indicates insertion of the peptide into the bilayer. Electrochemical results support this conclusion.

Of course, melittin gives rise to a strong amide I mode (see Fig. 3; DOI: 10.1039/d0fd00039f). This mode was not analysed in this paper. However, depending on the time of adsorption large changes in the intensity (concentration) and shape of the amide I mode were observed. Briefly, after 15 minutes of adsorption the amide I mode was centered around 1640 cm^{-1} indicating that a disordered peptide was adsorbed on the bilayer surface. However, after 1 h of interaction the amide I mode was centered at 1648 cm^{-1}, indicating its conformational transformation to α-helix. This structure is characteristic for membrane inserted melittin, thus we concluded that the peptide spans the membrane. Quantitative analysis of the amide I mode aiming at the determination of the average orientation of the long helix axis in the bilayer will be discussed in a separate paper.

John M. Sanderson enquired: In Fig. 3 of your article (DOI: 10.1039/d0fd00039f) you show polarization modulation infrared reflection-absorption (PM IRRA) data for the same system after 15 min and 60 min. There is a clear difference in the spectra, which indicates that the system had not reached equilibrium (or the endpoint of the binding process) after 15 min. Has equilibrium been reached after 60 min? I ask the question because in work we did with Alison Rodger to look at melittin binding to liposomes by dichroism methods, an endpoint was sometimes difficult to achieve.[1] We subsequently found that there was an acyl transfer reaction from the lipid to the peptide.[2]

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, DOI: 10.2174/092986611009011351.
2 C. J. Pridmore, J. A. Mosely, A. Rodger and J. M. Sanderson, *Chem. Commun.*, 2011, **47**, 1422–1424, DOI: 10.1039/c0cc04677a.

Izabella Brand replied: Our results show that we did not reach equilibrium, even after 1 h of interaction of the POPE–KLA bilayer with 10 μM melittin

solution. In my opinion, this is a dynamic system, which will finally lead to the membrane dissolution, disruption. Our aim was to elucidate the changes in the lipid molecules taking place during interaction with melittin and the impact of this interaction on the membrane electric properties. Currently, we analyse the amide I mode of melittin to elucidate the changes in the secondary structure of the peptide interacting for different times with the membrane. After 1 h of the interaction, independently of the melittin concentration, the amide I mode indicated that melittin adapted an α-helix structure. The average tilt of the long helix axis will be estimated. After exposure of the membrane for a given period of time to melittin solution (15 or 60 min), the sample was transferred into an electrolyte solution without peptide. The spectral changes were monitored for up to 20 h. In this time the average structure of the bilayer was preserved. We cannot prove if the process of melittin acylation by acyl chains in KLA and/or POPE took place. Our technique is not sensitive to the progress of this reaction. It is a very interesting point, and will be considered in our future investigations.

John M. Sanderson queried: You see desorption of material from the electrode after longer time periods – have you considered collecting this material and analysing it to see whether there have been any chemical changes?

Izabella Brand responded: *In situ* PM IRRAS experiments took *ca.* 20 h. In this time several positive- and negative-going potential scans were recorded. Next, the spectra of each cycle are checked, compared and if no major deviations over time occurred, averaged. During 20 h of the experiment no significant spectral changes were observed. Since we work with a single bilayer of lipids adsorbed on a *ca.* 1 cm^2 large Au electrode, and the volume of the electrolyte solution in the *in situ* cell is large (*ca.* 60 mL) eventually desorbed material will have a very low concentration. For this reason, I did not undertake the analysis of the analyte. Now, knowing that acylation of melittin can be a reason for the lack of changes in the spectra, I am considering the analysis of the solution after reaction. Thank you very much, your questions, comments and fantastic talk (DOI: 10.1039/d1fd00030f) will have a large impact on the planning of future experiments.

Evelyne Deplazes asked: The membrane is made on a supported monolayer, and the lipids will therefore have limited lateral movement compared to a bilayer in the fluid state. How do you think this will impact the interactions with the peptide? Can the membrane still adapt its structure to the peptide, *i.e.* can the lipids rearrange themselves in response to membrane binding? And how does this reduced lateral movement of the lipids affect the measurements that are being taken?

Izabella Brand answered: The lateral mobility of POPE molecules in the inner, Au surface facing leaflet is lower than in fluid floating bilayers. The lateral mobility of KLA molecules present in the outer leaflet, facing the electrolyte solution is not affected by the substrate. The outer leaflet provides the first contact to melittin. Thus, I assume, the adsorption of melittin on the bilayer surface and interaction with a lipopolysaccharide is not affected by the substrate. However, the insertion of melittin into the second leaflet of the bilayer may be affected by the reduced lateral mobility of POPE molecules.

Anand Srivastava enquired: With POPE being a H-bond donor, can you please comment on how the pH of the environment would play a role in the process?

Izabella Brand responded: We did not investigate the pH effects on the membrane structure and stability. The pH of the used electrolyte was 6.5. We are aware that pH affects not only the H bonding in POPE but also the protonation of carboxylate groups in the KLA molecule and in general in lipopolysaccharides. Thus, pH changes would affect the structure of lipid molecules forming the bilayer, which may further affect its stability in changing electric fields.

Kareem Al Nahas queried: How to differentiate a capacitance shift between peptides present freely only in the aqueous phase near the outer layer of the membrane rather than bound/inserted in the lipid membrane?

Izabella Brand replied: The capacitance of the Au electrode modified by a molecular assembly depends on the surface density, packing and compactness of the molecules adsorbed on its surface. The capacitance of a defect-free film adsorbed on an electrode surface is given in eqn (1):

$$C = \varepsilon_0 \varepsilon_r A/d \quad (1)$$

where ε_0 is the permeability of vacuum, ε_r is the dielectric constant of the molecules present in the film, d is the thickness of the adsorbed film, and A is the surface area of the electrode. When a defect-free film is formed on the electrode surface the dielectric constant of the adsorbing molecule has a large impact on the capacitance value. Lipid molecules are amphiphilic and contain long acyl chains. The dielectric constant of a hydrocarbon chain is $\varepsilon = 2$. Thus, lipid molecules have lower values of dielectric constants than proteins or peptides. Experimentally measured capacitance has a contribution from the capacitance of the lipid membrane (C_{lipid}), capacitance of eventually adsorbed protein film (C_{protein}) present on top of the membrane and of diffuse layer capacitance (C_{dif}):

$$1/C = 1/C_{\text{lipid}} + 1/C_{\text{protein}} + 1/C_{\text{diff}} \quad (2)$$

According to eqn (2) the adsorption of a peptide on the bilayer surface, will not significantly affect the measured capacitance, because it is determined by the lower capacitance of the system (in this case by the compact lipid bilayer). Thus, if melittin adsorbed only on the membrane surface the measured capacitance would practically not change compared to the bilayer of the intact bilayer. An increase in the capacitance indicated insertion of the peptide into the membrane which leads to an increase in the dielectric constant of the film adsorbed on the Au surface, and possibly caused some changes in the membrane packing and compactness.

Marie-Isabel Aguilar asked: You used 1 and 10 μM melittin. What P : L ratio did this correspond to in the systems you studied? Did you observe any capacitance changes as you approached a lytic concentration?

Izabella Brand answered: We did not perform our studies for a longer time than 1 h of a direct exposure of the KLA–POPE bilayer to melittin solution. In this time no lysis of the membrane was observed. If the membrane became unstable, the measured IR signals would change during the experiment and it would not be possible to obtain any answer. The P : L ratio, considering the supramolecular assembly of the outer membrane, has changed during protein adsorption. We were unable to determine the surface concentration of melittin; therefore we do not know the P : L ratio.

Paul O'Shea opened discussion of the paper by Ana J. Garcia-Saez: Can you comment on the targeting on the mitochondrial surface of the initial binding reactions?

Ana J. Garcia-Saez replied: The targeting of Bax and tBid to mitochondria is mainly mediated by the sequence of the C-terminal anchor of Bax, and in the case of tBid by Mtch2 and the cardiolipin binding domain identified in tBid.

The role of lipids in the membrane targeting has been mostly explored in *in vitro* reconstituted systems with model membranes. Here, the presence of cardiolipin at about 20% concentration is usually sufficient for efficient binding of tBid to the membrane. In the absence of tBid or another activator, recombinant, inactive Bax does not spontaneously bind to membranes, even if they contain cardiolipin. The presence of tBid induces Bax binding to cardiolipin-containing membranes. Bax can also bind to cardiolipin-containing liposomes if activated *in vitro* by incubation at 42 °C. The requirement of cardiolipin for binding is mainly due to electrostatic interactions, as it can be replaced by phosphatidyl glycerol at a similar density of negative charges.

Paul O'Shea asked: Binding seems to be related to negative charge on the surface of the membrane. Would you know if there is any localisation of the binding to say cardiolipin which can be localised in the membrane. So I guess I'm asking is there any correlation with cardiolipin regions?

Ana J. Garcia-Saez answered: Yes, the *in vitro* experiments suggest that membrane binding is due to electrostatic interactions with negatively charged lipids. Now, the question of cardiolipin regions is more complicated. In healthy mitochondria, the concentration of cardiolipin in the outer membrane is believed to be around 4–6%, while it is around 16% in the inner mitochondrial membrane. Although it has been proposed that the amount of cardiolipin in the outer membrane may increase during apoptosis, it still remains unclear whether cardiolipin plays a fundamental role in the targeting of tBid and Bax to mitochondria during apoptosis. One possible explanation is the higher local concentration of cardiolipin in potential cardiolipin domains, which could be sufficient for driving the targeting. However, strong evidence for these domains in the outer membrane of healthy mitochondria is missing. Furthermore, GFP-Bax presents a homogeneous localization at the microscopic level when it binds to mitochondria in healthy cells and in the initial stages of apoptosis.

John M. Seddon queried: Your model system contains a significant amount of cardiolipin, which has a strong tendency to form inverse non-lamellar phases in the presence of binding cations. Did you see any sign of this in your systems?

Ana J. Garcia-Saez responded: Not under the experimental conditions that we used. We usually work with giant unilamellar vesicle (GUV) preparations that present a high number of good quality GUVs. We cannot discard the formation of non-lamellar phases in the lipid layers attached to the electrodes, but we do not image them. I am also not sure whether we could unambiguously detect non-lamellar phases with the resolution of optical microscopy.

Patricia Bassereau asked: What do you think is the benefit to get such an indirect way to repress death (a 3-body, rather than a 2-body process)?

Ana J. Garcia-Saez answered: It provides additional levels of regulation. The decision to undergo cell death is the ultimate irreversible decision for a cell, as it ends the cell's existence and there is no way back from it. It thus makes sense that cells have developed sophisticated regulation processes with multiple levels of fine tuning to decide whether to die or survive. The proteins of the BCL-2 family are a very good example in this regard, because the activator, effector and inhibitor functions overlap between several family members with small differences in their mechanisms and functions.

Durba Sengupta enquired: Is there anything known at the structural level – especially about the residue-level specificity for Bax and tBid interacting with cardiolipins?

Ana J. Garcia-Saez replied: There is nothing really known at the structural level about a specific interaction of these proteins with cardiolipin. In the case of tBid, a cardiolipin-interacting domain could be localized within helix 6, which is also important for the pro-apoptotic activity of the protein.

David P. Siegel opened discussion of the paper by **D.** Peter Tieleman: Sorry if I am missing something, but my impression is that CHOL, POPC, POPS and DPSM are all enriched around AQP1 & OmpF, according to the probability scale beneath the figure (Fig. 2 in the paper; DOI: 10.1039/d1fd00003a). This seems to reflect a fairly general phenomenon, relatively insensitive to lipid structure. In the case of CHOL you show that there are specific CHOL–protein interactions. But for the phospholipids, what do you think this more general effect may be? Perhaps a length mis-match effect? Perhaps I merely misunderstand the definition of relative probability. I do see a patchiness in the association of POPC and POPS with the protein periphery.

D. Peter Tieleman responded: Emphasis on CHOL-protein interactions was guided by the relatively discrete nature of CHOL density around AQP1 and KcsA seen in Fig. 2, potentially suggestive of specific binding sites. As noted, some of the phospholipids are also enriched around a few of the protein entities, namely AQP1, OmpF, and KcsA, albeit more diffusely compared to CHOL. While these findings are consistent with previous studies, as discussed in the paper, the

causes and consequences of specific phospholipid enrichment are not clear in all cases. The comparably diffuse distribution of phospholipids compared to CHOL around some of the proteins suggests they don't form strong specific interactions in this annular shell. Though the molecular basis of annular lipid shells was not a focus of this investigation, they typically arise due to favorable, yet rather low affinity interactions between lipid head and tail groups and residues on the protein surface. We look forward to delving further into the atomistic details of such interactions in follow-up studies.

Durba Sengupta enquired: A more technical question. Did you calculate the potential of mean force (PMF) of cholesterol flip–flop in Martini 3 – and is there a higher penalty to flip–flop than in Martini 2 and what are the differences to the values calculated with atomistic force-fields?

D. Peter Tieleman replied: No, we haven't. There is a Martini 3 beta model at the moment but no finalized version. We have previously published PMFs with Martini, with the atomistic Berger/GROMOS87 combination and with the atomistic Slipids Force Field. There is quite a spread of values for the peak in the PMF. CHARMM37 seems to have much slower rates. Overall, however, the rates are still fast, in agreement with experimental difficulties to measure cholesterol flip flop rates and the notion that cholesterol equilibrates between leaflets quickly on macroscopic or biological time scales.

Robert Gilbert asked: It seems from your data that the smoother the structure of the membrane protein, the more likely the flip flop. In simple terms, if you have a protein that is "knobbly" on its intra-membrane surface, then it doesn't flip flop much. Could you comment on surface topography and the likelihood of flip flop. Also, is it possible to map the trajectory of the flip flop?

D. Peter Tieleman responded: I think that is a plausible hypothesis but it's hard to dis-entangle this from size and shape effects of the protein. To test it I'd consider artificial systems that look like walls (graphene sheet) with different levels of "knobbly-hood", but we haven't done that. You can certainly map the trajectory of flip flop. We have in the past, *e.g.* Gu *et al.*[1]

1 R.-X. Gu, S. Baoukina and D. P. Tieleman, *J. Am. Chem. Soc.*, 2020, **142**, 2844–2856.

Franca Fraternali questioned: Is Martini 3 clearly improving the description of protein flexibility and are the networks of contacts captured in the flexibility descriptors. How is the flexibility captured in this new force-field?

D. Peter Tieleman answered: Not in itself, in its current form, but there are a number of reasons why I'm optimistic that there is room for improvement. First, in addition to elastic networks the Go model approach to maintaining protein structure looks promising, and has been used a few times. This is not specific to Martini 3 but an alternative to elastic networks and in principle allows encoding more structural information because it's a potential function based on native contacts instead of an elastic network that just locks everything in place. Second, the choice of bead size in Martini 3 will allow redefining the protein backbone with more detail. The current Martini 2 protein force field is unusual in coarse-

grained protein force fields in that it has very accurate (relatively speaking) side chains but a very simple backbone, while foldable coarse-grained protein force fields tend to have very complex backbones to parameterize backbone conformations but very simple side chains. In Martini 2 there is not much room to make the backbone more accurate. We tried, with dummies and extra particles, but with the smaller particles in Martini 3 there is much more room to work on this in the future.

Franca Fraternali queried: The elastic network can be made variable in terms of membrane positioning? The flexibility and the rigidity are factors that could impact the position in the membrane.

D. Peter Tieleman replied: Yes, the elastic network definition and parameters can be adjusted. People have used some flexibility in definition to include or exclude loops, or have separate networks for domains or subunits, but generally I haven't seen very compelling results from this. We have used position restraints to anchor proteins in a specific location while the lipids and solvent are free to move, which means you don't define or pre-determine the position in the membrane (although there is an impact on possible tilting). For many purposes with the current version of Martini the preferred approach probably is default elastic network settings and no position restraints, although for specific questions that of course can change.

Georg Pabst enquired: The diffusion of cholesterol to the opposing leaflet leads to a mass imbalance in your system. As a consequence the differential stress within your system will change (specifically also because of the intrinsic curvature of cholesterol). Do you expect that these changes in bulk membrane properties couple back to your observations?

D. Peter Tieleman responded: They might, although you need quite a large imbalance to have observable effects on pretty much anything in a simulation. This might not be true for stress profiles but these are hard to calculate accurately. I do think this is an interesting and important direction for the next few years.

Paul O'Shea queried: How is your new modeling approach handling polarisation – *i.e.* polarisable force fields? Or are you using *via* massive parameterisation? Or something else maybe in terms of hybrids with atomistic models?

We find that there is a contribution of the water and a contribution of the lipid to say the membrane dipole potential and their mutual contribution differs markedly depending on the type of lipid. When we and others have tried to study this atomistically, the problem of equilibrium occurs in which we don't think the system has reached equilibrium with say 30 mol% cholesterol. So we have the problem of not being able to address biologically-relevant cholesterol-rich nanodomains and makes things very different. So it seems to me that the atomistic approach is some way behind where you are. Is there a way forward for this with the new Martini model or with a hybrid?

D. Peter Tieleman replied: A force field embodies specific approximations. An atomistic force field has no electronic polarization but has polarization effects due

to reorienting dipoles formed by fixed partial charges. A polarizable force field like Amoeba or a force field based on Drude oscillators does incorporate electronic polarization. Martini beads interact through parameterized potentials that have to incorporate polarization implicitly. Although it has explicit partial charges, on most beads these are zero because they represent a fragment with no net charge. The main advantage of Martini compared to atomistic simulations is its vastly increased sampling power, but if your scientific questions require both sampling of Martini and detail of an atomistic or polarizable force field you're out of luck. You'll have to wait for faster computers. Approaches to hybrid simulations for now focus on things like peptide or protein conformation described in detail with a Martini environment. Whether that is useful depends on the specific question of interest.

Paul O'Shea asked: We can download structures for atomistic modeling that are supposedly in some kind of equilibrium but many lipid structures are unavailable although they are observed in wet experiments to interact quite differently with peptides *etc* (see ref. 1). We observe in wet experiments that membranes containing all the ingredients to form micro-domains (cholesterol, sphingomyelin, *etc*) take a very long time to reach some kind of steady state structures and so are unlikely to satisfy equilibrium conditions suitable for atomistic modeling so is there information that coarse-grained (CG) is giving that atomistic cannot?

1 T. Asawakarn, J. Cladera and P. O'Shea, *J. Biol. Chem.*, 2001, **276**, 38457–38463.

D. Peter Tieleman responded: Structures in the protein data bank still have flexibility and a distribution of conformations at room temperature in solution or a membrane and proteins undergo changes in conformation as they go through their function. The notion of 'supposedly equilibrium' is problematic in this respect. The main power of CG force fields is that they allow better sampling, but at the cost of less chemical detail. It's a trade off you'll have to make for each specific research question.

Patrick Fuchs enquired: Now that MARTINI 3 has fixed the problem of "stickiness" between membrane proteins, have you tried to simulate more than one protein in the box? If yes, what is the lipid distribution like with more than one protein (in the case of multiple copies of the same protein or if there are different proteins)?

D. Peter Tieleman answered: We have published a number of simulations with multiple proteins but these were restrained in place (*e.g.* ref. 1 and 2). We have ongoing simulations on a larger number of proteins in a single bilayer but the time scales are so slow these systems are difficult to sample and we currently have no detailed comparisons between Martini 2 and 3.

1 V. Corradi, E. Mendez-Villuendas, H. I. Ingólfsson, R.-X. Gu, I. Siuda, M. N. Melo, A. Moussatova, L. J. DeGagné, B. I. Sejdiu, G. Singh, T. A. Wassenaar, K. Delgado Magnero, S. J. Marrink and D. P. Tieleman, *ACS Cent. Sci.*, 2018, **4**, 709–717.
2 B. I. Sejdiu and D. P. Tieleman, *Biophys. J.*, 2020, **118**, 1887–1900.

Izabella Brand queried: Can you please comment on the results of the incorporation of carbon nanotubes (CNTs) into lipid membranes? How did you select

the length of the CNT? Did you detect any interactions (hydrophobic/hydrophilic preference) between CNTs and lipid molecules?

D. Peter Tieleman replied: The nanotube was just a model for a mostly featureless object, without the geometrical features of side chains and the diverse chemistry of side chains. It was restrained to not move in the simulations, and its length is unrealistically short compared to actual CNTs but just chosen to be long enough to span the membrane. We have previously looked at a range of chemically modified CNTs of different thicknesses and lengths and their interactions with lipids.[1]

1 S. Baoukina, L. Monticelli and D. P. Tieleman, *J. Phys. Chem. B*, 2013, **117**, 12113–12123.

Ronald J. Clarke asked: If cholesterol flipping occurs on the microsecond timescale, then on a timescale relevant for many membrane protein conformational changes, *e.g.* ion pumping or ion channel gating, which occur over the millisecond-to-second timescale, one would expect the cholesterol distribution across the membrane to be in equilibrium, *i.e.*, with no asymmetric distribution across the membrane. Can you envisage a situation where an asymmetric cholesterol distribution across the membrane could persist for long enough to be relevant for an ion pump or the gating of an ion channel?

D. Peter Tieleman responded: I do expect the cholesterol distribution to be in equilibrium, but that does not mean it is equal. Cholesterol flips but other lipids do not flip, except on timescales of seconds to hours, and their distribution is tightly controlled by proteins and cellular processes. Equilibrium for cholesterol means equal chemical potential in both leaflets but the chemical environment in both is different and therefore the concentration that results from equal chemical potential is different. Protein conformational changes might well be coupled to local cholesterol concentration near the protein. I think that is an interesting suggestion you could look at with Martini simulations fairly readily. I don't think it will play a role in the overall cholesterol distribution between leaflets at a larger scale.

Ana J. Garcia-Saez opened discussion of the paper by Francisco N. Barrera: Related to the presence of negatively charged ions in the anti-apoptotic proteins – did you consider the helix5 of Bcl-xL, which in contrast to Bax naturally contains negatively charged residues?

Secondly, you presented studies in supported bilayers. Do you know if BAX can be pH regulated in other systems, like in liposomes with encapsulated dyes?

Francisco N. Barrera answered: We focused for these studies on BAX, seeking to harness the natural ability of helix alpha 5 to participate in pore formation. While we cannot predict with certainty the results of testing helix5 of Bcl-xL, we reasoned that BAX could be a more interesting target as it might be possible to extract biologically significant data on BAX poration from studies on BaxE5.

Ana J. Garcia-Saez remarked: That is interesting because the wild type peptide does not disrupt/dissolve membranes unless it is at a very high concentration.

Francisco N. Barrera responded: The differences between the WT peptide (Bax alpha 5) and the pH-sensitive peptide (BaxE5) are indeed surprising. One might imagine that the replacement of positive charges in the WT for negative charges in BaxE5 would be behind these differences, as lysine residues often influence peptide–membrane interactions.

Marcus Fletcher asked: In Fig. 1B of your submitted paper (DOI: 10.1039/d0fd00070a) there looks to be some membrane integrity disruption at pH 7.9 that is dependent on BaxE5 concentration, but in the AFM there is no observed disruption to the supported lipid bilayer. What do you think is happening there in the leakage assay to cause disruption?

Francisco N. Barrera replied: While we cannot rule out that supported bilayers are more stable than bilayers in a liposome, the lack of effect of BaxE5 in supported bilayers could be merely a kinetic effect, since a shorter incubation time was used than in the leakage experiments. BaxE5 at neutral pH displays a slow effect on membranes, but indeed it can attack membranes to some degree at long incubation times.

Johanna Utterström queried: Could the reason for the differences between leakage assay and AFM depend on that different lipids were chosen? POPC or DOPC? And what was the reason for using different lipids for the two experiments?

Francisco N. Barrera answered: Thanks for this question. POPC and DOPC, while similar in some respects, have different biophysical properties (like fluidity), and therefore it would not be surprising to me that the differences in acyl chain modulated the BaxE5 effect. The two lipids were used for simple utilitarian reasons.

Robert Gilbert asked: It seems to me, from your data, that the effect of the binding of protein is a key factor, aside from downstream effects. Could you comment on the extent to which your observations can be explained by the degree of protein binding in different conditions (*e.g.* pH).

Francisco N. Barrera replied: It would reasonable to expect that a major factor in the observed pH-responsiveness of the BaxE5 peptide is lipid partitioning. Specifically, at neutral pH the glutamic acid residues in BaxE5 will be negatively charged, precluding efficient membrane interaction/insertion. A pH drop will protonate these residues to increase the overall hydrophobicity of BaxE5. This effect will certainly increase the degree of peptide partitioning into the membrane, and increase the peptide concentration at the membrane surface. It is expected that in these conditions the peptide will be favored to self-assemble and disrupt membrane integrity.

Sreetama Pal enquired: I am curious to know whether the behavior of the Bax fragment could be reversed on increasing pH? If you observe some kind of hysteresis (similar to your work with the pH low insertion peptide), that might provide some interesting information about how protonation influences peptide–membrane interactions.

Francisco N. Barrera responded: This is an interesting question. We expect a pH rise to detach BaxE5 from the membrane. Hysteresis would be certainly expected, in no small part due to the changes in membrane state occurring as a result of the BaxE5 effect.

Sreetama Pal asked: It would be interesting to know the rationale for choosing this specific BaxE5 analog. Would you expect the behavior of the Bax fragment to change if you vary the number and relative position of the introduced glutamate residues? If yes, how?

Francisco N. Barrera replied: We introduced the smallest number of glutamic acids that we expected would provide control over membrane disruption by pH. However, changes in the number and position of the glutamic acid residues would probably affect the pH midpoint (pH_{50}) of membrane insertion, based on prior observations (see ref. 1).

1 H. L. Scott, J. M. Westerfield and F. N. Barrera, *Biophys. J.*, 2017, **113**, 869–879, PMID: 28834723.

Sreetama Pal queried: Is it possible to isolate or stabilize the BaxE5–membrane complexes at some or all the stages of membrane remodeling represented in the cartoon in the paper (Fig. 8; DOI: 10.1039/d0fd00070a)? That could represent a potentially useful system to understand more about the basic biophysics of how proteins shape membranes.

Francisco N. Barrera responded: That is a wonderful suggestion. The stabilization of some of the membrane remodeling stages could be probably achieved by raising the pH to physiological levels. This should de-activate or at least slow down BaxE5 action.

Burkhard Bechinger enquired: I understood from working on Bcl-xL and Bax proteins many years ago that these proteins remain largely folded as determined in their XR and solution NMR structures and anchor in the membrane merely through their hydrophobic carboxy terminus. Helix 5 would then remain sandwiched between other layers of helical domains and not be in contact with the membrane. Therefore, it is interesting that this domain alone has such potent activities. What can we learn from studies of helix 5 alone? Can this domain be used independently as a functional unit in therapeutic or diagnostic approaches?

Francisco N. Barrera replied: Data with BaxE5 (a variant of helix 5 of Bax) suggest that this sequence can forcefully remodel the membrane. A salient feature of BaxE5 is that its membrano-lytic effect is largely in check at neutral pH, while it is unleashed at acidic pH. Such pH-triggered membrane destruction could be potentially used to specifically target cancer cells, as they are often embedded into an acidic environment (see ref. 1). BaxE5 could therefore be a lead molecule for anti-cancer therapeutics. However, a tighter control of pH-responsiveness (*e.g.*, ref. 2), and a higher pH_{50} would be probably required for clinical promise.

1 J. C. Deacon, D. M. Engelman and F. N. Barrera, *Arch. Biochem. Biophys.*, 2015, **565**, 40–48, PMID: 25444855.

2 H. L. Scott, J. M. Westerfield and F. N. Barrera, *Biophys. J.*, 2017, **113**, 869–879, PMID: 28834723.

Ana J. Garcia-Saez added: The existing structures of the soluble forms of Bax and Bcl-xL, as well as of other family members, do not recapitulate the structures of these proteins in the active state in the membrane, at least for some of them.

In the case of Bax (and Bak), both proteins can retain the globular fold while anchored to the membrane *via* the C-terminus helix in an inactive state. Upon activation, they undergo a large conformational change and the central hairpin of helices partially opens, which is a fundamental step for the activation process. We and others have shown that the N-terminal part of the protein up to helix 5 is then involved in a stable symmetric dimerization domain *via* Bh3-into-groove interactions. From helix 6 on, the flexibility of the protein in the active conformation in the membrane is very high and so far it could not be represented by a unique structure. There is still a lot of work to be done regarding the structure of the active forms of these proteins and their complexes in the membrane.

Additionally, we also know that helices 5 and 6 are directly involved in interactions with the membrane which are interfacial, meaning, not classically transmembrane. It is well established that these helices in the protein are responsible for the pore forming activity. From the studies with the individual helices, we learned that indeed they can recapitulate the pore-forming activity of the entire protein, yet they miss the regulation of this activity provided by the other protein domains. There have been some efforts to use peptides derived from helix 5 for antimicrobial and anticancer purposes. Furthermore, understanding the mechanisms of the pore activity of Bax is important to develop small molecules that may directly activate it, which would be of interest for anticancer therapy, for example.

Ana J. Garcia-Saez asked: Did you test the pore activity of the modified Bax peptide in liposomes? Does it induce tubulation/curvature in giant unilamellar vesicles?

Francisco N. Barrera responded: The pore activity of the modified Bax peptide (BaxE5) was tested using a liposome cargo release assay. These results showed clearly enhanced cargo release at acidic pH, as shown in Fig. 1 of the manuscript (DOI: 10.1039/d0fd00070a).

Atomic force microscopy performed on supported bilayers did not hint at any effect of BaxE5 causing tubulation or curvature. However, we cannot rule these out, and additional fluorescence experiments using giant unilamellar vesicles could offer a clearer answer to this question.

Robert Gilbert enquired: Can you comment on how the purported role of dimerisation in Bax activity relates to your work with the isolated Bax-derived peptide you are working with?

Francisco N. Barrera replied: The BaxE5 peptide carves a membrane pattern (dubbed "lace"), which is characterized by a consistent width. We posit that laces are formed when a peptide oligomer, of well-defined dimensions, wraps around the lipid monolayer in the lace, transiently stabilizing it. This model implies that BaxE5 is stable with a particular self-assembly stoichiometry. Our results would be

compatible with an scenario where helix 5 of Bax participates in self-assembly of the protein, particularly at the membrane insertion stage where the pore is formed.

Paul O'Shea opened discussion of the paper by Stefano Vanni: I noticed in one of your slides (not included in your conference paper I think) that you have noise profiles when considering the free and bound structures. I wonder is there information in that noise which is not being used as it seems to change, so is probably not systematic uncertainty in the 'measurement'.

Stefano Vanni answered: If I understand the question correctly, it would be inappropriate to refer to this data as noise. These plots (also provided in the supplementary information of the paper; DOI 10.1039/d0fd00058b) represent the minimum distance between the protein and the membrane over the course of the simulations. The proteins chosen are known to interact transiently with membranes and hence the minimum distance varies between 0.5 and 3 nm as they bind and unbind continuously. From these minimum distance curves we extract the relevant information that we discuss in the article.

Evelyne Deplazes enquired: During the binding/unbinding event can you see the peptide tumbling on the membrane surface and are you thus sampling different binding interfaces on the protein including sampling favourable and unfavourable interactions? We often work with proteins that have hydrophobic patches and thus a more 'sticky' side that shows a strong preference for the membrane over other sides/faces on the protein. We have been struggling to find methods that sample the favourable and unfavourable interactions for these peptides and this is needed for calculating the free energy of binding. Do you think if the simulations are run for long enough (dozens of microseconds) the trajectory can be used to obtain and estimate the free energy of binding for both weak and strong binding peptides?

Stefano Vanni responded: Yes the protein does sample unfavourable interactions although transiently (with the exception of Evectin-2 PH domain in our dataset that binds strongly in an unfavourable conformation). In principle, one could indeed determine binding free energy from CG simulations with reasonable statistical accuracy by running long-enough simulations. Comparison with experiments (albeit not straightforward) would provide further information on the quality of the used force field.

Durba Sengupta asked: I am surprised by the large number of binding/unbinding events! Did you analyse the very short transient bound states and do they differ from the longer binding events? Both in terms of the protein orientations and the lipids interacting with the protein. And in that context, events such as lipid diffusion that haven't been sampled (*e.g.* clustering of the PS lipids) could play a role.

Stefano Vanni replied: Yes, we did analyse the membrane binding interface in the case of short transient states (binding duration < 50 ns) *vs.* longer binding events. For some proteins, the binding interface in the case of very transient binding is not the one that has been experimentally determined. This also sheds light on the

accuracy of the model as it is necessary to ensure that the protein does not stay trapped with a 'wrong' binding interface that has stronger electrostatics for example. As far as clustering of lipids is concerned, the timescale required for lipid redistribution to occur after protein binding exceeds the time for which some proteins stay bound to the membrane, making it hard to sample and analyse such events.

Madhusmita Tripathy said: The figures in the ESI are the crucial things we missed in the talk and paper (DOI: 10.1039/d0fd00058b). I find the binding trajectory of PDK1 PH domain at 30% DOPS to be quite interesting. In half of your simulations you see stable binding events and not in the other half. Why so?

Stefano Vanni answered: Our interpretation is that this is purely a sampling issue as all the binding events in the particular example are stable. Hence if the simulations were longer, one could have potentially seen unbinding and rebinding in the other half of the trajectories as well.

Madhusmita Tripathy enquired: Given that you do see the correct binding orientations at 30% DOPC, why are there no stable binding events over the 4 μs long trajectory? In other words, the proteins that approach the membrane in experimentally observed orientation, do they unbind less often?

Stefano Vanni replied: These proteins have an intrinsic tendency to bind and unbind/exhibit transient binding. We attribute this to the fact that they are not membrane residual proteins but rather membrane sensing proteins. The fact that we can reproduce this characteristic with the MARTINI model tells us that the free energy of binding is not over-estimated and the protein is not trapped in the membrane bound state.

Madhusmita Tripathy asked: I am curious because I see binding events to be stable in only half of the simulations with 30% POPS. Is it okay to see binding events that are not stable? Are there any other factors that take place? In the case where there are lower percentages, does the protein ever approach the correct membrane orientation or is it random?

Stefano Vanni responded: We consider all the binding events in PDK1 PH at 30% PS as stable binding events. The protein does approach in the correct orientation as can be seen with some proteins such as FVa-C2 where the membrane binding is only ~30% at 80 DOPC, 20 DOPS membrane composition.

Patrick Fuchs enquired: When you observe multiple binding/unbinding events, do you get the converged free energy of binding (or at least converged ratio of bound/unbound conformations)? If yes, do you get the experimental order of magnitude (for known cases)?

Stefano Vanni replied: We did not convert binding/unbinding events to free energy of binding, so we cannot comment on that. In principle, it is true that one could indeed determine binding free energy from CG simulations with reasonable statistical accuracy by running long-enough simulations. Comparison with

experiments (albeit not straightforward) would provide further information on the quality of the used force field.

Patrick Fuchs asked: Regarding amphipathic helices, do you see also multiple binding/unbinding events or irreversible binding? For ALPS from ArfGAP1, what about the flexibility of the peptide (especially around the two glycines)?

Stefano Vanni answered: The amphipathic helices bind irreversibly almost all of the time and only few unbinding events can be observed towards the beginning of the simulation. We did not take into account the flexibility of the peptide at specific positions, but we rather changed the overall force constant as a mean to increase flexibility. If one is interested in a partially disordered protein, a careful modelling of its secondary structure is clearly very important.

Robert Vácha enquired: Have you simulated ALPS with different amount/level of helicity (*e.g.* from CD experiments) to see if the helicity affects ALPS binding to membranes with different compositions?

Stefano Vanni replied: We did not test ALPS with different level of helicity, but rather simply changed the elastic network force constant, thus allowing for more/less flexibility for the peptide. We did so as we meant our analysis to be high-throughput and not system-dependent, but we expect that changing the level of helicity (also depending on the lipid bilayer composition) would affect the binding of the peptide to the bilayer.

Zack Jarin asked: Thank you again for the great paper and talk. If I recall correctly, you showed that some proteins did not reproduce the experimental behavior and did not bind more readily to PC/PS membranes than PC membranes. What were the net charges of the proteins you investigated? Could the truncated electrostatics in Martini model affect this result?

Stefano Vanni responded: The net charges on the proteins we studied vary significantly, from −3 (for PLA2) to +11 (for FVa C2). We can not exclude that the truncated/reaction field treatment of electrostatic interactions might partially affect the results. However, two observations seem to suggest that this shouldn't be the cause of the issue: (1) the proteins undergo fast tumbling motions close to the bilayers, and all regions of the proteins approach the membrane within the cutoff distance and (2) ultimately, the proteins bind to the bilayer with the correct interface.

Patricia Bassereau questioned: Do you add a particular interaction to keep your proteins next to the membranes and not let them diffuse away?

Stefano Vanni answered: No, we did not add any external potential or interaction to the classical energetic terms of the force field to prevent the diffusion away from the bilayer.

Anand Srivastava commented: Wanted to share some thoughts and see if they make sense. Though SPR and ITC gives different K_d – they are not generally orders of magnitude different – they are in the ball park. Similarly, one of the things that

can be tested here is "calculate" the K_d (using the kinetics off and on rates – like we do for SPR) and also do some umbrella sampling or other kind of advanced sampling and get PMF and from the PMF (after using standard state correction), calculate the K_d and compare the two K_d and see how the Martini3 simulations compare. That would be nice to see.

Stefano Vanni responded: Indeed, that would be very interesting. On the one hand, however, the lack of flexibility in protein secondary structure might compromise the ability of the model to reproduce the correct K_d. On the other hand, it would be interesting to investigate whether the performance of the model is similar for all proteins or if there are some (positive or negative) outliers, as this would indicate different binding mechanisms (or possibly the involvement of conformational changes only in some instances).

Anand Srivastava said: Related to the previous comment, it will be nice to see how much K_d calculated from PMF prescription and k_d calculated using k_{off}/K_{on} data are different.

Stefano Vanni replied: Agreed, but different enhanced sampling techniques could also lead to different K_d values, so this would require extensive testing.

Ronald J. Clarke communicated: Many peripheral membrane proteins are thought to interact with membrane surfaces *via* electrostatic interactions between negatively charged phosphatidylserine lipid headgroups and positively charged basic amino acid residues on the protein, *i.e.*, lysine and arginine, as described in your paper for the 3-phosphoinositide-dependent kinase-1 (PDK1). In your simulations you state in the Methods section (DOI: 10.1039/d0fd00058b) that you solvated the systems studied with 0.12 M NaCl. If a binding event is due to an electrostatic interaction one would expect the frequency of interaction to increase or the interaction distance to decrease as the ionic strength is decreased due to reduced electrostatic screening. Have you carried out any simulations at low salt concentrations or at zero ionic strength to check whether this is the case?

Stefano Vanni communicated in reply: We tested slightly different ionic strengths and we did observe small variations, but further studies would be required to properly characterise this behaviour. Indeed, the MARTINI developers indicate that the force field for ions is significantly improved in MARTINI 3 and we think our database could be a good test system.

Conflicts of interest

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