General Discussion

Dr Duax opened the discussion of Prof. Wallace's paper: It is not clear whether your references to the CD spectra of gramicidin in acetic acid in your contribution to this meeting are meant to question the accuracy of the X-ray structure determinations reported in our *PNAS* paper.[1]

One criticism of X-ray studies of gramicidin has been that crystals were grown from alcohols which have relative permittivities near 50. This is very high compared to the relative permittivities of lipids of 2 to 4. To address this problem we grew crystals from glacial acetic acid which has a relative permittivity much closer to that of lipids (5–6). We did not claim that acetic acid resembled lipids in other ways.

Our crystal structures of the Cs^+ complex of gramicidin from methanol and the hydronium ion complex of gramicidin from glacial acetic acid reveal the same right handed antiparallel double helix. These structures agree in all details with the model first proposed by Arseniev *et al.*[2] to interpret the NMR Noesy spectra of the Cs^+ complex of gramicidin in a methanol–chloroform mixture. Our structure is also compatible with ^{15}N NMR data on gramicidin in planar lipid bilayers.[3] Our structure is consistent with the standard characterization of the membrane active channel of gramicidin based upon CD spectra as being right handed.

While the significance and implications of an X-ray structure may be open to debate, the accuracy of a high resolution X-ray structure determination can and should be unambiguous. As you know the results of our X-ray analysis of Cs^+ and K^+ complexes of gramicidin are completely different from yours, different in molecular hand, ion content, nature of ion coordination, channel shape, and every single one of the 30 hydrogen bonds present in the antiparallel dimers. On the basis of published results we conclude that your reported structural models have chemical and stereochemical anomalies and crystallographic limitations not present in our refined structures.

The disparities between our structures and yours are all the more puzzling because the crystallization conditions were very similar and all the cell dimensions of the complexes agree to within 1%. In light of the controversy concerning the relevance of the X-ray results to solution spectra, transport properties and the mechanism of membrane transport of gramicidin we consider it vitally important to resolve the questions generated by the profound differences between our X-ray crystal structures and yours. There are three possibilities. (1) Our structures are wrong. (2) Your structures are wrong. (3) Both structures are right. Which of these possibilities is in fact the case can be quickly resolved by comparing the diffraction intensities that were used in our determinations and yours. We deposited the atomic coordinates and all of the intensities for our Cs^+ complex in the Protein Data Bank (PDB) when the paper was accepted in *PNAS* (two months before it was published in November 1998). You told me this morning that you attempted to access our intensities files through the PDB yesterday and could not. I am sorry to hear that the PDB is unable to cope with deposition and distribution in a timely fashion. We will gladly provide you with the intensity data on our Cs^+ complex directly. In the spirit of fairness and in the interest of science we would request that you reciprocate by sending us the intensity data for your Cs^+ complex within 10 days of receipt of our intensities data. We will be happy to do the same with our data on the K^+, Rb^+ and other complexes. Will you do this?

We hope that you will agree that it is important to resolve this ambiguity. If your structures and ours are correct it would be the first example that I know of where isomorphous forms of a structure with identical cell dimensions have entirely different conformations. Given the existence of 160 000 structures in the Cambridge Structural Database such an unusual phenomena is certainly worth characterizing carefully.

1 B. M. Burkhart, N. Li, D. A. Langs, W. A. Pangborn and W. L. Duax, *Proc. Natl. Acad. Sci. USA*, 1988, **95**, 12950.

2 A. S. Arseniev, I. L. Barsukov and V. F. Bystrov, *FEBS Lett.*, 1985, **180**, 33.

3 L. K. Nicholson, F. Moll, T. E. Mixon, P. V. LoGrasso, A. L. Lay and T. A. Cross, *Biochem.*, 1987, **26**, 6621.

Dr Wallace replied: You have raised a number of points in your comments, so I will try to answer them one by one: (1) The CD studies in acetic acid were done to address the statements in your *PNAS* paper that this form represented the conducting form in lipid membranes. They clearly show that the acetic acid form is not the conducting form found in phospholipids, but they in no way question the accuracy of your X-ray structure, merely the interpretation of it. (2) Some of the other evidences you cite for this being the conducting form are also controversial. While I am not an expert in solid state NMR, I understand that a paper disputing your claims that this structure is compatible with the spectroscopic data, has been submitted[1] that will address that issue better than I can in this limited space. (3) I agree that our X-ray structure of the CsCl form is completely different from yours. However, the crystals examined in each case are different, and given the polymorphic nature of this molecule, which can demonstrably form structures of different handedness, different stagger between the chains, and different hydrogen bonds in solution, it does not seem particularly surprising that a similar polymorphism is seen in the crystals.

1 T. A. Cross, A. Arseniev, B. A. Cornell, J. H. Davis, J. A. Killian, R. E. Koeppe, L. K. Nicholson, F. Separovic and B. A. Wallace, submitted.

Dr Duax further commented: You did not respond to the most important question. Will you release the intensities from your published structures to the PDB as we have?

Dr Wallace responded: We are currently re-refining our CsCl structure[1] using improved geometric parameters, as you have recently done[2] with the earlier Buffalo structure.[3] Once done, we intend to publicly release our structure *via* the PDB, in the standard fashion.

1 B. A. Wallace and K. Ravikumar, *Science*, 1988, **241**, 182.
2 B. M. Burkhart, R. M. Gassman, D. A. Langs, W. A. Pangborn and W. L. Duax, *Biophys. J.*, 1998, **75**, 2135.
3 D. A. Langs, *Science*, 1988, **241**, 188.

Dr Burkhart asked: How much interpretation can you make about the effect of side chain conformational change on the CD spectrum differences?

Dr Wallace responded: The CD spectra[1] do not show significant differences between gramicidin in different solvents. I know that your crystal structures[2] do show a difference in the orientations of the tryptophans between the methanol and ethanol or propanol structures. This may be due to the differences between solution and crystal structures, especially any constraints on the tryptophan side chains, which are located at the periphery of the moecule, due to packing in the crystals. That the two crystal structures crystallised in the same space group (ethanol and propanol) have similar tryptophan orientations and the one crystallised in another space group (methanol) is most different might tend to support the crystal packing as a source of these differences between solution and solid state studies.

1 W. R. Veatch, E. T. Fossel and E. R. Blout, *Biochemistry*, 1974, **13**, 5249; Y. Chen and B. A. Wallace, *Biopolymers*, 1997, **42**, 771.
2 B. M. Burkhart, R. M. Gassman, D. A. Langs, W. A. Pangborn and W. L. Duax, *Biophys. J.*, 1998, **75**, 2135.

Prof. Holzwarth asked: Why did you use a mixture of gramicidin A, B and C and not a pure sample? I would expect differences between a mixture and pure samples of gramicidin.

Dr Wallace responded: We used the commercially available mixture of gramicidin A, B, and C. Veatch had previously shown[1] that the mixture behaves spectroscopically in a very similar manner to pure gramicidin A.

1 W. R. Veatch, PhD Thesis, Harvard University, 1974.

Prof. Laggner commented: Could it be that both structures (Wallace and Duax) are right, but not really relevant to the structure in the bilayer system. Current ideas in this field suggest that the structure of peptides in the membranes may not be unique but rather dependent on various variables, *e.g.* the amount of peptide incorporated to the bilayer. In BLMs, monomer, it is rather difficult to control the amount of peptide actually present in the "black" part of the film.

Dr Wallace replied: I agree entirely. From all the physical and chemical studies done on gramicidin over the past 20 years or more, it is clear that the principle conducting form in membranes is the helical dimer, not any of the double helices seen in either Duax's and our structures. The studies presented in my paper at this meeting confirm this, and suggest there are only very specific conditions under which double helices may form conducting molecules, such as when there is a very severe mis-match of the lipid fatty acid chain length with the gramicidin size.

I should point out that the Duax CsCl crystals and ours are prepared under very different conditions: different peptide and ion concentrations and ratios, and different temperatures (which our recent CD studies[1] have shown produce very different spectra, and by implication, structures, in solution). The crystals have slightly different unit cell dimensions (but small variations in unit cell dimensions have significant consequences for this molecule.[2] Given the polymorphism seen for this molecule in so many different environments (solution, membranes, *etc.*) it is not surprising to find polymorphic crystal structures. It is very clear from our anomalous Patterson maps, for instance, that the Duax structure is not consistent with our data, and so must represent a different crystal form.

1 T. P. Galbraith and B. Wallace, unpublished results.
2 D. A. Doyle and B. A. Wallace, *J. Mol. Biol.*, 1997, **266**, 963.

Prof. Lee said: My question is about the effect of membrane thickness on the state of gramicidin. In di-$C_{22:1}$ PC, is gramicidin present as a $\beta^{6.3}$ dimer or monomer, or will the CD spectra of these two forms be indistinguishable?

Second, I thought that the thickness of a solvent-free bilayer and the equivalent solvent containing bilayer were very different, so that it seems surprising that chain length effects are the same in the two systems.

Dr Wallace responded: We cannot clearly distinguish $\beta^{6.3}$ monomers from $\beta^{6.3}$ dimers by CD spectroscopy. Both have similar backbone folds (which is what CD detects). CD studies we did some time ago[1] on gramicidin in which the N-terminal formyl group was replaced with an N-terminal acetyl group, which tends to destabilise the dimer and thus produce monomers, showed very little difference between the spectra, so I don't think we can say for certain whether the structures in C_{22} lipids are monomers or dimers from our work. However, the conductance studies of Mobashery *et al.*[2] suggest that a significant proportion of the population in this lipid is dimeric and conducting.

I agree with you that the bulk thicknesses of bilayers with and without solvent are quite different. But what I don't think we know is the actual thickness of the bilayer immediately surrounding and in contact with the gramicidin. In fact, I have to say that I was surprised that the "chain length effect" we are seeing only starts at C_{22}. I would have thought that much shorter lipids (even as short as C_{18}) would be a mis-match for gramicidin. But that is, again, based on thicknesses measured for bulk lipids in the absence of peptide, and the lipid surrounding the gramicidin may have substantially different properties.

1 B. A. Wallace, W. R. Veatch and E. R. Blout, *Biochemistry*, 1981, **20**, 5754.
2 N. Mobashery, C. Nielsen and O. S. Andersen, *FEBS Lett.*, 1997, **412**, 15.

Prof. Roux asked: It is not clear to my why the DH form gets stabilized in thick membrane relative to the HD form. Which one is stabilized, or, perhaps more justly, which one is destabilized?

Dr Wallace responded: I would think that really we are talking about the absence of stabilizing effects as much as anything. Numerous studies have suggested that interactions between tryptophan side chains and the interfacial region of the bilayer may be important structurally. In thin

lipids, the HD, with its tryptophans near the ends of the molecule, has them in a position that could interact favourably with the lipid headgroup region, which would have a stabilising influence; in the DHs, the tryptophans are spread along the length of the molecule, and so some of them would have to be buried deep in the bilayer, a much less favourable disposition. Thus in this case the equilibrium may be shifted towards the HD. However, in thick lipids, even the HD would have to bury some of its tryptophans below the bilayer interface, therefore this factor would be less likely to shift the equilibrium towards the HD. The other factor in the mismatch case could be the number of intermolecular hydrogen bonds holding the dimers together, which is 6 for the HD and between 26 and 30 for the DH.

Dr Burkhart commented: Given your own admission that gramicidin adopts many different structural forms and that the influences of Trp residue side chain conformation cannot be discerned, is it not dangerous to choose only four structural forms to describe each CD spectrum.

Dr Wallace responded: The control we have for whether the CD data is well represented by the reference data spectra used in the analyses is the NRMSD parameter. It is much like an *R*-factor in crystallography, in that it is a measure of the correspondence between the experimental data and the best fit to the reference data. A low value for the NRMSD indicates the reference data set reflects well the population of conformers present in the sample.

Dr Deber communicated: What are the linear (vertical) dimensions of the DH *vs.* the HD forms of gramicidin?

Dr Wallace communicated in response: The ion-containing DH is $\sim$26 Å long, whilst the HD form is $\sim$32 Å (see ref. 1).

1 O. S. Smart, J. M. Goodfellow and B. A. Wallace, *Biophysical J.*, 1993, **65**, 2455.

Dr Okazaki opened the discussion of Prof. Roux's paper: Within my knowledge, the interaction potential between protein cylinder and lipid chain carbon $u(r)$ is essential for this kind of calculation. The results must be very sensitive to the potential function between unlike particles. If you assume the strongly attractive potential between them, homogeneous mixing will be obtained but if you set the weakly attractive interaction, you will obtain a kind of phase separation, *i.e.* protein aggregation, as you show in your work. You might get even a double-well-like free energy profile for a particular potential function. The results including the free energy profile must change dramatically as a function of the protein–lipid interaction.

Now, my question is what kind of protein–lipid potential function did you assume or, in other words, how did you determine the interaction function?

Prof. Roux responded: The protein–protein potential of mean force $W(r)$ depends on several factors. One usually writes that $W(r) = U_{pp}(r) + \Delta W(r)$, where $U_{pp}(r)$ is the microscopic protein–protein potential energy and $\Delta W(r)$ is the lipid-mediated free energy potential. What you are saying is that the latter depends on the protein–lipid interactions, which is absolutely correct. In this preliminary study, we were mostly interested in exploring the magnitude of the forces arising from the influence of excluded volume of the hydrocarbon chains by the protein inclusion. Therefore, in the present calculation the protein–lipid potential was simply chosen as a repulsive hard cylinder. Of course more realistic interactions could be used (see for instance our answer to Prof. Smith below), but they would not allow an investigation of the excluded volume effect on the protein–protein lipid-mediated forces.

Dr Smart asked: Do your results have similarities to the results of Huang and coworkers[1] who have determined the 2D radial distribution function for peptides in lipid bilayers.

1 L. Yang, T. A. Harroun, W. T. Heller, T. M. Weiss and H. W. Huang, *Biophys. J.*, 1998, **75**, 641.

Prof. Roux responded: Huang has measured the in-plane distribution pair correlation function for the gramicidin channel. We intend to compare these data to the calculated lateral packing of cylinders corresponding to the size of a gramicidin channel.

Prof. Haymet commented: The level of theory seems nicely appropriate for the problem. The difficulty arises with the new calculations which you have discussed now and are not in the printed copy of the paper. The approximation for hydrophobicity which inspired your approach has long been known to break down the size of the solute increases beyond the solvent diameter. Hence calculation of the interaction of two cylinders are likely to be similarly qualitatively incorrect as the cylinder radium increases beyond the characteristic length-scale of the medium in which they are immersed. This is due to the fact that the solvent response-function is not allowed to relax in the presence of the solute.

Prof. Roux responded: In the paper were described preliminary results obtained for a hard repulsive cylinder of 5 Å diameter. We observe that the lipid-mediated potential of mean force has a complex structure, with an attractive well at contact and a repulsive barrier at a cylinder–cylinder separation distance of 20 Å. It is of interest to examine what is the dependence of this result on the size of the protein inclusion and that is the reason why we are investigating this matter. Nevertheless, I agree with you that the HNC integral equation theory for simple liquids formed by spherical particles has problems in dealing with very large differences in particle size, though I would add that the present case is more complex since there are several lengthscales in the lipid–lipid pair correlation function $\chi_{mm}(r)$ whereas the situation with monoatomic liquids is much simpler. Comparison with molecular dynamics simulations of atomic models will be done in order to assess the range of validity of the current theory for lipid bilayers. The extensive simulations performed in Prof. Klein's group will provide a good basis for that. Nevertheless, whether the theory is yielding semi-quantitative or qualitative results is secondary at this point. One must realize that an integral equation theory such as described here provides a unique route to gain some insights into lipid-mediated potential of mean force between protein inclusions and the influence of the hydrocarbon chains on the protein–protein interactions in bilayer membranes. There is presently no other theoretical approach to gain such information.

Prof. Holzwarth asked: Are you in a position to predict how far the influence of proteins could reach into the membrane; especially how many lipid layers around the protein will be influenced? We found experimentally for bacteriorhodopsin that as many as five to six layers of surrounding lipids are influenced.[1]

1 A. Böttcher, N. Dencher, R. Groll, F. Meyer and J. F. Holzwarth, *Reactions in Compartmentalized Liquids*, ed. W. Knoche and R. Schomäcker, Springer Verlag, Berlin, 1989, pp. 105–115.

Prof. Roux responded: In Fig. 2 we show that the density of the hydrocarbon core is perturbed over a distance of 30 Å around a 5 Å cylinder. The radius of one DPPC being roughly 4.5 Å based on a surface area per molecule of 64 Å^2 (using πr^2), this implies that a layer of 2 to 3 lipids are perturbed. The difference may be due in part to the fact that bacteriorhodopsin is much larger (it is a bundle of 7 transmembrane helices). Furthermore, in reality there is a direct attractive dispersion interaction between the protein and the lipids hydrocarbon chains whereas here we consider only the excluded volume effect. This may amplify the effect. Lastly, one should keep in mind that our theory offers an equilibrium statistical mechanical view of the perturbed density around an impurity. Ultimately, the number of observed lipids may depend upon the experimental method used to characterize the system (*e.g.*, magnetic resonance, fluorescence, infrared spectroscopy, differential calorimetry, etc.).

Prof. Klein commented: A liquid hydrocarbon against a hard wall will exhibit layering of methylene groups that extends 4 layers or so into the bulk liquid. Thus, the oscillation you observe in the hydrocarbon core around the 5 Å cylinder is reminiscent of this effect. It would be interesting to compare the DPPC lipid with, for example, hexadecane bulk liquid to see if there is any specific effect of the lipid. The main input in your calculations is the simulation data for the carbon–carbon density–density response function. These data were generated with a relatively small sample. How confident are you that the values are reliable in the range 15–20 Å and will uncertainties in this asymptotic region influence your results?

Prof. Roux responded: Fig. 1 shows that the carbon–carbon intramolecular correlation function $S_{mm}(r)$ has a large contribution followed by a peak up to 3 Å and then a slow decay over a

distance of 10–15 Å. Clearly, the intramolecular correlation contributes significantly to the response function of the lipid bilayer: the short range structure in the intramolecular correlation arises from nearest neighbor carbons along the acyl chains. The peak at $r = 0$ is indicative of the significant amount of short range order in the lipid chains perpendicular to the plane of the bilayer. The oscillations you are referring to arise from the intermolecular contributions in the response function. Those correspond partly to carbon–carbon contacts, and are reminiscent of liquid hydrocarbon. You are raising a very interesting question: to what extent does the response of the hydrocarbon differ from that of an isotropic liquid hydrocarbon? We will try to address that in the future by using a response function extracted from a liquid hydrocarbon simulation. Concerning your second question, the pair correlation function was calculated from a molecular dynamics trajectory of a lipid bilayer generated by Feller *et al.*[1] The atomic system that they simulated consisted of 72 DPPC molecules (36 in each leaflet). The physical length of the periodic box is approximately 48 Å. Thus, since the the correlation function decays almost to zero over a distance of 10 to 15 Å, it seems reasonable to assume that the dominant packing structure was captured by the molecular dynamics simulation.

1 S. E. Feller, R. M. Venable and R. W. Pastor, *Langmuir*, 1997, **13**, 6555.

Prof. Petersen asked: Please speculate on the interactions among several cylinders. Will there be an optimum size? Will a bundle of 3–5 cylinders of 5 Å radius behave as a single cylinder of ~9 Å and hence there might be no further aggregation?

Prof. Roux responded: The current calculations correspond to the infinite diluted limit of protein inclusion in a lipid bilayer. This assumption is necessary since we possess only the response function $\chi_{mm}(r)$ of the unperturbed bilayer from the simulation of Feller *et al.*[1] Nevertheless, one could try to investigate finite concentration and aggregation effects with the current theory, keeping in mind its limitations due to the response function.

1 S. E. Feller, R. M. Venable and R. W. Pastor, *Langmuir*, 1997, **13**, 6555.

Prof. Smith asked: What are the prospects for evolution of this approach towards an all-atom description of the helices?

Prof. Roux responded: The current theory is designed to address the influence of the lateral packing of the lipid hydrocarbon chains on the protein–protein potential of mean force. For this purpose the theory was kept as simple as possible. In particular, we considered only hard cylindrical protein inclusions with no details. Nevertheless, it is possible to construct a more sophisticated integral equation theory in which the detailed atomic structure of a membrane-bound protein will be used (*e.g.*, transmembrane helix or an amphipatic helix associated at the membrane/solution interface). This extended integral equation theory would require a more complete response function than the simple $\chi_{mm}(r)$ used in the present theory and which characterizes the lateral fluctuations. The extended approach would be the equivalent of integral equation theories such as those described in ref. 26. We are currently working on the development of this extended theory.

Dr Gilbert opened the discussion of Dr Bezrukov's paper. In the experiments on alamethicin reported larger pores appear to be favoured in their formation by the presence of lipids possessing a propensity for the formation of H_{II} phases (induced by pH modification). Does alamethicin itself promote H_{II} formation? Is this relevant to consideration of these data? Secondly, do the temporally co-existing hexagonal (H_{II}) and lamellar phases also coexist within a single lipid body—*i.e.* within the surface of a liposome? If so, what form would the interface between the lamellar and H_{II} phases take?

Dr Bezrukov responded: Yes, not only lipid monolayer spontaneous curvature modifies alamethicin channel behavior, but, in turn, alamethicin itself influences lipid curvature properties. According to an X-ray and NMR study by Keller *et al.*,[1] addition of as little as 1% of alamethicin to 1,2-dielaidoyl-*sn*-lycero-3-phosphoetanolamine introduces a large region of cubic phase into the

thermal phase diagram. This observation could be important for the molecular model of the phenomenon. To answer your second question, in the X-ray measurements that are reported in our paper the samples were prepared in excess water. Both lamellar and hexagonal phases are independent, three-dimensional structures. So necessarily they exist separately, and not on a two-dimensional surface, but within the same bulk sample. We do not know the structural nature of the interface between them.

1 S. L. Keller, S. M. Gruner and K. Gawrisch, *Biochem. Biophys. Acta*, 1996, **1278**, 241.

Dr Goñi asked: To what extent do your "change in pH" experiments answer the criticism, raised in relation to the PC/PE data, that you were changing the chemistry of the system? Your results look reasonable to me, but in my view protonation is also changing the chemistry of the host lipid towards alamethicin. Have you thought of changing curvature of PC bilayers by adding lyso PC? Or, conversely, adding polyunsaturated PC to saturated PC? Very little changes in the "chemistry" would take place in such an experiment.

Dr Bezrukov responded: The answer probably depends very much on what you mean by "chemistry". If changing head group interaction is chemistry, then yes, we are changing the chemistry of host lipid. In our opinion, however, changing the pH means less chemical modification of the system than admixing/substituting one lipid species by a distinct second lipid species. In response to your second point, in principle, this is a very good suggestion. In practice, it may be difficult to design a reliable experiment and to rationalize obtained results due to relatively high water solubility of lyso PC. Besides, an additional worry in experiments with the lipid mixtures is a possible "demixing" of lipids in the vicinity of the channel.

Prof. Laggner asked: Two related questions: What are the relative populations of lamellar and H_{II} phases in the coexistence region between pH 1.5 and 3.5. Why do you refer to these two states as "interstable"?

Dr Bezrukov responded: We can only estimate the relative quantities based on relative X-ray intensities. Qualitatively, the lamellar phase appears to be maximum between pH 2.3 and 2.9, where it coexists with the hexagonal phase, and appears to involve less than 50% of the lipid. The lamellar phase is nearly, but not quite, absent at pHs above and below 2.3 and 2.9. As for your second question, the X-ray scattering patterns are stable over periods of at least weeks.

Prof. Neumann asked: Does the dependence of the current intensity $\Delta i(t)$ on the electrolyte concentration reflect increasing charge screening of the phosphatidylserine (PtdSer) groups and thereby affect the spontaneous curvature?

Dr Bezrukov responded: The increase of single-channel current with the electrolyte concentration is mostly related to the increase in the average channel occupancy by ions. As seen in Fig. 6 of our paper, this increase mostly follows the bulk solution conductivity. The effect of increasing lipid charge screening that you mention is very small, because the initial contribution from lipid charge to the open channel conductance is tiny. This is not always the case though. For "small" channels it can be quite pronounced. Recently we published a study where the lipid charge effect on channel conductance was studied over varied charge densities.

1 T. K. Rostovtseva, V. M. Aguilella, I. Vodyanoy, S. M. Bezrukov and V. A. Parsegian, *Biophys. J.*, 1998, **75**, 1783.

Dr L. Fisher commented: I totally agree that membrane lipids must be functionally involved and have a controlling role in membrane protein behaviour. My question is whether we are looking at the right end of the lipid. Lipid packing studies to date have tended to focus on non-glycosylated lipids. It is a fact of nature, though, that the outer membrane lipids of mammalian cells are heavily glycosylated, and it may be that interactions between the headgroups, rather than the chains, of such lipids dominate their function. Would you care to comment on this, and on whether chain and charged headgroup interaction studies are of relevance for such lipids and the membranes containing them?

Dr Bezrukov responded: Yes, this is very interesting question, though I do not think that we, or anybody else for that matter, have enough evidence to state that interactions between the headgroups are more important than chain interactions. Many factors contribute to the balance of energies in channel conformational equilibrium. Glycosylation is likely to be important; at least the cells seem to think so. Whether head or tail "dominates" I would rather not decide in general.

Prof. Roux said: I fail to see a relationship between the propensity of forming the H_{II} phase and the stabilization of alamethicin multimers, which are a bundle of transmembrane helices. Could you comment on that please?

Dr Bezrukov responded: If you ask about the empirical relationship, it is in Fig. 3 *vs.* Fig. 10 of our paper. As for a detailed molecular mechanism, we are still far from any good idea. One possibility could be the "shape" of the channel to explain the increase in the number of stress-relieved lipid molecules as the channel goes to a state of higher conductance.

Prof. Lee commented: The difficulty you have, as you have said, is in separating effects of the chemical structure changes from effects on curvature. The transition temperature into the hexagonal H_{II} phase is higher for POPE than for DOPE. Have you compared the effects of POPE and DOPE on alamethicin channel formation?

Dr Bezrukov responded: No we have not. But this is definitely a good suggestion.

Dr Smart asked: Can your results be explained if the alamethicin molecule adopts a wedge-shaped bundle (and that this varies with bundle size)? Can Dr Sansom comment whether the models he has generated support this?

Dr Bezrukov responded: Yes, the shape of the channel exterior facing lipid matrix could be a key factor. I will leave this question for Dr Sansom who did extensive work modeling alamethicin channel.

Dr Sansom responded: The models of alamethicin helix bundles we have generated are, to a limited extent, wedge-shaped (if one takes a cross section down the pore *i.e.* bundle axis). This may provide a partial explanation of the result of Bezrukov and colleagues. However, to be certain of this probably requires a systematic series of simulations with *e.g.* different lipid headgroups or headgroup protonation states. I don't think "back of an envelope" approaches are going to work here.

Dr Kakorin asked: Did you change the pH value and NaCl concentration equally on the two sides of the lipid membrane?

Dr Bezrukov responded: Yes, we have varied the pH value and NaCl concentration equally on both sides of the membrane. In the future we are going to change the pH value on one side.

Dr Kakorin responded: If there were not a difference in pH value or NaCl concentration on the two sides of the membrane, then you cannot rationalise the variation of the conductance level of alamethicin channels with pH value or [NaCl] by the proton-induced increase in spontaneous curvature. The cartoon illustration of the curved monolayer (Fig. 9) is not relevant to the data. Actually, according to Helfrich's definition the spontaneous curvature reflects a possible asymmetry in the bilayer,[1] not in a monolayer as in Fig. 9. The physical–chemical origin of the asymmetry could be either a different chemical environment on both sides of membrane or a different chemical composition of the two monolayers.[2] Indeed, the variation of pH value and NaCl concentration may change only the Debye screening length and thereby the electrostatical interaction between charged lipid headgroups in two monolayers. This can lead to a change in the lateral membrane tension which may be operative to modulate the conductance level of alamethicin channels.

1 W. Helfrich, *Z. Naturforsch. C*, 1973, **28**, 693.
2 U. Seifert, *Adv. Phys.*, 1997, **46**, 13.

Dr Bezrukov responded: While we cannot rule out other factors, we correlate our findings exactly with what you suggest: with the pH-induced change in lateral pressure. As it is well known, lateral pressure in a monolayer of a symmetric bilayer is distributed differentially and changes along monolayer depth (*e.g.* see Fig. 1 of the paper by Templer *et al.* in this volume). Therefore, every attempt to reduce actual distribution with a single number is a simplification of the actual situation. We correlate probabilities of alamethicin channel states with the spontaneous monolayer curvature[1] which is related to the first moment of the lateral pressure.[2] The pH variation changes lipid charge thus changing lipid head–head interactions; the resulting modification in lateral pressure distribution is manifested by the change in lipid spontaneous curvature (Fig. 10). In our earlier work with alamethicin channel in PE–PC mixtures we were first to relate changes in the single-channel expression with lipid spontaneous curvature.[3] Recently Olaf Andersen and his colleagues[4] have shown that this parameter is also very important for the gramicidin A channel activity. Moreover, they showed that, at least in the case of gramicidin A and solvent-free bilayers, the channel expression is much more sensitive to changes in the first moment of lateral tension than to changes in its average value at lipid substitution.[5]

Thus, to say that the cartoon in Fig. 9 is not relevant to our channel data is, to put it mildly, an exaggeration. For further reading I would recommend the special issue of *Chemistry and Physics of Lipids*,[6] devoted to the functional role of non-lamellar lipids.

1 S. M. Gruner, *Adv. Chem. Ser.*, 1994, **235**, 129.
2 R. H. Templer, S. J. Castle, A. R. Curran, G. Rumbles and D. R. Klug, *Faraday Discuss.*, 1998, **111**, 41; M. M. Kozlov and V. S. Markin, *J. Chem. Soc. Faraday Trans. 2*, 1989, **85**, 261.
3 S. L. Keller, S. M. Bezrukov, S. M. Gruner, M. W. Tate, I. Vodyanoy and V. A. Parsegian, *Biophys. J.*, 1993, **65**, 23.
4 J. A. Lundbaek, A. M. Maer and S. O. Andersen, *Biochemistry*, 1997, **36**, 5695 and references cited therein.
5 C. Nielsen, M. Goulian and O. S. Andersen, *Biophys. J.*, 1998, **74**, 1966.
6 *Chem. Phys. Lipids*, Special Issue, 1996, **81**(2).

Dr Templer commented: The curvature elastic stress that is stored in a lipid bilayer is in general non-zero. For a flat bilayer, which has the same lipid composition on either side, the Helfrich Hamiltonian, as expressed in terms of the bilayer, would indeed be zero. However, if it is expressed in terms of each constituent monolayer it becomes evident that each monolayer wishes to bend to the same degree, but in opposite directions. Helfrich has called this the torque tension, and the frustrated curvature energy stored in each monolayer per unit area is proportional to the square of the spontaneous curvature of the monolayer and the bending modulus of the monolayer. Since the monolayer is held flat this curvature elastic energy must be stored in some way. This is done by increasing the area dilation at the interface.

Dr Sansom commented: Alamethicin helices are linked (by the glycine-X-X-proline motif) and thus, to a crude approximation, helix bundles are somewhat "hourglass" shaped in cross-section. Thus, lipids which prefer the H_{II} phase may pack better around the helix bundle.

Dr Bezrukov responded: Yes, I agree. This could be one of the possibilities for explaining the positive correlation between probabilities of higher conductance channel states and H_{II} lipid propensity.

Prof. Evans asked: Is the effect of 'spontaneous curvature' on channel activity altered by organic solvents that partition in the bilayer interior (*e.g.* as your group has shown for the H_{II} phase transition of DOPC)?

Dr Bezrukov responded: We did not study that. The present paper reports results for "dry" membranes obtained by Montal–Mueller monolayer opposition technique. No solvents were used in the X-ray liquid-crystal phase preparations.

Dr Bezrukov opened the discussion of Dr Smart's paper: What you are trying to do is very important for the central issue in molecular biophysics, the issue of structure–function relationship. For ionic channels their transport properties, in particular conductance, are of prime interest. However, the predictive power of your approach is not clear. Consider the following example. It is

well known (structural data, molecular models, permeation results) that the pore radius of the channel formed by gramicidin A is about three times smaller than the pore radius of amphotericin B channel. Using your approach we would expect about ten times higher conductance for amphotericin B channels. The single-channel measurements show that the actual situation is reversed: amphotericin B channel conductance is about three times *smaller* than the conductance of gramicidin A channel. Are you not worried about this 3000% discrepancy with your model prediction?

Dr Smart responded: The main point to emphasize is that the predictive power of the technique has been tested on all ion channels where an experimental high resolution structure was available.[1] In these tests prediction to within an average factor of 1.6 with a predictive r^2 (ref. 2) of 0.9 (6 systems tested). Extending the test set to include model structures with a reasonable certainty produced results to within a factor of 1.8 and a predictive r^2 of 0.46. In comparison to methods of forecasting binding affinities to enzymes on the basis of structure these figures are good, particularly for a first attempt.

The method has not yet been tested on amphotericin B, as the structure of the channel conformation has not been experimentally determined. Although models have been proposed[3,4] they are by no means certain, in particular the stoichiometry and role of sterol molecules is still unclear.[4] The pore dimensions of the models have been based on the fact that the antibiotic allows the diffusion of xylose or ribose, but not larger sugars through cell walls.[5] Experience has shown that such an identification is good for rigid molecules such as porins (see ref. 5 of our paper) but it is quite possible that the flexibility and/or changes of stoichiometry of amphotericin B may be involved in xylose transport. If the discrepancy is real then it may be due to the fact that amphotericin B is a polyene rather than peptide and is proposed to have a channel lumen lined by hydroxy groups, which makes it different in character from the channels used to parameterize the HOLE conductance prediction. In conclusion, the discrepancy may not exist but if it does then this is worthy of further investigation as this would mean that the amphotericin B has a structure activity relation which is 30-fold different from the many channels which fit within the HOLE procedure.

1 G. R. Smith and M. S. P. Sansom, *Biophys. J.*, 1998, **75**, 2767.
2 R. D. Cramer, D. E. Patterson and J. D. Bunce, *J. Am. Chem. Soc.*, 1988, **110**, 5959.
3 M. Bonilla-Marín, M. Moreno-Bello and I. Ortega-Blake, *Biochim. Biophys. Acta*, 1991, **1061**, 65.
4 M. Baginski, H. Resat and J. A. McCammon, *Mol. Pharmacol.*, 1997, **52**, 560.
5 B. de Kruihff, W. J. Gerritsen, A. Oerlemans, R. A. Demel and L. L. M. van Deenen, *Biochim. Biophys, Acta*, 1974, **339**, 30.

Prof. Roux asked: Ion channels generally exhibit saturation properties as a function of permeant ion concentration, *e.g.*, the conductance increases linearly at low concentration and then reaches a plateau at higher concentration. Sometimes it even decreases as the concentration is raised further. How is this Ohm's law approximation dealing with such phenomena?

Dr Smart responded: In short, HOLE does not attempt to deal with these phenomena. To date concentration effects have been ignored. In the original paper we state the desirability of making predictions in the low concentration range you mention. However, we are at present limited to the concentrations at which experimental data is available. The method is successful despite the problem of the diversity of concentration (for details see my earlier reply to Dr Bezrukov). Hopefully, in the future we will acquire data under consistent conditions for all channels with known structure. In many respects, although a prediction of absolute conductance in the expected range can provide a useful guide in model validation, the PEG addition experiment has the greatest potential to be of use yielding much more direct information. However, at present there is not a sufficiently large amount of data to make confident interpretations. This is why we are extending the method by collecting data for channels of known structure.

In passing, the situation is even more complex than you state. The surface conductance effects can cause higher than expected conductances at low ion concentrations (see ref. 50 of our paper) as the effective concentration of ions within a pore is increased with respect to bulk.

Dr Sansom asked: As you suggest the 6-meric and 7-meric α-toxin pores correspond to low and high conductance states, what is the timescale for switching between these two forms?

Are you suggesting that even for more complex membrane proteins, there is a similar problem to that discussed earlier for gramicidin *i.e.* that of relating different structure of a channel protein (static) to its biological function (dynamic)?

Dr Smart responded: Yes the problem of relating structure to function for ion channels is particularly acute. Even for gramicidin, where, not withstanding recent controversy, we know the structure for the conducting form, there is the problem of understanding the closure event. This is known to involve dimer breakdown into a monomeric form. However, the exact monomeric conformation adopted is still unknown. For more complex behaviours this problem is more acute. It must be remembered that in single channel recordings we are watching a single molecule or molecular assembly in action. Very often there is a massive excess of "silent" molecules present. In these cases most other experimental techniques can be expected to yield information on the conformation of the closed states.

The high and low conductance states may correspond to a difference in oligomerization but this identification is put forward as a working hypothesis worthy of further study rather than a firm conjecture. It must be remembered that a major piece of evidence is the fit between the structure-based HOLE prediction of the effect of PEG on conductance and that further work is required to make identification more firm (the data reported here on gramicidin is the first part of this process). A coauthor of the paper has an alternative explanation that the change in conductane is principally due to an alteration in the ionization state of the channel (see ref. 50 of our paper). The switch between the states is rapid in the timescale of single channel recordings. However, closure events are only very rarely observed in the absence of divalent ions. To further complicate the matter single channel recordings suggest that there are two low conductance states which differ in their own selectivity (see ref. 50 and 51 of our paper).

Prof. Smith asked: How was the diffusion coefficient calculated? Was it from the time dependence of the mean-square displacements, and if so did this function exhibit the required linearity?

Finally, in which way is the timescale of the motion thus quantified relevant to conductance?

Dr Smart responded: The diffusion coefficients were calculated by Tieleman and Berendsen from a molecular dynamics simulation of OmpF. A full description of the method used is given in ref. 10 of our paper, but the diffusion coefficients D_z are derived from the mean square displacement of atoms using:

$$\lim_{t\to\infty} \langle \{z(t) - z(0)\}^2 \rangle = 6D_z t$$

Each water molecule was assigned to belong to a slice of z coordinate space (1.2 Å thick) and its displacement measured over the next 5 ps. At the end of this time interval a reassignment was made. The diffusion coefficient was an average of all molecules within a slice. No information is given about the linearity you refer to.

The relevance of the timescale of the motion to conductance is an interesting question. Smith and Sansom[1] have shown that the diffusion coefficients of ions within channels are affected by factors of the same magnitude. My interest in using the data is in taking an empirical approach. A benefit of using the diffusion coefficient correction is that it results in the correct "boundary condition". As a channel gets sufficiently large to be regarded as macroscopic the diffusion coefficients of the water within it will tend to bulk values and therefore the correction will tend to 1 and the predictions will reduce to Ohm's law. The validity of this adaptation can only be proven by extending to all the systems analyzed with the original purely empirical correction and seeing whether it leads to improved predictions.

1 G. R. Smith and M. S. P. Sansom, *Biophys. J.*, 1998, **75**, 2767.

Dr Sansom responded: For the alamethicin simulations and for OmpF (ref. 1) the water diffusion coefficient was calculated from the mean square displacement over a 5 ps period. In an earlier study without a bilayer[2] we have looked at ion diffusion within pores and shown that diffusion

coefficients, again on a *ca.* 5 ps timescale, are similarly reduced. However, clearly these timescales are short relative to that of ion permeation. The relevance of such motions to conductance will depend *inter alia* on the strength of direct ion–pore interactions.

1 D. P. Tieleman and H. J. C. Berendsen, *Biophys. J.*, 1998, **74**, 2786.
2 G. R. Smith and M. S. P. Sansom, *Biophys. J.*, 1998, **75**, 2767.

Prof. Klein commented: With regard to diffusion of water molecules through ion channels I mention that our MD study of LS2 suggested two types of water molecules. The majority diffuse more or less unhindered through the pore but at about one-third of the bulk liquid value. But a few waters are hydrogen bonded to the inner wall of the channel with residence times of hundreds of ps. In layer channels, with more pore water, these long-lived bound waters may be less important.

Dr Smart responded: Presumably the diffusion coefficient I have used here reflects an average over these two types of water molecule. I think that the presence of tightly bound water may on occasions be important. Given a tight enough binding the water molecule may, in effect, become part of the channel. This could have some effect on the overall conductance but be very important in size selectivity. A possible example is the nicotinic acetyl choline receptor channel which appears from electron microscopic results to have a pore much larger than one would expect from its size selectivity.

Dr Gilbert asked: Does HOLE take account of the possibility of both surface and bulk conductance phenomena within a channel such as that formed by staphylococcal α-toxin.[1] Would the surface/bulk conductance dichotomy be relevant in seeking to explain high and low (and intermediate) conduction states of such channels (especially considering the expected dynamic nature of their effective diameters)?

1 Y. E. Korchev, C. L. Bashford, G. M. Alder, P. Y. Apel, D. T. Edmonds, A. A. Lev, K. Nandi, A. V. Zima and C. A. Pasternak, *FASEB J.*, 1997, **11**, 600.

Dr Smart responded: As discussed in my earlier response to Dr Sansom α-toxin has a difference in charge selectivity between the high and low-conductance states. This has implications for interpreting the results of the PEG addition experiments in terms of a small pore size. As discussed in detail elsewhere (in ref. 5 of our paper) this interpretation is thrown into question by data of PEG addition to other channels. For example the difference in the PEG addition curves observed between the $C = 1$ and $C = 3$ states of alamethicin, which differ roughly ten fold in conductance, is smaller than that between the 'high" and 'low' conducting forms of α-toxin. It is almost universally agreed that the different conductance states for alamethicin reflect different oligomerization numbers of the peptide rather than a difference in the charge state of the peptide. The assertion, therefore, that the data for α-toxin is incompatible with a difference in the size of the pore beween the states can be seen to be weak. The HOLE calculations provide an explanation for the difference without the need to invoke surface effects. It is quite possible that both effects contribute in reality.

HOLE takes such effects into account in an average fashion when making predictions of absolute conductance as they presumably play a role in the training set used for the derivation of the correction factors. Given a much larger set of data to work with it may be possible to explicitly include a correction function which incorporates the number of ionizable charges on the channel. However, at present, I have no way of representing the differences of such an effect between two systems.

You state in passing that α-toxin is expected to have a dynamic effective diameter. Given the fact that the channel lumen is provided by a β-barrel with strong hydrogen bonding it is likely that the channel is one of the least dynamic in terms of pore dimensions.

Prof. Klein commented: Our molecular dynamics studies[1] of small peptide bundles suggest that the dynamical behaviour of the bundle itself may play a role in conduction. That is, it could be important to take an ensemble average over the modes of vibration of the bundle. Radial breathing and torsional motion may be coupled to the passage of water molecules. This issue is not

usually discussed. In a recent paper involving a collaboration with the DeGrado group we draw attention to the possible role of dynamical fluctuations[2] at least for small bundles. Do you think this effect could have wider implications?

1 T. Husslein, P. B. Moore, Q. Zhong, M. L. Klein, D. M. Newns and P. C. Pattnaik, *Faraday Discuss.*, 1998, **111**, 201.
2 G. R. Diekmann, J. D. Lear, Q. Zhong, M. L. Klein, W. F. DeGrado and K. A. Sharp, *Biophys. J.*, 1999, **76**, 618.

Dr Smart responded: You make a good point. We know for instance that a caesium ion cannot fit through the gramicidin channel without a marked change in structure. However, the HOLE method does not try to account for the real behaviour of channels during the conductance of an ion. Rather an empirical approach is taken in which many factors are incorporated in an average way in the fitting process. A problem behind taking an empirical approach is that it does not lead to physical insights as to the actual processes involved. But it does have other advantages. In the area of prediction of binding affinities of ligands to receptors the "*ab initio*" approach whereby you start with a model, some interaction potential energy function and use chemical physics to understand the system has been shown to be of limited use. Rather Marshall and co-authors have shown that calculating relatively simple physicochemical properties for a set of complexes with known binding energies and applying a fitting procedure can result in high reliability methods for making predictions. There is not yet enough data to be able to take such a rigorous approach for channel conductance but HOLE is an attempt to start the process. To date results are good.

1 R. D. Head, M. L. Smythe, T. I. Oprea, C. L. Waller, S. M. Green and G. R. Marshall, *J. Am. Chem. Soc.*, 1996, **118**, 3959.

Prof. Roux commented: I agree with Prof. Klein's comment about the important structural fluctuations of channels formed by bundles of helices. But I would take this further by questioning the significance of the statistical fluctuations observed in MD in the absence of ions in the channel. The presence of ion in the channel may very much affect the structure of those flexible channels.

Dr Smart responded: As the questions in this discussion have revealed there are very many processes which affect the conductance of channels. The presence of an ion can be expected to have a marked effect which will vary as the ion moves through the channel. To understand and confidently predict this effect may eventually be possible. But my approach is to avoid it at present. It may be that solvated dynamics runs do not provide an improvement over a purely empirical approach because of the point you make.

Prof. Holzwarth asked: Can you predict dynamic changes in the micro- to millisecond time range caused by the mobility of membrane lipids next to channel forming peptides like gramicidin? We investigated the influence of peptides like gramicidin and an artificial peptide of 30 amino acids (2lys-gly-24leu-2lys-ala-amide) on the dynamics of the main phase transition of bilayer vesicles[1] and found very pronounced effects on the mobility and structure of the lipids near the peptides; I wonder how the mobility of the lipids might be reflected in the transport properties of the channel forming peptides.

1 A. Genz, T. Y. Tsong and J. F. Holzwarth, *Structure, Dynamics and Equilibrium Properties of Colloidal Systems, NATOASI Series C*, ed. D. M. Bloor and E. Wyn-Jones, Kluwer, Dordrecht, 1990, vol. 324, pp. 493–515.

Dr Smart responded: It may be possible to use molecular dynamics techniques to predict the effects that peptides have on membrane lipids and *vice versa*. However, at present simulation practicalities limit the area of the lipid bilayer considered. I have no direct experience in the area.

Dr Burkhart commented: Crystallographic structures are a space and time average of the molecule and contain in their B-factors many of these static and dynamic features inherent in the molecular motion.

Dr Smart responded: Temperature factors provide some information as to the ensemble average dynamics of the molecule within the crystalline environment. How relevant this is to the lipid bound form of a channel is a debatable point. However, the high resolution crystal or NMR structure of a channel in its active conformation provides information which is unobtainable in any other way. The recent excitement over the structure for the KcsA potassium channel disproves the view that as ion conductance is a dynamic phenomenon that "static" structures are of limited importance.

Prof. Roux opened the discussion of Prof. Klein's and Dr Sansom's papers: Most of the helices forming bundle channels are amphipathic which could, presumably, be lying parallel to the membrane/bulk interface. The application of a transmembrane potential together with the presence of permeant ions could be the microscopic factor driving the formation of the bundle. This could suggest that the current MDs that you described correspond to a metastable state of the channel. Could you comment please?

Dr Sansom responded: The application of a transbilayer voltage is needed to induce helix bundle formation, both for alamethicin and for other peptides (*e.g.* the LS peptide[1]). However, once open these channels are metastable on a *ca.* 10 ms timescale (*i.e.* 10^6 times the simulation timescale) even at zero mV transbilayer potential, as shown by open-channel current–voltage curves (*e.g.* Woolley *et al.*[2] and Kienker *et al.*[3]). The effect of the presence of permeant ions is more difficult to comment on—certainly there is evidence for increased channel open times at elevated ionic strengths.[4]

1 J. D. Lear, Z. R. Wasserman and W. F. DeGrado, *Science*, 1988, **240**, 1177.
2 G. A. Woolley, P. C. Biggin, A. Schultz, L. Lien, D. C. J. Jaikaran, J. Breed, K. Crowhurst and M. S. P. Sansom, *Biophys. J.*, 1997, **73**, 770.
3 P. K. Kienker, W. F. DeGrado and J. D. Lear, *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 4859.
4 W. Hanke, C. Methfessell, H. U. Wilmsen, E. Katz, G. Jung and G. Boheim, *Biochim. Biophys. Acta.*, 1983, **727**, 108.

Prof. Klein responded: The metastability referred to by Prof. Roux certainly applies to our MD calculations, and those of all other workers in the field.[1–3] The present situation has been likened to trying to ascertain how the human body functions by carrying out an autopsy on a cadaver.[4] Great strides were made in the early days of anatomy by studying non-functioning beings. Similarly, the study of an assembled bundle might be expected to yield clues as to the key elements of the functioning channel.

Naturally, we look forward to the day when larger systems with transmembrane potentials and ions will be amenable to study. For the present, we have a more modest aim and indeed focus on essentially pre-assembled bundles—with all of the many limitations this entails.

Prof. Roux is correct in pointing out that many helices that form channels are amphipathic in character and would thus likely prefer to be lying parallel to the membrane/bulk interface as isolated monomers. We have recently investigated the behavior of such a monomer—the Duff–Ashley M2 peptide and indeed find that on the nanosecond timescale, this 25-residue α-helical peptide is content to remain parallel to the interface albeit with some specific peptide–lipid anchoring interactions (see Fig. 1).

1 T. B. Woolf and B. Roux., *Proc. Nat. Acad. Sci. USA.*, 1994, **91**, 11631.
2 T. B. Woolf and B. Roux., *Proteins*, 1996, **24**, 92.
3 M. S. Sansom, *et al.*, *Biochem. Soc. Trans.* 1998, **26**, 438.
4 R. S. Eisenberg, 1998, personal communication.

Dr Bezrukov said: From your Fig. 2B I gather that you took great care over the ionization state of Glu18 sidechains and concluded that the net charge is rather small. What would be the effect of the higher charge on the channel structure? Also, what potential do you use to describe interactions between charges?

Dr Sansom responded: We have run simulations for $N = 6$ alamethicin bundles with either one Glu18 ionised[1] or with zero or six Glu18s ionised.[2]With six Glu18s ionised the bundle "falls

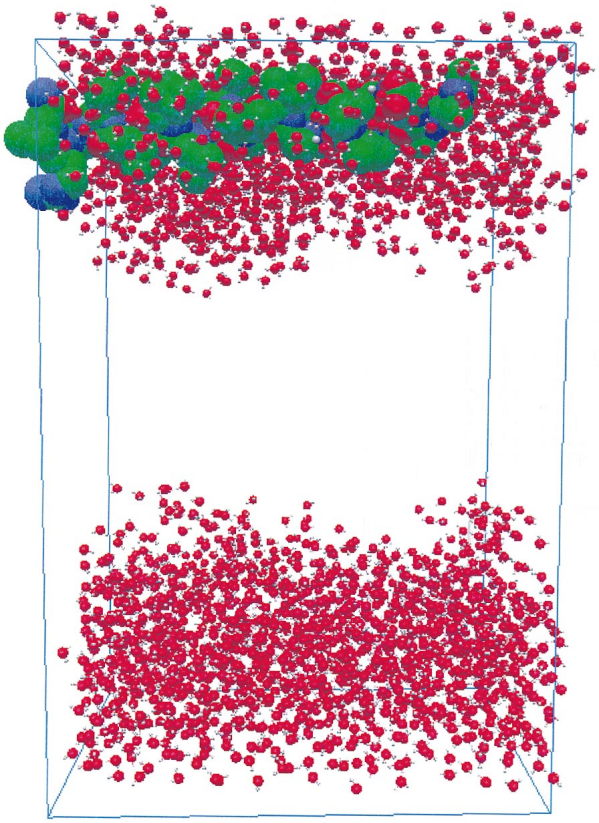

Fig. 1 The Duff–Ashley 25 residue peptide at the lipid/bulk water interface taken from an MD simulation initiated with the peptide lying parallel to the interface.

apart" during the simulation. With zero or one Glu18 ionised the bundle remains intact. This suggests that suppression of ionisation of sidechains may play a role in channel stability.

In studying the interactions between charges, in the pK_a calculations we used a screened interaction (by including a Debye length equivalent to 1 M KCl). But in the MD simulations a simple unscreened Coulombic interaction was used. Clearly this is an approximation, and may be a problem as it is known that *e.g.* melittin channels are stabilised by high ionic strength.

1 D. P. Tieleman, J. Breed, H. J. C. Berendsen and M. S. P. Sansom, *Faraday Discuss.*, 1998, **111**, 209.
2 D. P. Tieleman, H. J. C. Berenden and M. S. P. Sansom, *Biophys. J.*, 1999, **76**, in the press.

Prof. Neumann commented. There is no doubt about the importance of charged groups for the structure and for structural changes of macromolecules. Yet, for channel proteins the actual transport passage is usually hydrophobic without charged groups at some distance away from the channel part. See, for instance, Kukol and Neumann.[1]

1 A. Kukol and E. Neumann, *Eur. Biophys. J.*, 1998, **27**, 618.

Dr Bezrukov responded: Sometimes this is the other way around. See for example Forst *et al.*[1]

1 D. Forst, W. Welte, T. Wacker and K. Diederichs, *Nat. Struct. Biol.*, 1998, **5**, 37.

Prof. Klein responded: Current generation computers allow only *ca.* 5–10 nanosecond length trajectories, in most cases. However, there is one reported study by the Kollman group[1] spanning

1 μs. The next 3–5 years should see supercomputers reaching peak performance around 100 Teraflops, which should allow microsecond trajectories for membrane proteins. This will allow us in special cases to follow modest structural changes, caused for example by the passage of ions along channels. For less detailed models, the projected increase in CPU performance will enable coarse-grained models to study the assembly of model peptides into bundles and the response of the host membrane to external probes.[2]

1 Y. Duan and P. A. Kollman, *Science*, 1998, **282**, 740.
2 R. Lipowsky, *Progr. Colloid Polym. Sci.*, 1998, **111**, 34.

Dr Sansom said: I wish to comment on MD simulations of the tetrameric transmembrane (TM) helix bundle of the M2 channel protein from influenza A. We also have run such simulations,[1] albeit using a different lipid (POPC instead of diPhyPC) from that used by Klein and co-workers.[2] However, in contrast to the situation with model peptide channels (*e.g.* alamethicin) there is a problem with simulations of TM helix bundles from larger proteins, namely that of the exact extent of the helices. In the case of influenza M2, Duff and co-workers[3,4] have shown that a 25-residue peptide forms channels and is largely α-helical. However, it is not certain that all of the residues in the peptide form an α-helix, and to what extent the peptide mimics the intact M2 protein. We have used multi-nanosecond simulations of single TM helices of different lengths (from 18, 26 or 34 residues) in a phospholipid bilayer in order to determine the optimum length of the helix from M2. These simulations suggest that a region of length *ca.* 22 residues forms a helix stable throughout the simulation.[5]

The length of helix has a profound effect on the behavior of four TM helix bundle models in bilayer simulations. We have compared simulations with a bundle of 18-residue helices and with a bundle of 22-residue helices.[5] In both cases the helix bundles retained their left-handed supercoil structure, and gave a Cα rmsd of *ca.* 0.25 nm after a 4 ns simulation. However, the 18-residue bundle contained only 3 water molecules, which did not exchange with bulk water. Thus the 18-residue bundle looked like a "closed" channel. In contrast, the 22-residue bundle contained *ca.* 10 waters, and looked like an "open" channel. What is clear from these simulations is that one has to be a bit cautious in the choice of initial helix bundle model, as this may have a profound influence on the results of any subsequent bilayer simulation.

1 M. S. P. Sansom, D. P. T. Tieleman, L. R. Forrest and H. J. C. Berendsen, *Biochem. Soc. Trans.*, 1998, **26**, 438.
2 T. Husslein, P. B. Moore, Q. Zhong, D. M. Newns, P. C. Pattnaik and M. L. Klein, *Faraday Discuss.*, 1998, **111**, 201.
3 K. C. Duff and R. H. Ashley, *Virology*, 1992, **190**, 485.
4 K. C. Duff, S. M. Kelly, N. C. Price and J. P. Bradshaw, *FEBS Lett.*, 1992, **311**, 256.
5 L. R. Forrest, D. P. Tieleman and M. S. P. Sansom, *Biophys. J.*, 1999, **76**, in the press.
6 L. R. Forrest and M. S. P. Sansom, in preparation.

Prof. Okazaki said: It is interesting for me to see a decrease of membrane thickness and a decrease of alkyl chain order by the inclusion of the peptide. If you have reached some conclusion, please let me know about the mechanism. Are there any particular sites in the peptide which interact directly with the lipid molecule?

Prof. Klein responded: I am confident that our results are correct for the peptide concentration used in the MD simulation. Unfortunately, the ratio of peptide to lipid is only 1 : 8 in each leaflet of the bilayer. I am, therefore, concerned that the observed "thinning" of the membrane is related to channel–channel repulsions arising from interactions between the large helix dipoles. It would be useful to run more dilute samples, with say 128 and 256 lipids to quantify this effect more precisely.

Prof. Klein said: Did you find that the M2 (22-residue) peptide yielded a stable 4-helix bundle with a water pore or was it blocked at the His as suggested by various experiments and MD calculations[1] on a simpler system?

1 Q. Zhong, T. Husslein, D. Newns and M. L. Klein, *FEBS Lett.*, 1998, **434**, 265.

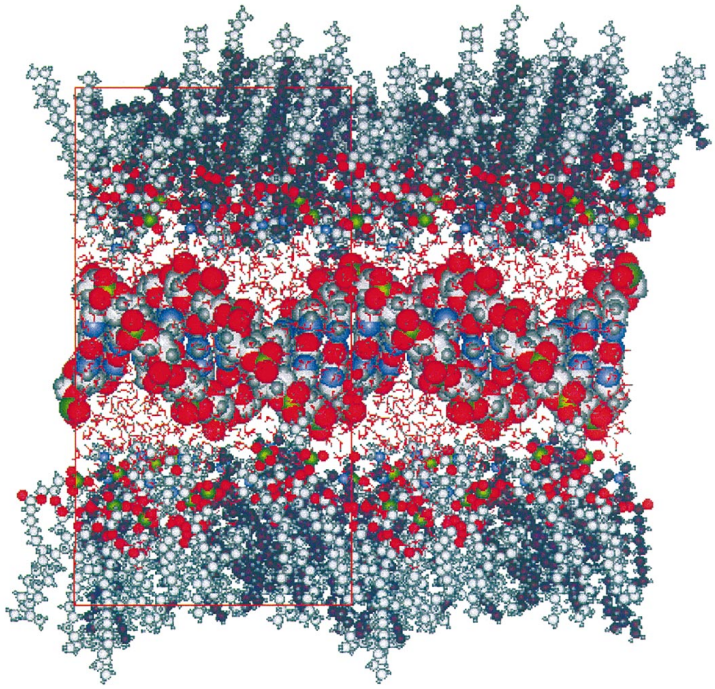

Fig. 2 A configuration taken from a multi-nanosecond MD simulation of the lipid–DNA complex. The two distinct lipids (DMTAP and DMPC) are drawn with light and dark shading.

Dr Sansom responded: The M2 (22-residue) helix bundle was "stable" in the sense that the left-handed supercoil was maintained, and the Cα rmsd at the end of 4 ns was *ca.* 0.25. Up to *ca.* 2 ns there was not a continuous water pore. Instead, there was a water-filled pore which was open to the surrounding environment at the N-terminal mouth but which was occluded towards the C-terminal mouth by the ring of His37 sidechains. However, after *ca.* 2 ns this pore opened up at the C-terminal as well. We suspect that this sort of behaviour might be rather sensitive (at least, on a 1 to 10 ns timescale) to the starting model used in the simulation, and so we are now exploring different starting structures.

Dr Amblard commented: Modelling the dynamics of macromolecules in solution or in membranes by coupling the elasticity of the macromolecule and viscous dissipation by the small surrounding molecules taken as a continuum could be a way of simulating molecular dynamics at much smaller frequency than the limit of MD classical simulation. On the other hand, Prof. Smith suggested earlier by MD simulation of bacteriorhodopsin, that this membrane protein could undergo dynamical transitions at well defined temperatures, revealed by the non-linearity of the crystal temperature factor with temperature. This suggests that bacteriorhodopsin does not behave as a set of harmonic oscillators, but in a much more complex and likely non-harmonic way.

To what extent could the complex elastic behavior of proteins be inferred from temperature-factor analysis, at least to provide a basis for modelling the dynamics at longer time-scales than with MD simulations?

Dr Sansom responded: I'm really not sure about using temperature-factor analysis for membrane proteins. My reservations are: (i) for a number of membrane protein structures the resolution is a bit low and so the B-factors may not be that reliable; (ii) most membrane proteins

are crystallised without lipids and so the relevance of the motions in the crystal to motions in the bilayer is uncertain.

Prof. Bayerl asked: Does the MD simulation of DNA in DMPC/DMTAP give any indication of a spatial confinement of the cationic lipids motion due to the electrostatic interaction with the DNA?

Prof. Klein responded: Our MD simulations[1] have been run long enough to begin to quantify the nature of the lipid–DNA interactions. The zwitterionic headgroup of DMPC competes effectively with the cationic lipid DMTAP in "neutralizing" the DNA phosphates. Fig. 2 and 3 show the overall structure of the complex and the distribution of nitrogen atoms around the DNA phosphates.[1]

1 S. Bandyopadhyay, M. Tarek and M. L. Klein., in preparation.

Prof. Holzwarth commented: The molecular dynamics simulations provide very interesting information about structural changes on an atomic level for times from femto- to several nanoseconds, but are not able to reach longer times. If one inspects the energy changes connected with membrane processes it can be shown that most of the important changes in biological systems are occurring at much longer times.[1] (1) Is there any chance in the near future to reach 100 nanoseconds or better the microsecond time range with MD simulations? (2) How do you rate the chances for approaches which avoid the enormous computer power needed for full nanosecond simulations by starting with some reasonable assumptions Could this be an acceptable approach and how can Monte-Carlo simulations be included into the solution? My personal ideas are circulating in a triangle of dynamic, structural and thermodynamic information, trying to connect all three types of available results to construct a simpler basis for MD simulations.

1 J. F. Holzwarth in *The Enzyme Catalysis Process*, ed. A. Cooper, J. L. Houben and L. C. Chen, Plenum, London, 1989, pp. 383–412.

Dr Sansom responded: There is every chance in the near future of reaching 100 ns simulations, given the increasing performance of computers, and the development of "smarter" MD algorithms. Indeed, we have run one alamethicin helix bundle simulation for nearly 20 ns (ref. 1). In the non-membrane field, simulations of the folding of a small protein fragment (*ca.* 40 amino acids) have been run for *ca.* 1 μs (ref. 2) although this is still out of the feasible range for membrane simulations.

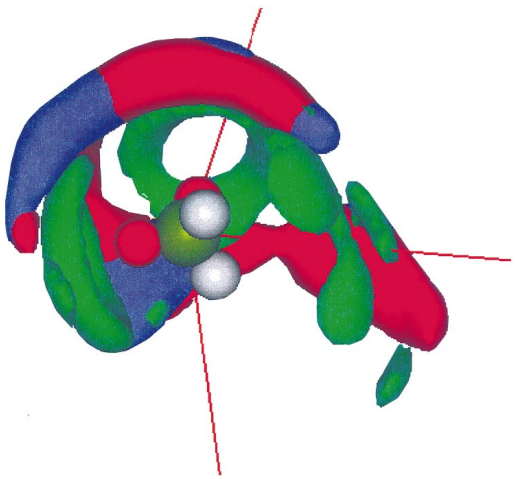

Fig. 3 Distribution of water molecules (green) and lipid N-atoms (red and blue) around a representative DNA phosphate group.

1 M. S. P. Sansom and D. P. Tieleman, unpublished data.
2 Y. Duan and P. A. Kollman, *Science*, 1998, **282**, 740.

Prof. Klein responded. As mentioned earlier, the Kollman group has reported on two microsecond long trajectories for a protein in water.[1] This was possible because of the availability of 256 CRAY T3E processors dedicated for a few months. The relentless progress in CPU performance should allow similar capability on a more routine basis in the 3–5 year time frame. The likely availability of these resources does not obviate the need to develop alternative approaches.

1 Y. Duan and P. A. Kollman, *Science*, 1998, **282**, 740.

Prof. Roux said: It is attractive to use a simplified description of the membrane environment in order to gain in computational efficiency and reach out to longer simulation times. However, it is important to keep in mind that one still doesn't know which details of the bilayer are going to be important in investigating the function of a membrane protein.

Dr Sansom responded: I fully agree with this. I think progress will be made by running more explicit bilayer simulations on a range of different membrane proteins, and then attempting a general analysis of which interactions are most important, at the same time as developing simplified descriptions as exemplified in the paper by Roux and co-workers.[1]

1 P. Lagüe, M. J. Zuckermann and B. Roux, *Faraday Discuss.*, 1998, **111**, 165.

Dr Amblard commented: When reading Dr Sansom's paper I was quite surprised by the fact that results of MD simulation are more often compared with results of other simulations than with experimental results. This is obvious in the 'Biological relevance' section of his paper. In the discussion of the last few papers dealing with MD simulation, not much was said about the connection with experimental data, about the kind of predictions generated by MD simulations at different time-scales, and about their experimental "testability" and the experimental models of interest. Could you and Prof. Klein comment on these points, and help non-specialists like me to grasp what the important questions and limitations are in this field, beyond the technicalities of MD simulations.

Dr Sansom responded: Maybe I was a bit too cautious in the 'Biological relevance' section of my paper, but I deliberately did not wish to over-interpret our results. I think some of the connections with experimental data for alamethicin have been described in Dr Smart's paper,[1] and so I won't duplicate them here. As I suggested in my paper, I think the motion of the pore-lining helices of alamethicin may have some relevance to gating of *e.g.* potassium channels such as KcsA. Indeed, recent spin-label studies of KcsA[2] provide experimental evidence of such helix motions, albeit on a much longer timescale.

1 O. S. Smart, G. M. P. Coates, M. S. P. Sansom, G. M. Alder and C. L. Bashford, *Faraday Discuss.*, 1998, **111**, 185.
2 E. Perozo, D. M. Cortes, L. G. Cuello, *Nat. Struct. Biol.*, 1998, **5**, 459.

Prof. Klein responded: You raise an excellent point. It is to be regretted if the results of a MD simulation are inaccessible to experimentalists. The predictive capability of the MD simulations is only useful to the extent that a range of experimental data can be accounted for. Alas, typically for membrane proteins, little is known beyond the basic structure. Even then, data is mostly related to the crystalline environments. The situation may change, as modern NMR methods achieve increasing success. Also, neutron and X-ray synchrotron experiments are likely to be important complements to modern NMR studies. I agree that it would be unfortunate if the focus shifted solely to technicalities of MD simulations.

Prof. Smith commented: Prof. Klein provides evidence here for a more fluid-like behaviour of the lipid in the presence of the protein and larger area per lipid, relative to the pure lipid. In preliminary results with Dan Mihailescu on gramicidin S binding to a DMPC bilayer we see the

opposite effect in the lipid fluidity. Would you like to comment on whether findings similar to yours[1] have been observed elsewhere and on the physical origin of these effects?

1 T. Husslein, P. B. Moore, Q. Zhong, D. M. Newns, P. C. Pattnaik and M. L. Klein, *Faraday Discuss.*, 1998, **III**, 201.

Prof. Klein responded: I am sorry to say that I cannot comment on the gramicidin/DMPC system. Prof. Okazaki has been interested in this system for some time.

Dr Sansom responded: Tieleman and co-workers[1] have analysed lipid properties simulations of six-helix bundles of alamethicin. He saw a decrease in order parameters, corresponding to increased tilt of acyl chains of lipids close to the helix bundle. I suspect different results may be found for different systems (see response to next comment), and so we need to be cautious in making generalisations at this stage.

1 D. P. Tieleman, L. R. Forrest, M. S. P. Sansom and H. J. C. Berendsen, *Biochem.*, 1998, **37**, 17554.

Prof. Roux commented: The presence of protein in a membrane has been shown to increase the ordering of the acyl chains of the lipids. Rice and Oldfield showed that the deuterium quadrupolar splitting of specifically labelled DMPC was increased in the presence of gramicidin. Our results from molecular dynamics simulations are in good agreement with this observation.[2] However, it should be stressed that averaging from five independent trajectories was required to get convergence.

1 Rice and Oldfied, *Biochemistry*, 1979, **18**, 3272.
2 Woolf and B. Roux, *Prot. Struct. Funct. Gen.*, 1996, **24**, 92.

Prof. Klein responded: Prof. Roux brings out an important point: The issue of convergence of NMR order parameters obtained from MD simulations. It is also our observation that these are difficult quantities to obtain reliably. Unfortunately, my group has no specific data for the gramicidin/DMPC system.

Dr Sansom responded: Also, I think it is important to note that different results may be obtained for different proteins. Tieleman and co-workers have analysed lipid properties in simulations of several different systems. For single, transmembrane helices, the effects on surrounding lipids are relatively minor. For four helix (influenza M2) and six-helix (alamethicin) bundles, and for the porin OmpF (a large β barrel) a decrease in order parameters, corresponding to increased tilt of acyl chains, of nearby lipids was seen. This suggests that we need to gather data from a wider range of simulations before we can attempt any generalisations. However, I agree that to obtain reliable statistics either long simulations or multiple independent trajectories are needed.

1 D. P. Tieleman, L. R. Forrest, M. S. P. Sansom and H. J. C. Berendsen, *Biochem.*, 1998, **37**, 17554.

Prof. Okazaki commented: First, the calculated order parameter of gramicidin was absolutely dependent upon the initial configuration. No conformational changes were observed for the gramicidin molecule within our preliminary ns order MD calculation.

On the other hand, the calculated order parameter of the DPPC alkyl chain in the pure DPPC bilayer in the liquid crystal phase was larger than the experimental one. But the cumulative average of the order parameter did not converge within our several ns order simulation. The convergence is very slow.

The order parameter is, thus, very difficult to evaluate from the limited simulation time of the MD calculation.

Dr Sansom responded: I agree that longer simulations are needed to get proper estimates of order parameters. Also, one suspects, careful attention to the setup of the simulation (*i.e.* lipid–protein packing) is needed.

Dr Bezrukov commented: I want to highlight the importance of molecular dynamics simulations in ion channel studies. Unfortunately analytical methods for description of channel transport

properties are far from satisfactory. For example, even a 'simple' question of how ionization of a single sidechain facing channel lumen influences channel conductance and selectivity is very hard to answer. Even in the case where all structural data is available (nothing to guess about here) I would not be surprised if the sign of the effect is difficult or just impossible to predict. Not only electrostatic *vs.* structural issues are involved here; the electrostatics itself at such short distances and high fields differs considerably from the continuous classic formulation. For this reason molecular dynamics simulations both of channel structure and its transport properties are much needed at present.

Dr Sansom responded: I completely agree that even "simple" questions are not resolved by simply looking at an X-ray structure. I feel one should be a bit more guarded about how quickly MD studies will progress. In principle, one should run a simulation which allows for dynamic protonation/deprotonation as an ion passes. I confess to not knowing how to do this at the moment, but I'm sure it will need long simulation times. Given the difference in timescale between current MD simulations (*ca.* 10 ns) and the permeation time of an ion (*ca.* 1 μs) I think we need to develop a hierarchy of theoretical descriptions of a channel in order to relate atomic resolution structures to physiological data from patch clamp experiments.

Prof. Holzwarth replied: Unfortunately the experimental techniques available are not specific enough to tackle the questions posed by Dr Bezrukov. On the other hand molecular dynamic simulations are not able to cover the time range beyond 10 ns. In summary I believe that the present experimental techniques are good in respect of the important time range from nanosecond to second but often lack molecular specificity; molecular simulations are fine for times shorter than a nanosecond but are not able to cover the really important "long time" phenomena. The future should improve this situation.

Dr Deber communicated: In the simulation of the four-helix bundles of M2, what were the features/motifs of interaction of the inter-helical faces/residues within the bundle (as depicted in Fig. 1 of your paper)? Are the interfaces predictable from primary sequence?

Prof. Klein communicated in response: To some extent, this issue has been discussed by my colleague, Prof. DeGrado and his collaborators,[1] in their analysis of mutagenesis data. Our MD study was designed to complement this experimental work and their analysis of the bundle structure. Although I am reluctant to speak for my colleagues on this issue, it is my impression that the inter-helical faces/residues do correspond to predictions based on the primary sequence of residues.

1 L. H. Pinto, *et al.*, 1997, *Natl. Acad. Sci. USA*, 1997, **94**, 1130.

Prof. Deber communicated: Is it possible to determine how helix–helix interactions vary (energetically, occurrences of residues at interfaces) as a function of alamethicin helical bundle size? A systematic approach here could enhance our understanding of the folding of polytopic membrane proteins.

Dr Sansom communicated in response: A very perceptive question! We are working on this, but haven't done such analysis as yet. One of the attractive features of these sorts of MD simulations is that they should allow analysis of helix–helix interactions in various "simple" bundles of transmembrane helices.

Dr Bhakoo communicated: There are a vast array of isolated biomembrane systems and their interactions are being studied by numerous methods by a number of excellent research teams. One particular worry I had is the lack of correlation of data between groups and between techniques utilised. Perhaps this could be addressed by having closer collaboration between research groups.

Another key issue that requires addressing is relating the simple isolated membrane systems to real biological membranes. A number of times the detailed understanding of model systems will not lead to the understanding of biological membranes. I believe that there is an urgent need for a

meeting between physical chemists, chemical physicists, biophysicists, biochemists, biologists, physiologists and microbiologists to discuss this issue.

Thus, a related question I have is as follows: How far do model membrane systems aid in providing understanding of real biological systems they are apparently mimicking?

Dr Sansom communicated in response: I guess this meeting is intended to address such worries. However, I think from a simulation perspective it is important that different groups work on similar systems so that one can get a feel for the extent to which the results converge. For example, going back to influenza M2, we have shown recently[1] that two independent modelling studies converge on a similar structure, which in turn is in agreement with experimental data.[2] This is encouraging.

In answer to your second question, clearly simple systems do not hold all of the answers. But, considering ion channels, gramicidin provided what turned out to be a good model for channel–ion interactions in the bacterial potassium channel.

1 L. R. Forrest, W. F. DeGrado, G. R. Dieckmann and M. S. P. Sansom, *Folding Design*, 1998, **3**, 443.
2 F. A. Kovacs and T. A. Cross, *Biophys. J.*, 1997, **73**, 2511.

Prof. Roux also communicated in reply to Dr Bhakoo: An important aspect of biophysics (experimental and theoretical) is the ability to use a reductionist approach in order to highlight the most important factors responsible for a phenomena. In doing so, it is useful to throw away as much detail as possible, as long as the essential elements are preserved. While I do not doubt that the complexity of biological membranes is much beyond our simple models, I think there is much to be gained by investigating simple model systems. Nonetheless, the intrinsic limitations of simple models should always be kept in mind.

In answer to your second question characterizing the magnitude of lipid-mediated protein–protein interactions is only a modest, though an important contribution to our understanding of the function of biological membranes.