

Peer Review File

Melittin can permeabilize membranes via large transient pores



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This study addresses an important task of deciphering the molecular mechanism of membrane permeation by host-defense. The authors use microsec-long atomistic MD simulations to study interactions of alamethicin and melittin with lipid bilayers. While a wealth of experimental evidence demonstrates that the action of these two representative peptides is different from each other (small stable pores for alamethicin vs larger dynamic lesions for melittin) the atomistic understanding of the permeation mechanism was missing. This study clearly captures the difference between the two and provides a compelling mechanism for the pore dissipation due to peptide transfer across the bilayer in case of melittin. The provided explanation of the mechanism of transient leakage, often observed for many host-defense peptides, is a valuable result that would justify the publication just by itself. The overall conclusions of the study are well supported, and only minor revisions are recommended, most of which can be addressed by rephrasing some of the passages.

1. The authors should exercise restraint while discussing the direct potential applicability of their approach "to discover new peptides for pharmaceutical and biotechnological applications in silico via high-throughput computational screens". The key issue in the design of the useful antimicrobial peptides is ensuring their selective action on bacterial vs mammalian cells. It has been suggested that lipid composition plays a role in ensuring such selectivity, thus studies with other lipid compositions are clearly required before the quoted claim from the abstract can be made. Moreover, it probably can only be made after truly selective peptides are used in the study, rather than melittin, which acts on both bacterial and mammalian membranes.
2. Continuing on the issue of lipid specificity, it would strengthen the manuscript if the authors were to examine behavior of melittin in the presence of anionic lipids. Specifically, it would be interesting to see if the simulations would result in the suppression of the pore formation, thus providing at least indirect evidence for the "detergent-like action" of melittin on such membranes. Another venue of comparison would be the effect of anionic lipids on stabilizing the conformation of melittin aligned with the plane of the bilayer, reported by the oriented CD.

Reviewer #2 (Remarks to the Author):

This reviewer is not a simulation specialist. I will assume that the authors' simulation procedure is valid. Indeed their results look reasonable to this experimentalist. However their interpretation of the simulations, particularly regarding the peptide's antimicrobial mechanism is erroneous and misleading. The authors performed simulations for two very different peptides, alamethicin and melittin. I will discuss melittin first because this peptide is in the title of this paper. I will leave my comment on alamethicin to the last paragraph.

Melittin is perhaps the most thoroughly studied membrane active peptide. Although it is the major toxin of the bee venom, it also exhibits antimicrobial activities; as a result, it has been extensively studied as a prototypical membrane active antimicrobial peptide (AMP). However its membrane activities critically depend on its concentration. At concentrations as low as a few nanomoles per liter, melittin can induce transient pores that allow transmembrane conduction of atomic ions but not leakage of glucose or larger molecules. At concentrations higher than tens of micromolar, it acts as a detergent disintegrating the membranes. If one is interested in its antimicrobial activities, it is well known that the minimal inhibitory concentrations of melittin and other AMPs are in the micromolar range. The phenomena of melittin-membrane interactions in these three different concentration ranges are entirely different.

The authors' simulation is of a single channel. As far as I can tell, it is consistent with the single channel activities induced by melittin at extremely low concentrations. It has long been recognized that melittin binding induces a local monolayer thinning effect at the binding site (called a wedge

effect by Terwilliger, Weissman, Eisenberg (1982)). A single channel of melittin (presumably of a toroidal type) is unstable in lipid bilayer, as exhibited by melittin single channel activities (reports of such data were scattered in refs in 1980's). However, at the minimal inhibitory concentrations, melittin always induces a large number of pores. Only a large number of toroidal pores distributed over a macroscopic area can achieve a uniform membrane thinning over a macroscopic area and attain equilibrium. Under this condition, one indeed observes stable melittin pores as demonstrated in many published papers (PNAS 110. 14243 (2013) and refs therein). The structure of such equilibrium melittin pores has also been determined, i.e., the toroidal model. Stable melittin pores were also observed in *E. coli* spheroplast membranes (Biophys. J. 112, 1663 (2017)). Thus the authors' assertion that melittin's permeabilization mechanism involves only transient pores is erroneous. The authors' simulations are not corresponding to melittin's antimicrobial mechanism.

In the introduction, the authors argued that "progress of pharmaceutical or biotechnological application has been hindered by a critical shortage in the detailed knowledge of the molecular mechanism of permeabilization and/or pore formation." This is not true. Melittin-like AMPs, such as magainin, have not been approved for pharmaceutical uses precisely because of the knowledge of their membrane lytic effects. So far the US FDA has approved three AMPs for therapeutic applications: gramicidin, daptomycin, and colistin. We still have problems understanding the mechanisms of the last two.

Finally, alamethicin is very different from melittin. While individual melittin binds to the interfacial region between the lipid headgroups and chains, with its peptide helix parallel to the plane of bilayer, thus inducing a local membrane thinning, alamethicin, being largely hydrophobic, inserts perpendicularly through the chain region and forms stable barrel-stave channels. There was a very extensive kinetic study on the alamethicin single channels by a Cornell group (Biophys J 69:2323 (1995); in contrast there are no systemic studies of melittin's single channels). The authors should compare their alamethicin simulations with these kinetic data.

This paper in its present form is not suitable for publication.

Reviewer #3 (Remarks to the Author):

Melittin has been found for more than fifty years. It is believed to permeabilize membranes thus equipped with extensive applications. However, the mechanism of interaction between the melittin and the membrane is not very clear to date. Experiments show that melittin can cause transient leakage of the membrane, which is different from the permanent pores in the membrane. An example is the permeabilization and resealing of *E. coli* membrane done by Weisshaar et. al. In this paper, the authors aimed to elucidate the details of the transient leakage of the membrane induced by the melittin by all atomistic simulations. As a comparison, they first showed that the alamethicin formed the equilibrium pores allowing the water and ions to pass through. However, melittin induced non-equilibrium and transient pores instead of equilibrium and stable pores. The authors then presented the formation and dissolution mechanism of the transient pores. The mass imbalance is considered as the driving force for the pores, and the translocation of peptides to balance the mass causes the dissolution of the pores. Next, the authors provided some information about the transient pores, including the transient timescale, the flux of water and ions, and so on.

However, I think the transient pores induced by melittin here are not of great novelty and surprise, because such concept has been proposed and experimentally observed for ten or more years. So, I think many improvements should be made before considering its publication.

(1) The comparison of pore formation of two types of peptides is important for understanding the mechanism of action of these peptides. For the membrane used in simulations, DOPC, DPPC/DPPG mixture and DMPC/DMPG mixture, the authors stated that the behavior of alamethicin in all these bilayers is almost identical. However, I find that the distribution of oligomer size is obviously different, and the fluxes of water and ions are different. In DMPC/DMPG the flux is not obvious. Otherwise the flux seems to occur mainly at the beginning. Since the pores are permanent, why the flux disappears

at the later time? What is the possible reason? Besides, the alamethicin is so hydrophobic as stated by the authors. So, could water pass through such hydrophobic pores? In addition, I think the hydrophobic alamethicin would be repelled by the membrane, why it immediately inserts TM? POPC is used in the simulation of melittin, as a direct comparison, why not provide the results of alamethicin in POPC?

(2) In the "Transient Pore Formation" section, should "Figure 2h" be replaced with "Figure 2e"?

(3) In the section "Transient pore formation", "We concluded that the likely mechanism is actually purely mechanical, driven by initial mass asymmetry", it is hard to think the mass imbalance is the driving force for opening the membrane. More supporting analysis is needed. Moreover, by this way, why do other AMPs not form the transient pores by mass imbalance? Are there any specific interactions related to the sequence of the melittin? According to the manuscript, the translocation of the peptides relieves the mass imbalance, thus transient pores dissolves. In my opinion, the membrane is flowing, thus leading to new mass imbalance and new transient pores?

(4) What's the difference between the Figure 2(a) second, fourth and Figure 2(e). Especially, I fell the mass distribution of Figure 2(a) second is the same with Figure 2(f).

In Figure 2(e), if the initial peptides are loaded on the lower-leaflet surface, would the pores form?

In Figure 2(d), some water flux is observed at the lower two panels, any pores formed?

(5) Figure 3, how to decide the diameter of the pore? In the section "Method-Pore radii", the authors stated that the pore is deformed strongly. They used the volume of water to estimate the volume of the pore. Hence, if the pore is not fully filled with water, the volume is underestimated.

(6) What's the linear correlation coefficient of the fit in Figure 5c? The parameter at room temperature is estimated by the Arrhenius model. When the temperature rises, does the state of water change?. Additionally, it would be helpful to give an explanation of the calculation of the 2D free energy surface in Fig. 5d.

(7) From my side, the set style of Figure 1 is not a good choice. At many places of the manuscript, the citation of the figure is just located at Figure X. To be precise, the location would be better in Figure X(x).

(8) To balance the mass is the driving force for the formation of transient pores. Wimley referred that "in addition to the stochastic, transient event that relieves transbilayer asymmetry of peptides and concomitantly causes complete release of entrapped contents, there can also be a silent non-permeabilizing pathway to equilibrium. Could such a "silent" pathway be "viewed" by simulations with specific initial conditions and boundary conditions?

(9) The authors should be more careful when comparing the simulations with experiments with real cells. For real cell membranes, lipid components are extremely complicated. It has been proved that lipid environments could totally change peptide or protein's activity. So, the simulations with realistic membranes would be helpful.

Answer to reviewer's comments (in blue).

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We agree that this was poorly phrased on our part and have toned down the corresponding statements in the abstract, introduction and main text. While high-throughput computational screens are the ultimate goal, the present work is only a starting point in this direction. Further investigation aiming for the mentioned selectivity are currently under way with several other peptides. We have rewritten many parts of the manuscript to make it more clear what is the advance here and what is the ultimate vision that will require substantially more effort.

2. Continuing on the issue of lipid specificity, it would strengthen the manuscript if the authors were to examine behavior of melittin in the presence of anionic lipids. Specifically, it would be interesting to see if the simulations would result in the suppression of the pore formation, thus providing at least indirect evidence for the “detergent-like action” of melittin on such membranes. Another venue of comparison would be the effect of anionic lipids on stabilizing the conformation of melittin aligned with the plane of the bilayer, reported by the oriented CD.

This is an interesting point. Our original simulations used only zwitterionic membranes (PC). As suggested, we have now performed melittin simulations in an anionic membrane. We chose pure DOPG instead of a mix (e.g. 3:1 PC:PG), to maximize the difference to PC and see the effects in the limited simulation timescale available for a revision, and added the results in new Figs. S7 and S8.

In brief, the strong dependence of the behavior of melittin on the initial mass imbalance is pronounced when compared to pure PC: when the bilayer imbalance is only slightly reduced by removing 2 lipids per peptide, melittin stays on the surface, even if the system is heated to 353K to speed up kinetics and make the membrane more fluid (Fig. S7). Deep insertions of peptides (and their attached lysine/arginine sidechains) are not observed. Melittin appears stuck to the surface, presumably due to the strong interactions of the cationic sidechains with the anionic lipid headgroups.

If the bilayer is not sufficiently overloaded on one side, melittin seems to prefer to stay on this surface rather than insert.

If we do not remove any lipids, the membrane is highly overloaded on the upper side. There are substantial membrane undulations as the bilayer tries to accommodate the excess peptide mass. We managed to capture one insertion event after 0.8 microseconds: 4 peptides insert to span the membrane (TM) to form a tiny water defect. This channel is filled with lipid PG headgroups and is very small (radius of 3-4 Angstroms) compared to the large toroidal channels we see in DMPC (radius 40 Angstroms). The water channel dissolves after 1.1 microsecond and individual TM inserted helices remain in a dry membrane, similar to the final state of the DMPC simulations.

This result contrasts strongly with PC, where channels are 10 times as large, made up of >10 peptides and stay open for long (up to 41 microseconds at the same 333K). We cannot (yet) find such large pores in DOPG. The short-lived water-defect, in lifetime and tiny size, is likely undetectable in experiment although a few ions leak.

For anionic membranes, comparison to experimental data is complicated by the large differences seen in the various experimental studies. It has been reported that PG lipids reduce membrane leakage (Benachir et al. *Biochim. Biophys. Acta.* 1235:452–460 (1995), Hinch et al. *Biochim. Biophys. Acta.* 1284:162–170 (1996)). Other studies in pure POPG see leakage of small as well as large molecules in a ‘detergent-like’ mechanism not necessarily involving pores (Ladokhin et al. *Biochim. Biophys. Acta.* 1514:253–260 (2001)). Transient permeabilization has been seen for mixed PC/PG membranes, also with reduced leakage if PG lipids are present. (Wiedman et al. *Biochim. Biophys. Acta.* 1828:1357–1364 (2013). Krauson et al. *Biochim. Biophys. Acta.* 1818:1625–1632 (2012)). Pores have not been detected in electron paramagnetic resonance experiments in 7:3 DPPC/PG (Gordon-Grossman et al. *J. Phys. Chem. B.* 116:179–188 (2012)). On the contrary, in 7:3 DOPC/PG, stable (i.e. non transient) toroidal pores have been directly observed (Lee et al. *Proc. Natl. Acad. Sci. USA* 110:14243–14248 (2013)).

The differences suggest that results for melittin likely strongly depend on the intricate details of each experimental setup. In a way this is similar in our MD simulations, where starting conformation (TM inserted vs. surface loaded), P/L, temperature and level of bilayer mass imbalance drastically determine the outcome of the simulation, such as pore formation or not.

It is important to note that the restraints of our MD simulation setup mean certain phenomena cannot be reproduced that may dominate the experimental measurements: for example, by loading a membrane strongly on one interface, we force the system mass to break open the membrane. An alternative large lateral expansion of the membrane is prevented by the pressure coupling algorithm, but may occur in an aligned bilayer stack, or a LUV. In the simulation box, a membrane cannot macroscopically curve much to relieve strain because of the periodic boundary conditions (PBC): the membrane is clamped to a planar 10nm x 10nm grid in the X and Y direction by the PBC. In the simulations, lipid flip-flop does not occur as the barrier is too high to see it in microseconds, but it may alleviate stress in an experiment going on for hours.

Our 2 control simulations are not enough to answer how leakage works in DOPG. For PC, we can repeat this over a wide range of temperature settings and even bilayer thickness, and the results match closely with the experiments. For PG, we yet need to determine the exact conditions under which bigger pores open up or the bilayer becomes permeable in other ways, which will require a much more substantial effort. Our two simulations here only cover a small subset of what melittin can do in PG and do not represent a converged picture.

What we can conclude from the 2 simulations is that the results in MD depend on loading, much more so than with PC. When we reduce the strain (i.e. mass imbalance) on the membrane only slightly, the peptides remain on the surface. PG seems to enhance the surface state. We wonder then what the exact loading is in the various experiments mentioned above. Did the peptides silently translocate in the much larger experimental timescales and thus the strain was already reduced? Did the method to prepare the samples lead to symmetric or highly asymmetrically loaded membranes?

We believe MD simulations will be able to solve the details of how melittin permeabilizes anionic membranes also, but it will require much more simulations using a wide range of setups, such as higher P/L, bigger membrane patches, different loading etc. Unfortunately, this could not be resolved in our 3 months revision. We are currently running new simulations to find out what is going on, but these studies are beyond the scope of the current manuscript.

We have added Fig. S7 and S8, and statement in the main text concerning melittin in anionic membranes.

Reviewer #2 (Remarks to the Author):

This reviewer is not a simulation specialist. I will assume that the authors' simulation procedure is valid. Indeed their results look reasonable to this experimentalist. However their interpretation of the simulations, particularly regarding the peptide's antimicrobial mechanism is erroneous and misleading. The authors performed simulations for two very different peptides, alamethicin and melittin. I will discuss melittin first because this peptide is in the title of this paper. I will leave my comment on alamethicin to the last paragraph.

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However, at the minimal inhibitory concentrations, melittin always induces a large number of pores. Only a large number of toroidal pores distributed over a macroscopic area can achieve a uniform membrane thinning over a macroscopic area and attain equilibrium. Under this condition, one indeed observes stable melittin pores as demonstrated in many published papers (PNAS 110. 14243 (2013) and refs therein). The structure of such equilibrium melittin pores has also been determined, i.e., the toroidal model. Stable melittin pores were also observed in *E. coli* spheroplast membranes (Biophys. J. 112, 1663 (2017)). Thus the authors' assertion that melittin's permeabilization mechanism involves only transient pores is erroneous. The authors' simulations are not corresponding to melittin's antimicrobial mechanism.

This is correct. We focused entirely on explaining and demonstrating the transient mechanism of melittin, as observed in many experiments (for example: Yang et al., Biophys J, 114(2) 368-379 (2018)). Melittin can behave very differently under various other conditions. We have altered the introduction, main text, and discussion to describe this in much more detail.

By predicting a single transient toroidal pore, we did not wish to jump to conclusions how melittin ultimately kills cells (eukaryotic or bacterial) at MIC. This is a much bigger, macroscopic effect. Too big to set up in MD: Current hardware limits simulations to membrane patches of $\sim 10 \times 10 \text{ nm}^2$ and $\sim 100\text{K}$ atoms, enough for 18 peptides and one pore. In addition, we simulate a synthetic bilayer, not a real cell membrane. The goal was to show in detail how melittin interacts with the membrane and the

exact steps needed to form and open large toroidal pores. This can explain a lot of the experimental data observed for melittin.

The uneven membrane loading is critical to this. There have been a lot of previous inconclusive MD simulation studies of melittin (including from us), none of which observed *ab initio* toroidal pore formation, predicted from experimental studies. This generated the impression that all-atom MD somehow cannot capture this, either due to insufficient sampling or model deficiency. Our study shows it is possible, but also provides a warning: drawing conclusions from MD simulations based on setups that add *a priori* information or pre-conceived arrangements. For example, from the ‘converged’ melittin simulations shown in Fig. 2g, one could erroneously conclude that melittin does not form pores at all. Instead what this simulation shows is that aggregation of transmembrane helices, as shown for alamethicin, is not the mechanism for melittin.

The point about the experimental studies that have reported stable melittin pores is very interesting, and we have added a discussion and rephrased parts of the manuscript to address these studies. In the case of in-plane neutron scattering on fully hydrated oriented multilayers of POPC (Yang et al., *Biophys. J.* 81:1475-1485, (2001)), the pore diameter (4.4 nm) matches nicely with what we see in our simulations (up to ~4 nm), although we have a thinner bilayer. Three other studies observed stable equilibrium pores, as well as their structures, determined via measurements performed in anionic GUVs made of 7:3 DOPC/DOPG (Lee et al., *PNAS* 110. 14243 (2013)), and in *E. coli* spheroplast membranes (Faust et al., *Biophys. J.* 112, 1663 (2017)), (Sun et al., *Biophys. J.* 111:132–139 (2016)). These stable pores are thought to form only after melittin monomers have redistributed to both sides of the bilayer, presumably by the transient poration mechanism we see in the simulations. Then, two monolayers exist with positive spontaneous curvature due to the wedge effect of melittin, building up a membrane strain because both monolayers are forced to be planar when they want to curve instead. Toroidal pores form to relieve this strain. The pores then persist.

This model can be tested by MD by loading both interfaces with peptides. Actually, in Fig. 3a we have tested this configuration for pure DMPC. However, no pores were found to form over the timeframe of the simulation. The most likely explanation is that the simulations are too short and there may also be too few peptides in the simulation box compared to the experimental system. Longer simulations, at different P/L ratios, temperatures, box sizes, and lipid combinations will likely be needed to reveal if stable pores form this way and how. This may be beyond the capability of current simulation technology, however, we are actively trying by running very long probe simulations.

Another interesting point is that only a large number of toroidal pores distributed over a macroscopic area can achieve a uniform membrane thinning over a macroscopic area and attain equilibrium, causing the stable poration. Such a macroscopic multi-channel setup is definitely beyond current simulation technology, although it may be feasible in 10 years. At present, we can reliably detect a single pore, because our system size is limited to ~100K atoms, with a bilayer of about 200 lipids containing ~18 peptides. This setup achieves 30 microseconds in half a year running continuously on state-of-the-art hardware, which is expensive to perform. Bigger systems are not technically feasible as they take years to run just a few microseconds in a whole year. We have clarified in the manuscript that we are currently limited in our study to provide insights into transient membrane poration of melittin only. This is the mechanism which we can now reliably reproduce under a variety of conditions, and it is consistent and adds insights to a large body of experimental studies.

It is intriguing, however, that the stable pores found experimentally are also toroidal and appear very similar to the ones we find here. Thus, it appears melittin may only form these toroidal pores, transient or stable.

We have updated the manuscript to discuss these topics in more detail.

In the introduction, the authors argued that “progress of pharmaceutical or biotechnological application has been hindered by a critical shortage in the detailed knowledge of the molecular mechanism of permeabilization and/or pore formation.” This is not true. Melittin-like AMPs, such as

magainin, have not been approved for pharmaceutical uses precisely because of the knowledge of their membrane lytic effects. So far the US FDA has approved three AMPs for therapeutic applications: gramicidin, daptomycin, and colistin. We still have problems understanding the mechanisms of the last two.

Our statement was indeed poorly phrased, and we have altered this section in the introduction. What we intended to say was that for a large number of membrane active peptides there is only indirect evidence on the mechanistic details of activity at a molecular level. High-resolution structures of channels are only available for a very small number of peptides. This is because it is very challenging to study these highly dynamic structures in membranes experimentally and the mechanistic details of membrane permeabilization is known only for a few peptides.

What this study suggests is that multi-microsecond MD offers a potential route towards that information. MD can display the atomic details of peptides aggregating, disrupting the membrane, and forming pores in a fluid membrane. Properly validated, MD simulations may be very useful as a tool in the future, i.e. informing medicinal chemists on how to tune sequences to alter pore distributions or membrane disruption. At the moment, MD and experimental measurements cover different scales in time and space (microseconds versus milliseconds, tiny bilayer patches of 10nm x 10nm in a box versus 100nm diameter LUVs). Simulations are still a long way off for a direct one-on-one comparison for most systems, but it is promising that for two very well known peptides, MD can now produce an atomic model that, without any initial assumptions, is consistent with a vast amount of experimental observations. These two peptides were chosen because there is so much experimental data available, to maximize the overlap for comparison. We have changed our text to make our motivation more clear.

Finally, alamethicin is very different from melittin. While individual melittin binds to the interfacial region between the lipid headgroups and chains, with its peptide helix parallel to the plane of bilayer, thus inducing a local membrane thinning, alamethicin, being largely hydrophobic, inserts perpendicularly through the chain region and forms stable barrel-stave channels. There was a very extensive kinetic study on the alamethicin single channels by a Cornell group (Biophys J 69:2323 (1995); in contrast there are no systemic studies of melittin's single channels). The authors should compare their alamethicin simulations with these kinetic data. This paper in its present form is not suitable for publication.

We now cite this study as well as other kinetics studies. We tried to condense too much information for alamethicin into too few figures, chiefly because we wanted to highlight the difference in the pore formation mechanism to melittin only.

The whole alamethicin section has been substantially updated, including a new Fig. 1. The kinetic study mentioned (Mak et al., Biophys J 69:2323 (1995)) and numerous prior electrophysiology studies since the 1970s reveal step-like conductance curves. It is currently not possible for MD simulations to generate the same curves because the time-scales differ by 3 orders of magnitude. For example, in Bert Sakmann's original 1979 paper (Nature 282, 1979, p336-339), plateaus can be seen with mean lifetimes of 20-130 ms at 18°-30°C. The study by Mak et al. shows similar kinetics in the multi-millisecond range. Our longest simulation for alamethicin is 27 microseconds only, a factor of 1000x shorter.

We have updated the main text and added a new Fig. 1 to demonstrate the strategy to nevertheless get converged statistics on pore formation for alamethicin. In Fig. 1, it can be seen that alamethicin quickly aggregates and forms initial pores when the system is heated to 333K. The final state is a hexamer and two tetramers, which remain stable and adhere to the barrel-stave models proposed since the 1970s, with the central Gln7 residue pointing to the pore interior. However, the kinetics are too slow for rearrangement of the aggregates, and no higher-order oligomers are observed.

So subsequently, simulations were repeated with higher temperatures to speed up the kinetics and obtain a much more converged equilibrium of oligomers. These higher-T simulations, shown in Fig. 2, Fig. 4 and Figs. S2-S5, manage to capture the bigger oligomers, such as the octamer shown in Fig. 2.

They also corroborate the experimental observations (Boheim, *J Membr Biol*, 19, 277-303 (1974)) that channels increase and decrease in size in steps of +1 or -1, i.e. individual peptides drift off and join other channels. We see this in our simulations, one example is shown in Fig. 1c at 2 microseconds.

Because of the hot conditions, these simulations are suitable only for demonstrating the alamethicin mechanism of pore aggregation. They are not suited for quantitative comparison of kinetics with experiments, because of the greatly reduced lifetime of the encountered pores at such hot conditions. An additional effect is that the pores can easily deform from circular geometry if running at high temperature, because they quickly drift laterally and collide with other channels. We now show more snapshots of typical channels encountered in these simulations in Fig. 2, Fig. 4, and in supplementary Figs. S2-S5. Channel deformations are not observed at lower temperatures, as seen in Fig. 1, which reveals circular tetramers and hexamers. In principle, we could have added an extensive alamethicin study here, by extracting the found channels from the simulations and simulating them in isolation (so they cannot collide with other channels) at room temperature to check for lifetimes and structural fluctuations. We did not, because the focus of the paper is melittin and alamethicin is mainly used to show that melittin does not assemble pores by lateral aggregation. Secondly, others have already published such alamethicin simulations before. For example, Perrin et al. used the Anton supercomputer to run a 14 microsecond simulation of a single hexamer of alamethicin in DOPC, using identical simulation parameters to ours (Perrin et al. *Biophysical Journal* 111, 1248–1257, 2016). The channel remained stable and symmetric. The hexamer was initially designed according to experimentally models, with the central Gln7 residue facing inwards. This is similar to the hexamer we find *ab initio* in our simulations. We have added a sentence in the main text to explain this.

Reviewer #3 (Remarks to the Author):

Melittin has been found for more than fifty years. It is believed to permeabilize membranes thus equipped with extensive applications. However, the mechanism of interaction between the melittin and the membrane is not very clear to date. Experiments show that melittin can cause transient leakage of the membrane, which is different from the permanent pores in the membrane. An example is the permeabilization and resealing of *E. coli* membrane done by Weisshaar et. al. In this paper, the authors aimed to elucidate the details of the transient leakage of the membrane induced by the melittin by all atomistic simulations. As a comparison, they first showed that the alamethicin formed the equilibrium pores allowing the water and ions to pass through. However, melittin induced non-equilibrium and transient pores instead of equilibrium and stable pores. The authors then presented the formation and dissolution mechanism of the transient pores. The mass imbalance is considered as the driving force for the pores, and the translocation of peptides to balance the mass causes the dissolution of the pores. Next, the authors provided some information about the transient pores, including the transient timescale, the flux of water and ions, and so on. However, I think the transient pores induced by melittin here are not of great novelty and surprise, because such concept has been proposed and experimentally observed for ten or more years. So, I think many improvements should be made before considering its publication.

(1) The comparison of pore formation of two types of peptides is important for understanding the mechanism of action of these peptides. For the membrane used in simulations, DOPC, DPPC/DPPG mixture and DMPC/DMPG mixture, the authors stated that the behavior of alamethicin in all these bilayers is almost identical. However, I find that the distribution of oligomer size is obviously different, and the fluxes of water and ions are different. In DMPC/DMPG the flux is not obvious.

There are several reasons. The main is lack of convergence. Especially the DMPC:DMPG simulation was too short. Secondly, the DMPC:DMPG simulation was run at a too hot temperature, so the membrane was a bit leaky. This simulation was actually our first simulation of alamethicin, run many years ago, when we tried to prove the kinetic model for alamethicin and experimented with very high temperatures. It is not needed, because alamethicin simulations work also at much lower temperature. In this revision here, we have replaced this simulation with a new one with 50K lower temperature (Fig. S3).

To show convergence is the main issue, we have continued the simulations of alamethicin in the other bilayers, and added a new simulation in POPC. All these results are now shown in newly designed Figs. S2-S5, including one of the two simulations in DOPC (363 K). It can be seen that alamethicin behaves qualitatively similar in all these membranes: 1. Channels and pores of 4-8 peptides are mostly populated. 2. 'Channels' with $N > 8$ are mostly smaller channels that have temporarily attached to each other, rather than a big single pore. However, the exact overall distribution has not converged, and especially bigger channels form infrequently. Presumably, only >100 -200 microsecond simulation or longer would result in converged oligomer distributions. That convergence is the issue can be best seen by comparing the two simulations in DOPC (Fig. 2 and Fig. S5). There is a rough match, but not identical distributions. The only difference in the simulations is the 30K lower temperature in Fig. S5.

On the other hand, if there was a dramatic effect of lipid type on the channel equilibrium, the plots would likely look significantly different - but they do not. We conclude from our simulations that alamethicin behaves roughly similar in all these membranes, i.e. pores assemble by lateral aggregation and actually look similar across all membranes. But details like transmembrane water flux and the exact oligomeric distribution are not converged in a few microseconds. Nevertheless, we believe that there will be a small effect of membrane type on alamethicin channel equilibria, but we do not want to speculate on this given that the simulations are likely not converged.

Otherwise the flux seems to occur mainly at the beginning. Since the pores are permanent, why the flux disappears at the later time? What is the possible reason?

There are two reasons why this appears so. The first is a display issue. We used water flux instead of water amount in channels in the membrane. The spontaneous (undirected) water flux is significantly higher in i.e. an octamer or hexamer than in a tetramer. Especially the smaller channels like tetramers contain only thin water wires with little spontaneous water flux (N_4 in Fig. 4b). Thus, if during a simulation at some point there are only pentamers and tetramers, it appears there are no water channels at all, but when then a hexamer forms there is a huge spike in flux.

For this reason, we have replaced the water flux panel in Fig. 4b with the water number density in the membrane core, which gives the number of waters contained in all channels in the membrane. It can be seen that there are continuous water channels throughout the simulation. The new Fig. 1 similarly shows the build-up of water in the membrane along the trajectory as pores form.

The second reason is a consequence of what we tried to determine. We heat up the system to speed up kinetics to get better convergence on the channel ensemble equilibrium and allowing bigger oligomers to form in a manageable simulation time. Secondly, we wanted to check for the gating mechanism of alamethicin first proposed in the 1970s (Boheim, *J Membr Biol*, 19, 277-303 (1974)), in which transitions are exclusively between adjacent levels, i.e. channels increase or decrease in size by $+1$ or -1 steps by individual peptides drifting on and off channels. The high temperature simulations directly confirm this, as can be seen in the oligomer population plots (Fig. 2, Figs. S2-S5).

As shown in Fig. 1, the kinetics are too slow at 333K to prove this. A consequence of running at 363K or even 393K is, however, that these simulations are not so suited to predict pore lifetimes and stability (especially pore geometry) as would be seen at 313K. Under hot conditions, channels can easily deform or even become dry bundles occasionally. Then the channels re-open. This is another reason for the flux panels having spike-like appearances. For $N > 8$, we usually see amorphous bundles. This has actually been observed by Boehm (1974), who suggested that larger N bundles are distorted away from an ideal circular cross-section towards a 'torpedo' shape, i.e. the bundles collapse within the plane of a membrane (Sansom, *Q Rev Biophys*, 26(4), 365-421, (1993)). We see this in our simulations.

Another point is that we have a tiny simulation box and a lot of peptide in it. So circular, water filled bundles are forced to constantly collide with other bundles. At high temperature, bundles diffuse quickly in the plane of the membrane, and when they touch, they tend to both deform, affecting the water flux. A proper flux study would involve extracting the predicted alamethicin channels of $N = 4,5,6,7,8$ from a high-T simulation, then cool down to 313K and remove all other peptides to simulate

them in isolation. We have not done this, because this has already been shown by Perrin et al, using identical force field parameters (Perrin et al., *Biophys J*, 111(6), 1248-1257 (2016)). In that study, a hexamer of alamethicin was constructed and remained perfectly circular and stable for 14 microseconds. The hexamer has the same structure as the one we predict here ab initio in our simulations. In summary, the flux curves here are affected by our high-T approach. We need hot conditions to show the equilibrium behavior of alamethicin and how this is very different from melittin. For proper analysis of lifetimes, flux and channel stability, additional simulations of each channel at 313K would be needed, but we have left this out, because the focus of our study is mainly on how melittin poration works and alamethicin is used as a control only.

All of these issues are now better explained in the revised manuscript.

Besides, the alamethicin is so hydrophobic as stated by the authors. So, could water pass through such hydrophobic pores?

The channels we find match almost perfectly to the ones proposed by the experimentalists decades ok. Tight barrel-stave pores with the polar Gln7 residue pointing into the water pore. This polar residue seems essential to attract water into the membrane. For example, our pores look very similar to the original Xray structure (Fox & Richards, *Nature* 300(5890), 325-330 (1982)) or the alamethicin models reviewed by Sansom 30 years ago (Sansom, *Q Rev Biophys*, 26(4), 365-421, (1993)). While we can predict these structures ab initio, as mentioned above, the high temperature we use to force equilibrium ensembles means our structures are more flexible and not always perfectly circular as the experimental models. But at the lower temperatures (Fig. 1) they are always circular and match very well.

In addition, I think the hydrophobic alamethicin would be repelled by the membrane, why it immediately inserts TM?

We have now added a new Fig. 1 and Fig. S1 to show the generation of the initial states of the peptides in the membrane. The monomeric adsorption and insertion of alamethicin and melittin are plotted for several lipid bilayers. Alamethicin first adsorbs to the membrane interface in a surface state parallel to the surface, but then rapidly inserts transmembrane (TM). This is due to the very hydrophobic AIB residues, which prefer the hydrophobic core of the membrane. Once TM inserted, it very seldomly switches back into the surface state at very high temperature (Fig. 2b, the small blue patches), but overwhelmingly stays TM inserted.

POPC is used in the simulation of melittin, as a direct comparison, why not provide the results of alamethicin in POPC?

There was no particular reason why we chose DOPC other than several experiments used that lipid. We have now added a new simulation of alamethicin in POPC also. The results are shown in supplementary Fig. S4. Behavior is similar to DOPC. This new control simulation has been run for the last 3 months, so is 8 microseconds long, not as long as DOPC. Full convergence of the oligomer distribution will require substantially longer simulations, but the behavior of alamethicin is qualitatively the same as in the other membranes: Lateral aggregation and formation of water filled channels, that look very similar. We have added a section discussing the behavior of alamethicin in all 4 membranes, including issues of convergence.

(2) In the "Transient Pore Formation" section, should "Figure 2h" be replaced with "Figure 2e"?

Thank you for spotting this, we have updated it. This is now Fig. 3.

(3) In the section "Transient pore formation", "We concluded that the likely mechanism is actually purely mechanical, driven by initial mass asymmetry", it is hard to think the mass imbalance is the driving force for opening the membrane. More supporting analysis is needed. Moreover, by this way, why do other AMPs not form the transient pores by mass imbalance?

We did not mean that the mass imbalance is the only mechanism observed for melittin. But it can explain a lot of experimental observations and is the only mechanism we can currently directly capture (with unbiased simulations) in MD simulations. We have since found this mechanism also with several related peptides: magainin2 and PGLa, both from Australian tree frogs and also suspected of being transient pore formers. These projects are still in progress and not included here. How far this mechanism can in general explain AMP activity is unclear. We believe this mechanism can explain transient leakage observed experimentally for some peptides, under certain conditions (bilayer composition, specific P/L). We have added sections to the results and discussion to discuss other mechanisms for melittin and the corresponding experimental evidence.

Are there any specific interactions related to the sequence of the melittin?

We have not yet been able to identify any specific interactions by melittin that are vital for transient poration. Neither for magainin2 and PGLa (unpublished results). The overall distribution of cationic residues seems to be important, but it will take us many more simulations to figure out how an exact sequence favors/disfavors this mechanism. Common at this point only seems to be that the peptides have a large hydrophilic angle, so toroidal pores are favored, and barrel-stave pores disfavoured.

The numerous aggregation simulations shown in Fig. 2 and Fig. 3, demonstrate that melittin does not aggregate and form pores due to specific helix-helix interactions. Peptides instead appear to repulse each other or only attach in very short-lived dimers or trimers held together loosely by non-specific interactions.

According to the manuscript, the translocation of the peptides relieves the mass imbalance, thus transient pores dissolve. In my opinion, the membrane is flowing, thus leading to new mass imbalance and new transient pores?

Once the mass imbalance is relieved (even only partly) the driving force appears to be gone. This is similar to our earlier work for PGLa, where once 1-2 peptides had translocated, there were no further translocations. The simulations do not allow for a new mass imbalance to build up: No peptide, once translocated, is seen moving back to the initially loaded leaflet. In the final state of the melittin simulations, a residual peptide mass imbalance remains, but it is not enough to cause any further events and peptides are stuck in position, as can be seen by the tilt angles in Fig. 5 and Fig. 6. No matter how long we continue those simulations, nothing further happens. We cannot for sure say that this is really the final state of melittin, as we cannot run MD for milliseconds yet. For the multi-microsecond range, the final state appears static.

(4) What's the difference between the Figure 2(a) second, fourth and Figure 2(e). Especially, I felt the mass distribution of Figure 2(a) second is the same with Figure 2(f).

Spontaneous toroidal pore formation does not occur for any mass imbalance, but the imbalance must exceed a critical threshold. The mass imbalance in the poration simulation (Fig. 3e, 3f) is actually bigger than the one in Fig. 3a (2nd and 4th). In those simulations, 2 lipids per peptide were removed in the upper leaflet. It is difficult to plot the imbalance visually. We have replaced the peak height dotted line of the lipid leaflets with a new dotted curve that represents the total mass density profile mirrored at the membrane center. Even with this new style, it looks like the poration simulation has roughly the same mass imbalance, but it is actually more, because no lipids were removed at all. So a smaller mass imbalance does not generate enough driving force to open a pore. It will be interesting to find out what the exact threshold is in the future. We describe this now in more detail in the main text. The whole figure has been further updated, with some simulations slightly extended, insertion plots better smoothed, and flux curves rescaled to show there is no water flux in Fig. 3a as compared to Fig. 3h.

In Figure 2(e), if the initial peptides are loaded on the lower-leaflet surface, would the pores form?

Yes. The only condition is that one leaflet is loaded with peptide, the other is not, and the lipids are symmetrically distributed across the leaflets. In MD, periodic boundary conditions are used, so

loading the lower membrane would actually be an identical setup, just all coordinates rotated by 180 degrees. For periodic boundary conditions to be acceptable in MD simulations of membranes, the water phase must be sufficiently large, so it acts as a barrier and there are no 'interactions' across it. In salty solutions, electric fields do not reach much beyond one Debye length (~7 Angstroms). The water phase is much bigger (50 Angstroms). So this condition is met in all our simulations, as no peptides ever move into the aqueous phase, but remain attached to the membrane, either in surface alignments or transmembrane inserted. A new Fig. 1 shows how single peptides rapidly adsorb to the membrane, and never detach back into solution.

In Figure 2(d), some water flux is observed at the lower two panels, any pores formed?

There are no pore or water defects in any of these simulations. The water flux is the residual water in the membrane. There is always some tiny amount of water in the membrane, as some water molecules can diffuse through the membrane. The thinness of DMPC is also contributing to this. It looks like the lower panels have stronger flux, but this is a display issue, as some simulations were run at a different MD frame output frequency, resulting in the flux analysis plots looking slightly different.

All these simulations never have even the smallest water wire or pore, which would manifest itself as a huge spike in the graph. This is a display issue. We 'zoomed' in too much, so the y-axis scale went up only to 1.0 (s⁻¹ nm⁻²). Fig. 3h, where the pore opens, has the y-axis up to 6.0 s⁻¹ nm⁻². If we would use this upper limit, all curves in Fig. 3d would not be visible. We now use 2.0 s⁻¹ nm⁻², to show how negligible the water flux is in absence of pores. We have updated Fig. 3.

(5) Figure 3, how to decide the diameter of the pore? In the section "Method-Pore radii", the authors stated that the pore is deformed strongly. They used the volume of water to estimate the volume of the pore. Hence, if the pore is not fully filled with water, the volume is underestimated.

We explain this in more detail now. Meant was that the pore is often not perfectly circular, but somewhat ellipsoid or elongated, which can be directly seen in Fig. 4a, 6a. The pore is always fully filled with water, never with a gas phase (i.e. 'dewetted'). We have dropped the word 'strong' as this suggested shapes which are not seen. In order to characterize the size of these various pores by a simple, clearly understandable parameter that lends itself to plotting, we arrived at the radius R, by back-calculating from the volume of the pore (estimated by the number of waters), the volume of an equivalent cylinder of radius R. This works quite well, as the pores are often of nearly circular shape. An alternative would be to use the cross-section of the pore, but radius feels more intuitive, as it informs on the size of molecules that could leak. We have updated the method section and the main text. Also, we now provide 3 supplementary movies, one for melittin (4 microseconds of the pore undulating), one for alamethicin (10 microseconds of pores forming and exchanging peptides), and a movie of the final moments of the melittin pore. In these movies it can be seen that both pores are mostly circular even at higher temperature.

(6) What's the linear correlation coefficient of the fit in Figure 5c? The parameter at room temperature is estimated by the Arrhenius model.

The linear fit in the Arrhenius plot has an $r^2 = 0.95$, which we now display on the plot. We have continued the 3rd simulation at 333K. The pore finally evaporated after a very long 41 microseconds. The Arrhenius fit has been updated. Pore lifetime estimate at 298K is now slightly higher at 0.85 ms. The long timescales illustrate why running these simulations at 298K or 313K would currently not be meaningful.

When the temperature rises, does the state of water change?.

Rigid 3-site water models like TIP3P and SPC do not show marked changes in water state and structure over a wide temperature range. The pressure and temperature coupling algorithms in MD with periodic boundary conditions enforce a liquid state of the system, even for temperatures far above the boiling point of water, i.e. the system behaves like a superheated liquid. As in many of our

previous publications, we can show that temperature affects the kinetics, but not the thermodynamics, so can be used to speed up events. Key to this is that peptides do not unfold in the membrane and remain helical, and that the membrane remains intact. In Fig. 2, the helix-helix dimerization free energy profile shows no temperature effect. Similarly, the toroidal pore of melittin looks exactly the same at all temperatures, but its lifetime is strongly affected (Fig. 6b). We now have added a new Fig. 1 to show why we chose hotter temperatures to predict the full ensemble of pores formed by alamethicin, and why just running at 333K does not result in convergence unless we could run for milliseconds.

Additionally, it would be helpful to give an explanation of the calculation of the 2D free energy surface in Fig. 5d.

The 2D free energy surface is simply calculated from the trajectory shown in Fig. 5 as $\Delta G = -kT \ln(p)$, with the lowest bin set to $\Delta G = 0$. This description has now been added to the method section and the main text. This free energy surface is an estimate only, used to visualize the 4 dominating orientations after pore closure. We do not see, even in very long simulations, individual peptides monomerically translocating their charged sidechains across the bilayer core, switching between these 4 states. Nor do we observe pores reforming. But all this could occur on longer timescales, i.e. milliseconds, which we cannot currently reach. An alternative are PMF calculations using e.g. umbrella sampling, for full peptide surface-to-TM insertion. We do not perform these, as we found they suffer from severe convergence and hysteresis problems and can be run only for a single peptide, not the multi-peptide system we have here. But cooperativity in pore formation is key, so a single peptide free energy surface would not be sufficient.

(7) From my side, the set style of Figure 1 is not a good choice. At many places of the manuscript, the citation of the figure is just located at Figure X. To be precise, the location would be better in Figure X(x).

We now cite all figures, i.e. Fig. 2d.

(8) To balance the mass is the driving force for the formation of transient pores. Wimley referred that “in addition to the stochastic, transient event that relieves transbilayer asymmetry of peptides and concomitantly causes complete release of entrapped contents, there can also be a silent non-permeabilizing pathway to equilibrium. Could such a “silent” pathway be “viewed” by simulations with specific initial conditions and boundary conditions?

Yes, this silent path can be directly visualized. We have shown it for the related antimicrobial peptide PGLa in 2017 (Ulmschneider, Biophys J 2017). Individual peptides – despite charge of +5 – can translocate monomerically in rare events occurring only every 10-30 μ s. This translocation does not involve formation of any large pore, and the membrane is immediately resealed after the translocation lasting < 100ns.

This work directly led to the present paper. We have found that this silent path can occur when (for PGLa) the bilayer mass imbalance was not large enough. For our simulations performed then, some lipids were removed in the bilayer leaflet containing PGLa. For (lipid) symmetric membranes, that have a large mass imbalance, we can now show transient pore formation also occurs with PGLa and with magainin (unpublished results).

We also found that if the mass imbalance is too little, then no further translocation occurs. In the 2017 paper, this happened after 1-2 PGLa peptides had translocated. This can explain why in many experiments, peptides are just seen on the surface. The driving force for either poration or translocation has likely gone.

For melittin, we did not have the time yet to run very long simulations to repeat what we did for PGLa, to see if it also can translocate silently. From our preliminary results, it appears the barrier for this is higher in melittin than PGLa. PGLa seems to have the cationic residues in a ‘better’ location to facilitate translocation.

Although this sounds like a very simple model, we find it can explain several aspects of many experimental results. We have added a sentence in the main text regarding 'silent' pathways.

(9) The authors should be more careful when comparing the simulations with experiments with real cells. For real cell membranes, lipid components are extremely complicated. It has been proved that lipid environments could totally change peptide or protein's activity. So, the simulations with realistic membranes would be helpful.

Yes, we did not explain very clearly why we do not (yet) use real bacterial or eukaryotic membranes. One reason is that there is a vast number of accurate experimental measurements for these peptides performed in simple synthetic bilayers with homogeneous lipids or mixtures, so we can do a direct, quantitative comparison to MD.

For real cell membranes, there would be a debate how realistic the membrane model and lipid composition in MD is, making such comparisons difficult. The bacterial outer membrane would be especially tough to model correctly. Also, experiments on real cells usually are sterilization assays that operate on timescales 7-9 orders or magnitude larger than what MD can do (minutes vs. microseconds), whereas we want to compare to i.e. simple vesicle leakage assays that are 'closer' to MD. We plan to use realistic membrane mimics in the future. The manuscript has been expanded to explain these reasons.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

All of my comments have been accounted for.

Reviewer #2 (Remarks to the Author):

(To make sure of completeness a pdf is attached below)

Review of the revised manuscript

Title:

First of all, I would like to suggest the title be modified to “Melittin can permeabilize membranes via large transient pores—a molecular dynamics study.”

Abstract:

I found the revised abstract inconsistent with authors' response to my previous critique. The sentence “For many sequences, transient leakage is observed, suggesting stable pores do not exist, but only transient structures.” is misleading. As the authors admitted in their response, this statement holds true only in very low peptide concentrations. In real-world scenarios, such as when a bee stings or when melittin is used to test its cellular effect, it operates within its MIC range, where stable melittin pores have been observed.

I believe the authors should explicitly state from the outset that their observations correspond to peptide activity at very low concentrations. Otherwise, claiming "agreement with experimental evidence" becomes problematic.

The last sentence of the abstract is a stretch. There is simply no basis for such a statement.

Introduction:

In the introduction, while the authors revised the next-to-last paragraph to describe the experimental findings on melittin more accurately, the overall tone of the paper still reflects observations based on low concentrations of melittin. Given that melittin's physiologically relevant activity cannot be extrapolated from low-concentration activities, the authors should make this clear at the beginning of the paper.

Transient pore formation:

I'd like to emphasize that transient pore formation doesn't elucidate the mechanism behind stable pore formation. The driving force for stable pores stems from a membrane-thinning effect caused by peptide binding at the interface. This phenomenon has been extensively studied, from alamethicin (BJ 68, 2361 (1965)) to melittin and other peptides (Biochemistry 43, 3590 (2004)). The distinction between the alamethicin pore and the melittin pore arises from their inherent properties. Alamethicin, being predominantly hydrophobic, easily integrates with lipid chains, while melittin, being amphipathic, remains confined to the lipid interface. Toroidal pores serve to expand the interfacial area, effectively alleviating the strain caused by membrane thinning.

I speculate that if the simulation fails to generate the membrane thinning effect, it is likely that stable pores will not form.

Final conclusion:

There is no basis for expecting a peptide sequence to distinguish between bacterial membranes and mammalian cell membranes. Many AMPs have been tested; they all attack mammalian cells as well as bacteria.

Reviewer #3 (Remarks to the Author):

The authors have addressed what I was concerned about, and the manuscript is improved. I support its publication.

Review of the revised manuscript

Title:

First of all, I would like to suggest the title be modified to “Melittin can permeabilize membranes via large transient pores—a molecular dynamics study.”

We have changed the title.

Abstract:

I found the revised abstract inconsistent with authors' response to my previous critique. The sentence “For many sequences, transient leakage is observed, suggesting stable pores do not exist, but only transient structures.” is misleading. As the authors admitted in their response, this statement holds true only in very low peptide concentrations. In real-world scenarios, such as when a bee stings or when melittin is used to test its cellular effect, it operates within its MIC range, where stable melittin pores have been observed.

I believe the authors should explicitly state from the outset that their observations correspond to peptide activity at very low concentrations. Otherwise, claiming "agreement with experimental evidence" becomes problematic.

We have changed the abstract text to avoid the misunderstanding that for many sequences, only transient poration is observed. What was meant: In leakage assays for many peptides, at low-medium P/L, often lower than the very high MIC where membranes are covered in peptide, transient leakage is observed. And we attempt to explain this transient leakage mechanistically using simulations.

For melittin, a recent example is the study by Yang, Choi, Weisshaar, Melittin-Induced Permeabilization, Re-sealing, and Re-permeabilization of E. coli Membranes, Biophysical Journal 114, 368–379, 2018).

For alamethicin, this is not observed at all, and we also show this directly.

The study provides a molecular detail picture for a vast body of experimental studies, which was the goal of our work here. A molecular picture of stable poration for melittin remains challenging to do, because the experimental setup needed to show this is difficult to reproduce in MD (see explanation below). The same is true for bacterial sterilization, which would need a much more realistic bacterial membrane model. The abstract has been altered to avoid any misunderstanding.

The last sentence of the abstract is a stretch. There is simply no basis for such a statement.

The last statement was intended to convey that if simulations can directly capture complex peptide-membrane phenomena – i.e. pore structures in transient leakage - then it is likely that in the near future they will work also for prediction of stable pores for melittin and other sequences. Given how hard it is to study peptides in membranes experimentally – and simultaneously the enormous increase in computer power - we believe all-atomistic computation has the potential to develop into a useful additional tool for experimental scientists to include in their peptide development pipelines. We have modified this statement.

Introduction:

In the introduction, while the authors revised the next-to-last paragraph to describe the experimental findings on melittin more accurately, the overall tone of the paper still reflects observations based on low concentrations of melittin. Given that melittin's physiologically relevant activity cannot be extrapolated from low-concentration activities, the authors should make this clear at the beginning of the paper.

We have altered this paragraph to clarify we are interested in explaining the experimental observations of transient leakage of melittin as observed in many experiments, and to determine the structure of the pores. Our simulations can explain these experimental results in detail.

The physiologically relevant mechanism is likely different, but currently not possible to capture in MD, as elaborated below.

Transient pore formation:

I'd like to emphasize that transient pore formation doesn't elucidate the mechanism behind stable pore formation. The driving force for stable pores stems from a membrane thinning effect caused by peptide binding at the interface. This phenomenon has been extensively studied, from alamethicin (BJ 68, 2361 (1965)) to melittin and other peptides (Biochemistry 43, 3590 (2004)). The distinction between the alamethicin pore and the melittin pore arises from their inherent properties. Alamethicin, being predominantly hydrophobic, easily integrates with lipid chains, while melittin, being amphipathic, remains confined to the lipid interface. Toroidal pores serve to expand the interfacial area, effectively alleviating the strain caused by membrane thinning.

I speculate that if the simulation fails to generate the membrane thinning effect, it is likely that stable pores will not form.

The experimental structural data obtained for stable melittin pores indicates they are also of toroidal shape and of similar size as our observed transient pores. Unfortunately, we cannot yet reproduce the presumed thinning effect of melittin and associated stable pores at much higher P/L in MD simulations. To test this, much larger simulation boxes, and many more peptides in the box would be needed: We would need to embed the predicted pores in large membrane patches, that both contain additional toroidal pores and many peptides on both surfaces of the membrane to induce the large-scale membrane thinning effect. And then check for the effect on pore lifetimes.

Such simulations would currently take several years to run due to the large system size (~0.5-1M atoms). This is a project for the future and beyond the scope of our study here. However, it is reassuring that our study is consistent with a large body of experimental evidence for melittin at low concentrations. Here, the toroidal pore formation of melittin at lower P/L, can now be captured fully.

In contrast, for alamethicin, we observe pores to be long-lived and stable. This matches directly with the many experiments.

We have updated the text in the result section and introduction to make this clear.

Final conclusion:

There is no basis for expecting a peptide sequence to distinguish between bacterial membranes and mammalian cell membranes. Many AMPs have been tested; they all attack mammalian cells as well as bacteria.

We have removed this sentence.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

I find the revision acceptable. I have no further comment.