The simulation studies of the interplay of peptides with lipids
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Chapter 3
Localization Preference of Antimicrobial Peptides on Liquid-Disordered Membrane Domains

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Abstract

We performed coarse-grained simulations of four antimicrobial peptides (Magainin 2, BP100, MSI-103 and MSI-78) on a phase-separated membrane to study their preference for different domains. All the peptides displayed a clear preference for the liquid disordered (Ld) phase over the liquid ordered (Lo) one. For all peptides but Magainin 2 there was a further preference for the domain interface over the disordered phase bulk. Two peptide concentrations were tested, at peptide-to-lipid ratios of 1/200 and 1/20. Similar results were observed at both concentrations, although Ld phase saturation at the higher concentration drove some of the peptide excess to the Lo phase. Interestingly, at high concentrations of Magainin 2, toroidal pores spontaneously formed in the Ld phase. We performed a series of additional simulations to characterize this phenomenon.

3.1 Introduction

Membrane lipid heterogeneity is crucial for various processes in living cells. Functions attributed to the membrane lipidome range from specific integral protein solvation (1) to signaling (2, 3) to formation of spatial domains of different local composition (4). Commonly used model systems are bilayers composed of ternary mixtures of cholesterol, saturated, and unsaturated lipids, which yield a rich phase behavior (5) at physiological temperatures. Over a range of component concentrations these ternary mixtures laterally separate into a liquid-ordered (Lo) phase, enriched in the saturated lipid and cholesterol, and a liquid-disordered (Ld) one, enriched in the unsaturated lipid (6-8). While such lipid segregation has not been observed at large scales in cellular membranes, the phase-separated ternary mixture model is still relevant, as there is plenty evidence of heterogeneity in lipid distribution, both in physiological membranes (9, 10) and in complex models thereof (11). Understanding the interplay between membrane proteins and such heterogeneous surroundings is central to shedding light on the function of both proteins and the associated lipids.

An important class of proteins interacting with lipid membranes is formed by antimicrobial peptides (AMPs). AMPs are typically short cationic peptides and diverse both in sequence and structure (12, 13). The amphipathic $\alpha$-helical structured AMPs are particularly abundant and widespread in nature (14). The net cationicity and the amphipathic character facilitates their incorporation into the negatively
charged microbial membranes. The activity of antimicrobial peptides (AMPs) has been observed to depend critically on the composition of target cell membranes. Many studies focus on model membranes containing negatively charged lipids, mimicking the bacterial membrane composition and allowing the characterization of the electrostatic component of the interactions between the cationic peptides and the host’s membranes (15-17). At the same time, the disruptions of AMPs on cholesterol-containing homogeneous membranes have attracted some interest since many AMPs are able to kill fungi, protozoa, and enveloped viruses, which have sterol-rich membranes (18-20). However, only a limited number of experimental studies (21, 22) have looked at AMPs interacting with phase-separated heterogeneous membranes.

In this work, we use molecular dynamics (MD) simulations to study the interactions of four AMPs — Magainin 2, BP100, MSI-103, and MSI-78 — with phase-separated model membranes composed of cholesterol, a saturated lipid (di-palmitoyl phosphatidylcholine; DPPC), and an unsaturated lipid (di-linoleyl phosphatidylcholine; DLiPC). The aim to select the four peptides is to have a little diversity in sizes, charges and sequences, although all four share the same α-helical secondary structure. Magainin 2 has a big size (23 residues) but low net charge (+3), while MSI-103 (21 residues) is highly positively charged (+7) but similar size as Magainin 2. BP100 is also rich in charge density (+6), but very short (11 residues). MSI-78 (+9, 22 residues) serves as a control; it has been investigated by McHenry et al. (22) experimentally on phase-separated lipid membranes.

We use the MARTINI coarse-grained force field (23), widely used in simulations of phase-separating bilayers (24). Previous simulation studies using this model have shown a typical preference for other compounds (e.g., transmembrane proteins (25, 26), sugars (27), aliphatics (28, 29), drugs (30) to partition into the Ld phase, or adsorb at the domain boundaries, and sometimes leading to domain remodeling. Here, our aim is to ascertain the phase preference of AMPs, and establish any effect it might have on the phase separation itself.

3.2 Methods

3.2.1 Force field and simulated systems

In this work, we employed MARTINI version 2.2 for our lipids, cholesterol, and peptide parameters (31-34). The representation of the simulation box, peptides and lipids are shown in Figure 3.1. We used the Avogadro software (35) to build the initial atomistic structures of each peptide, assuming an entirely α-helical secondary structure. The coarse grained MARTINI structures were obtained by using the Martinize script. The simulated bilayers were composed of DPPC, DLiPC, and cholesterol at a 42:28:30 ratio, and a total of 3628 lipids. A membrane patch was first
equilibrated in water for 2.3 µs until equilibrium Lo/Ld phase separation was reached, following the pioneering work of Risselada et al. (36). A rectangular patch shape of large aspect ratio was chosen so that phase domains could easily become continuous with themselves across the periodicity in the y direction, thus reducing the phase interface line tension. Each AMP was added to a separate copy of this patch, placed at the surface of the membrane. Two peptide:lipid (P/L) ratios were employed: 1:200 and 1:20. All systems were made charge neutral by addition of the appropriate amount of chloride ions. At the highest concentration case, peptides were added to both sides of the membrane, in equal numbers, to prevent bilayer disruption due to sheer tension mismatch and make sure the peptides have enough space on the surface of membranes. Prior to production runs, peptide orientation and depth were equilibrated for at least 200 ns under the following restraints: first and last backbone beads of each peptide were position-restrained in the xy plane to prevent lateral diffusion and untimely peptide-peptide association; a weak force in z was applied on the peptides, pulling them towards the bilayer and preventing dissociation into the aqueous phase. This setup allowed the peptides to rotate parallel to the bilayer plane to optimally face the bilayer, without getting trapped in pre-equilibrium aggregates. Production runs proceeded without any restraint on the peptides, for at least 60 µs.

Figure 3.1. System setup. Coarse-grained representation of simulation box, peptides, and lipids.

3.2.2 Simulation parameters

All the simulations were performed using the GROMACS 4.6.7 (37) software or version 5.1 when the use of flat-bottom restraining potentials was needed. Periodic boundary conditions were used. The temperature was coupled (coupling time 1.0 ps) to T=295 K, using the Berendsen thermostat (38). The pressure was coupled using the
Berendsen barostat (coupling time of 0.5 ps and compressibility of $4.5 \times 10^{-5}$), using a semi-isotropic coupling scheme in which the lateral and perpendicular pressures were coupled independently at 1 bar, corresponding to a tension-free state of the membrane. Non-bonded interactions were computed as Lennard-Jones (LJ) potentials, switched to zero from 0.9 to 1.2 nm (pair-list update frequency of once per 10 steps). Electrostatics were calculated as Coulombic interactions shifted to zero from 0 nm to the same 1.2 nm cutoff. A time step of 30 fs was used.

### 3.2.3 Buckling restraints

To prevent membrane buckling, one glycerol bead of each lipid was position-restrained in the $z$ direction with a weak quadratic potential. This strategy, also successfully applied to more complex membranes (11), allows the use of a box size of smaller height, without the risk of direct interaction between peptides and lipids of adjacent periodic images in $z$. In a reference simulation, we used as alternative a flat-bottomed potential restraint, confining headgroup particles to a $xy$ slab of defined vertical thickness. This potential, available in the recently published GROMACS 5.1 package, restrains the glycerol moieties to a slab 4 nm thick, and is harmonic at the slab edges but zero throughout. It therefore only acts on particles leaving the slab region, allowing the headgroups to move freely towards the bilayer core and even flip-flop.

### 3.2.4 Analysis

To identify different domains, a simple phase-assignment algorithm (39) was implemented using the NumPy (40), scikit-learn (41), scikit-image (42) and MDAnalysis Python packages (43). In this algorithm lipid positions were flattened in $z$ and smoothed using a Gaussian kernel with a 1.0 nm standard deviation. The first lipid tail beads (particles C1A and C1B) of DPPC and DLiPC were used to represent each species. The smoothed signal was sampled on a grid of cell dimensions $1 \times 1$ Å. The phase domain interfaces were determined by subtracting the DPPC signal from the DLiPC signal, and running the result through the scikit-image Canny edge-detection filter. The phase boundary perimeters were calculated as well by summing the pixels of edges, then normalized to a flat interface. Edge effects were avoided by extending the phase assignment area by 2 nm in each direction to include beads of the neighboring periodic images. Figure 3.2 schematizes the analysis of a system in the presence of low concentrations of BP100.
To quantify the domain preference of the AMPs, peptide backbone beads were assigned to each of three possible regions: Ld, Lo, or interface, based on their $z$-flattened position. Backbone beads in the Ld and Lo regions were the ones closest to a cell of the respective type. Interfacial beads were those within 1.5 nm of an interface cell, irrespective of Ld/Lo proximity. The counts of assigned backbone beads were normalized by the expected counts in each respective region were the peptides randomly distributed. The random expected counts were obtained by multiplying the total number of peptides by the area fraction of each region. The resulting value represents the ratio of enrichment of each region relative to a random peptide distribution. The reported averages were calculated over the last 20 µs of each production simulation, and the confidence intervals of 95% estimated using a bootstrap procedure with 1000 resamplings. The depth of peptides inserted into the membrane was measured by counting the distances of backbones of peptides with the PO4s of lipids in $z$ direction.

3.3 Results and Discussion

3.3.1 Peptide oligomerization

Figure 3.3 shows the lateral organization of the peptides after 60 µs simulation time, at 1:200 and 1:20 P/L ratios. Focusing at the level of peptide-peptide interactions, the oligomerization behavior clearly differs between Magainin 2 — mostly a dimer — and the other AMPs — mostly monomers. The oligomerization state is quantified in Figure 3.4, showing a much broader size distribution for Magainin 2, with a peak for the dimeric state. This is in good agreement with observed experimental behavior: Mukai et al. (44) and Wakamatsu et al. (45) found Magainin 2 formed dimers when bound to phospholipid membranes. We observed that MSI-103 does dimerize — transiently and to a low extent — at high concentrations (Figures 3.3F, 3.4B), in agreement with experimental evidence of environment-dependent MSI-103 aggregation (46). BP100 and MSI-78 remain as monomers independently of concentration. Although there is no direct experimental evidence that this is indeed
their preferred oligomerization state, these two peptides have cationic charge densities higher than those of either Magainin 2 or the weakly-dimerizing MSI-103. BP100 or MSI-78 aggregation is therefore an unlikely event, consistent with our observations.

**Figure 3.3.** Partitioning behavior of antimicrobial peptides. Snapshots of simulations at 60 µs at low (L:P 200, left column) and high (L:P 20, right column) peptide concentrations. DPPC, DUPC, and cholesterol are colored in blue, red, and yellow respectively. Backbones of each peptide are shown in a variety of colors: Magainin 2 in white, BP100 in pink, MSI-103 in cyan and MSI-78 in green. Two star-shaped aggregates can be seen for Magainin 2 in the Ld phase, where pores were observed to form (indicated by arrows).
Figure 3.4. Oligomerization analysis. Peptide oligomer size distributions over the last 20 µs simulations for different AMPs. A) low (L:P 200) and B) high (L:P 20) peptide concentration.

3.3.2 Peptide phase localization

According to the experimental data of McHenry et al. (22), MSI-78 has a preference for the Ld phase. Our simulations reveal the same behavior, as can be inferred from Figure 3.3. In fact, one can observe that all four AMPs prefer the Ld phase. The affinity for the disordered domain does not seem to be the same for all peptides, however, Magainin 2 is virtually absent from the ordered phase at low concentrations, whereas MSI-103 seems to distribute more homogeneously. Figure 3.5 shows the quantification of these observations over the last 20 µs of each trajectory. Indeed, there is a preference of all peptides for the Ld phase over the Lo one. It is the interfacial region, however, that is the most enriched, for all peptides but Magainin 2.
At high concentrations, and for all peptides, the enrichment ratio of each region is brought closer to 1 (Figure 3.5B). This seems to indicate that the preferred regions (interface and/or Ld) get saturated and the excess peptide is forced into the remaining phases. In the particular case of MSI-103 this effect was such that a preference for the interface region over the Ld phase is no longer discernible.

### 3.3.3 Driving forces

In case of transmembrane helices, we previously showed (25) that the main driving force for sorting of the peptides to the Ld phase is caused by changes in lipid enthalpy. The peptides are essential incompatible with the ordered nature of the Lo phase, disturbing the tight packing of saturated lipids and cholesterol. This leads to an enthalpic driving force for sorting into the Ld domains. In case of the surface adsorbed AMPs, one might expect little difference in the enthalpy of interaction of AMPs with either phase because the MARTINI models for DPPC and DLiPC have identical headgroups. A direct inspection of the peptide interaction enthalpies does however show a difference between Ld-adsorbed and Lo-adsorbed peptide potential energy (Figure 3.6A). Unexpectedly from the observed peptide localization, it is the Lo-adsorbed state that is the most energetically favorable — even if the peptides interact with shallower adsorptions (Figure 3.6B). To make sense of this enthalpic preference, which likely stems from the increased density of peptide–lipid interactions in the more compact Lo-phase, the peptide induced disturbance of lipid–lipid interactions is presumably the dominant factor also in case of AMPs. In agreement with this view that peptide-associated Lo-lipids are less packed, small islands of unsaturated lipids often accompany the few peptides that diffuse into the Lo phase (Figure 3.6).
Figure 3.6. Peptide interaction energies and penetration depths across domains. Only data for the less concentrated systems (L:P 200) is shown. A) Total nonbonded interaction potential energy in each region, averaged by the number of peptide particles in that region (intrapeptide nonbonded contributions were not included). B) Peptide backbone in-depth position, expressed as the z-position difference to the average of nearby phosphate beads, for each region. C) Magainin 2 peptide–peptide nonbonded interaction potential energy, averaged separately for each region (which is a component of the Magainin 2 energies depicted in A). Magainin 2 Peptide–peptide association is more energetically favorable in the Ld phase, somewhat less in the interface region, and energetically neutral in the Lo region (self-interaction energies were not plotted for the other AMPs, which do not dimerize and for which the involved energies are negligible).

The above argument of enthalpic competition between the Lo-lipid packing and peptide adsorption explains why the peptides display a further preference for the phase interface over even the Ld phase: at the interface the energetic cost of disrupting Lo-lipid packing is already partly paid for; peptides can then interact with Lo-lipids to some extent, and therefore have a lower global interaction energy, without incurring in the energetic penalty that drives them away from the Lo phase. Magainin 2 is an exception to the preference of the interface over the Ld phase. Magainin 2 peptides still become enriched at the interface, but clearly less than in the Ld phase (Figure 3.5). This seems to be a consequence of their preference for a dimerized state, which none of the other peptides share. Figure 3.6 shows that the peptide–peptide interaction energy of Magainin 2 is higher when at the interface or Lo phases, which suggests that these environments destabilize the dimers — perhaps due to a less compatible interaction depth profile.

3.3.4 Magainin 2 pore formation at high concentrations

At high P/L ratio, two Magainin 2 pores spontaneously formed in the Ld phase (Figure 3.3B). From the peptide’s mechanistic point of view, this is an expected event. However, it is a remarkable observation because simulation of spontaneous AMP membrane insertion and pore formation has remained elusive for coarse-grain models, and descriptions of the process are only available from atomistic simulations (47, 48).
For the MARTINI coarse-grain model the reasons behind the difficulty in simulating pore phenomena are not entirely clear, although it has been pointed out that MARTINI membranes often present an excessively high energy barrier to lipid flip-flop or crossing by polar moieties (49). Indeed, MARTINI membranes covered with Magainin 2 will sooner buckle and bud than form pores (50). In our simulations at high peptide densities membrane buckling was prevented by the application of a restraining potential in the $z$ direction on the lipid headgroups. Tension was further balanced by having equal amounts of peptide on each leaflet. Under this light, and given the reluctance of the MARTINI model for pore formation (49), we believe that the extreme buckling observed by others (50) is probably not the foremost mechanism. In reality pore formation will likely occur earlier, the more so as the presence of membrane proteins and actin filaments will prevent buckling.

Figure 3.7A depicts a step-by-step formation of one of the Magainin 2 pores. Preceding pore formation Magainin 2 dimers — mostly antiparallel — assemble in one of the leaflets in a roughly radial fashion (Figure 3.7B). For both pores formation involved the incursion of top leaflet peptides into the bottom leaflet. Two-to-three peptides, of mixed orientation and of different dimers, penetrate simultaneously end-first into the bilayer core (the mixed N- and C-terminal orientations presumably stabilizing the terminal charges), dragg­ing water beads along. Peptides from the bottom leaflet then tilt inward to meet the incoming top-leaflet peptides, after which the pore is established. In both cases, after initial radial aggregation, the process was quite fast, with the first peptide becoming transmembrane within 300 ns. Once inserted, peptide organization is tilted and no longer dimeric. The pore structure is also dynamic, with peptides frequently exchanging between the adsorbed and internalized states. However, no peptides were observed to fully translocate and adopt the adsorbed configuration in the opposing leaflet; there were, at most, cases where transmembrane peptides cross the membrane all the way and become anchored closer to the opposing leaflet than the starting leaflet. Regardless of peptide dynamics, once formed the pores themselves were stable for the remainder of the run (over 60 $\mu$s).
Figure 3.7. Process of Magainin 2 pore formation. A) Three side-view snapshots depicting peptide internalization from the top leaflet, contact with the bottom leaflet, and establishment of a stable pore (see the main text for more detail). Label times are relative to the beginning of internalization, with t=0 corresponding to 7.8 µs simulation time; for this system buckling was prevented by restraining vertical headgroup movement; the view at t=300 is rotated by 180° relative to the other two panels. B) Top and bottom leaflet views of the peptide organization at t=0, for the same system as in A. C) Pore structure obtained in a membrane held flat by a flat-bottomed potential, as opposed to the headgroup restraints in A and B. Peptide backbones are depicted in gray, with blue and red N and C termini, respectively; lipids are shown only as their PO4 bead, in orange (these are mostly, but not entirely, DLiPC lipids, since the pores formed in the Ld phase); the water beads closest to the pore core are shown in cyan; for clarity, all other system components were hidden, and in B the PO4 beads were also not shown.

As stated, the observation of Magainin 2 pore formation was facilitated by the restriction of lipid headgroup movement in the out-of-membrane-plane direction. Naturally, this raises concern about the significance of the pore structure since such potentials prevent the lipid headgroups from accompanying the internalizing peptides, as is expected to occur for toroidal pore models (47, 48). To test the influence of this bias we employed a different method to restrict membrane buckling, namely a flat-bottomed potential (see Methods). Under the less biasing flat-bottomed potential two Magainin 2 pores were also observed to form (Figure 3.7C), confirming that it is indeed the prevention of buckling — and not the specific restriction of headgroup
movement — that promotes pore formation. The final structure of these pores is quite similar to the pores obtained with the headgroup restraints, with peptides inserted in a tilted, monomeric fashion. Incidentally, though the membrane can become somewhat distorted near the pores when using the flat-bottomed potential, headgroups still do not completely follow the peptides into the bilayer core. This behavior may be connected to the high flip-flop energies under the MARTINI model (49).

### 3.4 Conclusion

In this work we were able to observe and assign the molecular bases for AMP preference for disordered phases. This extends and complements available experimental studies, in which a preference of AMPs for the Ld phase could be inferred, but only from indirect evidence (22). Contrary to what might have been intuitively expected, it is the favorable energy of the Lo lipid–lipid packing that diminishes the peptide affinity to that phase, since energetically the AMPs do prefer the high density of interactions in the Lo phase. The observed further preference of monomeric AMPs for the phase interface is a corollary of these energetic considerations.

In addition to Ld phase preference, in the case of Magainin 2 pores were also observed to spontaneously form there. The definition of the conditions required for this observation is a major breakthrough in the simulation of membrane-active peptides, and opens the door to a much more detailed characterization of determinants of pore formation.

Our observation of AMP accumulation in the Ld phase supports the view that phase preference can potentiate AMP activity by promoting localized high-density peptide regions in the membrane (22). Still, extrapolation of these conclusions to the much more complex bacterial or eukaryotic membranes, where sharp phase separation seems unlikely (11), should be done with caution.

### 3.5 References


