The simulation studies of the interplay of peptides with lipids
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Chapter 2
A Molecular Dynamics View on the Segregation Effect of Antimicrobial Peptides on Anionic Lipids

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Abstract
Antimicrobial peptides (AMPs), typically cationic in nature, have been proposed to preferentially interact with anionic lipids. Supporting this different AMPs have been shown to induce anionic lipid segregation in mixed bilayers, in a charge-dependent fashion. We present a multiscale molecular dynamics (MD) study of this binding and segregation mechanism, using the AMPs Magainin 2, BP100, and MSI-103 in interaction with a POPE/cardioplin bilayer. In agreement with experimental observations, the more cationic peptides caused a higher segregation of anionic lipids from zwitterionic ones. The detail afforded by MD simulations allowed us to confirm that this action is indeed mediated mainly by electrostatic interactions whereby the first lipid shell around the peptides becomes enriched in cardiolipin. We found no indication that AMP-mediated anionic lipid segregation extended further than this annular shell, i.e., no higher order organization into cardiolipin domains was observed. Consistent with experimental observations, BP100 falls outside the charge–segregation relationship. Contacts between cardiolipins and charged BP100 side chains did follow the same charge trend as for Magainin 2 and MSI-103. From these observations we were able to assign the outlying behavior of BP100 to its higher-than-average cationic density, which prevents maximal contact with cardiolipins.

2.1 Introduction
In recent years antimicrobial peptides (AMPs) have attracted much attention as promising natural antibiotics with potential to overcome the increasing threat of bacterial antibiotic resistance (1, 2). AMPs are relatively short (typically up to 30 residues long), positively charged (+6 charges are common), amphiphilic, and display a broad-spectrum of antifungal and antimicrobial activities. These short and cationic peptides have been shown to disrupt or permeabilize bacterial membrane via non-specific interactions leading to cell death.

In spite of their promising characteristics, the mechanism of AMP action is diverse, complex, and not yet fully understood. Several models of AMP action have been proposed (3-6), as well as a number of different ways to sub-classify these peptides (7). However, an increasing number of studies (8) show that these compartmentalizations might be too simplistic, and that instead several concurrent
factors—not necessarily the same across the AMP spectrum—might be responsible for antimicrobial action.

The AMP-mediated lateral segregation of anionic lipids in a membrane is one of the aforementioned disruptive factors. It has been observed for different peptides (9, 10) and, although probably not a lethal event by itself, it is a certainly destabilizing feature of AMP behavior that might act in synergy with other mechanisms (11). Wadhwani et al. have published experimental work on AMP binding to membranes containing anionic lipids and showed a correlation between the net charge of peptides and extent of anionic lipid segregation, regardless of the secondary structure of the peptides (12). Earlier work by Epand et al. (13) corroborates this conclusion. These observations were based on the changes in lipid phase transition temperature brought about by the demixing effect of the peptides, and therefore only provide a macroscopic and averaged view into the process. In particular, no hypothesis can be put forth regarding the topology of the anionic lipid clustering. Do peptides and anionic lipids cluster into larger-scale domains, phase-separated from the rest of the membrane? Or do they remain only locally clustered, dissolved in the bulk membrane lipids?

In this work we employ MD simulations to observe the precise molecular aspects of lipid binding and segregation by AMPs, in order to both explain the charge correlation and characterize the topology of peptide-lipid clusters. MD simulations constitute a powerful tool to observe the detailed mechanism of AMPs in action and have provided groundbreaking information in the field (14-23). Still, to the best of our knowledge, only a limited number of MD studies have focused on the mechanism of AMPs specific binding to anionic lipids (21, 24, 25). In these, coarse grained MD simulations were used to identify the driving force of AMPs to reorganize the lateral organization of lipid bilayers.

From the peptides experimentally studied by Wadhwani et al. three were chosen for our simulations: Magainin 2 for lying at the low end of the charge-segregation correlation, MSI-103 for lying at the high end, and BP100 for being an exception to this correlation with an observed induced segregation of cardiolipin lipids lower than that predicted by its high net charge. We performed MD simulations at both atomistic and coarse-grained (CG) levels: atomistic simulations were used to cross validate CG ones at shorter time and length scales, and CG ones were used to access longer equilibration times and sizes that might be relevant for the segregation event.
2.2 Methods

2.2.1 System setup

This work follows the lipid interactions of the peptides Magainin 2 (GIGKFLHSAKKFGKAFVGEIMNS, +3e), BP100 (KKLFKKILKYL-amide, +6e), and MSI-103 (KIAGKIAKIAGKIAKIAGKIA-amide, +7e). The histidine in Magainin 2 was considered to be deprotonated at all times. Peptides were added to both sides of the membrane at a lipid-to-peptide (L/P) ratio of 20.

A CG bilayer was built up with the same lipid composition as for the experimental observation of the charge-segregation relationship (12): a 3:1 mixture of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) and tetraoleoyl cardiolipin. The cardiolipin headgroup carries a double negative charge. In our smaller systems the membrane was composed of 58 cardiolipins and 172 POPE lipids; the system was solvated with water and enough Na\(^+\) ions for global charge neutrality, and equilibrated under NPT conditions to yield a box of dimensions 9.7×9.7×9.3 nm. Peptides were placed in contact with the bilayer. Atomistic systems were obtained via back-mapping (26) of these equilibrated CG structures.

Larger systems, generated by repeating the smaller ones 9 times in a 3×3 arrangement, were simulated only with the CG model. In the larger system Magainin 2 induced membrane buckling upon dimerization; extra water was added to this system to prevent the buckled membrane to interact with its periodic images in the z-dimension.

2.2.2 Simulation parameters

All the MD simulations were performed using the GROMACS 4.6 software (27) under periodic boundary conditions. The temperature was weakly coupled (coupling time 0.1 ps) to T=320 K, using the Berendsen thermostat (28). The pressure was coupled (coupling time of 1.0 ps and compressibility of 3.0×10\(^{-5}\)), using a semiisotropic coupling scheme in which the lateral and perpendicular pressures were coupled independently at 1 bar, corresponding to a tension-free state of the membrane.

The GROMOS 54a7 (29) force field was used to describe the atomistic system. A group-based twin range cut-off scheme was employed, using cut-offs of 1.0/1.4 nm and a pair-list update frequency of once per 5 steps. The time step was 2 fs. Particle mesh ewald (PME) (30) was used for long-range electrostatic interactions. The water was simulated using the SPC model (31), with bonds and angles constrained using the SETTLE algorithm (32). The length of all remaining covalent bonds was constrained using the LINCS algorithm (33).
The MARTINI force field version 2.2 was used to describe the lipids (34) and peptides (35, 36) in CG systems. This force field simplifies the system description by representing, as a rule, four heavy atoms as a single particle (37). Parameters for cardiolipin were taken from the work of Dahlberg et al. (38) and have been successfully used in a number of other protein-lipid binding studies (39, 40). The polarizable MARTINI water model (41) was used since both MARTINI representations of peptides and lipids carry explicit charges, and electrostatics can be expected to play an important role in their interactions. The properties of the polarized MARTINI water model were tested by Vögele et al (42) where it has been shown that this model is able to satisfactorily reproduce electrostatic properties of ionic solutions. The starting CG structures were equilibrated using the standard MARTINI water model. Non-bonded interactions were described by Lennard-Jones potentials, switched to zero between 0.9 to 1.2 nm as is common practice in MARTINI simulations; long-range electrostatics were treated with PME. A time step of 20 fs was used, with a pair-list update frequency of once per 10 steps.

2.2.3 Analysis

Lipid binding to peptides was quantified by counting peptide-lipid contacts for all the simulations. A contact was defined whenever lipid and peptide reference points lay within 0.7 nm of each other in the $xy$ plane—this distance was chosen to include essentially first neighbors (see the radial distribution functions in Figure 2.S3 and Figure 2.S4 in the Supporting Material). For CG simulations the reference points were the cardiolipin phosphates and the tip bead of the side chain of each peptide residue (or the backbone bead, in the absence of a side chain). To be directly comparable to the CG counting, in atomistic simulations the reference positions were the centers-of-mass of the atoms that map to the beads used as CG references. Contacts were only considered within the same leaflet. Only one contact per peptide-phosphate pair was counted (the closest), even if multiple reference centers lay within contact range. Counts were averaged per peptide and over time. Contacts were also calculated in the same fashion between lipid phosphate groups, within a 1.3 nm cutoff (this larger range encompassing the typical PO4-PO4 $xy$-distance between neighboring lipids—see Figure 2.S5 and Figure 2.S6 in the Supporting Material). When assigning cardiolipin contacts the following criterion was followed: if only one phosphate group of a cardiolipin was in contact with a peptide residue, then both phosphates were considered in contact with that residue, counting as a full cardiolipin contact. If each of a cardiolipin's phosphates were in contact with a different residue, then each residue was assigned a half cardiolipin contact; if both residues belong to the same peptide that cardiolipin was considered to be shared. When monitoring cardiolipin interaction with the peptides’ cationic charges contacts were considered only with Lys tip beads or N-terminus backbone beads.
2.3 Results and Discussion

The phenomenon of AMP-induced cardiolipin clustering and segregation was simulated at the AA and CG levels of detail. AA systems comprised 10×10 nm membrane patches with peptide added to both leaflets, and were simulated for at least 973 ns. CG systems, which can sample much longer time and length scales, were 9-fold larger than AA ones and were simulated for at least 1.5 µs. The representations of the systems, peptides, and lipids in both atomistic and CG models are shown in Figure 2.1.

**Figure 2.1.** AA and CG representations of the lipids and peptides used in this work. Magainin 2 is shown in AA and CG bilayer systems at the actual relative sizes and compositions at which they were simulated. POPE tails and phosphate group are colored gray. Cardiolipin tails are colored blue and phosphate group red. Peptide backbone is colored green, cationic side-chains blue, anionic side-chains red, and apolar side-chains gray.
A concern in this study was to ensure the CG model yields reliable information compatible with that of the atomistic model. For this reason the results are presented also in a CG/atomistic comparative fashion. The two models do overlap on most results. Specific discussions are presented below for the cases where they do not agree.

### 2.3.1 Peptide organization

In the short time scale of the AA simulations peptides diffuse only a very short range. Oligomerization, when it happens, generally involves first encountered neighbors and lasts for the remainder of the simulated time. The CG systems are, conversely, much more dynamic, with peptide-peptide interactions forming and breaking up along the simulation. In spite of these differences the peptide organization in both models is largely in agreement—in particular oligomerization propensities (Figure 2.S1 and Figure 2.S2)—validating the use of CG in this respect. Snapshots of the peptide organization are presented in Figure 2.2 and Figure 2.3.
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**Figure 2.2.** Top view of atomistic systems after at least 850 ns simulation time. A) Bilayer without peptides. B) Bilayer with Magainin 2. C) Bilayer with BP100. D) Bilayer with MSI-103. The phosphorus atoms of cardiolipin are depicted in red, peptide backbones in green and, cationic sidechains in blue. Sodium ions within 1 nm of cardiolipin phosphorus atoms are depicted in cyan. PE lipids were omitted for clarity.

**Figure 2.3.** Top view of CG systems after at least 1 µs simulation time. A) Bilayer without peptides. B) Bilayer with Magainin 2. C) Bilayer with BP100. D) Bilayer with MSI-103. The color scheme is the same as in Figure 2.2, with the difference that the red beads now represent a full phosphate group.
Magainin 2 dimerized in the two models, in further agreement with experimental work that reported the dimerization behavior of Magainin 2 in phospholipid bilayers (43, 44). During the atomistic simulation, peptides aggregated into mostly dimers and trimers. Occasional interactions between these aggregates led to temporary formation of larger-order aggregates (Figure 2.1, Figure 2.2). In the CG simulations dimers and trimers could also be observed. In this case, the long simulation timescales also allowed the observation of subsequent dissociation events. Magainin 2 dimerization occurred essentially via its exposed nonpolar patches, leaving its charged residues free to interact with lipids.

BP100 peptides did not dimerize in either model. To the best of our knowledge no studies on BP100 oligomerization have been reported; nonetheless, the behavior observed in both CG and atomistic models is expected given the very high charge density on these short peptides.

In AA simulations of MSI-103 a single dimer was observed to form, while the rest of the peptides remained unassociated. In CG simulations, sporadic peptide association was also observed, but not as long-lived. MSI-103 dimerization in phospholipid bilayers has been studied experimentally, and monomers and dimers were shown to coexist in membranes, although this can be affected by subtle changes in peptide concentration, sample hydration, and lipid composition (45, 46).

2.3.2 Peptide – lipid contacts

In contrast with the limited peptide diffusion in AA systems, lipids exhibit enough freedom to move around the system, often exchanging with one another in preferred interaction regions.

A clear preference for cardiolipin to locate in the vicinity of the peptides is visible in both models (Figure 2.2 and Figure 2.3). This interaction is essentially electrostatic in nature, arising from the high cationic content of the peptides, and the anionic charges on cardiolipin. This can be inferred from the cardiolipin association behavior in the presence and absence of peptides: in Figure 2.2A and Figure 2.3A, where a bilayer without peptides is shown, small clusters of cardiolipin form, in spite of the strong anionic repulsion. Clustering happens mostly due to cationic counterions coordinating multiple cardiolipin headgroups. This is in line with other reports (47) on cardiolipin-counterion binding. Upon addition of peptides to these membranes cardiolipin headgroups interact preferentially with the cationic sidechains resulting in cation displacement.

Figure 2.4 shows the average number of contacts per peptide with lipid phosphate groups, for atomistic and CG simulations. The contact plots clearly show a preference for the peptides to bind cardiolipin over POPE. This preference is such that in all cases there are more cardiolipin phosphates than POPE ones bound to peptides, even
though in our simulated systems POPE phosphates outnumber cardiolipin ones 3-to-2 (taking into account that each cardiolipin carries two such groups).

It should be noted that cardiolipin binding was restricted to the immediate shells around the peptides. No large-scale clustering or domain formation was observed with either model. In this case AMP action on the membrane is limited to the modulation of bulk lipid composition, rather than phase miscibility/separation behavior.

It is also visible from Figure 2.4 that, while for the most part in very good agreement, CG results indicate a larger number of contacts between PE and MSI-103 than atomistic ones. We ascribe this difference to the dimer that formed in atomistic simulations where the entire dimerization interface of the involved peptides became screened from lipid interactions—particularly interactions with PE since the cationic sidechains were still free to interact with cardiolipins. The small number of peptides in the atomistic system and relatively shorter simulation time caused this single long-lived dimerization to take a disproportionate weight on the contact counts. The exact same type of peptide-PE screening happens, in a larger scale, with Magainin 2. In this case atomistic and CG contact counts agree because the dimerization behavior is similarly described by both models.

![Figure 2.4](image)

**Figure 2.4.** The number of lipid phosphate contacts with peptides after AA and CG simulations, averaged over time and the number of peptides. Error bars represent the standard deviations over simulation frames. Green: PE-peptide neighbors. Red: Cardiolipin-peptide neighbors.

Interestingly, peptide-PE contacts also seem to increase with peptide charge, especially in the CG results. This, however, reflects more the organization of the peptides than a direct effect of charge: Magainin 2 is the least cationic of the peptides, and while also the largest (23 residues) it is mostly dimerized in the membrane. The number of its sidechains exposed to interaction with lipid headgroups is, therefore, roughly halved. MSI-103, which has a size similar to that of Magainin 2 (21 residues) but the most cationicity and a lower propensity for dimerization binds a significantly larger amount of lipids. Furthermore and as discussed in section 3.1 Magainin 2
dimerization occurs through its nonpolar patches and does not hinder the preferential interaction of basic sidechains with cardiolipin. As a result, Magainin 2–PE contacts become selectively decreased.

### 2.3.3 Lipid – lipid contacts

 Preferential binding of cardiolipin to peptides is the likely mechanism behind the observed changes in phase-transition temperature reported by Wadhwni et al (12). As can be seen from Figure 2.5, peptide sequestering of cardiolipin indirectly causes a decrease of PE-cardiolipin contacts. The resulting bulk bilayer composition is thus cardiolipin-depleted and its phase transition behavior can be expected to be closer to that of pure POPE, just as hypothesized and observed by Epand et al. and Wadhwni et al (9, 10, 12).

![Figure 2.5](image.png)

**Figure 2.5.** The average number of phosphate neighbors of PE heads after AA and CG simulations with the three peptides, normalized by the counts in the absence of peptides. Error bars represent the standard deviations over simulation frames. Green: PE-PE neighbors. Red: PE-cardiolipin neighbors.

Figure 2.6B shows the ratio of PE-to-cardiolipin lipids in the membrane bulk (i.e. of lipids not in contact with any peptide). The dependence of this ratio on peptide charge ultimately explains the trend reported by Wadhwni et al (12) (reproduced in Figure 2.6A) in which more cationic peptides yield a bulk phase behavior closest to pure PE. A somewhat discrepant behavior between AA and CG bulk ratios can be seen for MSI-103. While this can be partly attributable to the aforementioned increased MSI-103–PE binding in the CG simulations, the AA model of MSI-103 was indeed able to deplete cardiolipin from the bulk more efficiently than the CG model. This is evident when comparing Figure 2.2D and Figure 2.3D, and may reflect the simplified treatment of electrostatic interactions by the CG model.
Figure 2.6. A) Reproduction, from Ref. 12, of the relationship between peptide charge and melting temperature shift—indicative of lipid demixing—obtained experimentally with 3:1 POPE:cardiolipin mixtures. B) The bulk ratio of POPE-to-cardiolipin lipids averaged over time (atomistic simulations after 500 ns, CG after 800 ns) as a function of peptide charge. Error bars indicate distribution standard deviations. In both AA and CG models the bulk ratio in the presence of BP100 is below the Magainin 2–MSI-103 trend (an effect smaller in the CG case, and rendered less visible due to the long y-axis scale imposed by the AA results). C) Number of specific
cardiolipin contacts with peptide charges (empty bars), and the fraction corresponding to lipid contacts shared by residues of the same peptide (shaded bars). Blue: AA, red: CG.

2.3.4 Outlying behavior of BP100

The reason to include BP100 in this study was its outlying behavior regarding anionic lipid clustering. Experimentally, BP100 was observed to promote a weaker cardiolipin clustering than predicted by its charge (12) (Figure 2.6A). Wadhwani et al. suggest that this observation arises from the short size of the peptide: either due to its inability to penetrate deeply into the membrane, or due to the entropic penalty of gathering many cardiolipins within its small immediate solvation area. In our results (Figure 2.6B) the same weaker cardiolipin segregation was observed for both AA and CG systems—the former yielding a more prominent outlying BP100 behavior. Our results show that the lower BP100–cardiolipin contacts result from a crowding effect that forces both phosphates of cardiolipins to coordinate different peptide charges. This is clear in Figure 2.6C, which plots the number of cardiolipin contacts with charged peptide groups and the fraction of those cardiolipins that is shared among same-peptide residues. The number of shared BP100–cardiolipin contacts is the highest among the three peptides, both in relative and absolute terms, and for both AA and CG models (see the Methods section for details on the assignment of shared contacts).

The increase in cardiolipin sharing by BP100 residues is a consequence of its high cationic density: sharing cardiolipin contacts satisfies the peptide's anionic interactions while avoiding the enthalpic and entropic penalties involved in maintaining a locally dense cluster of anionic cardiolipins. Figure 2.7 schematizes this view. In agreement with our explanation, the other obvious outlier in the charge-clustering trend reported by Wadhwani et al. (12) is the also highly charge-dense HIV-TAT peptide (a 13-mer with +8e total charge).
Figure 2.7. Schematic depiction of cardiolipin contact sharing at high peptide cationic densities. Yellow: peptide. Blue: cationic peptide charges. Red: pairs of cardiolipin phosphate groups.

2.4 Conclusion

In this work we observed, using MD simulations at atomistic and CG scales of detail, how anionic lipids bind to AMPs. Both atomistic and CG simulations reproduced the experimentally observed trend of increased lipid segregation by more cationic AMPs. Lipid binding was also observed shown to be local to each peptide, not extending further into formation of domains.

Our observations are valuable in interpreting the consequences of AMP action at the lipid-segregation level: it can now be hypothesized how the depletion of anionic lipids from the bulk membrane phase, rather than a phase separation, can affect bacterial processes and contribute towards cell death.

We were also able to observe and explain the outlying behavior of BP100, which clusters fewer anionic lipids than predicted by its charge. These observations ultimately led to the precise understanding of how peptide charge density affects anionic lipid clustering.

2.5 References


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2.6 Supporting Material

This section contains peptide aggregation analyses and a set of xy-plane radial distribution functions (RDFs) for both atomistic and coarse grained systems.

Figure 2.S1. Peptide oligomerization along atomistic simulations after 500 ns.

Figure 2.S2. Peptide oligomerization along coarse grain simulations after 800 ns.
Figure 2.S3. xy-plane RDF of cardiolipin phosphorus atoms around peptides after atomistic simulations. Reference atom groups in the peptides were the same as used for contact counting (see the main text).

Figure 2.S4. xy-plane RDF of cardiolipin phosphate groups around peptides after CG simulations. Reference beads in the peptides were the same as used for contact counting (see the main text).

Figure 2.S5. xy-plane RDF of cardiolipin phosphorus atoms around PE ones, after atomistic simulations.

Figure 2.S6. xy-plane RDF of cardiolipin phosphates atoms around PE ones, after CG simulations.