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Concentration-dependent modes of melittin-induced membrane destabilization: an integration of experimental and computational tools

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Abstract

Melittin is a well-known antimicrobial peptide whose mechanism of action is still not entirely understood. In this study, we combine complementary experimental techniques with molecular dynamics simulations to determine melittin-membrane interactions at different scales. Using 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE):1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG) membranes that mimic the *Escherichia coli* inner membrane, this integrative approach reveals a clear transition in melittin's mode of action with increasing loading. At low concentrations, melittin primarily perturbs the membrane interface, inducing modest surface expansion and subtle lipid reorganization. At higher concentrations, the membrane response shifts toward increased hydration, lipid disorder, membrane thinning, and morphological remodeling. Simulations support these observations, linking concentration-dependent membrane responses to enhanced lipid mobility and stronger effects on anionic DPPG. Melittin, therefore, affects the membrane in a concentration dependent-manner, transitioning from surface-bound carpet-like permeabilization at low loading to transient pore formation and membrane reorganization at higher concentrations.

Introduction

Antimicrobial peptides (AMPs) are short molecules found in a wide range of bacteria, animals, and plants, serving as part of the host's immune defense with a broad spectrum of activity against pathogenic bacteria, viruses, and fungi. AMPs possess an overall cationic charge and a significant proportion of hydrophobic residues¹, enabling selective interaction with the negatively charged microbial membranes and disrupting their integrity. Their primary mode of action involves membrane disruption, while secondarily targeting intracellular moieties and key cellular processes. Several mechanisms of action have been proposed², including pore formation (through the barrel-stave model and the toroidal-pore model), surface accumulation (carpet-like model) and membrane aggregation. AMPs are a promising alternative to conventional antibiotic therapy to address antibiotic resistance³. Their rapid and multifaceted mechanisms of action make it difficult for pathogens to develop resistance, being advantageous in combating multidrug-resistant infections. Several AMPs have already reached clinical and commercial use, such as gramicidin⁴,

daptomycin⁵, and polymyxins⁶, while others, including pexiganan and omiganan⁵, are in advanced stages of development for therapeutic applications.

Despite their promise, the natural diversity and versatility of these peptides also means that their mechanisms of actions are difficult to determine, which limits their broader application and accessibility. This is exemplified by melittin, a well-characterized AMP derived from bee venom⁷. While its primary mechanism of action has been studied, significant gaps remain in the understanding of its behavior at the molecular level. Composed by a 26 amino acid chain, featuring a hydrophobic amino-terminal region and a positively charged carboxyl-terminal region⁸, it exerts its activity primarily by disrupting bacterial membranes through a multi-step mechanism. Initially, melittin binds to the membrane surface via electrostatic interactions between its positively charged residues and the negatively charged phospholipids of bacterial membranes. Upon sufficient accumulation, melittin adopts an amphipathic α -helical structure, inserting into the lipid bilayer and inducing structural perturbations^{9,10}. Its most accepted mode of action involves the formation of transmembrane pores or micelle-like aggregates, which compromise membrane integrity, leading to leakage of cellular contents and, ultimately, cell death². Models such as the barrel-stave, toroidal-pore, and carpet-like mechanisms describe these disruptions, with the exact pathway depending on peptide concentration, membrane composition, and environmental conditions¹¹. As a result, questions persist regarding the precise structural arrangement of melittin during membrane disruption, as well as the factors that govern its selectivity and potency across different membrane types. These unanswered questions stress the broader challenges faced in harnessing the therapeutic potential of AMPs, as the complexity of their mechanisms hinders the ability to design and optimize them for clinical use.

Understanding the mechanisms by which AMPs interact with cellular membranes, remains a significant challenge, largely due to the methodological constraints of early studies. The latter often relied on simplistic models, such as single-component lipid vesicles or basic antimicrobial assays, to infer AMP activity. However, these methods often yielded conflicting conclusions, as is the case with melittin. For instance, while vesicle leakage assays initially suggested that melittin disrupts membranes solely by forming stable barrel-stave pores¹², later studies under different conditions proposed a detergent-like solubilization mechanism^{13,14}. Such discrepancies arise because simplistic models overlook critical variables (e.g., lipid composition, peptide concentration, and intracellular targeting), underscoring the need for more comprehensive methodologies.

Herein, we investigate the interaction of melittin with a simplified *E. coli* inner membrane model, at two different lipid-to-peptide ratios (L:P 30:1 and 10:1) across three model membranes, with complementary spectroscopic and microscopy techniques. DOPE and DPPG were used to produce different membrane models: *i*) Langmuir monolayers to carry out surface pressure (π) versus area isotherms and BAM to assess morphological changes, with parallel PM-IRRAS measurements identifying key functional groups involved in air/water interface interactions; *ii*) thin lipid films analyzed by AFM to characterize melittin-induced nanoscale reorganization of lipid stacking in the dry state; and *iii*) lipid bilayers studied by ssNMR to resolve atomic-level peptide-membrane interactions under near-native aqueous conditions. This multi-technique strategy provides a comprehensive view of melittin membrane activity, correlating peptide structure with membrane composition and dynamics. Cross-validating results from interface-sensitive, nanoscale, and atomic-resolution methods, we overcome the limitations of single-technique approaches and establish a robust mechanistic framework for the membrane interactions with melittin.

Materials and methods

Materials

Melittin from honey bee venom (>85%, M2272) was purchased from Sigma Aldrich. The lipids used for preparation of the model membranes were 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). They were supplied by Avanti Lipids Inc. and stored at -20 °C until utilization. Stock solutions with a final concentration of 10 mg mL⁻¹ were prepared by dissolving DOPE in chloroform and DPPG in a 4:1 (v:v) chloroform:methanol mixture. The stock solutions were stored at -20 °C between uses. The buffer solution consisted of 5 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES) and 20 mM sodium chloride (NaCl) in a 60:40 D₂O:H₂O mixture, adjusted to pH ~ 7.2. The buffer was stored in sealed glass bottle in the fridge at 4 °C between uses.

Preparation of liposomes

The liposomes used for the DLS and ssNMR studies were prepared using the thin film hydration method¹⁵. The lipids were dissolved in chloroform, mixed in the desired ratios, and dried under a continuous stream of nitrogen to form a dry lipid film on the vial walls. Complete solvent evaporation was ensured by overnight vacuum drying. The lipid film was then hydrated with water or buffer solution, at a temperature above the highest phase transition temperature (T_m) of the lipids in the sample, followed by ~ 5 minutes of sonication to reduce aggregation. The lipid suspension was stirred until liposome formation, cooled, and stored at 4 °C overnight for complete hydration. For the samples containing melittin, the peptide was first solubilized in a chloroform/methanol mixture and added to the lipid solution before solvent evaporation. The solvent drying and lipid hydration steps were performed following the protocol described for the peptide-free samples.

Dynamic light scattering (DLS)

The hydrodynamic diameter, particle size distribution and polydispersity index of the vesicles were measured by DLS using a Zetasizer Nano-ZS equipment (Malvern Instruments). The measurements were performed at 25 °C with a scattering angle of 90 °C.

Atomic force microscopy (AFM)

Thin lipid films, with and without melittin, for AFM experiments were prepared on glass microscopy slides using a Spin Coater Model P6708D Series (Specialty Coating Systems). Spin-coating was performed by dropping 400 µL of the lipid solution on the slowly rotating glass slide followed by acceleration to 2000 rpm for 60 s. The samples were then stored in a desiccator under vacuum overnight to ensure complete solvent evaporation. The AFM characterization was performed at air pressure below 1 mbar and room temperature with a Park HX-Hivac microscope (Park Systems). The scanning was done at a rate of 0.05 Hz with a silicon tip Multi75E-G (Budget Sensors) with a force constant of 3 N m⁻¹, operated in non-contact mode at a resonance frequency

of 81.7 kHz and an amplitude of free vibrations 15 nm. The set point was 80% of the free vibrations.

Langmuir films, PM-IRRAS and BAM

Langmuir films were prepared in a clean room using a Mini KSV Langmuir trough (KSV Instruments Ltd). Surface pressure–area (π –A) isotherms were recorded at 22 ± 1 °C using a Wilhelmy plate (Whatman Chr1) connected to an electrobalance. Lipids and melittin stock solutions (1 mg mL^{-1}) were prepared in chloroform/methanol. The films were formed by spreading the desired solution at the air–water interface using a microsyringe (Hamilton, 25 mL). The compressibility modulus (C_s^{-1}) was calculated from the compression isotherms using the following equation:

$$C_s^{-1} = -A \left(\frac{d\pi}{dA} \right)$$

The collapse pressure (π_{col}), lift-off area (A_{LO}) and mean molecular area at 30 mN m⁻¹ (A_{30}) were determined directly from the compression isotherms.

The Langmuir monolayers were equilibrated for 10 min before being compressed to the desired surface pressure at a rate of 10 mm min⁻¹. PM-IRRAS were recorded with a KSV PMI 550 instrument (KSV Instruments Ltd) over the Langmuir trough, with polarization between p and s modulated at high frequency. The incidence angle was set to 81°, and spectra were acquired at 30 mN m⁻¹ with 600 scans and 8 cm⁻¹ resolution, using the corresponding subphase spectrum as background. BAM images were recorded with a EP4 imaging ellipsometer (Accurion) at the air–water interface of a Langmuir Mini trough from KSV. The BAM instrument was fitted with an Ultraobjective camera, set at an incidence angle of 53.1°, with the polarizer at 10°, analyzer at 2° and compensator at 0°. The images were corrected with Accurion's Data Studio software.

Solid-state nuclear magnetic resonance (ssNMR)

The liposomes prepared by the thin-film method previously described were separated from the bulk aqueous phase by centrifugation and packed into a 4.0 mm ssNMR rotor using a modified version of T. Cross protocol¹⁶. ¹H, ¹³C and ³¹P spectra were acquired on a Bruker Avance III 400 spectrometer operating at B₀ field of 9.4 T, with Larmor frequency of 400.130 MHz for ¹H at a temperature of 300 K. All experiments were performed on double-resonance 4 mm Bruker magic-angle-spinning (MAS) probes at a MAS frequency of 10 kHz (except for the ¹H-¹³C INEPT experiments, done at 5 kHz) and under room temperature conditions. Samples were packed into 4 mm MAS rotors with inserts and caps made of Kel-F. For the ¹H single-pulse experiments, the initial 90-degree pulse had a duration of 3.0 μs and a recycle delay of 1.0 s. 1D ¹³C spectra were acquired under high-power proton decoupling using the SPINAL64 sequence, with a recycle delay of 4.0 s and a 90-degree pulse length of 4.0, 3.75 and 5.0 μs for the DOPE:DPPG, (DOPE:DPPG):MLT 30:1 and (DOPE:DPPG):MLT 10:1, respectively; the amplitude of the decoupling pulses was 45 kHz. For ¹³C INEPT experiments, the ¹H 90-degree pulse length was set to 5 μs; non-selective polarization transfer was achieved using an INEPT pulse sequence with

evolution delays τ_1 and τ_2 of 2.0 ms and 1.2 ms respectively. The recycle delay was 1.75 s and decoupling was employed at a radiofrequency-field strength of 45 kHz during signal acquisition.

For the 1D ^{31}P experiments, also under high-power proton decoupling, the 90-degree pulse length was 4 μs , with recycle delays of 3.0 s, and with an amplitude for the decoupling pulses of 45 kHz. The longitudinal relaxation time (T_1) values for ^{31}P were obtained using an inversion recovery pulse sequence. The ^{31}P 90-degree pulse length was set to 4.0 μs employing a 1.0 s recycle delay, and a high-power proton decoupling pulse of 45 kHz of amplitude; 14 inter-pulse delays were used varying from 1 ms to 32 s. The different contributions for each ^{31}P spectrum were deconvoluted using the Solids Lineshape Analysis module in TopSpin 4.2.0 program (Bruker).

Molecular dynamics (MD)

The interaction of melittin with the model membrane was studied with molecular dynamics simulations for three different systems containing a fully hydrated lipid bilayer in the absence or presence of melittin peptides. In these systems, both upper and lower leaflets of the bilayer consisted of 100 DOPE molecules and 25 DPPG molecules, maintaining the 4:1(v:v) ratio used for the experimental analysis. For every system, a thick layer of water molecules was added to minimize the effect of inter-bilayer interactions, with 60 water molecules per lipid molecule. The systems consisted of I) membrane only, II) membrane with 2 melittin chains, and III) membrane with 4 melittins chains. The initial configuration was prepared using CHARMM-GUI^{17–21}. Each of the melittin peptides in α -helical conformation (PDB ID: 2MLT) were placed side-by-side on the bilayer-water interface of one of the leaflets. All simulations were performed using the GROMACS package (version 2023.2)²². The CHARMM36m force field was employed to model the melittin peptides and lipid molecules while the TIP3P model was used for water. The bilayers were equilibrated for 6 ns and the production phase ran for 250 ns. For the production phase, the temperature was maintained at 323.15 K using a Nosé-Hoover thermostat. The pressure was set at 1 bar using a Parrinello-Rahman barostat, and a semi-isotropic coupling scheme was employed to control the pressure in the directions parallel and normal to the bilayer, independently. Electrostatic interactions were calculated with the particle mesh Ewald (PME) method. Lenard-Jones interactions were truncated at 1.2 nm with a smooth switching function applied in the range of 1.0–1.2 nm. The LINCS algorithm was used to constrain all bonds involving hydrogen atoms. The time step size was set to 2 fs. The density and order parameters for the acyl chains were calculated using the corresponding GROMACS scripts. The analysis of area per lipid, membrane thickness and diffusion coefficient were performed with MDAnalysis^{23,24}, LiPyphilic²⁵ and freud²⁶. The snapshots were visualized using PyMOL²⁷.

Results

Impact of Melittin on Membrane Fluidity, Lipid Packing, and Structural Integrity

Langmuir monolayers composed of DOPE:DPPG in an 8:2 molar ratio were prepared to model a simplified *E. coli* inner membrane. Their interaction with melittin was studied at L:P molar ratios of 10:1 and 30:1. Compression isotherms and compressibility modulus of these monolayers were obtained by using the Langmuir-Blodgett technique. The shape of the π -A isotherms shown in Figure 1a indicate that no phase transition occurs for any of the monolayers, and all of them exist in the liquid-expanded phase²⁸, as evidenced by the maximum compressibility modulus (C_s^{-1}) below 100 mN m⁻¹ (Figure 1b). The presence of melittin causes monolayer expansion, with lift-off areas increasing proportionally to the peptide concentration (Table 1). The consistent rise in mean molecular areas across all isotherms up to the collapse point suggests full integration of melittin into the monolayers²⁹. Despite this incorporation, the large-scale phase behavior of the monolayers at the air-water interface remains unchanged, with the monolayers staying in a liquid-expanded phase at both L:P ratios. The reduced collapse pressure at the 10:1 L:P ratio hints to a decrease in monolayer stability, which does not occur at the 30:1 ratio. The compressibility modulus analysis revealed that all three monolayers have similar in-plane elasticity behavior up to ~20 mN m⁻¹, differentiating at this point between the low and high melittin concentrations. The DOPE:DPPG and (DOPE:DPPG):MLT 30:1 monolayers show a similar trend, suggesting comparable stability and packing organization. In contrast, at a 10:1 ratio melittin disrupts the monolayer, increasing membrane fluidity and reducing structural organization, as demonstrated by the smaller maximum C_s^{-1} values (Table 1).

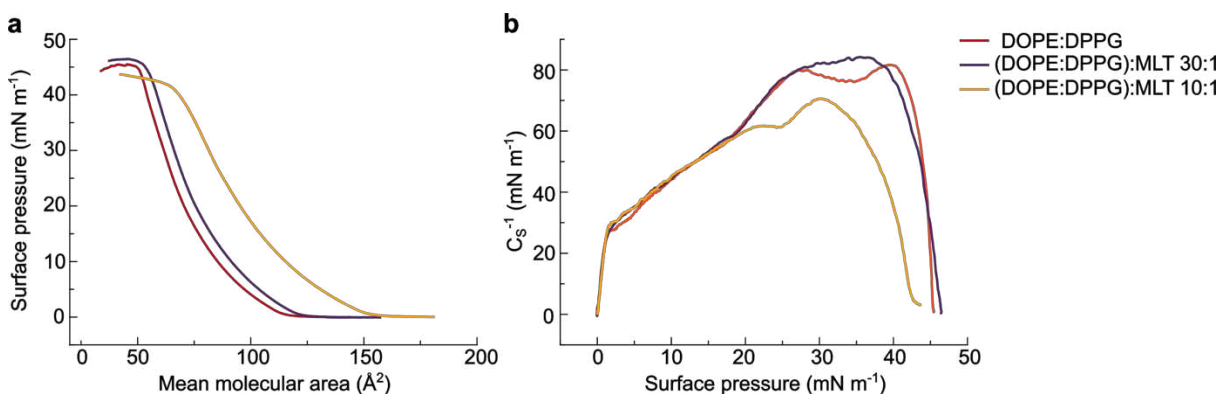


Figure 1. Interaction of melittin with lipid monolayers. a. Surface pressure–area isotherms of Langmuir monolayers on HEPES buffer subphase of DOPE:DPPG (red), (DOPE:DPPG):MLT in ratios 30:1 (purple) and 10:1 (yellow). The area per molecule was calculated assuming that molecules of DOPE, DPPG and melittin remain at the interface. **b.** Compressional moduli of the mixed (DOPE:DPPG):MLT monolayers on the HEPES buffer subphase.

Table 1. Parameters for the pure DOPE, DPPG, melittin and mixed (DOPE:DPPG):MLT monolayers on the HEPES buffer subphase, where A_{LO} = lift-off area, A_{30} = area at 30 mN m⁻¹, C_s^{-1} =compressional modulus and π_{col} = collapse pressure.

Composition	A_{LO} (Å ²)	A_{30} (Å ²)	Max C_s^{-1} (mN m ⁻¹)	π_{col} (mN m ⁻¹)
DOPE	125	74	101	46
DPPG	138	48	174	65
DOPE:DPPG	118	62	82	45
(DOPE:DPPG):MLT 30:1	127	66	84	46
(DOPE:DPPG):MLT 10:1	157	82	71	43
MLT	-	175	48	37

The effect of melittin on the interfacial properties of the lipid monolayers was also studied using PM-IRRAS spectroscopy, providing insights into the molecular organization of the model membranes. *Figure 2* presents the PM-IRRAS spectra of DPPG, DOPE, DOPE:DPPG and the (DOPE:DPPG):MLT monolayers at a surface pressure of 30 mN m⁻¹ in 5 mM HEPES buffer subphase. Lipid packing in monolayers was examined by analyzing the asymmetric (ν_{as}) and symmetric (ν_s) stretching vibrations of methylene groups, which describe the conformational order of lipid alkyl chains. Highly ordered hydrocarbon chains show asymmetric and symmetric CH₂ bands around 2920 and 2850 cm⁻¹, respectively³⁰, with higher frequencies indicating decreased conformational order. The spectra in *Figure 2* show that DOPE:DPPG monolayer has a more disordered hydrophobic core than DOPE and DPPG monolayers, as evidenced by an increase in the $\nu_{as}(\text{CH}_2)$ frequencies from 2918 to 2928 cm⁻¹ (*Table S1*). The lipid mixing induces disorder in the acyl chains due to their different chain lengths and headgroups, increasing the fluidity of the monolayer³¹, as confirmed by the decrease in the compressional modulus (*Table 1*). The presence of melittin at 30:1 L:P ratio slightly increases the acyl chains order, shifting the CH₂ stretching frequencies to lower values. In contrast, at a 10:1 ratio, the $\nu_s(\text{CH}_2)$ band broadens and decreases in intensity, indicating a decrease in the monolayer order³². This change in CH₂ frequencies, along with the changes in maximum C_s^{-1} and collapse pressure values in Table 1, are the first indicators of melittin acting via different mechanisms depending on its concentration.

Another interesting spectral region in *Figure 2* is between 1800–1000 cm⁻¹, where the phosphate group signals appear between 1300-1000 cm⁻¹ and the ester carbonyl band is seen around 1700 cm⁻¹. These bands are sensitive to changes in the interfacial region of the monolayer: frequency variations indicate different hydrogen-bonding states of the functional groups and are therefore related to changes in headgroup hydration³³. All mixed monolayers show a band around 1740 cm⁻¹, corresponding to non-hydrogen-bonded carbonyls buried in the hydrophobic region of the lipid arrangement. The 10:1 L:P monolayer shows additionally a second $\nu(\text{C=O})$ band around 1714 cm⁻¹. This lower-frequency peak is associated with hydrogen bonding between the carbonyl groups and water molecules^{34,35}, and is not observed for the sample with lower melittin concentration. The PO₂⁻ stretching bands are highly sensitive to phosphate group hydration. The

shift to lower frequencies of the $\nu_{\text{as}}(\text{PO}_2^-)$ band from 1227 cm^{-1} in DOPE:DPPG to 1224 cm^{-1} at the 10:1 L:P ratio, along with the shift of the $\nu_{\text{s}}(\text{PO}_2^-)$ band from 1065 to 1060 cm^{-1} indicates a higher degree of hydration of the phosphate group. This suggests that the interaction with melittin at this concentration changes the lipid headgroup environment, facilitating water penetration around the phosphate and carbonyl groups³³. In contrast, at a 30:1 ratio the $\nu_{\text{as}}(\text{PO}_2^-)$ band remains unchanged while the $\nu_{\text{s}}(\text{PO}_2^-)$ increases significantly from 1065 cm^{-1} to 1080 cm^{-1} . Because the asymmetric PO_2^- stretching mode is more sensitive to changes in hydration and local electric fields than the symmetric mode, the selective upshift in $\nu_{\text{s}}(\text{PO}_2^-)$ suggests that interaction with the melittin induces conformational or orientational changes on the phosphate group, such as a reduction in the motional freedom of the polar headgroups^{36,37}. The upshift is also consistent with dehydration of the phosphate groups, possibly due to competition between peptide and water molecules for bonding sites. Overall, these results indicate that melittin modulates the lipid headgroup region in a concentration-dependent manner, promoting increased hydration at higher peptide loading while inducing local reorganization at lower peptide concentrations.

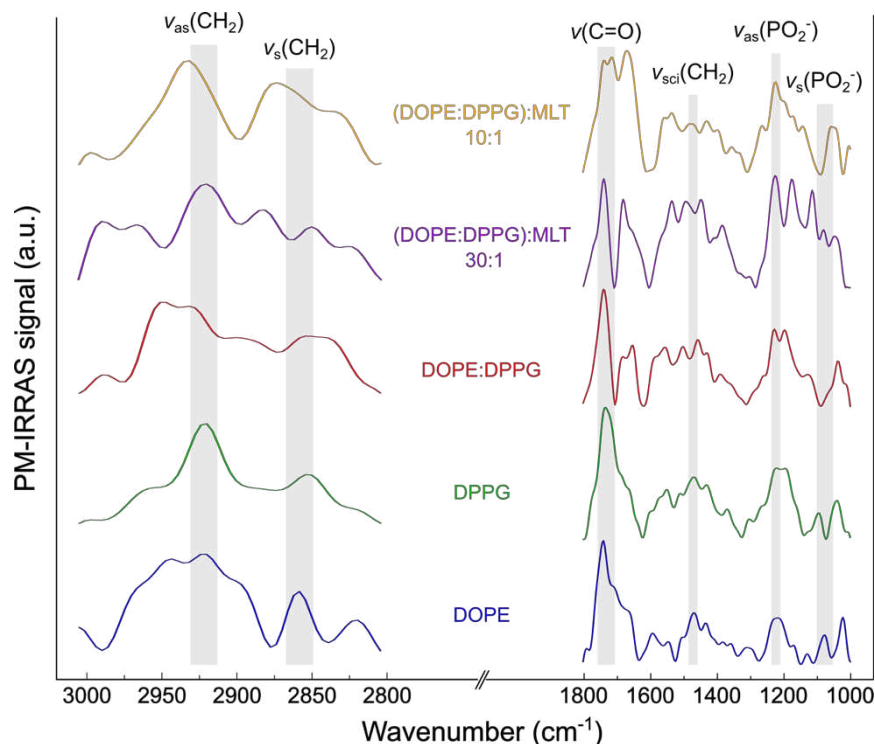


Figure 2. PM-IRRAS spectra of the DOPE (blue), DPPG (green), DOPE:DPPG (red), (DOPE:DPPG):MLT 10:1 (purple) and 30:1 (yellow) monolayers on the 5 mM HEPES subphase at $\pi = 30 \text{ mN m}^{-1}$, in the 3000–2800 and 1800–1000 cm^{-1} ranges.

Melittin-induced morphological changes at the microscopic scale

BAM was used to capture morphological changes of the monolayers. Figure S1 shows BAM images taken at various surface pressures for the three Langmuir monolayers. In the absence

of melittin the lipid mixture forms a film with bead-like domains, which become more noticeable near monolayer collapse. These brighter spots in Figure S1 correspond to condensed microdomains where the saturated lipids interact more strongly while coexisting within the LE phase favored by the majoritarian unsaturated DOPE composition. The general morphology remains unchanged with melittin incorporation, consistent with the surface pressure-area results, as all samples exist in the liquid-expanded phase^{38,39}. However, at a 30:1 L:P ratio, the bead-like domains appear earlier (*i.e.* at a lower surface pressure) and in higher numbers than they do for DOPE:DPPG and the 10:1 L:P, indicating a slight increase in lateral rigidity. This observation is in accordance with the very small increase in C_s^{-1} described previously and the increase in alkyl chain order observed by PM-IRRAS. In the case of the 10:1 L:P monolayer, the domains first appear at higher pressures than for the other two, and seem to be flattened and less contrasting, giving the monolayer a more uniform appearance. These features align with the increased acyl chains disorder and in-plane elasticity revealed by the compressibility modulus changes. At this higher concentration melittin seems to act as a fluidizing agent, as higher compression is required to induce lateral homogeneities that form easier at a lower melittin concentration³⁶.

AFM was used to further investigate the surface topography and nanoscale structural features of lipid thin films, providing information on film thickness and lateral heterogeneity that complements the insights from BAM. In addition to height imaging, AFM phase imaging was employed to probe local variations in viscoelastic and adhesive properties of the films. Although BAM and AFM probe different systems, their combined use provides a more detailed interpretation of melittin-lipid interactions under varied conditions, including AFM peptide-induced changes in the membrane nanomechanical properties⁴⁰. *Figure 3* shows that the lipid film thickness decreases drastically with increasing melittin concentration, indicating a thinning effect on the dry lipid film⁴¹. The effect of melittin is also evidenced in the topographical and phase images of the film (*Figures 4* and *S2*). In the absence of melittin, the surface morphology matches what is expected from a thick phospholipid dry film⁴², with multiple stacked lipid bilayers forming a heterogeneous surface with minimal height variations. As melittin concentration increases, the film undergoes progressive reorganization, reducing the lateral inhomogeneities. This change is observed as the fusion and homogenization of what were originally separate “stacks”, evidenced in *Figure 4*. The images for the 30:1 L:P and especially the 20:1 L:P ratio resemble reported ones for intermediate stages of a temperature-induced phase transition^{43,44}. This suggests that increasing melittin content could drive a phase transition from the gel to the liquid-crystalline phase, thereby leading to morphological changes in the films akin to those observed in temperature-driven transitions where there is fluidization of the bilayers. The thinning of the films with increasing melittin content suggests a homogenization effect, consistent with reports on binary and ternary lipid films interacting with melittin⁴⁵. Moreover, at the highest melittin content the average film thickness is in the range of 4 to 8 nm, similar to a single hydrated lipid bilayer⁴⁶. This observation provides further evidence of the disruptive effect of melittin on multilayered lipid stacks. However, it is less effective at perturbing a continuous bilayer supported on a solid substrate, most likely due to stabilizing adhesion forces.

AFM phase imaging is done simultaneously with topography imaging in tapping mode, and it allows to resolve features in soft samples that would otherwise be hidden. In the case of lipid samples, it can help identify compositional heterogeneities on the same surface that arise from different lipid domains or the presence of proteins or peptides^{47,48}. For the DOPE:DPPG sample, the film exhibits the coexistence of gel-fluid domains, characteristic of binary lipid mixture with distinct phase transition temperatures⁴⁹. This behavior is perturbed by the melittin at lower concentrations, and completely disrupted at higher concentrations. The phase image for the 30:1 L:P film in *Figure 4* shows that the system retains some of the original phase organization, similar to the peptide-free sample. However, when the peptide concentration is higher, the original compositional organization is severely disrupted. Furthermore, at the highest peptide concentration, small aggregates are also identified in the phase image that were not observed for lower concentrations. Hence, low melittin concentrations cause localized phase disturbances, as observed in BAM images, while simultaneously affecting film large-scale organization. Similar effects have been reported for pore-forming toxins from bacteria⁴⁹.

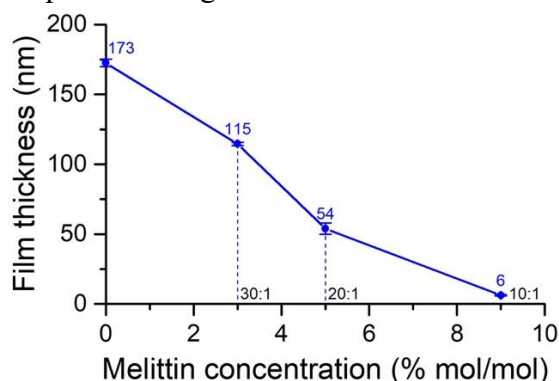


Figure 3. Effect of melittin on the thickness of solid lipid films. Lipid film thickness as a function of the molar concentration of melittin in the precursory solution. The equivalent L:P ratios are indicated at each concentration point.

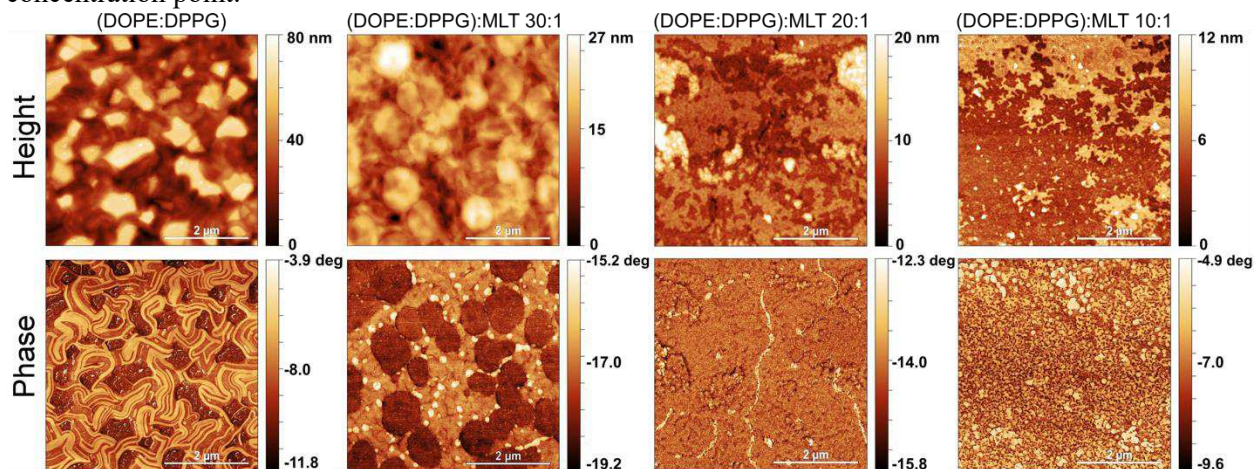


Figure 4. Interaction of melittin with thin lipid films. AFM topographical and phase images of the lipid films of DOPE:DPPG, (DOPE:DPPG):MLT 30:1, 20:1 and 10:1. Each subset corresponds to a 5x5 μm scan area, scale bar is 2 μm .

Specificity of melittin binding and molecular interactions

To further investigate melittin's interaction with phospholipid membranes, we employed multilamellar lipid vesicles (MLVs) as a model system. These vesicles were prepared in the same ratios as for the other membrane models to mimic the multilayered structure of cellular membrane in an aqueous environment. Using DLS and ssNMR we can correlate large-scale hydrodynamic changes with molecular level interactions, respectively. DLS analysis (*Figure S3* and *table S2*) showed that the DOPE:DPPG MLVs have a predominant population with an average hydrodynamic diameter of 890.9 ± 498.7 nm, with minor contributions from additional populations indicating some heterogeneity. For the 30:1 L:P ratio, the vesicle size distribution remains largely unimodal, with the major peak accounting for 86 % of the scattering intensity and a reduced average of 687.1 ± 311.8 nm. The 10:1 L:P MLVs exhibit a pronounced decrease in the dominant hydrodynamic diameter to 331.1 ± 105.1 nm, accompanied by an increase in the contribution from larger aggregates (up to 8.2% of the intensity). The progressive narrowing of the main population indicates increasing uniformity in vesicle size with increasing melittin concentration, while the intensification of the larger-sized population could be due to vesicle aggregation. This behavior is consistent with previous findings, where melittin has been shown to induce vesicle fusion or aggregation in L:P ratios as low as 250:1, particularly in liposomes containing above 70 % DOPE⁵⁰. The pronounced reduction of the hydrodynamic diameter with increasing melittin content is consistent with the bilayer disruption observed by AFM.

Solid-state NMR spectroscopy was used to investigate the effect of melittin on the MLVs at an atomic level, focusing on the ¹H, ¹³C, and ³¹P resonances. For the sake of comparison, all ¹H and ¹³C chemical shifts are listed in *Table S4*. Comparison of the ¹H spectra for MLVs with and without melittin reveals the most pronounced chemical shift changes for resonances associated with the phospholipid headgroups, glycerol necks and the atoms C2 and C3 (*Figure 5*). Analysis of this data suggests that melittin exhibits a stronger interaction with the polar regions than with other parts of the phospholipid molecule. The ¹³C spectra further confirms that melittin primarily interacts with the glycerol ester region and the DPPG headgroups of the phospholipids (*Figures 6*). The chemical shift change plot in *figure 6b* shows that the most significant changes ¹³C perturbations occur for the C2 and C3 positions, the g2 carbon, and the DPPG headgroup carbons, with all affected resonances following a similar trend for both melittin concentrations. Another important observation is the pronounced chemical change of the carbonyl signal (*Figure 6a, inset*), along with the appearance of a second component for the 10:1 L:P MLVs, indicating the presence of distinct local environments. In all cases, the magnitude of the chemical shift changes is larger at the 10:1 L:P ratio, indicating a stronger perturbation of the bilayer at higher melittin concentration.

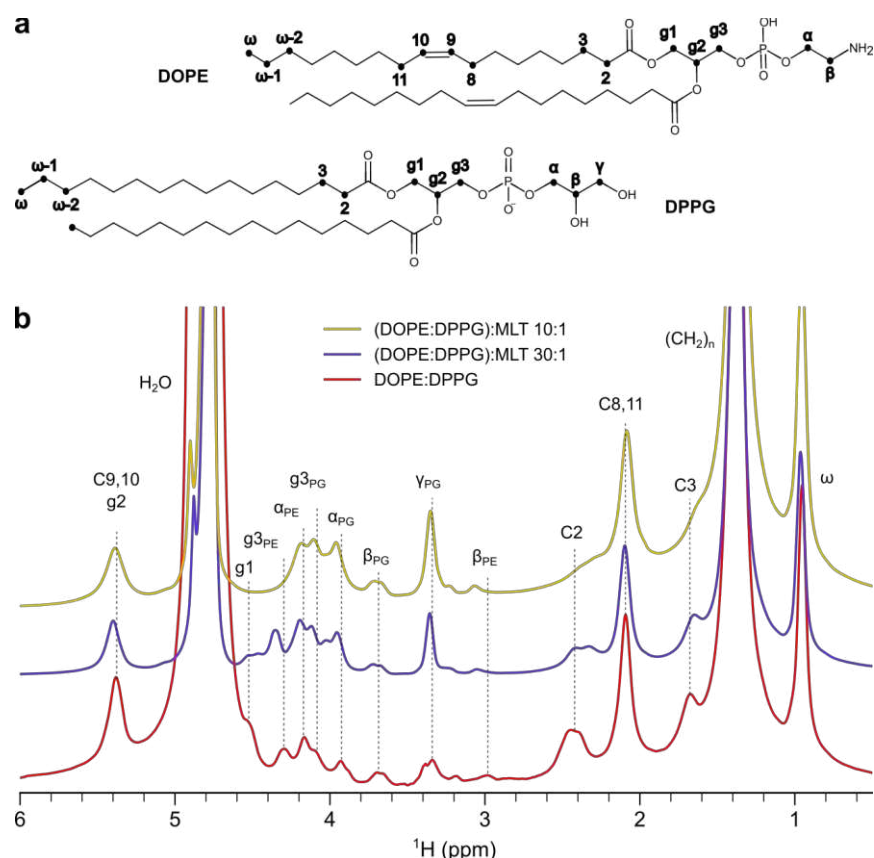


Figure 5. a. Structures of DOPE and DPPG phospholipids. **b.** ^1H MAS spectra of DOPE:DPPG, (DOPE:DPPG):MLT 30:1, and 10:1 L:P MLVs.

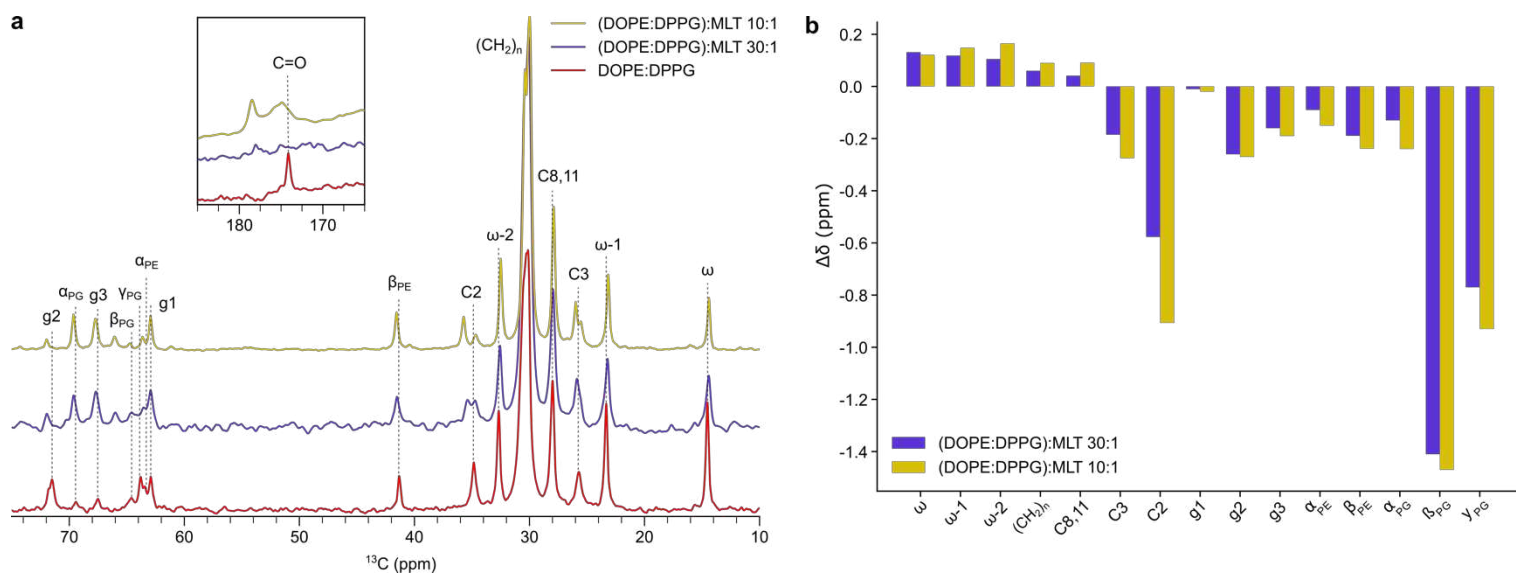


Figure 6. Effect of melittin complex MLVs. a. ^{13}C INEPT spectra DOPE:DPPG, (DOPE:DPPG):MLT 30:1, and 10:1 L:P MLVs; the inset shows the carbonyl-region from HPDEC spectra. **b.** Plot showing the relevant changes in the MLVs ^{13}C chemical shifts caused by their interaction with melittin.

The ^{31}P HPDEC spectra in *Figure 7* reveal noticeable changes in both intensity and band shape upon peptide addition. Deconvolution of the signals was carried out for all samples and in every case the resulting components were consistent with the composition of the prepared MLVs (80 % DOPE and 20 % DPPG). For DOPE:DPPG MLVs without melittin, the broad band arises from the overlap of the two phosphorus signals, with the DOPE contribution centered at -0.02 ppm and the DPPG contribution at 0.30 ppm. Upon addition of melittin at both concentrations, the spectra become asymmetric, and a distinct shoulder appears at higher chemical shift. For MLVs at both the 30:1 and the 10:1 L:P ratios, the DOPE and the DPPG signals move to higher chemical shifts; however, the magnitude of this change is more pronounced for DPPG. This observation is consistent with ^{13}C results, which indicate a stronger interaction of the peptide with PG headgroups compared to PE. This conclusion is further supported by ^{31}P T_1 results, which show that at a 30:1 L:P ratio the interaction with melittin leads to a reduction in the mobility of the DPPG phosphate group, evidenced by an increase of its T_1 . Previous works have shown that an increase in the phosphorus T_1 is commonly associated with increased lipid rigidity, reflecting a more restricted motion of the phosphate group relative to the glycerol backbone^{55,56}. At a ratio of 10:1 L:P this effect becomes less pronounced, although the T_1 value remains higher than for the melittin-free MLVs, indicating a persistently increased rigidity. The opposite tendency can be observed for DOPE, albeit with substantially smaller changes in T_1 compared to DPPG, reflecting a much weaker perturbation of the DOPE phosphate environment.

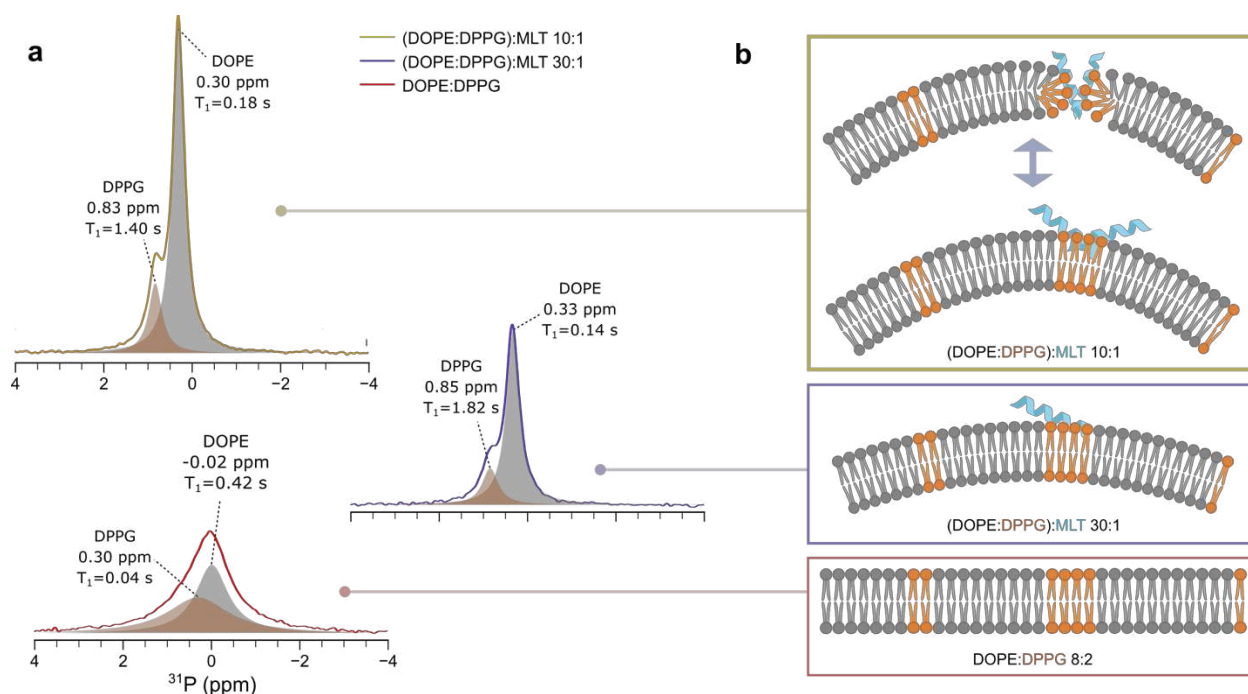


Figure 7. Melittin's preferential interaction with DPPG. a. ^{31}P HPDEC spectra of the DOPE:DPPG, (DOPE:DPPG):MLT 30:1, and 10:1 L:P MLVs. Each subset shows the chemical shift and T_1 values of the deconvoluted components assigned as DOPE and DPPG. **b.** Pictogram description of the preferential interaction of melittin with DPPG at different melittin concentrations.

Computational insight into melittin-lipid interactions

Molecular dynamics simulations were carried out on three models: a DOPE:DPPG (8:2) lipid bilayer (System I), the same bilayer with two melittin chains (System II), and with four melittin chains (System III) (Figure S4). These simulations were intended to capture trends in bilayer behavior as a function of peptide content. While direct comparison *between silico* models and experimental systems probed by different techniques is not straightforward, the simulations provide additional insight into the observed melittin-lipid interactions. Table 2 summarizes the MD results of the bilayers structural parameters for each system. Consistent changes are observed in membrane fluidity, bilayer thickness, area per lipid, and overall bilayer organization with increasing melittin content. The increase in membrane fluidity, reflected in the diffusion coefficients trend, indicated a progressive fluidization of lipid packing as peptide concentration increases^{51,52}. A gradual decrease in bilayer thickness is also observed as melittin concentration increases. This disruptive behavior is consistent with the AFM measurements showing reduced film thickness (Figure 3) at higher melittin content, as well as DLS results indicating a decrease in hydrodynamic diameter.

Table 2. Comparison of structural parameters of three lipid bilayers with zero, 2 and 4 melittin peptides, obtained by MD simulations (200 ns of sampling).

System	System description	Area per lipid ($\text{\AA}^2$)			Membrane thickness ($\text{\AA}$)	Diffusion coefficient ($\times 10^{-7} \text{ cm}^2/\text{s}$)
		Total lipids	DOPE	DPPG		
I	Lipid bilayer	73.01 ± 1.46	72.51 ± 1.04	75.00 ± 1.14	38.37 ± 1.13	3.22 ± 0.18
II	Lipid bilayer + 2 MLT	73.81 ± 6.61	72.73 ± 4.12	78.13 ± 11.27	37.76 ± 1.09	3.42 ± 0.19
III	Lipid bilayer + 4 MLT	74.68 ± 8.93	73.59 ± 7.96	79.06 ± 11.02	37.14 ± 1.07	3.64 ± 0.21

Another significant finding is the increase in the area per lipid, with a stronger effect observed for DPPG compared to DOPE. For DPPG, the area per lipid increases by approximately $4 \text{ \AA}^2$ from system I to system III, whereas DOPE exhibits a smaller increase of about $1 \text{ \AA}^2$. This trend in area per lipid obtained from the bilayer simulations mirror the one observed in Langmuir monolayers, where increases of $4 \text{ \AA}^2$ and $20 \text{ \AA}^2$ were measured for the 30:1 and 10:1 L:P ratios, respectively. Furthermore, the standard deviation of the area per lipid increases more drastically for DPPG, indicating that a higher sensitivity of this lipid to the presence of melittin; this preferential perturbation of DPPG is in agreement with the ssNMR observations. The two-dimensional projections of the area per lipid evolution over the simulation time shown in Figure 8 reveal that the largest increases coincide with melittin binding sites, consistent with localized lipid reorganization driven by peptide association. Increased membrane undulations were observed with higher melittin content, as shown in the snapshots in Figure 8. This structural phenomenon can also be pointed out in the density plots of the DOPE glycerol ester group, where the internal leaflet profile deviates from a standard Gaussian distribution and shifts toward the bilayer center (Figure S6).

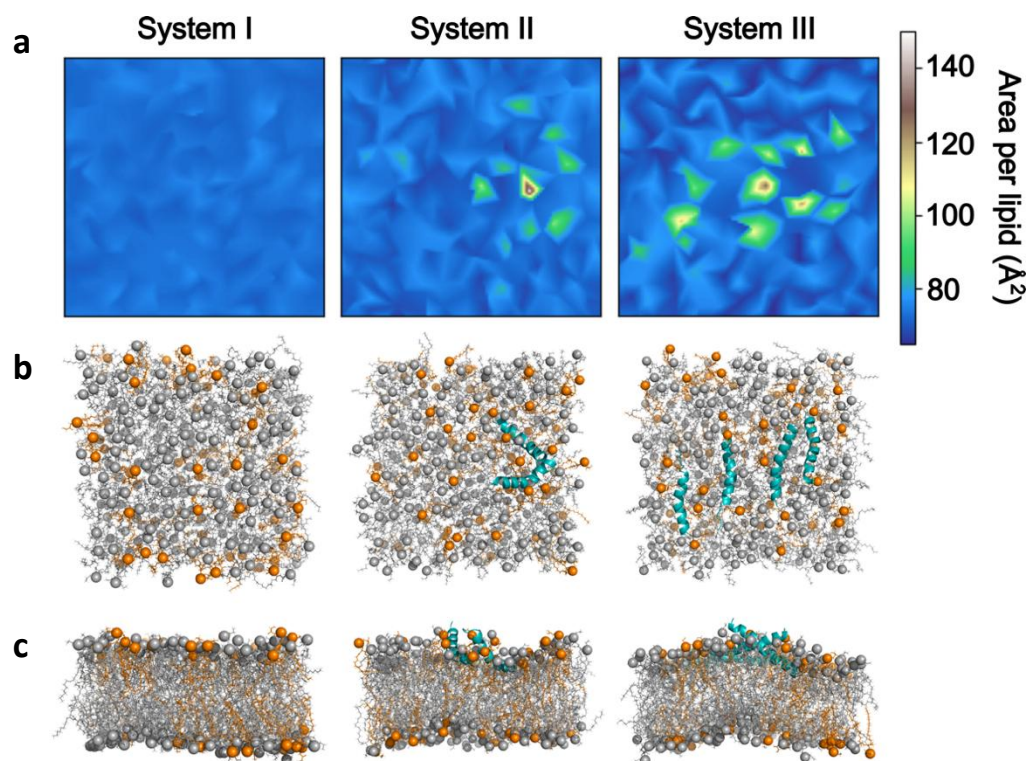


Figure 8. a. Projections of the area per lipid onto the xy plane for systems I, II and III. The areas per lipid were averaged over 200 ns of sampling based on the center of mass of each lipid. **b.** Snapshots from the top and **c.** transversal view at 150 ns of the MD runs for systems II and III. DOPE and DPPG phospholipids are shown in grey and orange, respectively. The P atoms shown are spheres. Water molecules and ions are omitted for clarity.

Figure 9 shows the acyl chain order parameters for all three systems, depicting the decrease in the order for all atoms with increasing melittin content. However, the magnitude of these changes is not sufficient to assume significant disruption of the bilayer hydrophobic center, consistent with ssNMR results and the unchanged chemical shifts observed for the signals corresponding to the acyl chains.

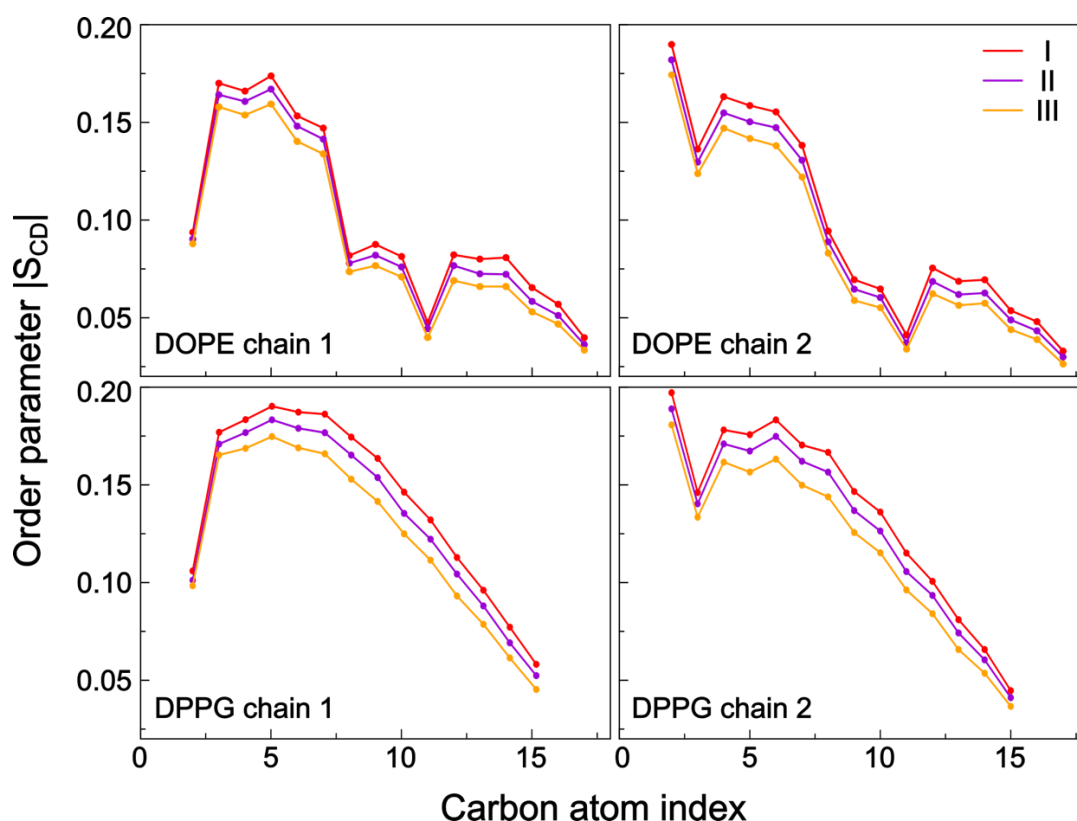


Figure 9. Order parameters $|S_{CD}|$ of systems I (red), II (purple) and III (orange) for both chains of DOPE and DPPG at 323.15 K as a function of the carbon atom indexes (as assigned in *Figure 5a*). The order parameters were averaged over 200 ns of sampling.

Discussion

We combined complementary experimental techniques and molecular dynamics simulations to study how melittin interacts with anionic membranes comprising DOPE:DPPG (8:2). By using membrane models that span different length scales and hydration states, we connect interfacial behavior, membrane morphology, and molecular-level dynamics to peptide concentration and, ultimately, to melittin mechanism of action. At low peptide concentration (30:1 L:P), melittin associates with the membrane without inducing major structural changes. Langmuir monolayers remain in the liquid-expanded phase, showing only slight expansion and minimal changes in compressibility. Brewster angle microscopy reveals morphologies very similar to peptide-free membranes, with only subtle changes in lateral organization. Spectroscopic measurements indicate that melittin primarily interacts with the interfacial region: PM-IRRAS and solid-state NMR (ssNMR) show preferential interaction with DPPG headgroups and reduced molecular motion of phosphate groups, without evidence of increased hydration or deep bilayer penetration. In particular, PM-IRRAS reveals a decrease in order in the upper acyl chain region together with a slight increase in order in the lower acyl chains, a pattern that has been associated with the carpet model, where peptides bind parallel to the outer leaflet and induce compensatory

ordering deeper in the bilayer⁵³. AFM, DLS, and MD simulations further support this picture, showing mild thinning, modest vesicle size reduction, and localized lipid rearrangements near melittin binding sites, while overall membrane continuity is preserved. The increased constraint of the phosphate groups and the stronger interaction with the interfacial region indicate that, at this ratio, melittin adsorbs parallel to the membrane surface and causes non-specific permeabilization without stable pore formation, consistent with a carpet-like mechanism⁵⁴ (as represented in Figure 7b).

At higher peptide concentration (10:1 L:P), the nature of the interaction changes. Langmuir monolayers exhibit stronger expansion, reduced collapse pressure, and increased acyl chain disorder, indicating enhanced membrane fluidity. BAM shows delayed and flattened lateral heterogeneities, while AFM reveals pronounced thinning and homogenization of lipid films. PM-IRRAS detects increased hydration of carbonyl and phosphate groups, suggesting water penetration into the interfacial region, and ssNMR reveals larger chemical shift changes and multiple local environments, particularly involving DPPG. MD simulations are consistent with these observations, showing increased lipid diffusion, larger area per lipid, enhanced bilayer undulations, and reduced acyl chain order. These combined features are compatible with the formation of toroidal pores, where peptides insert more deeply into the membrane and stabilize water-filled defects through lipid curvature and headgroup rearrangement. The toroidal-pore model has been long associated with melittin mechanisms of action. However, the absence of strong acyl chain chemical shift perturbations in ssNMR suggests that, rather than long-lived pores, melittin may induce highly dynamic and short-lived defects. This behavior is consistent with proposed kinetic variants of the carpet model, in which peptide adsorption continues until a transient pore forms, allowing peptide translocation across the bilayer before the membrane reseals^{55,56}. In this context, the increased hydration detected by PM-IRRAS and the marked increase in membrane fluidity would facilitate transient water penetration and curvature, while the lack of persistent acyl chain perturbations reflects the short lifetime of these pore-like states (Figure 7b). In our system, this scenario is further supported by DLS results, which show a much larger decrease in MLV size at higher melittin concentration, consistent with enhanced membrane remodeling and vesicle reorganization following peptide translocation.

The analysis of ³¹P spin-lattice relaxation times (T_1) provides insight on the peptide binding to the membrane. The molecular motion that dominates phosphate T_1 relaxation does not correspond to the motion of the lipid molecule as a whole, but rather to reorientation of the phosphate group relative to the glycerol backbone⁵⁷. This distinction is crucial, as it shows that melittin strongly constrains the motion of the phosphate headgroup through specific interactions at the membrane interface, without necessarily restricting global lipid mobility. To our knowledge, this type of relaxation-based analysis of peptide-lipid interactions has not been applied to antimicrobial peptides, and it provides a sensitive probe of headgroup-level dynamics that complements chemical shift and structural measurements.

Taken together, the results indicate that melittin acts via a concentration-dependent mechanism in DOPE:DPPG (8:2) membranes, a behavior that has been observed as well for

membranes of different compositions^{50,58–60}. At low peptide loading, melittin remains surface-bound. It perturbs lipid order asymmetrically across the bilayer and induces non-specific permeabilization consistent with the carpet model. At higher concentrations, increased hydration, fluidity, and membrane remodeling point toward the formation of transient toroidal-pore-like defects or a kinetic carpet mechanism involving short-lived pores and peptide translocation. Across all techniques DPPG is more strongly affected than DOPE, consistent with what the literature reports, and highlighting melittin selectivity towards anionic membranes. More broadly, the mechanism of action of antimicrobial peptides must be evaluated on a case-by-case basis, as it depends not only on the peptide but also on membrane composition, including headgroup chemistry, acyl chain length, degree of unsaturation, and lipid complexity. For the DOPE:DPPG (8:2) system studied here, the combination of interfacial, spectroscopic, microscopic, and computational techniques provides a coherent, multi-scale picture of melittin–membrane interactions. This integrated approach offers a robust framework that can be extended to other antimicrobial peptides and membrane systems, where different compositions may lead to varying mechanistic outcomes.

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