



# Disturbing Lipid Phase Equilibrium in Model Membrane Induced by Lytic Peptides

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## Abstract

Lytic peptides are rich in cationic and hydrophobic residues that form amphipathic  $\alpha$ -helix when in contact with lipid bilayers. Evidence showed that they act on the lipidic phase of the cell membranes, inserting into the lipid core and disturbing the lipid-packing. The insertion creates unbalanced elastic stresses in the outer and inner membrane leaflets that would be relieved by the opening of pores and/or defects and the consequent loss of the cell content. Here we compile results obtained in the investigations of the effects of three peptides on the lipid organization in model membranes whose compositions mimic the target plasma membrane of bacteria and cancer cells to which these peptides demonstrated lytic activity. These peptides have in their sequence the presence of both acidic and basic residues distributed such that they are third and fourth neighbors. We compiled results obtained from previous investigations with these peptides, using two main experimental techniques: lipid monolayers at the constant area and compression isotherms and differential scanning calorimetry. Here, we showed that the peptide-induced lipid packing perturbation was dependent on the structure of the polar head group and on the acyl chain.

**Keywords** Lytic peptides · Lipid-packing perturbation · Domain formation · model membranes

## 1 Introduction

Lytic peptides with antimicrobial properties are short amino acid sequences of up to 40 residues. These peptides belong to the innate immune system of almost all living species and act as the first line of defense [1, 2]. These sequences are rich in hydrophobic and basic residues, distributed along the sequence forming an amphipathic structure, mostly  $\alpha$ -helix or  $\beta$  structure when in contact with lipid membranes. Their cationic nature provides the selectivity for anionic membranes, the main characteristic of the outer leaflet of the bacteria plasma membrane. Experiments evidenced that their antimicrobial activity did not require specific membrane receptors [3, 4]. Molecular dynamic simulations, coarse-grained [5, 6] or all atoms [7, 8], and experimental results [9–11] evidenced that they only act on the lipid phase of the cell membrane.

Their mode of action remains elusive, despite several models proposed in the last decades reviewed in [10, 12, 13]. These peptides adsorb on the membrane–solvent interface, insert the non-polar face into the membrane core perturbing the lipid membrane organization. As a consequence, they induce unbalanced elastic stresses between the outer and inner membrane leaflets. Opening pores and defects relieve these induced stresses giving rise to the lytic process, the loss of the cell content [14]. From all the proposed models for the lytic activity of the peptide adsorption to the membrane solvent interface, the insertion of its hydrophobic face into the membrane hydrophobic core and the consequent perturbation of the lipid-packing are the most significant events that determine the peptide lytic efficiency.

In the present work, we compiled experimental results obtained in the investigation of the perturbation of lipid-packing and lipid phase equilibrium induced in model membranes by three antimicrobial peptides (AMP) Polybia-MP1 (MP1), L1A, and its acetylated analog, acL1A. The main characteristic of these peptides is the simultaneous presence of acidic and basic residues that are third and fourth neighbors in the sequence. The simultaneous presence of acidic and basic residues occurs among AMP with low frequency, and the low net charge at physiological pH confers very low

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cytotoxicity to MP1. MP1 (IDWKLLDAAKQIL-NH<sub>2</sub>), isolated from a native wasp [15], displays potent antimicrobial [15, 16], antifungal [17, 18], and antitumor [19, 20] activities and discriminate leukemic from healthy lymphocytes [21]. L1A (IDGLKAIWKKVADLLKNT-NH<sub>2</sub>) is a synthetic peptide with selective bactericidal activity to Gram-negative bacteria without being hemolytic [22, 23]. We compiled results obtained from the association of three main experimental approaches: lipid monolayers at both variable and constant area and differential scanning calorimetry (DSC). The model membrane lipid composition mimics the target plasma membrane of bacteria and cancer cells to which these peptides demonstrated lytic activity; for Gram-positive bacteria, anionic phospholipid phosphatidylglycerol (PG), and for Gram-negative bacteria, zwitterionic mixed with anionic phospholipids: PC/PG and PE/PG. PC is phosphatidylcholine, and PE is phosphatidylethanolamine. For cancer cells, PC/PS once cancer cell membrane loses the asymmetric lipid distribution of the healthy cells and exposes PS (phosphatidylserine) in its outer leaflet [24].

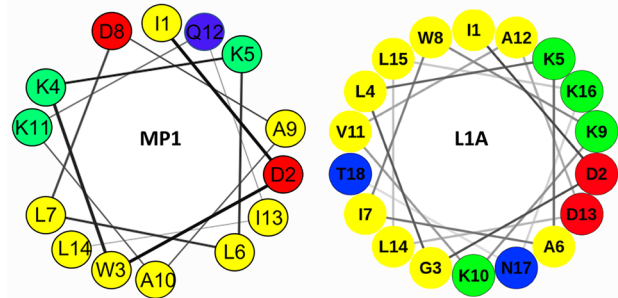
## 2 Peptide Insertion Into Lipid Monolayers

Lipid monolayer is an important and simplified model for the membrane solvent interface [25–29]. Experiment at a constant area peptide injected into a subphase beneath a lipid film provides valuable information about the peptide adsorption and the induced effects on the monolayer. Peptides with interfacial activity insert into the lipid film leading to an increase in the film surface pressure.

Experimental evidences suggest that the peptides MP1 [30], L1A, and acL1A [31] display significant surface activity as also observed for other antimicrobial peptide [25, 32]. The similarity of the peptides studied here is the concomitant presence of both acidic and basic residues distributed such that they are third and fourth neighbors (Fig. 1). The synthetic sequence, L1A, is four residues longer and has an extra lysine providing higher net charge. The relative positioning of acidic and basic residues is maintained the same to that of MP1 (see Fig. 1). The neutralization of the positive charge of the N-terminus by acetylation (acL1A) significantly enhanced its lytic activity in mixed anionic vesicles [23]. In this section we compiled results showing the effect of lipid charge and packing on peptide ability to insert into lipid film.

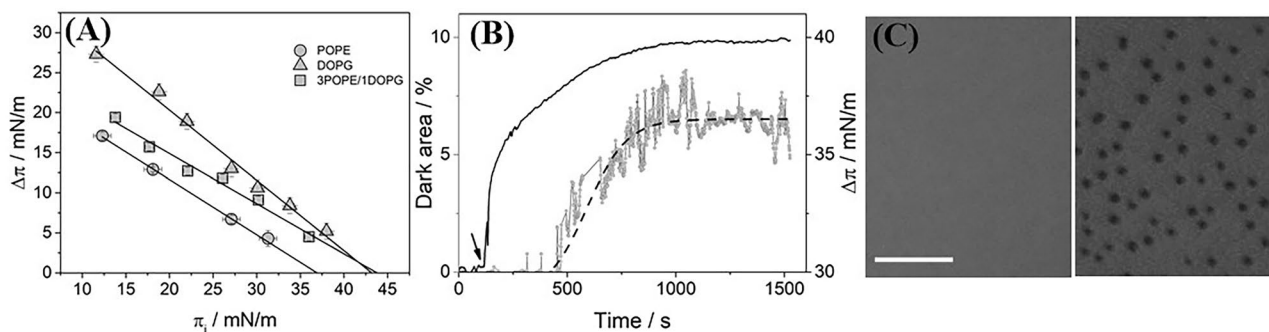
For lipid films with different initial surface pressure ( $\pi_i$ ), the injection of the same peptide concentration provides different changes of the maximal surface pressures ( $\Delta\pi_{max}$ ). A  $\Delta\pi_{max}$  vs  $\pi_i$  plot is in general linear as shown in the Fig. 2A. The negative slope in this plot indicates that as the lipid film becomes more densely packed (higher surface pressure) is more difficult for peptides to insert into the film.

	Sequence	Q	N <sub>R</sub>	<H>
MP1:	IDWKLLDAAKQIL-NH <sub>2</sub>	+2	14	-0.11
L1A:	IDGLKAIWKKVADLLKNT-NH <sub>2</sub>	+3	18	-0.11
acL1A:	ac-IDGLKAIWKKVADLLKNT-NH <sub>2</sub>	+2	18	-0.10



**Fig. 1** Structural features of the peptides and helical wheel plot of amphiphatic helix: amino acid sequences of the peptides, the net charge at physiological conditions (Q), number of residues (N<sub>R</sub>), and the hydrophobicity (<H>) [23, 33]. The helical wheel representation was created from the webserver Netwheels (<http://lbq.uncb.br/NetWheels/>) [34]; green represents positively charged; red, negatively charged; blue, polar uncharged and yellow, hydrophobic residues

The extrapolated  $\pi_i$  value to zero change in the surface pressure ( $\Delta\pi \rightarrow 0$ ) defines the film maximum insertion pressure (MIP) above which insertion is no more observed. MIP quantifies the peptide capability to adsorb and insert into a real plasma membrane whose lateral pressure ranges from 30 to 35 mN/m [35]. As MIP exceeds these values, the peptide probably will insert into the lipid membrane [28]. The plots in Fig. 2A shows that acL1A was able to insert into 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol (DOPG), and 3POPE/1DOPG lipid films. This mixture contains the main phospholipids of the Gram-negative bacteria plasma membrane. MIP was similar for both peptides and higher in the presence of anionic lipids indicating that peptide incorporation was favored by negatively charged surfaces [36]. Molecular dynamics simulation [23] and experimental [22, 31] results evidenced that this peptide adsorbs to the lipid membrane by inserting its N-terminus. Electrostatic plays a central role in the peptide insertion into a lipid film. L1A, acL1A, and MP1 discriminate the zwitterionic from the anionic lipid monolayers, reflecting the low affinity and low lytic activity of these peptides in neutral membrane [22, 31]. These peptides also discriminate the type of polar head group. Although PG and PS have the same net charges (−1), these polar head groups have different structures. PG has one discrete charge (phosphate). PS has two negative charges (phosphate and carboxyl) and one positive charge (amino group) and forms two electrical dipoles. The affinity and lytic activity of MP1 is significantly higher for PS-containing membranes [37, 38]. The peptide capability in inserting into a lipid film is also dependent on the acyl chain. The insertion



**Fig. 2** Adsorption of peptide onto lipid–water interface: **(A)** Representative plots for the maximum change of surface pressure ( $\Delta\pi$ ) upon injection of acL1A beneath the monolayers, measured as a function of initial surface pressure ( $\pi_i$ ) of pure POPE (circle), pure DOPG (triangle) and 3POPE/1DOPG mixture (square) at 20 °C. Continuous lines represent linear regressions. **(B)** Representative adsorption kinetics of acL1A (black line) and change in the dark area (light gray circles connected) upon injection of the peptide (indicated by arrow)

into a lipid film of unsaturated acyl chains 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS)) and the same polar head group has significantly higher MIP in comparison with lipid films with saturated chain (1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (DPPS)) two carbons shorter than oleoyl chain [30].

In this regard, Hadicke and Blume investigated the effect of polar head group charge, acyl chain length, and saturation on the cationic peptide (KL)4K penetration [39]. They showed that peptide binds to the interface of negatively charged lipid monolayers due to electrostatic interaction in a way that depends on the lipid head group structure, size, and acyl chain length.

The insertion of peptides into a lipid film disturbs the liquid expanded-liquid condensed equilibrium that can also be investigated using a small home-made circular trough equipped with an optical window and observed under an optical microscope. Depending on surface pressure, the monolayers of phospholipids with saturated chains can present two distinct phases: liquid and condensed. Fluorescence microscopy is a means to visualize the microscopic condensed domains and the effect of peptides in disturbing these two phases. Figure 2B shows the kinetics of surface pressure change of 3POPE/1DOPG film at  $\pi_i = 30$  mN/m. The arrow indicates acL1A injection into the subphase. This figure also shows that the total dark area in the image, corresponding to the condensed domains area, increases due to the penetration of peptides. Figure 2C shows fluorescence images of the lipid film in the absence of peptide and 1000 s after the peptide injection. At this surface pressure, the acetylated analog induced the formation of solid domains, the dark spots, that will only be visualized at above 30 mN/m (see next section). The gel-to-liquid crystalline phase transition

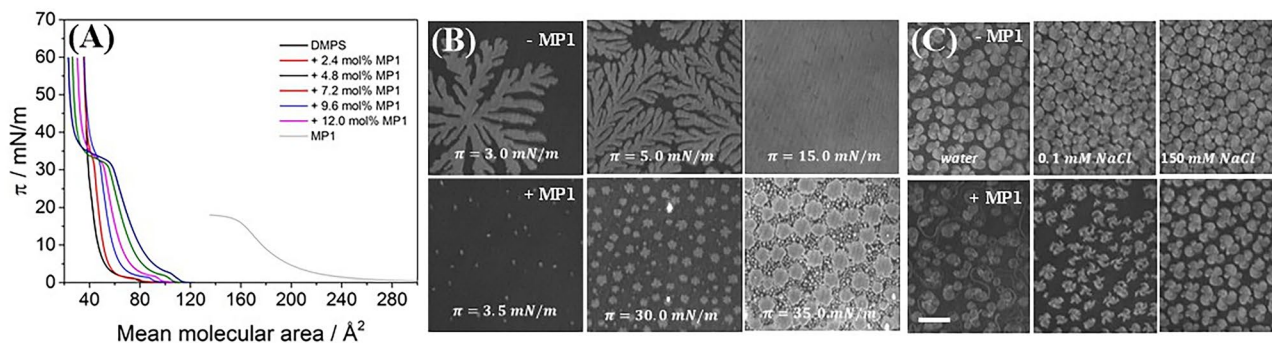
into a 3POPE/1DOPG monolayer, spread at  $\Delta\pi = 30$  mN/m. The dark area percentage was determined by a sigmoidal function (black dashed line). **(C)** Representative fluorescence microscopy images at 1000s before (left) and after (right) acL1A injection into the sub-phase. In these experiments, the lipid film contained a small fraction of fluorescently labeled lipid (Texas-Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (TR-DHPE)). The scale bar represents 50  $\mu$ m. Adapted from [36] with permission

temperature of POPE is 25 °C while DOPG is below zero and that of the 3POPE/1DOPG mixture is around 15 °C. The temperature of experiments of Fig. 2B and C was 20 °C. The condensed phase is hardly observed at this temperature even at higher surface pressure. The dark spots induced by the peptides indicate that the peptide induces lipid segregation most likely sequestering the DOPG lipids resulting in a peptide/DOPG-rich liquid phase, POPE-rich liquid phase, and a pure POPE-rich condensed phase.

### 3 Peptide Effect on the LC-LE Equilibrium in Lipid Films

Liquid-condensed/liquid-expanded phase equilibrium characterizes the lipid-packing in monolayers as a model membrane. In lipid monolayers, especially those with saturated acyl chains phospholipids, the hydrophobic chains get more regularly ordered under compression, characterizing a transition from a liquid-expanded phase (LE) to a liquid-condensed (LC) one [40, 41]. Compression of lipid films is a powerful tool to investigate how the peptide affects the membrane lipid-packing. Here, we choose 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (DMPS) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) lipids that displays the LE-LC coexistence plateau and explored the effect of MP1 on the phase behavior. PS was found in the outer leaflet of cancer cells and PC is a neutral lipid characteristic of healthy cells.

Figure 3A shows compression isotherms, surface pressure vs mean molecular area ( $\pi$  vs A) for films of pure DMPS and in mixtures with different molar fractions of MP1. For larger molecular area and near-zero surface pressure, the



**Fig. 3** Peptide-lipid co-spread onto the interface: (A) surface pressure-area compression isotherms for DMPS co-spread with increasing amounts of MP1 onto the water surface at  $T = 20^\circ\text{C}$ . (B and C) Representative BAM images for monolayers of pure lipid (above), and for

mixtures of DMPS/MP1 (B) or DPPC/MP1 (C) (bottom) spread onto pure water and registered during compression at the indicated surface pressures. Image size in (B):  $200 \times 200 \mu\text{m}^2$ . The scale bar represents  $50 \mu\text{m}$  in (C). Adapted from [30] and [43] with permission

lipid film is in a highly disordered phase (gas phase) up to molecular areas around  $90 \text{ \AA}^2/\text{molecule}$ . Increases in surface pressure accompany the compression up to approximately  $3.0 \text{ mN/m}$  and characterize a liquid-expanded (LE) phase. Increasing compression, the pressure is leveled at this value while the molecular area decreases from  $80$  to  $60 \text{ \AA}^2/\text{molecule}$ . This plateau, which resembles a first-order phase transition, corresponds to the coexistence of LE and LC phases. The surface area increases quickly for further compression. Lipid film becomes less compressible that characterizes a condensed (solid-like) phase. Peptides co-spread with lipids stabilizes the disordered phase, and the extent of the coexistence plateau decreases. At pressures around  $32 \text{ mN/m}$ , a peptide-enriched-phase gives rise to a second plateau that is  $15 \text{ mN/m}$  higher than the collapse of the pure peptide film. Above this second plateau, the molecular areas are smaller than the pure lipid film area, indicating that the monolayer loses part of lipid and peptides. Visualization of film compression assessed by Brewster angle microscopy (BAM) revealed large leaf-like or dendrite-like condensed domains whose sizes increase in proportion to the surface pressure. MP1 significantly affected the size and shape of condensed domains, probably inducing a decrease in the molecular diffusion to the domain growth and/or lowering the line tension [42]. These results indicated that electrostatic attraction between MP1/PS film appears as a membrane remodeling factor, inducing phase equilibrium displacement and thinning of the membrane as estimated from BAM images [30]. Additionally, MP1 partitions preferentially into the liquid expanded (LE) phase.

MP1 showed diverse effects in DPPC films. For these monolayers, the coexistence plateau occurs at  $5 \text{ mN/m}$ , and the peptide displaced the compression isotherms for larger molecular areas as those observed for DMPS. Figure 3C shows BAM images at surface pressures around that of the LE-LC plateau. BAM images showed triskelion-shaped

condensed domains for water and salt subphases (up to  $150 \text{ mM NaCl}$ ). MP1 affected the shape and size of these domains dependent on both subphase conditions salt and pH. In pure water and  $0.1 \text{ mM NaCl}$ , MP1-induced smaller long thin branches in the triskelion-shaped domains.

The shape of domains is determined by the competition between the line tension and dipole moment difference between LE and the domain phase [44]. In the case of elongated and curved domains formed in chiral molecules, Krüger and Lösche introduced a term in the domain free energy indicating that these shapes occur due to preferential orientation of molecules inside the domain. In these subphases, we hypothesized that the peptide co-crystallizes with lipids. The polar face of a peptide makes electrostatic interaction with a neighbor peptide, and the acidic and basic residues establish saline bridges while their non-polar faces are in contact with the lipid acyl chains [43].

The effect of the synthetic peptides, L1A and its acetylated analog, was also explored in lipid films composed with neutral and/or anionic lipids with different head groups (PC, PE and PG) and acyl chain (DPPG and POPG).

L1A and acL1A induced a similar impact in DPPC films as evidenced by MP1 [31]. In the presence of the anionic lipid, 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG), both peptides disrupted the anionic lipid monolayer to a greater extent when compared to DPPC. Visualization of DPPG/peptide mixed films by fluorescence microscopy revealed that, in pure water subphase, DPPG is in a condensed state. Both peptides were able to drag lipid molecules to a more expanded phase, increasing lipid disorder. This effect is more pronounced for the acetylated analog. In saline condition ( $150 \text{ mM NaCl}$ ), DPPG-pure monolayers display LE-LC phase transition due to change in the ionization state of the PG groups. Both peptides induced an increase in the LE-LC pressure transition indicating stabilization of the LE phase. These experiments revealed that the configuration

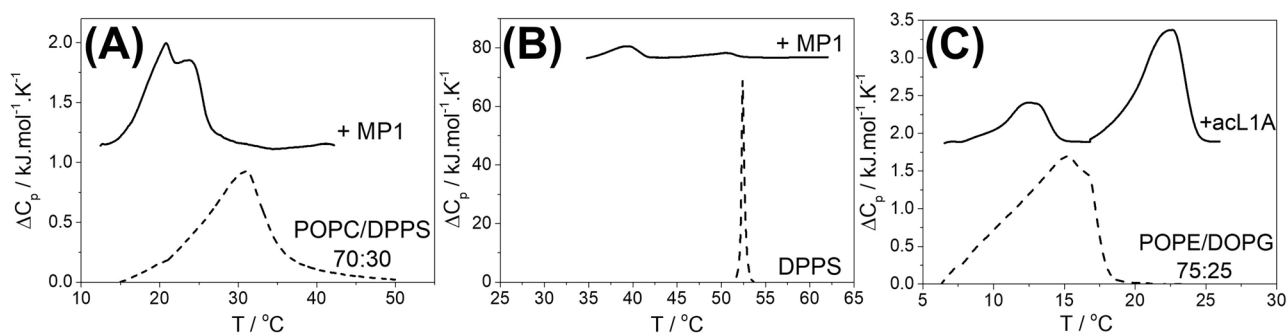
adopted by the peptide in which salt bridges between the acidic and basic residues occur plays a role in the effect promoted by the peptide inside the membrane. When peptide/peptide interaction is favored, peptide molecules coexist with lipids in a more dense phase. Ionic strength disfavors the salt bridges, and the peptide molecules stay in a more fluid phase. Results obtained from peptide/PC/PG isotherms showed that incorporating both peptides into the monolayers induced an increased lipid disorder and prevented the formation of stiff films.

L1A and acL1A also affected POPE, DOPG, and 3POPE/1DOPG mixed lipid monolayers [36]. Compression isotherm of pure POPE display LE-LC phase at 35 mN/m (pH 7.4, 150 mM NaCl, 20 °C). The dense regions induced by L1A and acL1A remained at the interface up to the peptide squeeze from the interface. The area occupied by these dense regions was similar to the theoretical area occupied by acL1A, while L1A showed the lowest values. This result suggests that acL1A demixes from POPE while L1A could be partially mixed. Interestingly, this effect remained at a higher temperature (30 °C), in which the POPE monolayer displays only the LE phase. The analysis of the mixture compression isotherm revealed that both peptides interacted strongly with DOPG and remained at the interface up to film collapse pressure. These isotherms showed only a liquid-expanded phase consistent with the liquid-crystalline state at this temperature (see next section). The absence of dense regions in the PE/PG/peptide system suggests that both peptides could mix with lipids and stabilize at the interface, provoking film collapse at lower surface pressure. Considering the system with LE-LC phase transition (at 10 °C), both peptides induced a reduction in the surface pressure of LE-LC phase transition at a pressure above the peptide exclusion to subphase. We hypothesized that film compression squeezed DOPG and peptide molecules from the interface remaining attached to the water/lipid head group interface. The

exclusion of PG molecules from the interface would lead to an increase in the amount of PE and then provoke a decrease in the surface pressure of the LE-LC phase transition. L1A and acL1A were soluble in DOPG while they did not mix with POPE promoting lipid segregation by recruiting PG away from PE.

#### 4 Effect of Peptides on the Gel-liquid Crystalline Phase Transition

Differential scanning calorimetry (DSC) is a sensitive technique that reveals information about the effect of small molecules on the lipid phase transition [45, 46]. We explored the effect of MP1 in the thermotropic behavior of the mixture POPC/DPPS that mimics the outer leaflet of cancer cells. The phospholipids POPC and DPPS have very different gel-to-liquid crystalline phase transition  $T_m = -10$  and 52.5 °C for POPC and DPPS, respectively. The thermogram of the mixture 7POPC/3DPPS showed a single broad peak centered at 35 °C. The broad peak observed for the mixture indicates stabilization of the fluid phase. MP1 induced the displacement of the main transition to 20 °C and two shoulders centered at 22 and 24 °C, as shown in Fig. 4A. The POPC/DPPS/MP1 thermogram indicated peptide-induced lipid segregation. The preferential binding of MP1 to DPPS induced a rich DPPS+MP1 phase and a POPC-rich phase, reducing, consequently, the main transition temperature. The decrease in the phase transition temperature also indicates destabilization of the lipid membrane due to the insertion of the peptide into the hydrophobic region of the membrane, disturbing the lipid packing. MP1 also impacted the phase equilibrium in DPPS vesicles. Pure DPPS thermogram shows a narrow peak at 52.5 °C and  $\Delta H_m = 38.2$  kJ/mol. In the presence of MP1 at [L]/[P] = 15 ratio, the thermogram shows two peaks: one at 50.6 and the other around 39.0



**Fig. 4** Change in the lipid thermotropic behavior in anionic vesicles induced by the peptides. DSC heating thermograms of MLVs composed of 7POPC/3DPPS (A), pure DPPS (B), and 7POPE/3DOPG in the absence (dashed line) and presence (continuous line) of indicated peptide at [L]/[P] = 15 acquired at 0.5 °C/min. The MLVs were

obtained by hydrating the lipid film with a buffer (20 mM HEPES, 1 mM EDTA, 140 mM NaCl, pH = 7.4) (details of preparation see [36]) (C) was extracted from [36] with permission. The data showed in (A) and (B) were not published

°C shown in Fig. 4B with enthalpies 6.7 and 14.2 kJ/mol, respectively. This result suggests that MP1 binds to DPPS and induces two phases in the bilayer. One of pure DPPS with higher transition temperature and another of MP1 and DPPS stabilizing the fluid phase. This result is in line with that observed in compression isotherms under the same conditions in which the monolayer compressibility module was less than 100 mN/m for pressures of 30 mN/m [30].

The preferential interaction for anionic lipid was also evidenced by other peptides such as magainin 2 [47], buforin II [48], gramicidin S [49], and LL-37 [50].

We also explored the effect induced by L1A and acL1A on monolayers DPPC, which mimic the lipid composition of mammalian membranes, and PC/PG or pure PG, which mimic the plasma bacterial membrane of Gram-negative bacteria in order to investigate the effect of the anionic charge.

The peptide acL1A also impacted the thermotropic phase behavior (the phase transition) of pure DPPG and 8DPPC/2DPPG mixture. The reduction of N-terminal charge by acetylation influenced the peptide effect on the lipid organization [31]. AcL1A induced a decrease in the enthalpy of the gel-to-liquid crystalline transition of DPPG,  $\Delta H_m \sim 19$  kJ/mol at  $T_m$  1.7 °C higher than for pure lipid. AcL1A significantly reduced in 13 kJ/mol the enthalpy of the 8DPPC/2DPPG mixture transition. The most charged peptide L1A affected only marginally the enthalpy and the temperature of both pure DPPG and the mixture. These results indicate that the acetylated analog was more efficient in disturbing the lipid-packing of DPPG and 8DPPC/2DPPG, inserting more deeply into the hydrophobic core [51–53]. This result agrees with the evidence from the compression isotherms that the analog drags PG molecules to the LE phase and stabilizes this phase. Furthermore, this result is consistent with both a deeper insertion observed in unilamellar vesicles and the effect on the mechanical properties of giant vesicles [31].

L1A and acL1A also affected the phase equilibrium of the 3POPE/1DOPG mixture, the main component lipids of Gram-negative bacteria plasma membrane, such as *E. coli*. As evidenced in Fig. 4C, AcL1A induced the gel-to-liquid crystalline transition peak to split into two. One at low temperature and another at high-temperature corresponding to liquid phase rich in DOPG/peptide and another of pure POPE. AcL1A induced symmetric phase separation with lower enthalpy in comparison to L1A, indicating that the acetylated analog induced segregation of the anionic lipid more efficiently than L1A. Again this result agrees with monolayer experiments, showing that acL1A mixed with DOPG and demixing from POPE. It is worth noting that lipid segregation is, in general, observed for highly charged peptides [54]. Interestingly the less charged peptide was more efficient in inducing lipid segregation. Using

this model system a similar behavior was also observed for LL-37 [50], HNP-2 [55] and PGLa [56] peptides.

Similarly to PE/PG/acL1A system, the addition of the antimicrobial peptide cWFW also induced a reduction on membrane fluidity followed by the domain formation *in vivo* and *in vitro* assays [11]. The same authors evidenced the cWFW peptide can segregate PE from PG model membrane [57, 58].

## 5 Concluding Remarks

In this work, we gathered experimental evidence of the effect of lytic peptides on lipid-packing in model membranes. These peptides present intense antimicrobial activity. One showed a selective affinity to cancer cells displaying intense inhibitory activity in the proliferation of leukemic lymphocytes and did not harm the healthy ones. Despite their low net charges, the experiments in lipid monolayers and differential scanning calorimetry evidence that they induced lipid segregation a liquid phase enriched by the peptide and another gel or solid-like pure lipid. Lipid segregation was dependent on the structure of the lipid polar head and the length of the acyl chain. The perturbation induced in the lipid-packing correlates with the lytic efficiency of these peptides.

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**Author Contributions** D.S.A. and J.R.N contributed to conceptualization, methodology, investigation, writing—original draft preparation, and writing—review and editing.

## Declarations

**Ethics Approval** Not applicable.

**Consent to Participate** Informed consent was obtained from all individual participants included in the study.

**Conflicts of Interest** The authors declare that they have no conflict of interest.

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