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Membrane-peptide interaction: Focusing on membrane properties

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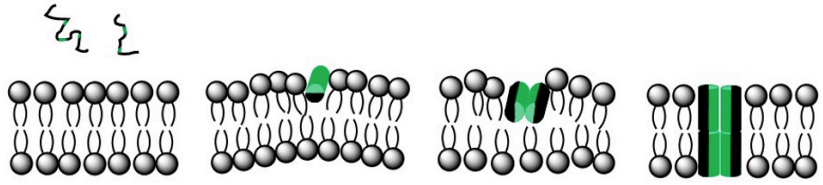
Abstract

Cellular membranes compartmentalize cells, comprise a permeability barrier, and are the starting place for several signaling cascades and processes in which lateral diffusion of molecules is a key factor. Although it has been shown that organisms adapt the lipid composition of their membranes in order to maintain these in a mainly fluid state, several studies point to the coexistence of regions with different compositions and mechanical properties. In this context, while proteins have been related to solid docks, sterols are accepted as liquid-ordered phase state inducers. Thus, the current model for membranes is a patchwork-like surface, with the different regions being highly variable in size and very dynamic.



Many peptides, like cationic antimicrobial peptides and cell penetrating peptides, target cell membranes. The affinity of these soluble peptides to membranes depends on membrane features such as composition, charge density, compaction, and fluidity. As a consequence of the patchwork-like character of the membrane, regions with a broad spectrum of properties are available to interact with these peptides. Therefore, it is important to know how peptide-membrane interaction depends on membrane properties, and also what happens with the membranes after the interaction.

Here, we summarize our contribution to understanding how the interaction of peptides with membranes is modulated by membrane properties. The influence of the phase state, electrostatics, and chemical composition of the membrane on peptide binding is described using biomimetic systems. The effect of peptide association on membrane properties is also revisited. Finally, possible extrapolations to cells are discussed.

Keywords: Membrane adaptation, membrane physicochemical properties, Antimicrobial alternative drugs, Membrane-active peptides, pore formation

Introduction

Cell membranes are composed of lipids and proteins, and the accepted model proposes that they are formed by a lipid matrix in which proteins insert. This model dates from 1972 [1] and is known as the Fluid-Mosaic Membrane (FMM) model[1]. It was envisioned as a basic framework model for cell membranes that could explain existing data on the behavior of membrane proteins and lipid structures, and their dynamics. Within a few years of its introduction, it became obvious that the FMM model needed to be modified to reflect the emerging data on extracellular and intracellular mechanisms. Therefore, with the FMM model as a basis, novel cell membrane models emerged, proposing a much less homogeneous view.

The current model considers the interaction of the extracellular matrix and membrane-associated cytoskeletal components with cell membranes, and their potential influence on the mobility and distribution of trans-membrane glycoproteins, as well as the possibility that less mobile lipid-protein or lipid-lipid domains might exist in membranes, like frozen or semi-frozen islands of less mobile lipids in a sea of fluid phospholipids [2]. This model has been tested in recent decades with single particle tracking experiments and super-resolution microscopy, among other techniques, and it has been concluded that, although fluidity is detected at the mesoscale, membranes are very heterogeneous and dynamic, and several obstacles hinder the free motion of the membrane components [3].

The plasma membrane forms a dynamic multi-dimensional architecture that can quickly respond to intracellular (and extracellular) events. Kusumi et al. have proposed that plasma membranes are organized for this purpose into dynamic hierarchical structures [3]. Within these hierarchical structures, membrane components (macroscopically) diffuse from 5 to 50 times slower than when the same components are reconstituted into artificial membranes without membrane-associated cytoskeletal or other structures. Conversely, the macroscopic diffusion rates can also be increased (20-fold) through disruption of membrane-associated cytoskeletal networks.

Thus, cell membrane properties are variable in time and space, making them very plastic in the sense that they can change local properties in order to rapidly adapt to a specific requirement. The presence of transient heterogeneities also implies that the environment interacts with membranes with a great variety of compositions, and consequently, different characteristics.

Of particular interest are the mechanical properties. Membranes cannot be too easily deformable, nor too rigid. They may have to change from rigid to soft depending on the environment or cell metabolism. Three different kinds of deformations of mechanical properties must be distinguished. Membranes can be considered as a quasi-two-dimensional system that can be compressed or expanded (changes in the area), can be deformed (shear stress), or curved (bending stress) [4]. Each kind of stress may have an immediate and a delayed response, since membranes are usually viscoelastic.

In our research group, we have studied membrane rigidity/softness under the presence of membrane active peptides (MAPs). MAPs are able to translocate membranes or permeabilize them by pore/defect formation, and even cause membrane rupture through different mechanisms [5]. Briefly, in the carpet model, peptides disrupt the lipid bilayer, forming lipid-peptide soluble aggregates. Peptides can also insert into the membrane and aggregate to form toroidal or barrel pores. Membrane permeation may also be increased by an electroporation-like mechanism, driven by the adsorption of highly charged peptides on the lipid-solution interface, or by locally thinning the membrane. Finally, peptides can translocate through the formation of a lipid-peptide complex inside the membrane, which is known as the adaptative mechanism.

Two kinds of MAPs can be distinguished: Cell-Penetrating Peptides (CPPs) and Anti-Microbial Peptides (AMPs). The difference between these is mainly functional [6], since AMPs induce membrane rupture at low proportions, while CPPs are able to translocate the membrane without damaging it [7,8]. Due to their ability to translocate cell membranes, CPPs are used as a tool for incorporating molecules inside cells as cargoes bound to the peptide (CPP-molecule chimeras).

AMPs are proposed as substitutes for traditional antibiotics as bacteria do not acquire resistance to these as easily as to traditional antibiotics. The reason for this is that the target of peptide action is the lipid region of the membranes, and thus bacteria have to change lipid composition in order to become resistant [9]. However, changes in lipid composition lead to changes in membrane properties, and these properties have to remain within an optimal range for cells to survive [10].

MAPs may have a high positive charge or be slightly charged, and they may be amphipathic, or completely hydrophilic. Conversely, many peptides that act at lipid-water interfaces assume an amphipathic secondary structure, induced by the anisotropy of the interface. In recent years, there has been increasing interest in this kind of peptide, and much effort has been put into analyzing the peptide properties that optimize membrane-peptide interaction and the subsequent translocation/pore formation/lysis. However, less work has been done in understanding the membrane factors that modulate the processes. Even fewer studies have examined the effect of the peptide on the membrane properties at sublytic doses.

The studies performed so far indicate that the specific mechanism, as well as the fate of the membrane after peptide-membrane interactions, is commanded by both peptide and membrane characteristics. Since membranes are heterogeneous and in constant change, a clear picture of which membrane properties are key, as well as which properties may change, is crucial. Therefore, we aim to understand which of the membrane properties are able to direct peptides towards the target membrane, and which are the main properties of the membrane that become affected by the peptide-membrane interaction. The results found up to now are summarized here.

This review has been structured into five sections. The first gives a brief description of the biomimetic systems that can be used for membrane-peptide interaction studies. The second to fourth sections focus on the findings about the influence of electrostatics, phase state, and lipid composition of the membrane. The final section includes possible extrapolations to cell membranes. Results with two peptides are presented: the poly-arginine KR9C (net charge +10, see Figure 1) as a CPP example [11,12], and Polybia-MP1 (or simply, MP1) peptide (IDWKKLLDAAKQIL-NH_2, net charge +2, see Figure 1) as an AMP example [13]Polybia-CP: I L G T I L G L L K S L-NH2 (1239.73 Da.

The poly-arginine peptide is hydrophilic and does not acquire secondary structure or amphipathicity upon binding to membranes [11,12,14]. MP1 is also unfolded in solution, but acquires an alpha helix structure in contact with lipid bilayers, with the corresponding secondary amphipathicity [15,16].

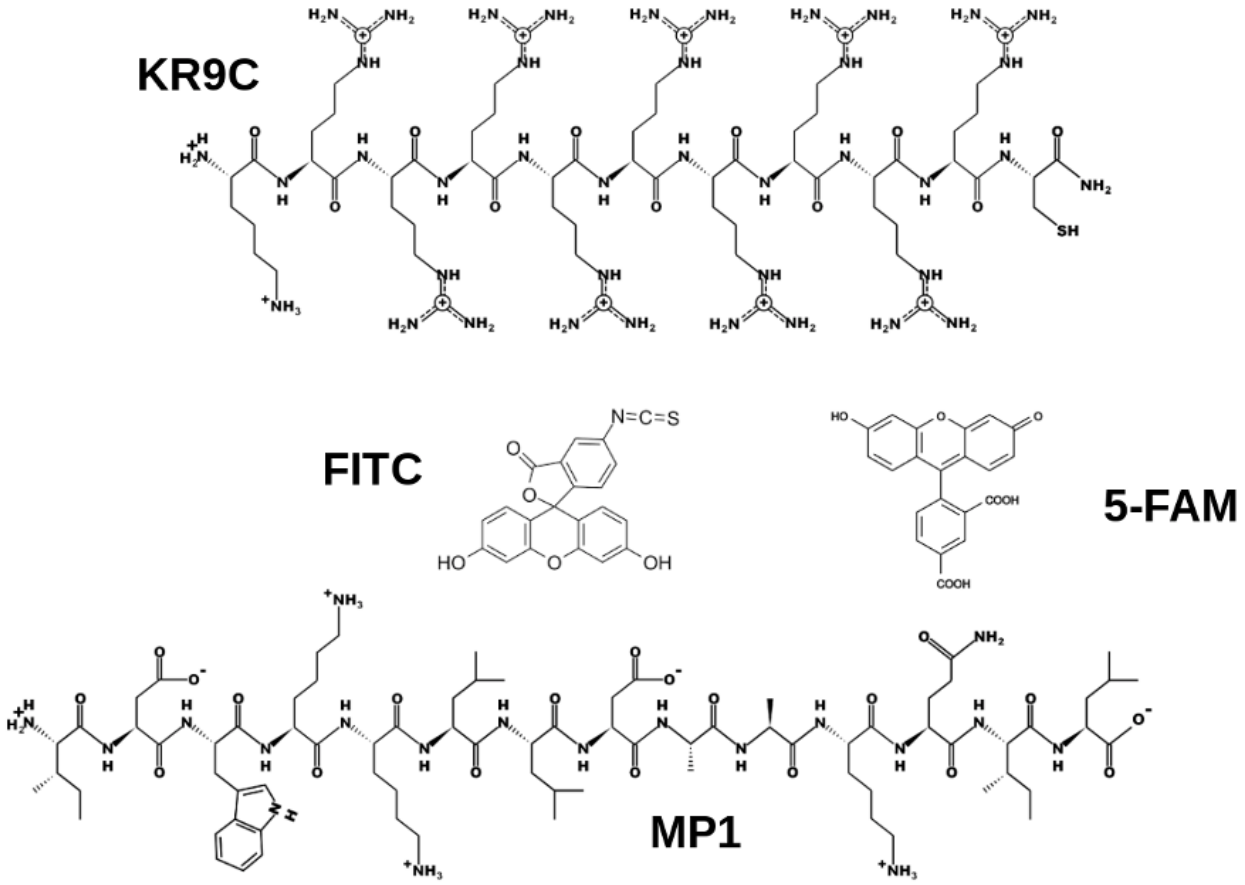


Figure 1 Chemical structures of the peptides used (KR9C and MP1) and the fluorescent moieties (FITC and 5-FAM).

1- Biomimetic systems and techniques used for studying peptide-lipid interactions

Biological membranes are complex open systems and, due to their dynamic composition, understanding the principles that rule their lateral organization and the relation with their functioning is a challenging task. However, with the revolutionary work of Mueller et al. creating ‘‘black lipid membranes’’ [17] and the discovery of liposomes by Bangham et al. [18], the

study of lipid bilayer properties has grown at an exponential rate, and many other areas have been developed from the application of these model membrane systems [19].

Several lipid systems have been successfully used as model membranes for studies on peptide-lipid interactions. All of them are oversimplifications of the real cell membrane, since the only perturbation performed on the system is the presence of the peptide. In a cell membrane, different processes occur simultaneously with the one studied, making interpretation difficult. Therefore, performing experiments in simplified artificial membranes is a very useful tool for understanding membrane-peptide interaction, despite the extrapolation of the results to systems *in-vivo* not being straightforward.

Each model system has certain advantages and disadvantages, depending on the situation in which it is more suitable to be used. Related to this, cell membranes in biological systems cannot necessarily be considered similar to free-standing bilayers, since they are often interacting with (and supported by) cytoskeletal proteins, neighboring membrane stacks, and extracellular matrices, with these interactions affecting the native lipid phase behavior. Therefore, in order to fully exploit the existing membrane model systems to retrieve biologically relevant information, it is important to have as clear as possible an understanding of the influence of preparation conditions on the lipid film.

It was demonstrated that results obtained with different models may be compared qualitatively, but not always quantitatively [20,21]. Even when working with a simple phospholipid mixture, the properties of the phases change between the different model membranes. Therefore, it is always desirable to use as many model systems as possible, with the choice of a given biomimetic system depending on which property is under study.

Having said that, we will now briefly describe the techniques and systems used for the results and summarize them here, along with the information that can be obtained from the results in relation to peptide-membrane interactions.

Aqueous peptides first interact with the external hemilayer of the membrane, adsorb, and eventually incorporate into the hydrophobic region. Afterward, membrane disruption by the peptide or peptide translocation without membrane rupture may occur.

The first step in peptide-membrane interaction can be followed using Langmuir monolayers, that is, a monomolecular film at the air-water interface. Langmuir films can be compressed, and thereby the molecular density can be varied, while the surface tension (γ) and electrostatic potential (ΔV) are registered. Additionally, the membrane can be simultaneously observed by Brewster angle microscopy or fluorescence microscopy [22,23]. The electrostatic potential determined in Langmuir monolayers is calculated as the change in the Volta potential difference of the air relative to the aqueous solution after generating the monomolecular film. For this determination, two electrodes are used: a reference electrode inside the aqueous solution, and a second electrode in the air. Two methods exist for determining electrostatic potential, depending on the characteristics of this second electrode: a radioactive electrode is used in the ionizing method, and a vibrating electrode in the kelvin method [24,25].

Since the only change in the system before and after generating the monolayer is the presence of the film at the air-water interface, changes in Volta potential differences are assigned to changes at the interface. Therefore, the DV has contributions from the water molecules and ions that orient around the lipid polar head-group, and from the molecular dipoles that orient perpendicular to the interface.

The interaction of the monolayer with soluble peptides is usually studied by adding the molecule to the solution underneath the film, which is previously prepared at the desired molecular density. The film compaction is defined by the surface pressure $\pi=\gamma_0-\gamma$, where γ_0 is the surface tension of the clean interface and g is the surface tension in the presence of the amphiphile.

Surface tension decreases (and thus, π increases) when the peptide penetrates the monolayer, with the change in π ($\Delta\pi$) being a measure of the strength of the perturbation promoted by the peptide on the film. It is important to remark here that $\Delta\pi$ depends on both the degree of peptide penetration and the compressibility of the host film. In a stiff monolayer, a small change in the area caused by peptide insertion will promote high changes in π, while in a more compressible film, lower π changes may be recorded as a consequence of the same area change. Therefore, to obtain a film parameter independent of the host film stiffness, changes in film area (ΔA) can be calculated from $\Delta\pi$. This is performed assuming that the film can be tessellated into two types of region: those where the peptide penetrates and disrupts the monolayer structure, forming a lipid-peptide structure, and those that are formed by pure lipids. Thus, the pure lipid regions get progressively compressed as the peptide penetrates and a peptide-lipid structure is formed. ΔA corresponds to the compaction of the lipid molecules inside the pure lipid regions, which is a better parameter than $\Delta\pi$ for comparing different monolayers [26].

Another important parameter that can be obtained from experiments of peptide adsorption into lipid monolayers is the exclusion surface pressure (π_e), which is the maximal surface pressure at which peptides penetrate the film. This

parameter can be obtained from extrapolations to $\Delta\pi$=0 mN/m of $\Delta\pi$ plots as a function of the initial surface pressure of the film (corresponding to the molecular density of the lipids before the addition of the peptide). High π_e indicates that the peptide is included in the film even at high compaction. In order to compare the results found with monolayers with those in bilayers, it has to be considered that the molecular density of the lipids in bilayers is similar to that in monolayers at high surface pressures (30 mN/m or higher) [27,28].

Some peptides adsorb to the membrane in the polar head-groups region without deeply penetrating the monolayer, resulting in no detectable changes in surface pressure. This behavior can be detected following the reflectivity of the interface at the Brewster angle, which depends on the interface thickness [26], or through ΔV measurements for charged peptides [26].

Aside from peptide adsorption and penetration from the solution into monolayers, premixed lipid-peptide films can be prepared and studied. These experiments allow the effect of a controlled amount of peptide on film properties to be studied and the maximal surface pressure at which peptide stays at the interface to be determined. However, these experiments have the drawback that only one hemilayer is present in the system. Therefore, the peptide structure in these experiments may differ from that in a bilayer. Langmuir films cannot be used for peptide translocation, or for measuring changes in membrane parameters such as permeability or bending rigidity.

Peptide-membrane interactions can also be studied using vesicles of different sizes. Large unilamellar vesicles (LUVs) enable the determination of zeta potential, which is the potential at the slipping plane. This potential is sensitive to the net charge at the membrane-solution interface under conditions of low ionic strength. Therefore, the adsorption of charged peptides to LUV surfaces can be followed by changes in the zeta potential under these conditions [29].

Giant unilamellar vesicles (GUVs) have the advantage of being large enough to be observed with optical microscopy. Using peptides labeled with a fluorescent moiety (FITC, 5-FAM, etc.), peptide adsorption can be followed in GUVs using confocal microscopy [29,30]. With GUVs combined with labeled peptides, the internalization of the peptide into the vesicle lumen can also be followed [30]. For these experiments, the GUVs must be large enough for the signal from the membrane to be split from that from the lumen. The drawback of this method is that it requires a labeled molecule, and the size of the fluorophore is not negligible in comparison with the peptide size for small peptides like those described in this review (Figure 1). To minimize this effect, a low amount of the labeled peptide is used, combined with unlabeled peptide. Experiments can be performed with different labeled/unlabeled ratios to check the influence of the fluorophore.

Deformations of the GUV's shape can be used for studies of membrane deformability, as will be explained in Section 5. Permeability can be conveniently studied using GUVs filled with sucrose and immersed in glucose. In these experiments, the loss of contrast is followed using transmission microscopy. The contrast is due to the sucrose/glucose gradient that leads to a different refractive index in the GUV's lumen compared to the exterior. This gradient is lost when the membrane becomes permeable [31].

Alternatively, permeability can be determined in LUVs pre-loaded with carboxyfluorescein [30,32,33], which is a fluorescent molecule that self-quenches at high concentrations. In these experiments, the detection of emitted fluorescence indicates a dilution of carboxyfluorescein due to its escape from the vesicle's lumen. Since the loss of content of the vesicle also occurs when the vesicle breaks, controls of vesicle size using dynamic light scattering are mandatory. The results obtained with a fluorometer following the loss of carboxyfluorescein in LUVs come from a vesicle population, while loss of contrast in sucrose-filled GUVs corresponds to single-vesicle data, thus requiring the observation of several vesicles.

Translocation of charged peptides can be detected as ion currents induced by the application of an electric field using black lipid membranes (BLMs). In this system, the conductivity of the membrane is determined as the slope of current vs potential difference plots. Current is originated from the migration of charged species through the BLM formed in a micro-metric hole that separates two chambers in an electrochemical cell [29].

In summary, several membrane models coupled with different techniques can be used for determining the different parameters of interest. We now describe the influence of different membrane properties on membrane-peptide interaction, using results from all the above systems.

2- The influence of membrane electrostatics in peptide-membrane interactions

Membranes can be characterized by three different electrostatic potentials: transmembrane potential (Ψm), surface potential (Ψs), and dipole potential (Ψd). These potentials have been previously described and their effect has been discussed in detail [25,34,35].

We give a brief description here, using the scheme in Figure 2A. Ψm is the difference in Volta potential between the aqueous solutions at both sides of the membrane. It depends on the ionic composition of the solutions and is one of the driving forces for ionic conductance through the membrane.

Ψs is caused by the presence of charges in lipids and proteins at the membrane surface and is screened by the ions in the solutions. Ions are attracted by the electric field generated by the charged membrane, but are also subjected to thermal motion, giving rise to a diffuse layer of ions that generate a drop in potential (Ψdl, the diffuse layer potential). Gouy-Chapman or Stern models are used to describe this potential drop, and the Debye length (λ_D) emerges as an important parameter, being the distance at which the electric potential decreases in magnitude by 1/e.

Finally, a nonlinear change in electrostatic potential occurs inside the membrane, defining Ψd. This is due to the presence of highly ordered molecules with a charge distribution characterized by a dipole or higher order multipoles inside the membrane.

It is important to clarify here that the electrostatic potential determined in Langmuir film experiments is usually called "surface potential". However, since it is a change in Volta potential differences, ΔV has contributions from all the electrostatic changes induced by the presence of the film. As previously mentioned, ΔV includes potential changes due to the dipoles from the water molecules. These molecules change their orientation from that at the clean air-water interface to that adopted when they hydrate the lipid polar head-groups. Ions also adopt different orientations in the presence of the film, especially for charged lipids, where Ψdl develops. When the film is composed of charged surfactants, Ψs contributions are also included in the electrostatic potential measured. Finally, the surfactant molecules orient their dipoles, generating Ψd, and contributing to the change measured in Volta potential difference.

The interaction of soluble ions with membranes may be affected in different manners by all the potentials mentioned. Surface potentials foster the adsorption of cationic peptides to membranes, as expected. This is demonstrated in Figure 2B for lipid monolayers and KR9C. This figure shows that the poly-arginine peptide induces higher perturbations in charged monolayers (pH > 7) than in neutral monolayers (pH < 6) [26]. This was also observed for the less charged MP1 peptide. As shown in Figure 2C, the maximal surface pressure at which the peptide inserts into monolayers (exclusion surface pressure) is higher for lipids with charged polar head groups (PS) than for neutral lipids (PC), considering the same hydrocarbon chains [36]. This preferential interaction was observed under conditions at which the Debye length is large, and thus, the surface charge is screened at large distances from the membrane, for instance, in pure water (red bars in Figure 2C), λ_D = 300 nm, implying that at 300 nm the potential is 1/e times that of the surface. The attractive effect of the membrane charge is reverted in the presence of salt. At 150 mM NaCl (green bars in Figure 2C), λ_D decreases to less than 1 nm, and the electrostatics becomes negligible, so that other effects emerge (phase state in this case, as will be discussed in the next section).

Regarding the transmembrane potential, computational simulations demonstrated that an imbalance caused by changes in the ionic composition of the aqueous solutions bathing the membrane promotes the translocation of a cationic peptide [37,38]. This is in line with the effect promoted by externally applied potentials using electrodes. The application of a potential difference between each side of a membrane promotes the migration of charged species through the membrane. The velocity of ion migration is determined by measuring the current, and the membrane conductivity is obtained, which depends on the particular ion, the membrane composition, and phase state.

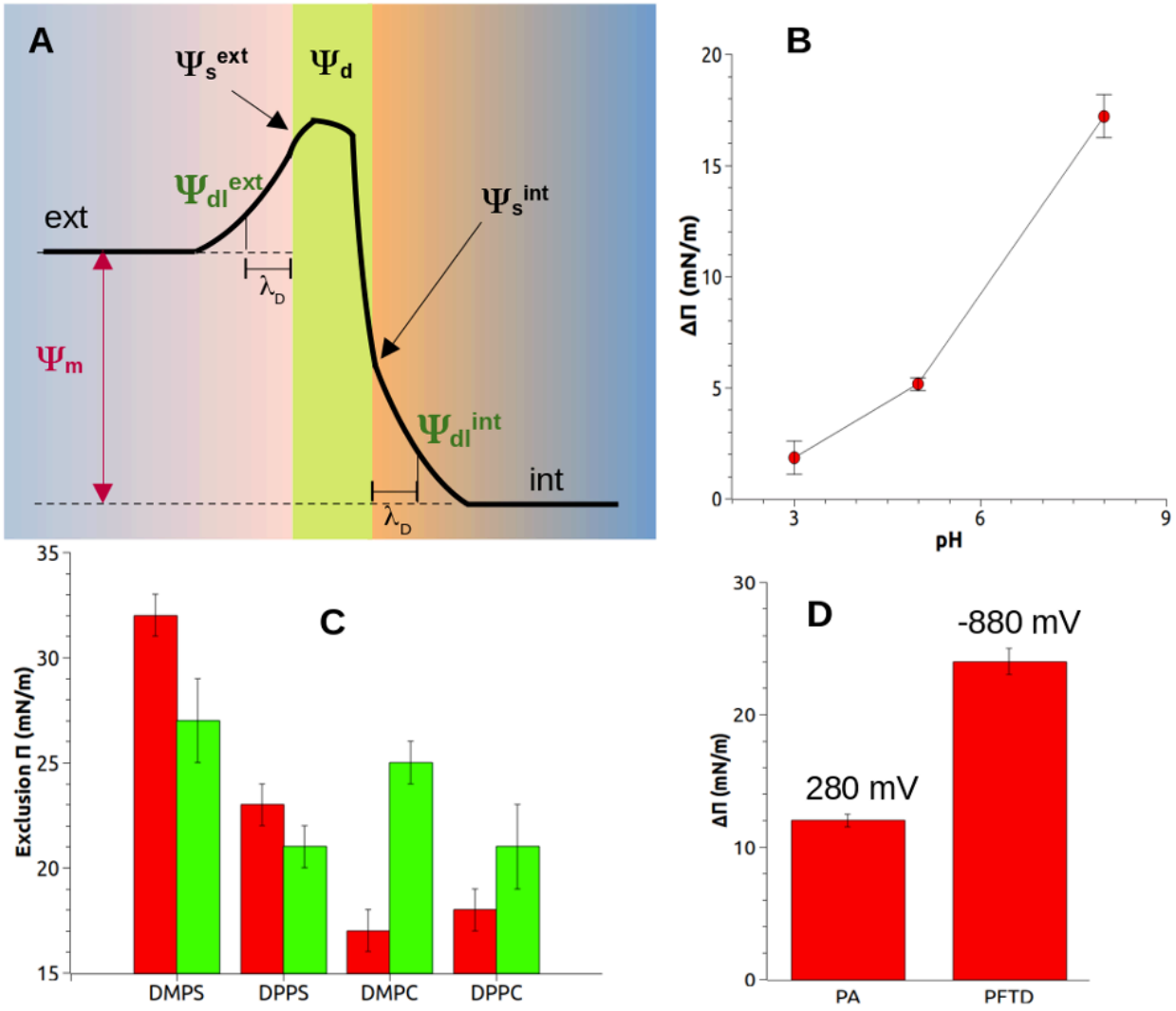


Figure 2. Membrane electrostatics and peptide-membrane interaction. A) Scheme indicating the transmembrane potential (Ψm), surface potential (Ψs), dipole potential (Ψd), potential drop at the diffuse layer (Ψdl), and Debye length (l_D). B) Increase in surface pressure due to the insertion of KR9C peptide into monolayers of palmitic acid, initially at 10 mN/m as a function of the pH of the aqueous solution. Adapted from [26]"ISSN":"15205827","abstract":"Cell-penetrating peptides (CPPs, with permission. C) Exclusion surface pressure for the peptide MP1 in monolayers of the indicated lipids over water (red) or NaCl 150 mM (green). Adapted from [36], with permission. D) Increase in surface pressure due to the insertion of KR9C peptide into monolayers of ionized palmitic acid or perfluorotetradecanoic acid initially at 20 mN/m. Adapted from [26]"ISSN":"15205827","abstract":"Cell-penetrating peptides (CPPs, with permission.

Both potentials discussed so far (transmembrane and surface potentials) markedly affect the adsorption and translocation of cationic peptides, as expected due to basic electrostatics. The influence of the dipole potential, however, has been less studied, despite it being known since 1969 that the permeation of hydrophobic cations and anions is affected by this potential [25]. Liberman and Topaly [39] compared the conductivity changes induced by the addition of fat-soluble anions or cations and discovered that to increase the conductivity of the membrane to a certain value required an approximately 10^5 times greater concentration of cations than anions. They attributed this very significant difference to the partition coefficient of fat-soluble ions, the coefficient for anions being 10^5 greater than that for cations, and hypothesized that the inner part of the bilayer membrane must be positively charged. Now it is accepted that the interior of lipid bilayers has a more positive charge density than the region of the polar headgroups, i.e., that Ψs have positive values for all lipid bilayers, which should be unfavorable for the insertion of cationic peptides. In order to test this, the insertion of KR9C into monolayers of different dipole potentials was studied. Figure 2D shows the increase in surface pressure due to peptide insertion into monolayers formed by two different fatty acids with similar surface charges and compaction. PA is a hydrogenated fatty acid, with dipole potentials of 200 – 300 mV, while PFTD is a perfluorinated

fatty acid, with negative values of the dipole potential (-200 – -900 mV) [38]. The results show that this little-explored membrane parameter largely affects peptide insertion, and thus, should be studied in depth. Dipole potential depends on membrane composition, phase state, and compaction.

3- Influence of membrane phase state in peptide-membrane interactions

In lipid bilayers, molecules interact through Van der Waals, hydrogen bonding, electrostatic and steric interactions, giving rise to an optimal molecule-molecule distance. Depending on the strength profile of the interaction, which defines the intermolecular spacing, the hydrocarbon chain and the polar head group may move and rotate to a greater or lesser degree, defining membrane mechanical properties such as fluidity, compressibility, and deformability [40,41]. Lipids with a long and saturated hydrocarbon chain (16 C or more) and an intermediate-sized polar head group, such as DPPS, DPPC, DPPG, or DPPA, form very compact bilayers, with low lateral motion and low fluctuation in the positions of the carbon atoms in the hydrocarbon chains. These membranes are in the so-called gel phase (G) at room temperature and have melting temperatures (Tm) from G to liquid-disordered (LD) phases of 40°C or higher (see Tables for Tm on the web page of Avanti Polar Lipids). In contrast, unsaturated lipids such as DOPC, or those with short saturated hydrocarbon chains (such as DLPG), form LD fluid phases at room temperature, with Tm values lower than 20°C.

In the presence of most sterols (cholesterol, ergosterol or phytosterols), membranes are also fluid but very compact, giving rise to the so-called liquid-ordered (LO) phase state. The characteristics of this phase state have been described [42]. Briefly, these membranes are fluid, with the diffusion coefficient of lipids being similar to those in the LD phase, but are very difficult to compress or bend, presenting low permeability. Hopanoids, a family of lipids synthesized only by some bacteria, are also able to induce a LO phase state in these sterol-lacking bacteria, and are thus sterol surrogates [43].

Soluble molecules are generally observed to be less prone to penetrate G or LO membranes than LD membranes, since the inclusion of the molecule implies a local compression of the membrane. This explains the low values for the exclusion pressures of MP1 into DPPS monolayers when the electrostatic attraction is screened (green bars in Figure 2C), as DPPS monolayers are very stiff with Tm = 54°C. Similarly, KR9C showed higher exclusion pressures for DPPC than for DOPC monolayers [29].

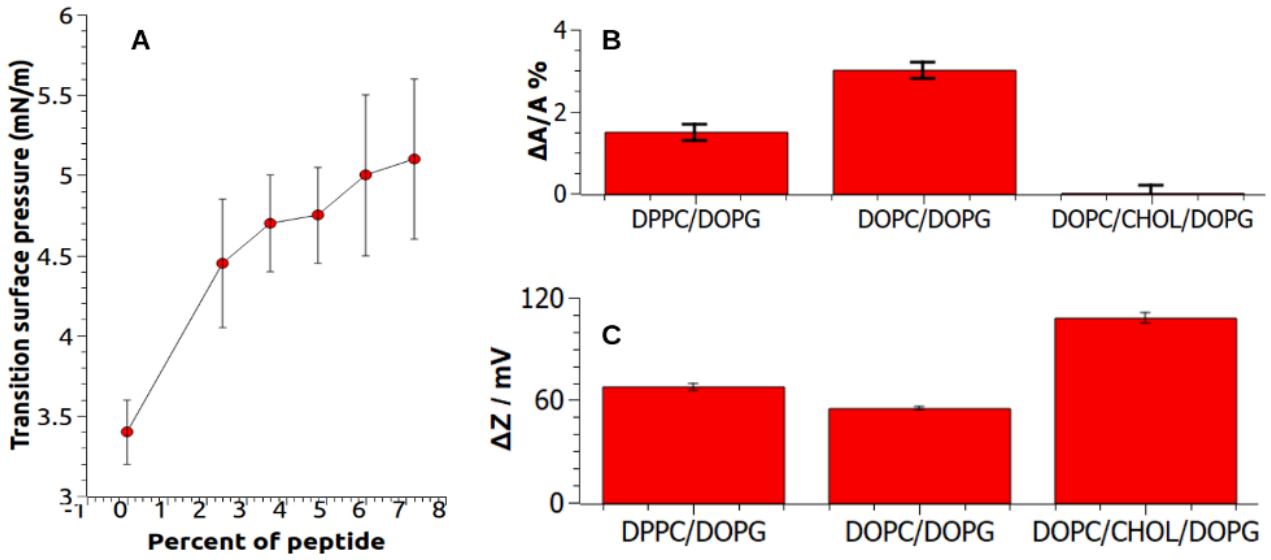


Figure 3. Membrane phase state and peptide-membrane interaction. A) Increase in surface pressure for the phase transition from a fluid to a rigid phase state induced by compression of DPPC monolayers with increasing amounts of MP1. Adapted from [44], with permission. B) Changes in monolayer area due to the insertion of KR9C into monolayers of the indicated compositions. C) Changes in the zeta potential of large unilamellar liposomes after adding KR9C to the solution. B) and C) were adapted from [29] with permission.

Besides the lower inclusion of molecules from solution, when peptides insert in membranes with phase coexistence they localize preferentially in the LD phase. Figure 3A shows that the transition surface pressure from expanded to compact phase state for DPPC monolayers increases as the amount of peptide in the monolayer increases, indicating that the peptide stabilizes the more expanded phase, and thus mixes preferentially with lipids in the expanded phase [44].

Despite the slight insertion of peptides into compact membranes, they may adsorb on the membrane-solution interface and alter membrane properties. As an example, Figure 3B shows the area change induced on monolayers at 30 mN/m due to the insertion of KR9C. The three lipid compositions present similar charge density. The peptide is added to the solution under monolayers initially at 30 mN/m. The larger the change in the area, the larger the perturbation promoted by peptide incorporation, which is detected in monolayers composed of unsaturated lipids (DOPG/DOPC) forming LD membranes. Membranes with cholesterol (LO phase) render no peptide incorporation, and consequently, no film area change is observed upon adding peptide to the media [29]. However, liposomes composed of DOPC/CHOL/DOPG show the greatest changes in zeta potential, which goes from negative to positive values due to peptide adsorption [29]. The preference for peptide adsorption to rigid membranes may be explained by considering electrostatics, since local charge density is usually higher in compact regions of the membrane than in relaxed regions. Another factor affecting the preferential adsorption of peptides on rigid membranes is the smaller decrease in entropy upon adsorption, as rigid membranes are already ordered before the adsorption, while order is induced by adsorption in disordered membranes [45,46].

4- The influence of the chemical composition of the lipid bilayer in peptide-membrane interactions

There is a great variety of lipids, and these are chemically diverse and present in various amounts and proportions. The question of why the universe of chemical structures of these molecules is so vast is currently unanswered, but it is clear that this is required since alterations of membrane lipid homeostasis are linked to various diseases [47].

Thus, not only is the phase state of membranes important but also their specific chemical identity, and even their minority components are subtly regulated. This is very relevant in the context of AMPs, since there is indisputable evidence that bacteria modify their membranes to reduce the lytic effects of these peptides. The physiological response of bacteria to AMPs involves changes in their lipid composition in terms of both head group and acyl chain structure, which significantly impact membrane properties such as net surface charge and membrane fluidity [9,48].

We therefore evaluated possible differences caused by lipid composition, using MP1 and LO membranes. The importance of the LO regions was first proposed in eukaryotic membranes. The formation of this phase state depends crucially on the ordering properties of sterols (cholesterol in mammals, ergosterol in fungi and phytosterols in plants). The induction of LO regions in eukaryotes has been considered a fundamental step during the evolution of cellular complexity. Conversely, it was suggested that bacteria and archaea do not require such a sophisticated organization of their cellular membranes. However, it was later discovered that many signal transduction, protein secretion, and transport processes in bacteria depend on the presence of LO phases [49]. This led to the search for sterol surrogates in bacteria, and hopanoids have been indicated as candidates. Hopanoids are diverse pentacyclic molecules derived from the cyclization of squalene, which shares the same hydrocarbon skeleton as the compound hopane. It was shown that diplopterol, the simplest bacterial hopanoid, is able to induce a LO phase with similar properties to that induced by other sterols [50–53], and hopanoids are now accepted as bacterial sterol surrogates, with the ability to order saturated lipids and to form a LO phase in model membranes.

We studied the interaction of the peptide MP1 with membranes containing cholesterol or diplopterol, in order to test the possible chemical sensitivity of the peptide in LO membranes. We found that MP1 permeates membranes with diplopterol more effectively than those with cholesterol (Figure 4A) [30]. Furthermore, monolayer perturbation by the peptide was stronger in membranes with diplopterol than with cholesterol, and the adsorption of the fluorescently labeled peptide was faster and more extensive on bilayers with diplopterol than those with cholesterol. In other words, MP1 is able to discriminate the chemical composition of LO membranes and to distinguish between mammal-like and bacterial-like compositions.

It has been proposed that in systems in which electrostatic effects can be neglected, as in this case (neutral membrane and low charged peptide), peptide aggregation in the membrane is driven by membrane deformations [54–56]. Therefore, subtle differences in membrane mechanical properties (which are not distinguished using biomimetics systems) may lead to differences in the mechanism of peptide-membrane interactions, and thus in the consequences of the interactions, as will be discussed in the next section.

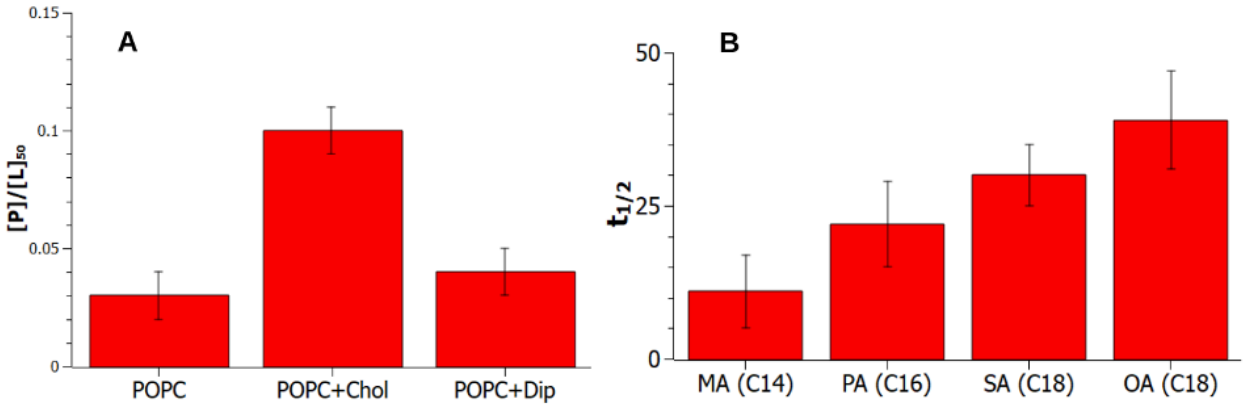


Figure 4. Membrane composition and peptide-membrane interaction. A) MP1-to-lipid molar ratio at which carboxyfluorescein leakage is 50%. Adapted from [30], with permission. B) Time for half insertion of KR9C into lipid monolayers. Adapted from [26]"ISSN":"15205827","abstract":"Cell-penetrating peptides (CPPs, with permission.

The polar groups of the lipid molecules are also important for the activity of cationic peptides. It has been shown that poly-arginine CPPs are chelated by the phosphate or the carboxylic acid of the polar head-groups of lipids, forming complexes that decrease the energy cost needed to internalize the peptide inside the membrane [11]. Thus, poly-arginine peptides show an increased affinity for membranes with lipids that contain these functional groups exposed for interaction. Aside from the lipid class, the hydrocarbon chain affects peptide-membrane interaction, influencing phase state, lipid motility, and the exposure of the functional groups. For instance, poly-arginine peptides were shown to penetrate POPG monolayers [57] and only adsorb without penetrating into DMPG monolayers [26]. We observed that the penetration rate for KR9C into membranes is faster when the membranes are composed of lipids with one hydrocarbon chain rather than with two [14]. In this regard, comparing different fatty acids, peptide insertion into monolayers is faster, the shorter the hydrocarbon chain, as shown in Figure 4B [26]). This may be explained as an adaptative mechanism for the insertion of this peptide inside the membrane, being favorable in energy terms to form a lipid-peptide complex with single-chain lipids than with lipids with two hydrocarbon chains.

5- Consequences of peptide-membrane interactions on the membrane properties

A clear consequence of the interaction of the peptide with membranes is the dramatic change in surface electrostatics when anionic membranes acquire a positive surface potential. This effect occurs even when the peptide does not incorporate into the membrane, permeate, or damage it, as shown in Figure 3C for DOPC/CHOL/DOPG membranes and KR9C.

Another clear effect that is detected in almost all membrane compositions for most AMPs is a change in the deformability of the membrane upon shape change. This property was studied in our laboratory for different membrane compositions faced with KR9C or MP1. Two methods were employed, both using GUVs: registration of thermal shape fluctuation, and kinetic retraction of membrane nanotubes.

Briefly, the analysis of shape fluctuations of membranes and vesicles is based on collecting time sequences of snapshots obtained by optical microscopy. The thermally induced fluctuations around equilibrium form are monitored, and the mean square values of shape deviations are determined. This method is less demanding from an experimental point of view because it is based on direct video microscopy observation of giant vesicles. A disadvantage is that, as the vesicles should exhibit visible fluctuations, this method cannot be applied to rigid vesicles. The difficulty of the method is in the analysis of the videos: first, the vesicle contour in the equator must be determined, and from the contour, the radius is obtained as a function of the polar angle for each frame in a video (Figure 5). This allows the amplitude of the shape fluctuation to be quantified, from which the bending rigidity is obtained. This procedure requires a software for image processing, which was developed by S. V. Amante and P. E. Scurti and is available for use at https://github.com/GatitoNegro/Determinacion-de-rigidez-de-biomembrana-usando-GUVs [58].

In biology, it is not only the energy cost of a process that is important, but also the velocity at which the process will happen. Therefore, as well as bending rigidity, viscosity terms are of interest. In this regard, membranes can be deformed with stress, and the kinetic relaxation will give an insight into both membrane rigidity and viscosity. For this analysis, we prepare membrane nanotubes from GUVs attached to the cover glass, separating the membrane from a micro-sphere adhered to the membrane by means of optical tweezers. The nanotube is stretched until the desired length is reached,

and then the laser is turned off. Dynamics for nanotube relaxation can be determined from videos of this process, by determining the position of the center of the GUV and the micro-sphere in each frame, giving rise to plots of nanotube length as a function of time. This allows us to determine a characteristic time for nanotube relaxation, as shown in Figure 6 [29,30].

In the case of KR9C, we observed different behaviors in the composition of the three membranes studied. Membranes in the LO phase were not affected by peptide adsorption, while rigid membranes ruptured after peptide addition. In contrast, in LD membranes, where peptides adsorbed and subsequently translocated, a softening of the membrane was detected using the latter method [29]. This implies that the CPP is not as innocuous for the membrane as is desirable for a CPP.

MP1 showed more drastic results, leading to membrane rupture for LD membranes and LO membranes with cholesterol. Membranes in LO phases induced by diplopterol showed an interesting behavior: immediately after adding the peptide, a fast softening was observed, and subsequently, an increase in rigidity was detected. Therefore, in the longer term, the membranes were similar to those in the absence of the peptide. This was detected using thermal fluctuations for high MP1 concentrations (visible fluctuations were registered for 6 μM peptide, see Figure 5), and by the kinetics of nanotube retraction for 0.6 μM peptide, as summarized in Figure 6.

Based on these results (Figures 5 and 6) and others, we propose the mechanism schematized in the graphical abstract. At short times, a low amount of peptide accumulated in the external hemilayer in a diluted regime. This first stage is followed by the formation of peptide dimers/multimers until pores/defects form, allowing vesicle leakage and the passage of the peptide to the vesicle lumen. The pores/defects may be formed by lipids and peptides (disordered toroidal pores), or with one peptide at each hemilayer, since the thickness of the bilayers is twice the length of the peptide. When peptides are in the outer hemilayer in a diluted regime, they may increase the membrane's spontaneous curvature because, at this stage, membranes would be asymmetric, with peptides only in the external leaflet. When peptides reach a threshold concentration at the interface, they would form dimers/multimers, and eventually, pores. This is accompanied by a decrease in the characteristic time for nanotube retraction, which could be due to an increase in the spontaneous curvature (since membrane symmetry would be recovered). In addition, a decrease in shear viscosity and an increase in the bending rigidity of the membrane may occur in the presence of pores.

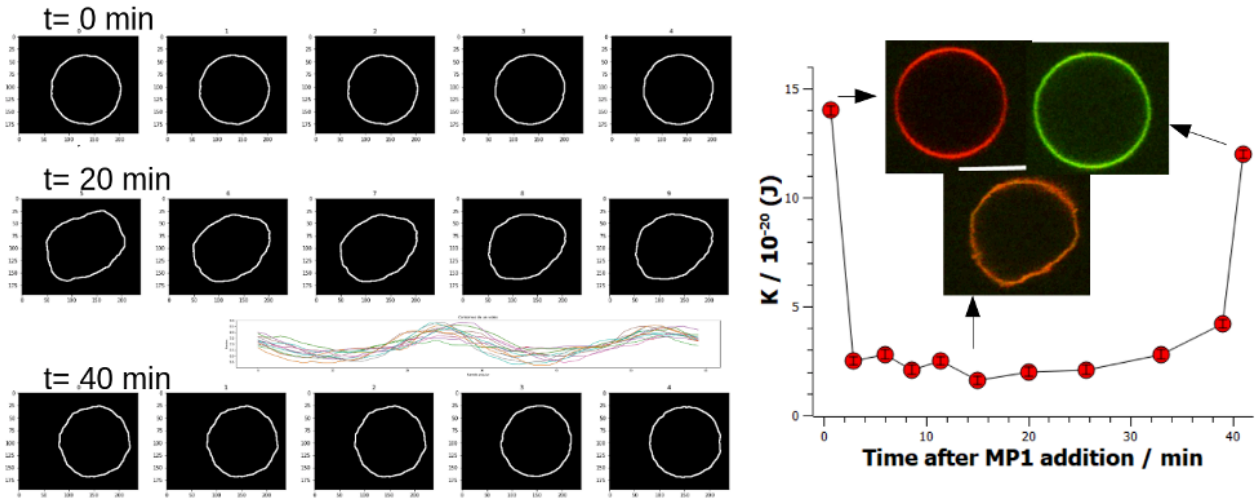


Figure 5. Consequences of membrane-peptide interaction on membrane bending. Vesicles are composed of POPC/diplopterol (6:4) in the presence of 6 mM MP1. Left: Sequential images recorded immediately before peptide addition and at 20 or 40 min after adding the peptide. Resolution: 0.1 mm/px. Central plot: vesicle radius as a function of the polar angle for each frame at 20 min after adding the peptide. Right: Bending rigidity as a function of time after peptide addition, determined from the thermal shape fluctuations. Inserted images: three selected frames for different times after peptide addition. Images are the merge of red and green channels, representing membrane and peptide, respectively. Scale bar: 10 mm.

In summary, membrane behavior in the presence of the peptide MP1 is very different in the case of cholesterol-containing membranes than in that of diplopterol-containing membranes, despite both membranes being initially similar. This is important since it may be one of the reasons for the different action of peptide in mammal than in bacterial cells and this is now being studied deeply.

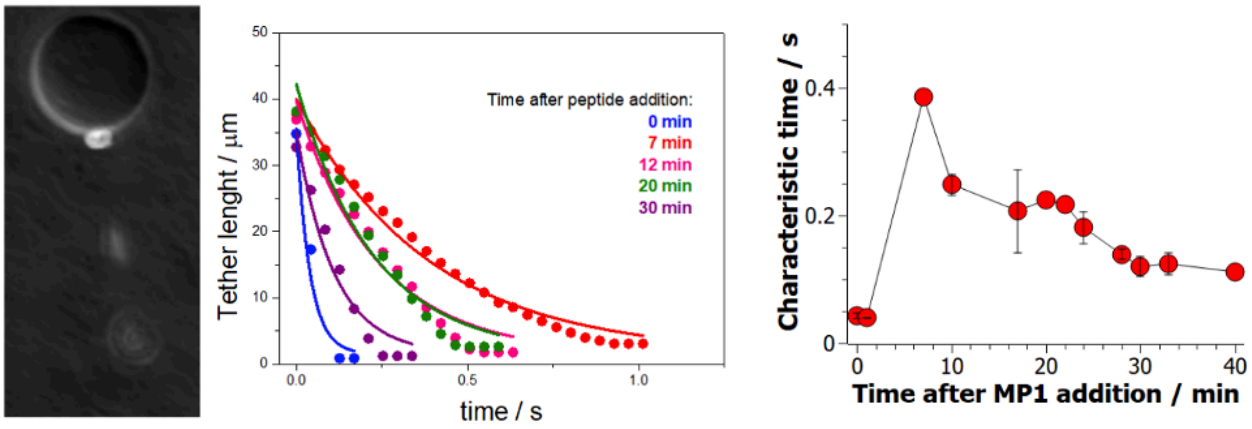


Figure 6. Consequences of membrane-peptide interaction on membrane bending. Vesicles are composed of POPC/diplopterol (6:4) in the presence of 0.6 mM MP1. Left: Accumulated images showing the motion of a bead attached to the tip of a membrane nanotube. Center: Representative data for nanotube retraction of tethers extracted from a GUV after the indicated times of adding the peptide. Right: Characteristic time for the kinetics of nanotube retraction as a function of time after peptide addition. Adapted from [30], with permission.

6- Cell membranes

As already mentioned, it is proposed that cell membranes are very dynamic and heterogeneous in regard to their composition, and consequently, local electrostatics and mechanical properties will dynamically vary from one region to another of the membrane. It was shown in our laboratory that hopanoid-lacking and sterol-lacking bacteria increased their permeability, as well as their tolerance to traditional antibiotics that act at the membrane level, upon preincubation with the sterol, cholesterol, or the hopanoid, diplopterol. This indicates that the lipids are incorporated from the media by the bacteria and included in their membranes, affecting membrane properties [52]. These bacteria also showed an increased tolerance toward MP1 (Figure 7), and this effect was higher in bacteria incubated with cholesterol than with diplopterol [30]. Our studies indicate that both lipids protect cells from the action of MP1, but not to the same degree. This result is in line with those found in the biomimetic systems previously discussed, and points to the importance of cholesterol as a key factor for cell distinction by AMPs. The presence of this sterol may help to explain the high resistance of red cells to the lysis induced by MP1, with 50% hemolysis at peptide concentrations above 0.1 mM. In comparison, the peptide concentration needed for provoking the death of 50% of a *Pseudomonas aeruginosa* culture is 1.5 µM (unpublished results). Concentrations as low as 2 µM MP1 cause an 80% decrease of *Pseudomonas aeruginosa* population, but not hemolysis (Figure 8).

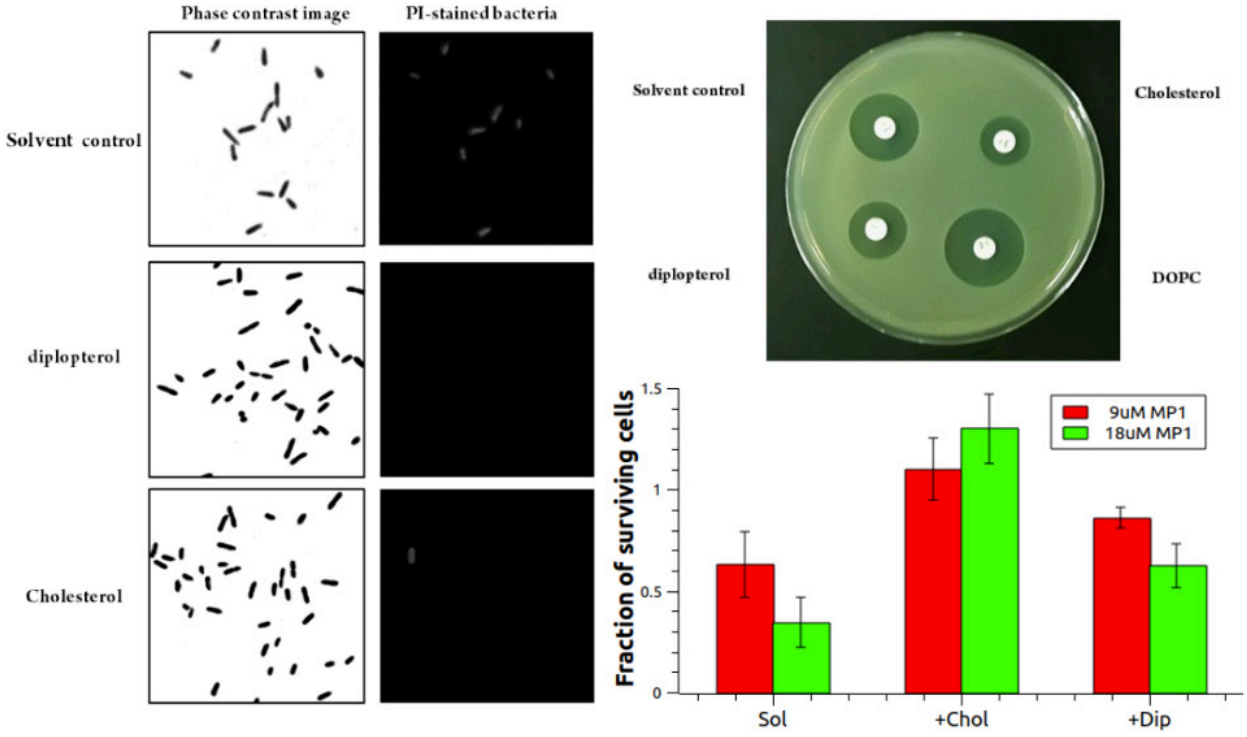


Figure 7. Cell membranes and peptide-membrane interaction. Left images: permeability of *P. aeruginosa* exposed to chloroform:methanol, diplopterol or cholesterol dissolved in the solvent. Permeability was determined with propidium iodide. Right image: disk diffusion test with imipenem embedded with cholesterol, diplopterol, DOPC or the solvent mixture (chloroform:methanol). Plot: Fraction of surviving cells incubated in the solvent (Sol), or a solution of cholesterol (+Chol), or diplopterol (+Dip) after 3 h of exposure to 9 μM (red) or 18 μM (green) of MP1. Adapted from [30] and [52], with permission.

It is interesting to note here that, unlike reports from other AMPs, MP1 appears not to sense membrane electrostatics. Bacteria have a higher proportion of anionic lipids than mammal cells, and it has been proposed that AMPs differentiate membrane targets from others due to electrostatic interactions. However, MP1 slightly alters the membrane surface charge upon adsorption to neutral membranes [30], and does not alter the surface charge of *Pseudomonas aeruginosa* (unpublished results). Besides, bacteria death is similar in media with high salt content to that in media without salt (Figure 8), also suggesting a non-electrostatic peptide-membrane attraction.

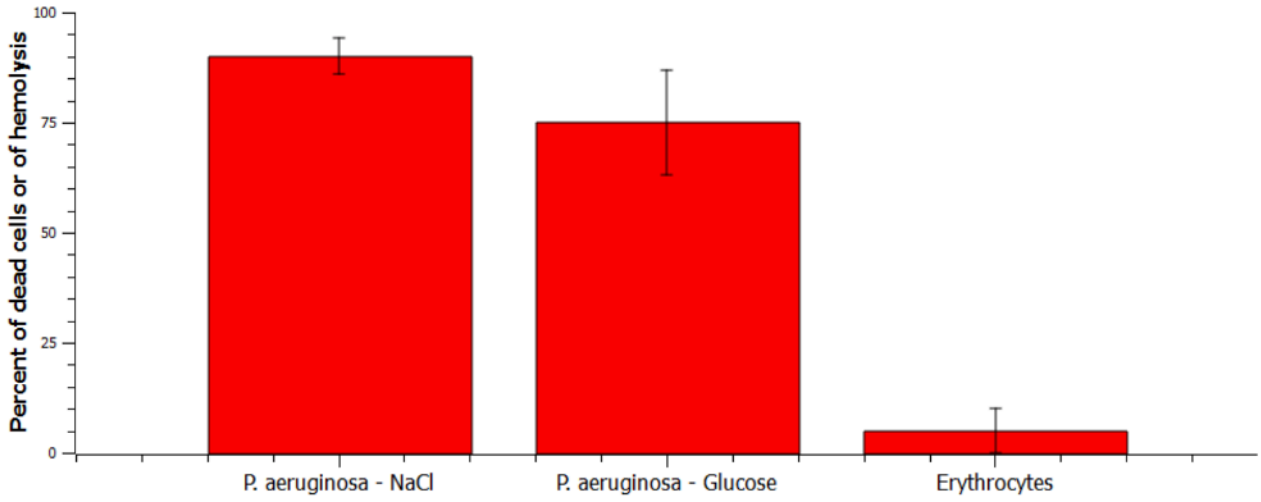


Figure 8. Cell membranes and peptide-membrane interaction. Percentage of cell death or hemolysis for cells exposed to 2 mM MP1. Cells correspond to *P. aeruginosa* suspended in 0.15 M NaCl or 0.3 M glucose, or to human erythrocytes.

Conclusions

Peptide-membrane interactions have been widely studied using both biomimetic systems and cells. Despite some progress, details about the mechanisms involved are still unknown. Of particular importance are the reasons for the higher interaction and consequent peptide-induced lysis of bacteria compared to mammal cells, since this is the most important parameter for using peptides in therapy.

A second important factor to be understood in order to use peptides in therapy is what happens at sub-lethal peptide concentrations. This is of paramount importance to avoid the acquisition of resistance by bacteria.

Our studies show that all membrane properties modulate peptide affinity. Anionic membranes show an enhanced interaction with cationic peptides due to electrostatic interactions but, at physiological ionic strength, the phase state becomes equally or even more important. Phase state defines the degree of incorporation of the peptide, and although the surface affinity may be higher to liquid-ordered membranes, peptides are usually not able to penetrate these membranes. For membranes with comparable charge density and in the same phase state, chemical composition subtly modulates peptide affinity.

Not only do membrane-peptide affinity and the degree of peptide penetration depend on membrane properties, but also the fate of the membrane subjected to the presence of the peptide. The same peptide can exert a softening effect, promote membrane lysis, or not affect the membrane, depending on membrane characteristics.

Since cell membranes are not homogeneous, but with patches of different characteristics coexisting in a dynamic fashion, peptides may cause different effects simultaneously, and the whole picture is complex. Therefore, an understanding of the mechanism and regulation of peptide-membrane interactions in a simple system is required, as well as the potential extrapolation of these results to cell membranes in living organisms.

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Bios

Natalia Wilke

I am Bachelor in Chemistry 1998 ("Premio Universidad" best average award), and PhD in Chemical Science in 2003 at the National University of Córdoba. I was awarded Fellowships by the Fullbright Foundation (2007, USA) and by the Von Humboldt Foundation (Germany, 2015-2017) to carry out studies on assembling and use of optical tweezers in biophysics, I implemented these techniques in Córdoba in 2010.

I am Principal Researcher (CONICET). I lead a research group that inquires about how different organisms regulate the biophysical properties of their membranes, and how they adapt to stress situations. We use several biophysical techniques (optical tweezers among them), and our research focuses on both artificial biomembranes and living micro-organisms.. In 2021 I received the "Premio "Houssay 2021 (biological and biochemical science)" from the National Department for Science, Technology and Innovation. A complete list of my publications can be obtained at:

https://www.conicet.gov.ar/new_scp/detalle.php?keywords=&id=24476&articulos=yes or https://scholar.google.com/citations?user=2Fnxr8wAAAAJ&hl=es&oi=ao

Together with research, teaching was important in my carrier. I am currently Full Professor and vice-director of the Bachelor's degree in Chemistry (National University of Córdoba).