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Review

Complementary biophysical tools to investigate lipid specificity in the interaction between bioactive molecules and the plasma membrane: A review

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ABSTRACT

Plasma membranes are complex entities common to all living cells. The basic principle of their organization appears very simple, but they are actually of high complexity and represent very dynamic structures. The interactions between bioactive molecules and lipids are important for numerous processes, from drug bioavailability to viral fusion. The cell membrane is a carefully balanced environment and any change inflicted upon its structure by a bioactive molecule must be considered in conjunction with the overall effect that this may have on the function and integrity of the membrane. Conceptually, understanding the molecular mechanisms by which bioactive molecules interact with cell membranes is of fundamental importance.

Lipid specificity is a key factor for the detailed understanding of the penetration and/or activity of lipid-interacting molecules and of mechanisms of some diseases. Further investigation in that way should improve drug discovery and development of membrane-active molecules in many domains such as health, plant protection or microbiology.

In this review, we will present complementary biophysical approaches that can give information about lipid specificity at a molecular point of view. Examples of application will be given for different molecule types, from biomolecules to pharmacological drugs. A special emphasis is given to cyclic lipopeptides since they are interesting molecules in the scope of this review by combining a peptidic moiety and a lipidic tail and by exerting their activity via specific interactions with the plasma membrane.

Contents

Abbreviations: Aβ peptide, Beta Amyloid peptide; AD, Alzheimer Disease; AFM, Atomic Force Microscopy; AMP, Antimicrobial Peptide; ATR-FTIR, Attenuated Total Reflection Fourier Transform Infrared Spectroscopy; AZT, Azithromycin; BAM, Brewster Angle Microscopy; BM, Big Monolayer; BODIPY, Boron-dipyrromethene; C16BC, Hexadecylbetainate chloride; CG, Coarse Grained; CD, Circular Dichroism; Chol, Cholesterol; CLP, Cyclic lipopeptide; CMC, Critical Micelle Concentration; DHE, Dehydroergosterol; DIG, Detergent-Insoluble Glycolipid-(enriched complex); DHPC, 1,2-dihexanoyl-sn-glycero-3-phosphocholine; DMPA, 1,2-dimyristoyl-sn-glycero-3-phosphatidic acid; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPE, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine; DODAB, Dioctadecyldimethylammonium bromide; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DPPG, 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol; DPPS, 1,2-dipalmitoyl-sn-glycero-3-phosphoserine; DRM, Detergent Resistant Membrane; FRET, Forster Resonance Energy Transfer; GIPC, Glycosphingolipid; GM1, Monosialotetrahexosylganglioside; GUV, Giant Unilamellar Vesicle; GS, Gramicidin S; HM, Hypermatrix; IR, Infrared Spectroscopy; IRRAS, Infrared Reflection Adsorption Spectroscopy; ITC, Isothermal Titration Calorimetry; LB, Langmuir–Blodgett; Ld, Liquid disordered; LGP, Lipophilic Glutathioine Peptide; Lo, Liquid ordered; LPS, Lipopolysaccharide; LUV, Large Unilamellar Vesicle; MD, Molecular Dynamics; MP, Membrane Protein; NBD-(DP)PE, N-7-nitro-2-1-3-benzoxadiazol-4-yl (dipalmitoyl)phosphatidylethanolamine; NMR, Nuclear Magnetic Resonance; NR, Neutron Reflectivity; PA, Phosphatidic acid; PC, Phosphatidylcholine; PDB, Protein Data Bank; PE, Phospatidylethanolamine; PG, Phosphatidylglycerol; Phi, Hydrophilic; Pho, Hydrophobic; PI, Phosphatydilinositol; PLA1, Phospholipase A1; PL, Phospholipid; PMF, Potential of Mean Force; PM-IRRAS, Polarization Modulation Infrared Reflection Adsorption Spectroscopy; PM, Plasma Membrane; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; PS, Phosphatidylserine; QCM-D, Quartz Cristal Microbalance with Dissipation; R18, Octadecyl Rhodamine B chloride; RhBG, Rhamnose-Based Glycolipids; Rh-PE, Rhodamine-phosphatidyleth-anolamine; SF, Surfactin; SIMS, Secondary Ion Mass Spectrometry; SIV, Simian Immunodeficiency Virus; SLB, Supported Lipid Bilayer; SM, Sphingomyelin; SPR, Surface Plasmon Resonance; SUV, Small Unilamellar Vesicle; TOF-SIMS, Time-of-Flight Secondary Ion Mass Spectrometry; TR-DPPE, Texas-Red Dipalmitoylphosphatidylethanolamine

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1. Plasma membrane

1.1. General concept

As compared to nucleic acids, responsible for the genetic information and proteins, that perform most of the functional and enzymatic tasks, lipids often appear as "Cinderella" in the biomolecules family, being considered as just sitting there passively.

The basic principle of the organization of membranes looks very simple, formed by lipid bilayers where the polar headgroups are facing the aqueous environment and the hydrocarbon tails facing the interior of the bilayer, yet the details are surprisingly complex. Hence, plasma membranes (PMs) are complex dynamic entities which delimit the cell from its environment. They are the point of exchange with adjoining cells, and between the cell and the external medium. They are the primary place where signal recognition and transduction into intracellular responses for nutritional uptake, environmental responses, and developmental signalling occur [1,2]. Over years, it has become increasingly clear that if they are laterally fluid, they also can adopt a fascinating range of spatial organizations like the formation of transient local ordered clusters which are biologically important for several functional states of membrane proteins [2].

The cell membrane is a carefully balanced environment and any change inflicted upon its structure by a bioactive molecule must be considered in conjunction with the overall effect that it may have on the function and integrity of the membrane [3]. Understanding the mechanism at the molecular level by which bioactive molecules interact with cell membranes is therefore of fundamental importance.

1.2. Plasma membrane composition

PMs are composed by three main classes of lipids: glycerolipids (mainly phospholipids—PL), sphingolipids and sterols [2,4]. However, between species or cell types within a species, the lipid composition of PM can show a high degree of diversity; Table 1 illustrates this complexity.

In eukaryotic cells, the major structural lipids are glycerophospholipids, such as phosphatidylcholine (PC), phospatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidic acid (PA) [5–8]. Their hydrophobic tails, with chain length varying mostly from 14 to 22 carbons are either satured or *cis*-unsaturated. PC is the most abundant, accounting for more than 50% of PL [9]. The backbone of sphingolipids is constituted by a ceramide with saturated or *trans*-unsaturated hydrophobic chains. In mammalian cells, sphingomyelin (SM) and glycosphingolipids are the most abundant. Concerning sterols, cholesterol (Chol) is predominating in mammals and has a preferential interaction with sphingolipids, forming the so-called rafts domains (see below).

It is worth noting that the variation in headgroups and aliphatic chains permits the existence of more than a thousand different lipids.

Table 1
Lipid composition (in molar %) of different cell membranes in eukaryotic or prokaryotic organisms.

Lipids	Eukaryotic cells					Prokaryotic cells			
	Human erythrocyte	Human alveolar macrophage	Rat liver	*A. thaliana* leave	*S. cerevisiae*	*B. megaterium Gram +*	*S. aureus Gram +*	*P. aeruginosa Gram −*	*E. coli Gram −*
PC	16	30	25	17	25				
PE	15	21	12	18	10	73		60	82
PS	7	21	2	3	3				Traces
PG				4		27	58	21	6
CL					~2		42	11	12
PI	0.5		4	5	9				
PA	1				5				
SL[a]	14 (SM)	7 (SM)	13	7 (GIPC[a])	10–20 (MIPC)				
Sterol[a]	46 (Chol)	8 (Chol)	43 (Chol)	46 (sitosterol)	30–40 (Ergosterol)				
Others	0.5	13	1						
Ref	[5,6]	[7]	[8]	[1,10,11]	[13,15,17]	[8]	[19]	[19]	[19]

PC: phosphatidylcholine, PE: phosphatidylethanolamine, PS: phosphatidylserine, PG: phosphatidylglycerol, CL: cardiolipin, PI: phosphatidylinositol, PA: Phosphatidic acid, SL: sphingolipid, SM: sphingomyelin, GIPC: glycosyl inositol phosphorylceramides, MIPC: mannosyl inositol phosphorylceramides.
[a] The most abundant lipid of these categories is indicated in brackets.

Compared to mammalian membranes, plant PM is always highly enriched in sterols (sitosterol and stigmasterol mainly) and sphingolipids [1,10,11], which are packed at a higher density than glycerolipids and resist to mechanical stress [1]. Although the results of lipid analyses from several plant species are available in literature [12], a complete characterization of plant PM is still lacking, more particularly concerning the negatively charged glycosphingolipid (GIPC), specific to plants and fungi [1,13,14]. In the case of fungi, GIPC with mannose, called mannosyl inositol phosphorylceramides (MIPC) are the main sphingolipids [13,15]. Other particularities of fungi PM is the presence of ergosterol as sterol [16,17] and the wide variability in phospholipid composition according to the culture medium supplementation [18].

Bacterial membranes, in contrast to eukaryotic membranes, present a significant higher population of negative intrinsic curvature lipids (PE and cardiolipin) and negatively-charged lipids (phosphatidylglycerol and cardiolipin) [8,19,20] and an absence of sterol [20]. As shown in Table 1, diversity between Gram + and Gram − bacteria is also very important.

In parallel to the huge diversity in their global composition and lipid asymmetry between the two leaflets, membrane lipids also occur in different lipid phases depending on their structure and environment (for a review, see [2]). Globally, for the lamellar phases, three different behaviors can be observed: (i) the liquid-crystalline or liquid-disordered phase notably formed by glycerophospholipids with unsaturated acyl chains, (ii) the solid gel phase due to SM-rich lipid mixture; (iii) a third remarkable phase, liquid-ordered, is formed by the association of sterol and bilayer-forming lipid. The latter is related to 'raft' domains, having a high order parameter and a high diffusion coefficient [2]. Since the 90s, the notion of lipid domains, notably the raft domain model, has been put forward [21]. Those detergent-insoluble glycolipid-enriched complexes or DIGs (or DRM for detergent resistant membranes) are richer in sphingolipids and Chol that are close-packed. It has been proposed that these lipid domains anchor specific proteins involved in signalling and membrane trafficking [21,22], playing a role in the activity of those proteins. The size and the thermodynamic stability of these entities are not fully determined, but their dynamic nature has been stressed [23,24]. Some evidence indicates that the clustering of lipid rafts into more active signaling platforms depends upon interactions with and dynamic rearrangement of the cytoskeleton [25]. While the biological existence of such domain is still under debate, raft domains have been suggested to be implied in a number of specific interaction between biomolecules (peptides, proteins, drugs) and the membrane [23].

Very recently, the existence of micrometric lipid domains (in comparison to rafts that are considered as nanometric structures) enriched in glycerophospholipids and sphingolipids has also been visualized using fluorescent lipids, while still controversial in a biological context [26,27].

1.3. Models of membranes

The existence of lipid domains, lipid asymmetry, coexistence of phases and diversity in lipid composition are the reasons why membranes are extremely intricate structures. The complexity of this system is further increased by their association with proteins and carbohydrates. This complexity makes the biophysical interactions with bioactive molecules very difficult to investigate in a 'real' situation [28]. Therefore, simplified artificial membrane systems, which mimic the natural bilayer lipid membrane, have been developed [28,29].

Model membranes are systems in which the organization best mimics the lipid arrangement found in natural plasma membranes. Three systems are widely used, i.e., lipid monolayers, supported bilayers and liposomes (Fig. 1). While each of these systems exhibits advantages and disadvantages, they all mimic to a certain extent the lipid arrangement of natural cell membranes (for review see [22]).

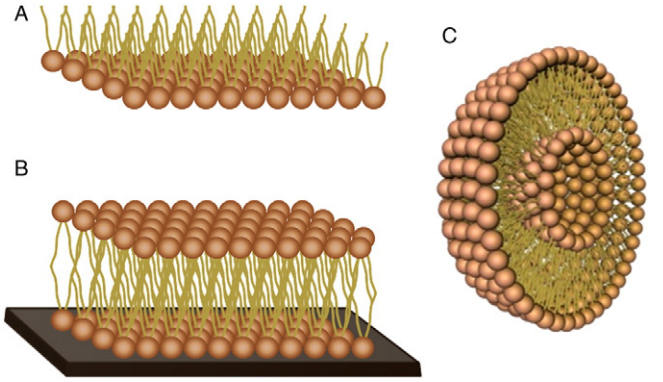


Fig. 1. Schematic representation of membrane models described in the text: (A) lipid monolayer, (B) supported lipid bilayer, (C) liposome.

Lipid monolayers provide a simple model considered as half the bilayer of biological membranes. They can be used to study the ability of compounds to penetrate into the outer leaflet of the membrane and to characterize the interactions of the molecule of interest with lipids [30,31]. They are formed at the air–water interface of a Langmuir trough by spreading lipids of the membrane under consideration. Parameters such as the nature and the packing of the spread molecules, the composition of the subphase (pH, ionic strength) and temperature can be varied in a controlled way and without limitation [32].

Supported lipid bilayers (SLBs) are biomimetic model membranes constituted of a flat lipid bilayer supported onto a solid surface such as mica, glass or silicon oxide wafers. These biomimetic systems typically allow the investigation of interactions with lipid head groups but also to predict the phase behavior and the molecular lateral organization of biological membranes [28]. They can also be suitable to investigate membrane-integral protein interactions in a functional manner [33]. They can be prepared by Langmuir–Blodgett technology, by fusion of lipid vesicles or by surfactant depletion from micellar solutions composed of a mixture of surfactants and phospholipids [34–36]. Since several years, tethered or cushioned bilayers made up of a lipid bilayer spaced from the solid surface by spacer molecules have been developed in order to enhance the mobility of the lipids onto the support [33,37]. Supported lipid bilayer models can be prepared quite easily and are much more stable than lipid vesicles. When SLBs are prepared by Langmuir–Blodgett technology, the lipid asymmetry can be controlled while it is not the case when using vesicular model systems. In addition, as these membrane assemblies are confined to the surface of a solid support, they can be characterized much easier than free-floating vesicles using a large variety of surface sensitive techniques such as AFM [38–40], secondary ion mass spectrometry (SIMS) [41], fluorescence microscopy [38], optical ellipsometry [42], quartz-crystal microbalance [43], X-ray reflectivity [44] and neutron reflectivity [45].

Lipid vesicles or liposomes are versatile biomimetic model membranes commonly used for studying membrane phase behavior and membrane processes such as membrane fusion, molecular recognition, cell adhesion, membrane trafficking and pore formation. These lipid assemblies enclose a small aqueous compartment and are produced from the aqueous dispersion of membrane lipids (pure or in mixture). Whereas lipid monolayers are constituted of only one lipid leaflet and therefore do not reflect the complexity of biological membrane structure, lipid vesicles are composed of two leaflets, which are arranged in a way that is similar to that of biological membranes. However, they are metastable structures offering poor long-term stability [46–48]. Depending on the mode of preparation, liposomes could be multilamellar or unilamellar (a single bilayer), and according to their size, are classified as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) or giant unilamellar vesicles (GUV) [49–52]. For a recent review about their preparation modes, the reader can refer to [24].

1.4. Molecules interacting with model membranes

Those models have been used to study the interaction of many molecules with the membrane. Among those molecules, peptides such as cell penetrating peptides (CPPs), antimicrobial peptides (AMPs), viral fusion peptides, lipopeptides or amphiphilic pharmacological drugs have been widely studied. For the latter, since they usually have intracellular targets, it is inevitable that they must cross the PM (and possibly other intracellular bilayers). The use of biophysical approaches and model membranes has shed light on a better understanding of drug/lipid interaction, which is of critical importance for pharmacological science (for a review, see [3,28]). A variety of such biophysical studies have been published and we list below some of them in a non-exhaustive way.

With models as simple as DPPC/DOPC or DPPG/DOPC monolayers or liposomes, we have highlighted differences in lipid penetration and affinity of fluoroquinolone antibiotics that are correlated to *in vivo* data, such as cellular accumulation [53,54]. The effects of lipid composition were also investigated for azithromycin (AZT) using simple membrane models. The effects of AZT is lipid-dependent, inducing notably modifications of elastic properties of DOPC [55], but not with SM and Chol [56,57].

Liposomes as model membranes are also used to estimate drug efficacy, notably in the determination of partition coefficient that measures the amount of molecules entering into/through the biological membrane [58]. Some studies using liposomes have also evidenced transport mechanisms [59], or help understanding toxicity of some drugs [60,61].

The activity of antimicrobial peptides (AMPs) has also been widely studied using model membranes. These peptides are a very promising alternative to conventional antibiotics that suffer from increasing bacterial resistance to their action. Recent publications [4,62,63] have focused on those molecules, so we will not extensively review this area. Briefly, using model membranes and biophysical approaches, it was shown that AMP affinity for bacterial membrane is generally triggered by electrostatic interactions; AMPs are mostly positive cationic peptides that interact preferentially with negatively charged lipids such as PG, cardiolipin or LPS present in bacterial membranes [19,64,65]. AMP activity also depends on the lipid structure (headgroup and acyl chains) [4], and the presence of Chol is suggested to play a role in AMP selectivity, while this is still under debate [66].

Models of membranes are also extensively used to characterize and investigate the lipid interactions of nanosystems like liposomes [67,68], nanoparticles [69,70], dendrimers [71–74] and also amphiphilic block copolymers [75].

Most of the biophysical studies mentioned above or in recent reviews decipher about the interaction of drugs, AMPs or other biomolecules with rather simple model of membranes, composed of two or three different lipid types, few are discussing about lipid specificity, as for AMPs, but rarely in a systematic manner.

However, lipid specificity should be an important factor for the understanding of mechanisms of lipid penetration and/or activity of the interacting molecule or of mechanisms of some diseases, and is hence of critical importance in life science. Further investigation in that way should improve drug discovery and development of membrane-active molecules in many domains such as health, plant protection or microbiology.

In the following paragraphs, we will present practical information and complementary biophysical approaches that can give information about lipid specificity at a molecular point of view. Those methods are applicable to any kind of molecules that can be purified and for which 3D structure is accessible in the PDB database or by models.

2. What kind of lipid to choose?

Since model membranes should be representative of natural membranes, their reconstitution must reflect this "real" composition as best, taking practical and experimental details into account. It is hence important to soundly consider the choice of the lipids, of the model (monolayer vs bilayer, type of liposome,… see above) and of the complexity of the lipid mixture.

- Choice of lipids: This choice will be done according to different criteria. The first one consists in choosing an appropriate lipid for the organism under consideration [76,77]. For example, concerning model sterol, it is better to choose Chol for a study with red bood cells [78,79], sitosterol when working with plants [80,81], or ergosterol in the case of fungi species [82,83]. The second criterion is the availability of the lipid. Some of them are commercially available or could be easily extracted and purified using well-known protocols, others are not. As an example, plant sphingolipids are not commercially available and are very difficult to purify from plant cell extracts. In the same way, the predominant ceramide in yeast is 4-hydroxysphinganine that is not commercially available. In order to overcome this difficulty, the use of available models as close as possible to the considered lipid in terms of structure, charge, chain length and (un)saturation should be considered. Table 2 gives the main commercially available model lipids that can be used to prepare model membranes for each organism.
- Choice of membrane model: each system has its advantages and disadvantages and will be chosen according to the information needed, considering the feasibility of the experiment. In order to analyze the first steps of the interaction of a bioactive molecule with a membrane, the most suitable model will be Langmuir monolayers which mimic the external leaflet of the membrane bilayer [79,83–85]. Similarly, for the analysis of the insertion of a membrane-active molecule within the membrane, liposomes or supported bilayers will be preferred [79,86]. The limitation of the models must also be considered: actually, it is impossible to prepare liposomes containing only sterols.
- Choice of lipid mixture: The lipid composition of the model membrane is one of the most important points. As expected, more the composition of the model membrane is close to the reality, more the experimental results will be biologically relevant. However, increasing the complexity of the model membrane will in turn render the biophysical observations difficult to interpret. It is therefore important to design carefully the experiments taking into account this balance between complexity and feasibility. Most of the studies using model membranes adopt generally a step-by-step approach, by modifying gradually the lipid composition, for example going from PC membranes to PC/sterol or to PC/sphingolipid/sterol systems. In the literature, the most complex systems contain generally three lipid types (PC, sphingolipid, sterol).

Table 2
Commercially available model lipids for different species.

Species	Glycerophospholipids	Sphingolipids	Sterols	References
Plant	PLPC	GlcCer[a]	Sitosterol	[80]
Fungi	DOPC POPE	Phytosphingosine[b]	Ergosterol	[77,82]
Bacteria	POPE POPG CL			[76]
Mammalian	POPC	Sphingomyelin	Cholesterol	[78]

[a] C16 Glucosyl(ß) Ceramide (d18:1/16:0) D-glucosyl-ß-1,1′ N-palmitoyl-D-*erythro*-sphingosine.
[b] (2S, 3S, 4R)-2-amino-1,3,4-octadecanetriol.

3. Which method for which information?

We have divided the biophysical methods into two groups (that are non-exhaustive), depending on the kind of information you get:

1. "Global molecular" methods giving information on the global effects of the molecule of interest on the lipids. These include (among others): Langmuir monolayer technique, isothermal titration calorimetry (ITC), fluorescence spectroscopy and imaging, atomic force microscopy (AFM), neutron reflectivity (NR), surface plasmon resonance (SPR) or electron paramagnetic spectroscopy (EPR).
2. In the second group, we have considered techniques giving information at the molecular/atomic level, that we call "molecular-specific" techniques. In that group, we gathered infrared spectroscopy (FTIR and PM-IRRAS), nuclear magnetic resonance (NMR) on lipids (^{31}P, ^{2}H, ^{13}C NMR), secondary ion mass spectrometry (SIMS) and "in silico" approaches.

All those methods are summarized in Fig. 2.

3.1. "Global molecular" biophysical techniques

3.1.1. Langmuir trough

The Langmuir trough technique applied on monolayer model at an air–water interface has been extensively used since the beginning of the 20th century [87] for characterizing lipid–lipid, protein–lipid or drug–lipid interactions at a micrometric level. More recently, the combination of Langmuir trough with highly sensitive techniques like atomic force microscopy (AFM), polarization modulated infrared reflection adsorption spectroscopy (PM-IRRAS), Brewster angle microscopy (BAM), fluorescence microscopy, ellipsometry, nonlinear optical spectroscopy, synchrotron based X-ray scattering techniques has emerged as a powerful tool for visualizing lateral segregation and for obtaining structural information in phase-separated lipid monolayers at both micrometer and nanometer scales [88]. Combination of two or more complementary techniques makes the Langmuir technique one of the most suitable platform for learning about the mechanism occurring at the cell surface [89,90].

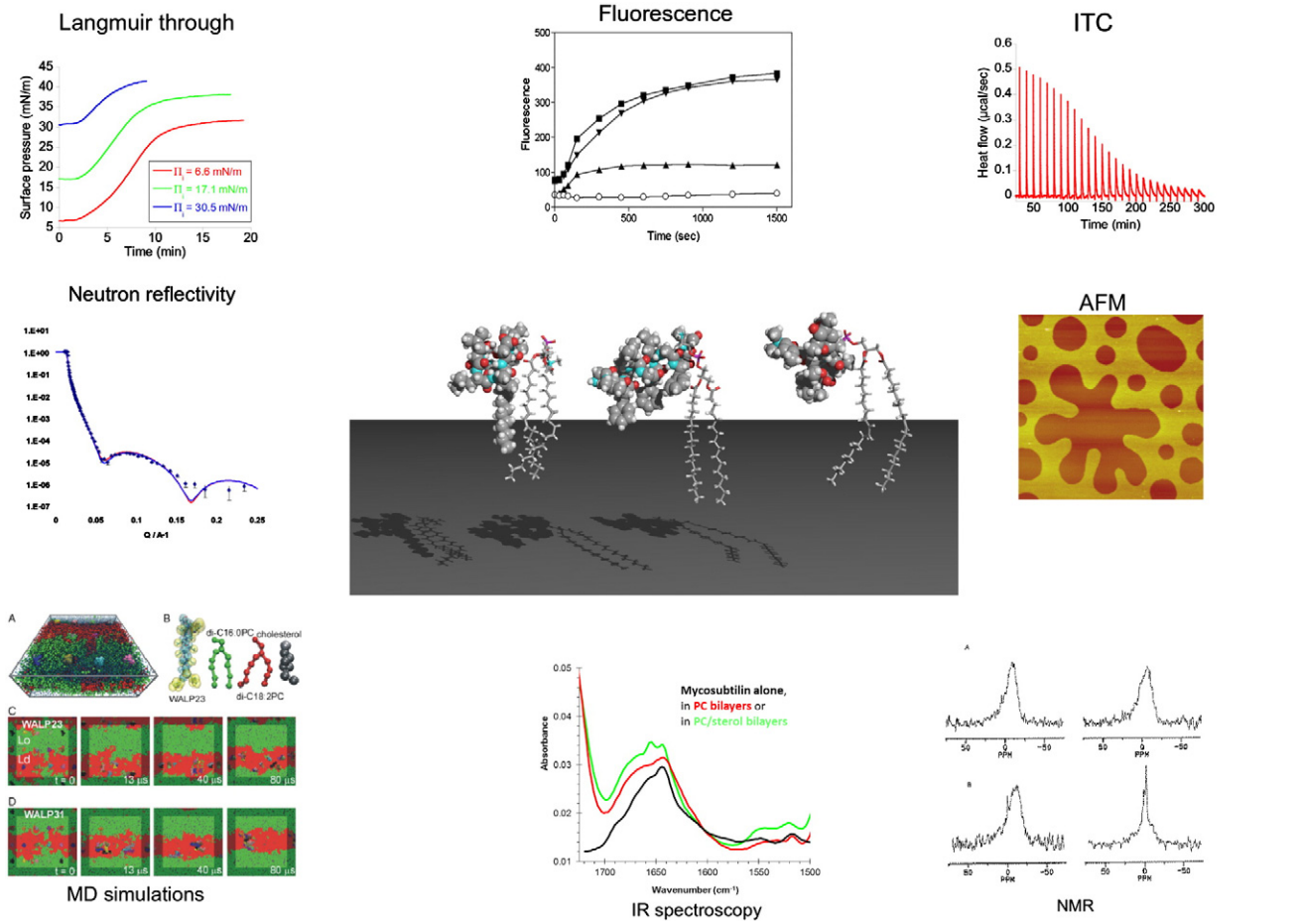


Fig. 2. Schematic representation of most of the different biophysical tools described in this review and used to study specific interaction between lipids and bioactive molecules. Three main classes of molecules, pictured at the centre of the figure, are under consideration: 1. cyclic lipopeptides represented left by surfactin in interaction with POPC, 2. peptides like SIV tilted peptide interacting with DPPE in the middle, 3. pharmacological drugs such as azithromycin (right) associated to DOPC. The molecules are CPK-represented and phospholipids as sticks (blue atoms: nitrogen; grey atoms: carbon; red atoms: oxygen). Biophysical techniques are considered here as two groups: one group describing global effects of bioactive molecules on membranes (Langmuir trough, fluorescence, ITC, NR, AFM), the other methods giving information at the molecular (atomic) level (NMR, IR spectroscopy, molecular modeling). In this figure, one example is giving for each method: Langmuir trough assays represent the penetration kinetics of 1-18-bis-octadec-9-enyl-α-L-rhamnopyranoside into DMPC at different initial surface pressure (Π_i) (unpublished results). Fluorescence experiments show the effect of Aβ29-42 tilted peptide on fusion of lipidic phases of liposomes (lipidic fluorescent probe: R18) in the presence of increasing peptide:lipid ratio (adapted from [337]). ITC profiles are obtained by successive injections of POPC vesicles into a solution of 1-18-bis-octadec-9-enyl-α-D-xylopyranoside (unpublished results). NR profiles are recorded 3 h after the injection of 20 μM fengycin on a d-P-h-O-PC bilayer in D_2O.The line results from the fitting (unpublished results). AFM topography image [*z-range 10 nm*] is obtained for mixed monolayers prepared at 0.25 fengycin molar ratio and ceramide. The image size is 15 μm × 15 μm. Lighter levels in the images correspond to higher height. Adapted from Eeman et al. [146]. IR spectra represent the Amide I band of mycosubtilin with or without lipids. ^{31}P NMR spectra at 101.3 MHz of multilamellar vesicles made of PC:PE:PI:PS:SM:Chol in the presence of Aβ29-42 (adapted from Mingeot-Leclercq et al. [337] JCP). MD simulations of the sorting of WALP 23 and 31 peptides in model membrane with coexisting fluid phases made of DPPC, DOPC and Chol. Adapted from Schäfer et al., [313].

Three main types of experiments leading to different relevant information can be performed with the Langmuir trough technique.

The first one is the basic compression isotherm assay which is a plot of the changes in surface pressure that occur when reducing under a pseudo-equilibrium condition the area occupied by insoluble molecules spread at the air–liquid interface [88]. It gives information about monolayer formation, molecular area, monolayer phases, monolayer stability, two-dimensional compressibility and interaction of species from the subphase with the monolayer [88]. By performing a thermodynamic analysis (calculation of the excess free energy of mixing and the free energy of mixing), compression isotherm data give the evaluation of the mixing behavior and hence the molecular interactions occurring between molecules of different natures (i.e. drug-like molecule and a phospholipid representative of a cell membrane) spread at an air–liquid interface [29,30].

The second type of measurement is the adsorption/penetration experiments which aim to evaluate the ability of surface-active molecules to adsorb at an air–liquid interface or to penetrate into a more or less ordered lipid monolayer formed at the air–water interface. The increase of surface pressure resulting from the interaction of the bioactive compound with the lipid monolayer over time gives rise to the adsorption/penetration kinetics [79,86,91,92]. By plotting the maximal surface pressure increase observed at the steady state as a function of the initial surface pressure of the lipid monolayer, the exclusion surface pressure (ΠIe)—also called the maximum insertion pressure—as well as the synergy factor can be determined. These parameters are useful to determine the extent of bioactive molecules that bind to different lipid monolayers [84].

The third way to work with the Langmuir trough technique is to form a monolayer on the water surface with a subsequent transfer onto a solid support to obtain lipid monolayer or bilayer representative of a biomembrane [88,93–95]. The so-called Langmuir–Blodgett (LB) technology gives the opportunity to apply techniques like AFM (see below) not suitable for *in situ* analysis at the air–water interface.

3.1.2. Fluorescence spectroscopy and imaging

Fluorescence can be used in different ways to explore the interaction between a biomolecule and liposomes. Several experiments can probe the effect of the molecule on the membrane integrity. The modification of the environment of the fluorescent probe following the interaction with the bioactive molecule and the subsequent change in fluorescence intensity is indicative of membrane disruption, membrane fusion or aggregation (for a review, see [96]). In the case of peptides, the intrinsic fluorescence of amino acids such Trp or Tyr can also be used to probe membrane insertion [63,97,98].

To study leakage process, fluorescence increase of self-quenched carboxyfluorescein or calcein [99] encapsulated into lipid vesicles is followed over time. Another technique consists in using fluorescent dye/quencher pairs such as ANTS (HPTS)/DPX, encapsulated in the same liposome population [100]. When leakage occurs, concomitant dequenching of ANTS (HPTS) is observed. For fusion and aggregation, the intermixing of the lipid leaflets of the liposomes and that of aqueous contents (for fusion only) can be observed. For the aqueous mixing, fluorescence quenching assays using ANTS/DPX [101] or Tb^{3+}/DPA [102] pairs are usually carried out. In this case, the fluorescent dye and the quencher are encapsulated into two different liposome populations. Mixing of the aqueous compartments induces a fluorescence decrease. For the lipid mixing assays, dequenching or FRET (Forster Resonance Energy Transfer) assays are used [103,104]. The latter provides a measure of the average distance between donor and acceptor fluorescent molecules, the fluorescence energy emitted by the donor molecule being transferred to the acceptor molecule if both molecules are close enough (typically $1 < d < 10$ nm) [105]. The two fluorescent probes, for example NBD-PE (donor) and Rh-PE (acceptor), are incorporated into one liposome population and mixed to non-labelled vesicles. When lipid mixing occurs, the dilution of the dyes decreases the FRET efficiency. Variation of this method is also suitable to detect the mixing of the liposome inner monolayers [96]. Another method consists in labelling the lipid phase with R18, a lipophilic fluorescent probe, at a selfquenched concentration. Dilution of the probe due to fusion/aggregation induces an increase of the dye fluorescence [106].

By modifying the lipid composition, the global influence of one specific lipid can be studied. We have notably shown the influence of PE on the membrane fusion and disruption induced by tilted peptides from viruses but not for tilted peptides involved in the interaction with bacterial or mitochondrial membranes, that are more influenced by acidic PL [107]. Fluorescent lipid-like probes further allow the visualization of lateral structures of membranes. Lipid domain formation can notably be visualized by probes able to specifically label lipid phase. The well-known LAURDAN [108] displays unique properties when inserted into membranes, i.e. it distributes equally into ordered and disordered-like lipid phases but shows a phase-dependent emission spectral shift, bluish in the ordered phase and greenish in the disordered one [109,110]. It means that it can give information directly from fluorescent images by using the proper set of emission filters. Other probes such as di-4-ANEPPDHQ [111] or newly designed probes [112] able to measure the degree of membrane order in artificial liposomes as well as in living cells are also useful tools to quantify lipid order.

Other probes such as TR-DPPE (Texas-red dipalmitoylphosphatidylethanolamine) and NBD-DPPE (N-7-nitro-2-1-3-benzoxadiazol-4-yl dipalmitoylphosphatidylethanolamine) partition respectively into Ld and Lo phases [113]. Fluorescent sterols like dehydroergosterol (DHE) can also be used to assess the importance of sterols in domain formation [114,115]. These probes have been very recently used in simple binary DMPC/chol liposomes to demonstrate that α-hederin, a molecule from the saponin family, is able to have a specific interaction with sterol and to induce lipid domain formation, in contrast to its aglycone homolog, hederagenin [116].

BOPIDY (boron dipyrromethen) fluorescent lipid analogs of sphingolipids or PC have also recently been used to visualize micrometric domains [26,27].

3.1.3. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) measures the heat released or adsorbed when two interacting components are brought together in the same environment and initiate a reaction. One binding partner is titrated into a solution containing the other partner at a constant temperature [117,118]. The changes in heat energy are recorded over time and allow the evaluation of enthalpy changes due to the interaction between titrated and titrating solutions. This technique provides a complete thermodynamic description of binding processes [119].

ITC has a large panel of pharmaceutical applications and in the field of biological membranes. This technique is a high-sensitivity method suitable to provide a complete thermodynamic characterization of an interaction at equilibrium conditions (e.g. interaction between receptor–ligand, enzyme–substrate, antibody–antigen, cell–ligand, drug–DNA…), without requiring a specific labelling [120].

In the field of biological membranes, ITC is the method of reference for studying interactions between a bioactive molecule (surfactant/peptide/protein) and model lipid membranes (vesicles or micelles) [121–124]. Different processes such as membrane formation, membrane binding, peptide aggregation at the membrane surface, peptide conformational change, membrane partitioning, and membrane solubilization can be investigated using ITC. Parameters like binding affinities, binding enthalpies, and binding stoechiometries as well as relevant concentrations such as critical concentrations for the onset and end of membrane solubilization can be determined [118]. These data can be used to provide valuable insight into the origin and nature (electrostatic or hydrophobic) of bioactive molecule–membrane interactions and the factors that influence them.

By varying the composition of the liposome, specific interactions with a particular lipid [120,125,126] or a specific physical structure or organization of the lipid bilayer [127] can be highlighted.

This calorimetric technique is also a convenient tool for determining the critical concentration (CMC) at which surfactant micelles are formed and the corresponding heat of micelle formation.

3.1.4. Atomic force microscopy

The atomic force microscopy (AFM) belongs to the local probe microscopy methods. The local probe (usually an oxide-sharpened microfabricated Si_3N_4 tip) interacts directly with the sample surface. The measurement is based on the sensing of interaction forces between the tip and the surface via the electronic clouds of their external atoms. A complete description of the technical aspect of AFM can be found in [128].

AFM can operate in different modes, which differ mainly in the way the tip is moving over the sample, in order to visualize surface structures and lateral organization (contact or tapping mode) or to study intra- and intermolecular forces (force spectroscopy) [129–131,130,131]. The lateral resolution is in the nanometer range and the vertical resolution is around 0.1 Å [132]. AFM can be performed in aqueous solutions making it suitable to study biological samples under physiological conditions [131,132]. Nevertheless, the technique requires attaching the sample onto a solid support which can limit the degree of freedom of the molecules [133]. Tethered lipid bilayers [134] and bilayers on holes [135] can overcome this problem but are still under development.

Since its invention in 1986 [136], AFM has been largely used for providing three-dimensional images of single molecules (e.g. proteins, DNA, RNA…) adsorbed on surfaces of two-dimensional protein crystals, of living cells and for studying the lateral organization of supported lipid membranes with a nanometer resolution [137–139]. Besides, this technology has been shown to be very useful for probing quantitatively physical properties such as surface forces, surface charge and hydrophobicity, for measuring inter- and intramolecular interactions, as well as for molecular recognition studies with specific chemical probes or probes bearing biologically active molecule [140–144]. In the field of biomembranes, this technique is a powerful tool to observe phase-separated domains of supported lipid bilayers on the micro and nanoscales, and to monitor membrane remodeling and alteration upon interaction with exogenous agents like solvents, (lipo)peptides, proteins, detergents and antibiotics. The reader can refer to references [133] and [145] for some examples.

AFM has also been largely exploited to obtain information about the lateral organization of phase-separated lipid monolayers containing a bioactive molecule [146,147].

Real-time imaging of bilayer formation and of interactions of bioactive molecules with model lipid membranes have been performed [39,57,148–152]. Recent progresses in AFM technique and set-up like the high-speed AFM [153] and the continuous flow AFM set-up [154] offer promising perspectives in terms of rapid biological molecular processes monitoring.

AFM has extensively been used to study the lipid selectivity of molecules interacting with biological membranes. Although AFM is not yet suitable for single-lipid mapping [155], the technique is useful to determine the preferential binding site/lipid phase of exogenous agents [57,138,156–161]. By varying the composition of the supported lipid bilayers, it was observed for example that azithromycin perturbs more strongly DPPC domains than SM or SM-Chol domains [57]. SIV tilted peptide has also been shown to induce a preferential destabilization of DPPC domains by forming nanoholes [138] while cyclosporine A targets SM lipid [156].

3.1.5. Neutron reflectivity

Specular neutron reflectivity (NR) is a method of choice to provide information on average structure and composition of ultra-thin film layered systems along the direction perpendicular to the interface down to a nanometric scale (~2 nm) [162–164].

The principle is based on neutron scattering from materials by interacting with the nucleus of the atoms [162]. The sensitivity of neutron reflection to the layer structure depends on the contrast in neutron refractive indexes of the layer components and the surrounding media, which can be achieved by the contrast variation method and/or the use of perdeuterated components [165]. The structural parameters (thickness, surface coverage, refractive index) of the layers are derived from variation in reflected intensity as a function of the wave vector (related to the angle of incidence and the neutron beam wavelength) [166]. As for AFM, the sample has to be supported on a planar substrate, which can be a limitation for some sample analysis.

Since a decade, NR has shown its great potential for life science studies and more particularly for characterizing biomimicking membranes and for probing their interaction with various biological molecules. This method has already been applied for quantifying the presence and vertical location of small amounts of exogeneous molecules within a supported phospholipid bilayer and for observing their effects on the bilayer in terms of thickness and roughness [166–168]. By varying bilayer composition, some specific aspects of the interaction can be highlighted. As an example, in the case of Antennapedia homeodomain, it has been shown by NR that its vertical repartition within the bilayer is dependent on the phospholipid charge [167]. Another example is the phospholipase A2 which penetrates into the bilayer in increasing order for DOPC, POPC and DPPC [166].

3.1.6. Surface plasmon resonance

Surface plasmon resonance (SPR) is a surface sensitive technique powerful for monitoring in real-time affinity, selectivity and kinetics of biomolecular interactions without requirement of a specific label [169,170].

SPR occurs at the boundary of two materials of different dielectric functions [171], e.g. glass covered by a thin metal (typically gold) film and air or water. A p-polarized light is emitted by the light source, reflected on the gold-coated sensor surface and detected by a diode array detector. Typically, to study the interaction of a biomolecule with a biomimetic membrane, a lipid monolayer or bilayer is immobilized onto the gold layer and the biomolecule is injected over the surface in a continuous flow. If it adsorbs onto the biomembrane, changes of the incidence angle due to refractive index modification is observed over time (Hall et al. [107]). From this sensorgram, binding events can be visualized and kinetics of the interaction can be calculated. Besides monitoring in real-time and use of free-label molecules, another main advantage of the SPR technique is the high throughput of samples that can be performed.

This technique has been largely used to study the membrane-binding properties of proteins aiming at determining their lipid specificity [169,172,173]. It was also applied to investigate the direct interaction of antimicrobial peptides with model membrane systems to gain insight into their mechanism of action [170,174]. It can, for example, detect multiple steps of the membrane-binding process. It is also a powerful tool to analyze ligand binding by integral proteins supported into a lipid film [171]. To our knowledge, this technique has been applied to study the membrane interaction of bioactive small molecules like cyclic peptides [175]; however, it is little used for the study of other biomolecules and was never applied to lipopeptides or glycolipid biosurfactants.

3.1.7. Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance spectroscopy (EPR) is a spectroscopic technique that detects species that have unpaired electrons. For the study of exogenous molecules–membrane interactions, different variants of the technique (continuous wave EPR, electron spin echo envelope modulation and double electron–electron resonance) can be combined to obtain information on ordering, mobility and polarity of phospholipids and mobility of exogenous agent within the membrane

[176]. To obtain full set of data, the technique requires spin-labelled compounds (exogenous agent and/or phospholipid labelled with nitroxide for example) and deuterated phospholipids and water. Moreover, by using spin-labelled phospholipid with the spin-label attached at different positions along the lipid, it is possible to probe membrane packing at different depths. With this technique, we can also access to information about the insertion depth, orientation and aggregation state of the exogenous molecule within the membrane.

EPR technique has been applied to study the effect of the presence of surfactants [177], of lipopeptides [178], of drugs like clomipramine [179] and of dendrimers [180] on the structure of lipid bilayers or biological *stratum corneum* membrane [181,182]. It was also used to determine the location and dynamics of basic peptides [183] and to study lipid nanodomain formation [184,185].

Other techniques like ellipsometry [161,186–190], quartz cristal microbalance with dissipation (QCM-D) [43], X-ray reflectivity [191–193] can also be useful to obtain average information about specific interaction between bioactive molecule and biomimicking membranes, but are not further discussed in this review.

3.2. "Molecular-specific" biophysical approaches

3.2.1. Infrared spectroscopy

Infrared (IR) spectroscopy is a well-established method to study the interactions between lipids and bioactive molecules that provide relevant information at the molecular level. Since excellent papers or book chapters presenting a detailed view of the IR techniques have been published [194–198], we will focus exclusively on the use of IR techniques for analyzing lipid specificity in the interaction between molecules of interest and membranes. In the IR methods, different membrane systems can be used, such as liposomes (FTIR-ATR) or Langmuir monolayers (IRRAS or PM-IRRAS). In general, model membranes made of one or two different types of lipids have been used. Usually, the spectra of model membranes are measured alone and in the presence of the membrane-active molecule.

Three principal regions are analyzed in the IR spectra:

- 3000–2800 cm^{-1}: This region corresponds to the absorbance of the alkyl chains derived mostly from the phospholipids [199,200].
- 1800–1400 cm^{-1}: It corresponds to the absorbance of C═O ester and phospholipid alkyl groups. In the case of a membrane-active protein or peptide, the amide I and II bands also appear in this region [196,199,200].
- 1300–1150 cm^{-1}: This region corresponds mainly to the absorbance of PL phosphate groups [199,200].

Tables 3A and 3B present a detailed view of different bands from respectively DPPC, a phospholipid commonly used in model membranes (Table 3A) and of the secondary structures found in peptides, based on the absorbance of the amide I group (Table 3B).

As an example for the use of IR technique in lipid specificity, the interactions of a small antiparasitic peptide named LGP (lipophilic glutathioine peptide), with different monolayers have been investigated using PM-IRRAS spectroscopy [201,202]. The monolayers made of DPPG, DPPC, DODAB (dioctadecyldimethylammonium bromide, positively charged) and Chol or a mixture of Chol and sphingomyelin to simulate lipid rafts, were used [201]. The authors suggest that the charge state of the phospholipids has no influence on the peptide conformation (which is unfolded) and orientation since the spectra obtained with DPPG (negatively charged), DPPC (neutral) and DODAB monolayers are similar. However, in the presence of Chol or "raft" monolayers, LPG conformation shifted from an unfolded state to a folded turn structure, concomitantly with an orientation parallel to the interface, suggesting a lipid-specific interaction of the peptide.

Table 3A
Attribution of the IR bands for DPPC vesicles hydrated with deuterated water [199].

Wavenumber (cm^{-1})	Attributions
2957	CH_3 stretching, asymmetric
2924	CH_2 stretching, asymmetric
2871	CH_3 stretching, symmetric
2853	CH_2 stretching, symmetric
1732	C═O stretching, ester
1467	CH_2 bending
1380	CH_3 bending, deformation, symmetric
1233	PO_2- stretching, asymmetric
1171	C–O stretching, single bond
1159	C–C stretching, skeletal
1060	R–O–P–O–R
1082	PO_2- stretching, symmetric

Table 3B
Amide I band frequencies and assignment to secondary structures for a peptide (protein) in deuterated water [196].

Wavenumber (cm^{-1})	Secondary structure
1637–1624	Beta sheet
1644–1641	3_{10} helix
1649–1641	Random coil and turns
1657–1649	Alpha helix
1671–1650	Turns
1690–1675	Beta Sheet

For mycosubtilin, a natural antimicrobial lipopeptide acting on the plasma membrane, many papers have been devoted to study its interactions with different membrane systems, mostly using FTIR and PM-IRRAS techniques [79,86,92,203–207]. When the peptide is inserted in monolayers and liposomes constituted by DMPC, mycosubtilin is structured as γ-turn and interacts mainly with the lipid alkyl chains [86]. When DMPC/chol liposomes are used, significant changes have been observed [79]. The presence of Chol modifies the secondary structure of the lipopeptide from γ-turn to β-turn. Furthermore, thanks the absorbance of its phenol ring, the involvement of the tyrosyl residue in the interactions with Chol-containing liposomes has been observed [79]. This result is particularly important because a previous study showed that the methylation or acetylation of the phenolic OH group of Tyr residue abolishes the biological activities of mycosubtilin [208]. The authors concluded that the lipopeptide activity could be modulated by the interactions between the phenolic component of Tyr and sterols present within the membrane. Sterol could therefore be the favored partner of mycosubtilin, facilitating its incorporation in the membrane.

Contrarily to mycosubtilin, Chol does not seem to favor membrane insertion of gramicidin S (GS), a powerful antimicrobial peptide with bactericide and fungicide activities [209,210]. The effect of Chol on the interactions between gramicidin S and liposomes has been analyzed by FTIR spectroscopy, notably at the level of the amide I band of the peptide [211]. Measurements were performed at 0 °C and 50 °C, with DMPC liposomes containing or not Chol; at 0 °C, lipids are in gel state whereas at 50 °C, they are in fluid state. In the presence of DMPC liposomes, when the temperature increases from 0 °C to 50 °C, gramicidin can steadily penetrate into DMPC bilayer, as assessed by gradual conformational changes of the peptide [212]. In the presence of Chol, the absorbance of GS amide I band is the same as that observed in buffer, indicating that Chol induces the exclusion of gramicidin from the bilayer. At higher temperatures, GS molecules penetrate into the bilayer, but to a lesser extent than into DMPC bilayers, underlying the "exclusion" effect of Chol [211].

The analysis is also applicable to non peptidic molecules such as glycolipids. Very recent studies were carried out in our laboratory on the membrane properties of rhamnose-based glycolipids (RhBG). In the presence of POPC liposomes, rhamnolipids interact mainly with the phospholipid alkyl chains, C═O and phosphate groups. When Chol is added, the interactions between the alkyl chains and RhBG are less marked, suggesting again a modulating effect of Chol on membrane insertion (unpublished data).

It should be noted that ATR-FITR is also used to determine the relative orientation of phospholipids and peptides, as it was done for the SIV peptide and fragments derived from apoliproproteins [213,214].

In summary, IR spectroscopy is a powerful technique to study the interactions of bioactive molecules with lipid systems at the molecular level. Information on the conformation of the molecules and their orientation as well as on the chemical groups involved within the interactions can easily be obtained.

3.2.2. Nuclear magnetic resonance

Solution and solid-state NMR of lipids has been largely reviewed by the group of E. Dufourc [215,216], so we will only give a brief description and mention the information we can get from this technique applied to lipids. Solution (for lipids dissolved in organic solvents) and solid-state (for lipids in a hydrated membrane state) NMR is one of the most powerful techniques to decipher structure and dynamics of lipids. NMR relies on the presence of active atomic nuclei constituting the lipids: ^{1}H, ^{31}P, ^{14}N essentially; lipids can also be deuterated (^{2}H) or fluorinated (^{19}F).

The analysis of the chemical shift anisotropy $\Delta\sigma$ in a ^{31}P NMR spectrum is notably useful to determine specific interactions between phospholipids and a bioactive molecule at a molecular level. PLs organized in bilayer give in a ^{31}P spectrum a typical asymmetric shape with a peak at high field and a shoulder at low field. The difference between the two peaks is called chemical shift anisotropy, $\Delta\sigma$. This parameter is particularly sensitive to phase transition and temperature. In the case of aminoglycoside antibiotics, the measurement of $\Delta\sigma$ has suggested that these molecules are able to decrease the motional freedom of the phosphate groups of PL, and more particularly those of acidic lipids like phosphatidylinositol [217,218].

The analysis of the ^{14}N in DMPC/DHPC bicelles can serve as probe for changes in electrostatic environment of the polar headgroups of lipids, by the study of the symmetry of the quadrupolar splitting of the ^{14}N nucleus of magnetically aligned bicelles. This was notably done for curcumin, a natural compound interacting with membranes: it was shown to be anchored to the bilayer by a hydrogen bond near the phosphate group, like Chol [219].

NMR can also help analyzing the structure of the molecule that inserts into the lipids; this is widely used for peptides interacting with model membrane systems [63,220–222]. On the other hand, NMR spectral characteristics of a non peptidic biomolecule (such as antibiotics) labelled with ^{15}N for example, alone or in the presence of lipids can also shed light on the molecular group involved in the interaction [217].

3.2.3. Secondary ion mass spectrometry

Secondary ion mass spectrometry (SIMS) combines sample imaging with the unambiguous identification of molecules by their mass [223]. A primary ion beam is bombarded onto the sample which generates secondary ions with a very characteristic fragmentation pattern, allowing the compounds to be identified [223,224]. It measures directly, without partitioning of dye, the mass of components or isotopically labelled fragments from the components [225]. For a detailed description of the technique, the reader can refer to the review of Boxer et al. [223].

This technique is still in its infancy concerning its application for membrane lipid composition and distribution analysis because mass spectra of membrane lipids are rich in acyl chain fragments, which are largely identical for many phospholipids [224]. Other limitations are insufficient sensitivity, spatial resolution and preservation of the sample without bulk water, as the analysis is done in high-vacuum conditions [223]. SIMS technique has a great potential to image the distribution of specific molecules within complex biological samples and to measure the amount of each molecule within a specified region of the sample [223] but still needs instrumental development to fully achieve these goals. A higher spatial resolution (down to 50 nm), a higher sensitivity and a higher mass resolving power have been obtained by the development of new instruments like high-resolution dynamic SIMS (NanoSIMS from CAMECA) [225]. By incorporating isotopically labelled lipids into the lipids of interest, NanoSIMS can facilitate differentiation of lipid signals and enhance their secondary ion yield [225]. Nevertheless, SIMS has a poorer spatial (lateral and vertical) resolution comparatively to AFM but provides more significant molecular information [223].

SIMS technique has already been applied to in situ analyses of lipids in tissues or individual cells for biomedical purposes (for recent reviews see [182,226]). It has also been used to analyze phase separation of lipid on monolayer [227] or bilayer models [225,228,229] for acquiring new insight into the molecular interactions involved in lipid organization in membranes.

The distribution of exogenous agents like amphiphilic block copolymers [230] or surfactant peptide [231] into a lipid monolayer has already been explored by SIMS after Langmuir–Blodgett transfer onto a solid support.

To date, only one study deals with the use of SIMS to characterize the effect of an antimicrobial peptide on supported lipid bilayers [232].

The recent development of microfluidic devices that permit to analyze hydrated samples with SIMS [182,233] should provide new opportunities to study the interaction of exogenous agent with biological membranes in real-time.

3.2.4. "In silico" approaches

A number of theoretical and computer simulation approaches have been developed to describe bioactive molecule/lipid interactions, which vary in the way the system is modelled, and hence in the type of information that can be obtained from each particular model. Among others, two "in silico" approaches are interesting. The "docking" method consists in the systematic analysis of a huge number of positions of the lipid molecules around the molecule of interest, mimicking one leaflet of the lipid bilayer. Another approach is molecular dynamics (MD), in which the dynamics of the molecule into the lipid bilayer and its effects on surrounding lipids is investigated.

3.2.4.1. Docking methods. In the 80s, we have developed a simple theoretical method, called Hypermatrix (HM), that allows to surround a biomolecule like a drug or a peptide with lipids [234,235]. The molecule of interest is positioned and fixed for the whole calculation at the centre of the system, oriented at the hydrophobic (pho)/hydrophilic (phi) interface. The lipid molecule is also oriented at the pho/phi interface and, by rotations and translations, more than 10^7 positions of the lipid around the central molecule are calculated. The energy values together with the coordinates of all assemblies are stored in a matrix and classified according to decreasing values (Fig. 3A). The most stable matching is used to decide the position of the first lipid. The position of the second lipid is then defined as the next most energetically favorable orientation stored in the matrix taking steric and energetic constraints due to the presence of the first lipid molecule into account. The process ends when the central molecule is completely surrounded with lipids. This process simulates the interaction of a biomolecule with a lipid monolayer but the interfacial behavior of any amphiphile molecule can also be calculated with this method. In that case, the surrounding molecule is the same as the central one, forming a monomolecular layer.

In order to account for the presence of two phases, we have developed an empirical equation for the hydrophobic energy; this equation is part of the forcefield described in [236]. One limitation of this method is that the structures of the biomolecule and of the lipids do not change due to the interaction. However, even with that limitation, and thanks to the fact that the two phases are considered, parameters such as the interfacial area of the molecule (lipid or bioactive molecule) are in very good agreement with the parameters measured experimentally. For different types of molecules (natural molecules, drugs, lipids, lipopeptides,…), we have shown a very good agreement (less than 5–10% difference) for the values of the interfacial area either calculated

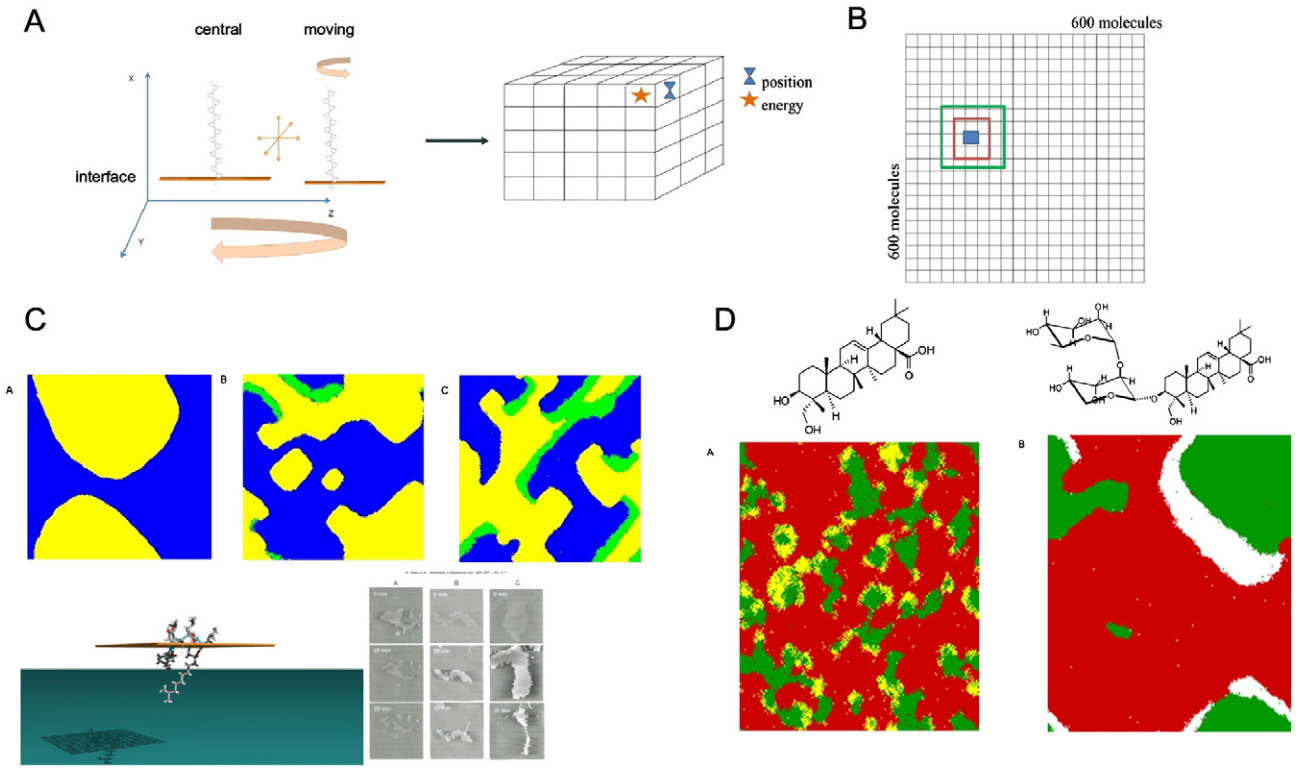


Fig. 3. Schematic explanation of HM and BM docking methods. A. In HM method, the central molecule stays still while the partner is moving towards and around the central molecule by rotations and translations (more than 10^7 positions are tested); both molecules are oriented at pho/phi interface (orange plane). The position and corresponding energy are stored in a matrix. The molecules are then assembled as described in the text. B. In BM method, the interactions between each pair of molecules are calculated as in HM. The molecules are then positioned at random in a 600x600 molecules grid. The interaction energy is calculated based on the energy matrix and takes into account two layers (red and green squares) of molecules around the molecule of interest (blue patch). The molecules are permuted until the system energy reaches a minimum by using a Monte Carlo minimization procedure. C. Effect of surfactin concentration on model membrane structure (DOPC/DPPC). Each pixel represents a molecule: DOPC: yellow; DPPC: blue; SF: yellow. The modeling and AFM experiments show that surfactin inserts at the boundary of DOPC/DPPC domains at low concentration. At higher concentrations, the shape of the lipid domains is significantly modified (adapted from [161]). a. DOPC/DPPC 1:1 mol/mol. b. DOPC/DPPC/SF 1:1:0.1 mol/mol. c. DOPC/DPPC/SF 1:1:0.3 mol/mol. D. Calculation of the interaction between hederin derivatives and lipids in a monolayer. Each pixel represents a molecule. The chemical structure of corresponding molecule is shown above. Alpha hederin was shown experimentally to induce phase separation in the presence of Chol, while for hederagenin, the shape of the domain formed with Chol is very different. Monolayer formation between molar 67% DPMC (red), 23% Chol (green) and 10% of A. Hederagenin (yellow): B. α hederin (white) (adapted from [116]).

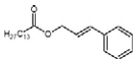
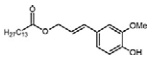
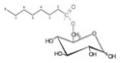
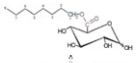
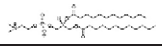
or measured by Langmuir monolayer technique, even for molecules differing only by the location of the carbonyl group in a ester bond [237,238] (Table 4). It should be noted that the calculated value corresponds mostly to the experimental molecular area in weak compression conditions.

This docking method is particularly useful to compare the specific interaction of the molecule of interest with different lipids and hence to understand its membrane activity. As an example, the interaction of hexadecylbetainate chloride (C16BC), belonging to a novel class of green surfactants, with different mammalian lipids was recently studied: the molecule was shown by calculations and experimentally to preferentially interact with SM as compared to POPC [239].

In the antibiotics field, calculations of the interaction of different aminoglysoside molecules with lipids have shown a more favorable interaction energy with PI as compared to other phospholipids. This preferential interaction has been suggested to be related to their nephrotoxicity [217,218].

We have also evidenced a preferential interaction of the nootropic agent, piracetam, with PE, a non-lamellar lipid necessary for membrane destabilization and fusion [86]. By specifically interacting with this lipid,

Table 4
Comparison between the molecular area calculated by HM method and the values determined experimentally (corresponding to A_0, i.e. the initial molecular area without compression of the monolayer film) by the monolayer Langmuir technique. Cic14 and Coc14 correspond to synthetic phenolic molecules.

Molecule	Calculated Molecular area (Å^2)	Experimental molecular area (Å^2)	Chemical structure	References
Surfactin	157	154	See Fig. 4A	[238]
Cic14	52	49		Sainvitu, Deleu, Lins, unpublished
Coc14	62	65		id
Glucose octanoate	38	37		[237]
Octyl glucuronate	45	44		[237]
DMPC	60	65		Lins, unpublished

piracetam could stabilize the membrane structure, in relation with its potential beneficial effect for Alzheimer disease [240].

Other docking methods are also available, such as that used by the group of Fantini [241,242]: they show a preferential interaction between Chol and some tilted peptides, identified by our team as potential lipid destabilizing motives [107,243–246].

Very recently, we further improved the HM docking approach, by increasing the number of interacting partners and the total number of molecules in the system. We called this new version as "Big Monolayer" (BM) method. This is a two-steps procedure, in which the first step consists in the calculation of paired interactions between the molecules, derived from the HM method described above. The main difference is that we calculate the interaction between each molecule under consideration. As an example, in a system composed of DPPC, DOPC and a lipopeptide (such as surfactin), for each pair of molecules (surfactin/DPPC, surfactin/DOPC, surfactin/surfactin, DPPC/DPPC, DOPC/DOPC, DOPC/DPPC), the interaction energies are calculated for a huge number of positions, as for HM. Another difference is that, in this improved version, the statistical Boltzmann energy is considered for each pair of molecules, rather than the energy classification. The Botzmann energy is calculated taking into account a Boltzmann statistics corresponding to the sum of the interaction energy of each relative position tested multiplied by the probability of the position. These interaction energies are then used in the second step, which consists in the construction of a grid of $n \times n$ molecules (n between 200 and 600) (Fig. 3B), and in the minimization of the system using the values calculated in the first calculation step. If $n = 600$, a grid of 360 000 molecules, initially positioned at random, is constructed and the energy of the system is calculated (Fig. 3B). The energy of one molecule is equal to the sum of the energies with its 24 closest neighbors in the grid, i.e. two layers of molecules around the molecule considered (Fig. 3B). Random permutations are made and the energy of the new configuration is calculated. By a Monte Carlo procedure, this new configuration is kept or not, as a function of the energy difference between the two states. For a grid of 360 000 molecules, one calculation step consists in 360 000 permutations; 50 000 to 100 000 steps are carried out. For the molecules at the border of the grid, the molecules at the opposite border are considered as their closest neighbors, avoiding border limits. Graphically, each molecule type is represented by a colored point and all the points are represented on the grid (Fig. 3C,D).

This method permits to visually observe the preferential interactions and phase separation between the molecules under consideration. Lipid phase separation can be simulated, as for DPPC/DOPC equimolar mixture (Fig. 3C). When surfactin is added to that lipid system at different molar ratios, we observe that the lipopeptide is localized at the edge of DOPC domains, changing their morphology; this was in very good agreement with the AFM experiments (Fig. 3C) [161]. In the case of α-hederin, a member of the saponin family, the molecule is able to interact specifically with cholesterol in a DMPC:Chol system, forming domains, as observed on the BM picture (Fig. 3D) and by fluorescence experiments [116]. This effect is not observed with its related analog, hederagenin (Fig. 3D).

3.2.4.2. Molecular dynamics. Molecular dynamics (MD) is a valuable tool to study interactions between biomolecules and membranes because it gives access to the atomistic details of the interaction as well as energetics and dynamics of the observed processes. MD is based on the use of the motion equations of Newton and on a forcefield to simulate how an ensemble of atoms moves relative to each other. Forcefields include potential equations and parameters to reproduce stretching, bending and rotations of covalent bonds, to maintain planarity and chirality of several groups as well as to simulate Van der Waals and electrostatic interactions. The parameters which depend on defining atom types have been calibrated to reproduce a wide range of experimental values. For lipid parameters, these values notably include area and volume per lipid, bilayer thickness and order parameters. Due to the increase in computational power and the improvement of MD softwares, methodologies and forcefield parameters, the use of MD is becoming an increasingly popular method to study lipid and membrane-related questions. Typical simulation times are now in the order of 50–100 ns for a hydrated bilayer of ~100 lipids but simulation times as long as 1 μs have also been reached [247,248]. The simulation time can also be increased, typically ranging from microsecond to millisecond by using coarse grained (CG) forcefields, in which groups of atoms are represented by one bead. Time and length scales are then approaching experimental resolutions [249]. Several CG forcefields have been developed for lipid molecules like Klein [250], MARTINI [251] or ELBA models [252]. The popular MARTINI forcefield allows mixed simulations with proteins [253], carbohydrates [254] and has been notably used to study the formation of lipid domains [255], membrane fusion [256,257], self-assembly of surfactant [258,259] or membrane protein oligomerization [260]. Parameters for lipids are now available for the main MD softwares (Table 5), i.e. Charmm [261–263], Amber [264–267], Gromacs [251,255,268–285], NAMD [286], and improved parameter sets are frequently added and compared [264,273,287–291]. These parameters are generally dispersed over different websites and servers [292,293] but most of them can also be found on the Lipidbook repository website [293]. Bilayer patches with different compositions are available on internet [292–295] but building its own composition is also possible and requires an equilibration time around ~100 ns. It can also be done by self-assembly in CG with or without a reverse transformation or through the use of several membrane builders which are now available (GCGS [296], MemBuilder [297], Charmm-GUI [295,298], CELLmicrocosmos [299]). The insertion of biomolecules within the membrane can be carried out notably through a self-assembly procedure [300,301] or by using methods such as g_membed [302] or inflategro [303].

Molecular dynamics is used to compute a broad range of values (position, orientation, structure, dynamics) of the inserted molecule and its effect on the surrounding lipids that can be correlated with experimental values. The orientation in the bilayer has notably been followed for different classes of peptides such as fusion peptides, WALP peptides, amphiphile or transmembrane peptides; these results were correlated to ATR-FTIR, NMR or neutron diffraction data [300,304,305]. MD is also useful to test the stability of peptide conformation into the membrane [300,306] in correlation with CD, IR or NMR

Table 5
Lipid molecules for which MD parameters are accessible, depending on the forcefield.

Forcefield	Lipids	References
CHARMM36	DLPC, DMPC, DPPC, POPC, DOPC, SDPC, DAPC, POPE, Chol	[262,263,290]
GAFF (AMBER)	DLPC, DMPC, DPPC, POPC, DOPC, POPE	[265]
Slipids (AMBER)	DLPC, DMPC, DPPC, POPC, SOPC, DOPC, DLPE, DPPE, POPE, DOPE, DLPG, DMPG, DPPG, POPG, DSPG, DOPG, DOPS, SM, Chol	[266,267]
Berger lipids (Gromos)	DMPC, PLPC, DPPC, POPC, DOPC, POPE, SM, Chol	[269–272]
Gromos 43A1-S3	DLPC, DMPC, DPPC, DOPC, POPE, SM, Chol	[273]
Gromos 53A6 CKP	POPC, DMPG, POPG, DPPE, POPE, CL, lipid A, PIP2	[274–276]
Gromos 53A6 kukol	DMPC, DPPC, POPC, POPG	[277]
Gromos 53A6 Poger	DLPC, DMPC, DPPC, POPC, DOPC	[278]
OPLS	DPPC, POPC	[279]
Martini	DHPC, DLPC, DPPC, POPC, DSPC, DOPC, DAPC, DUPC, DHPE, DLPE, DPPE, POPE, DSPE, DOPE, POPG, DOPG, POPS, DOPS, SM, CL, Chol	[251,255,280–285]

experiments and to analyze lipid destabilization induced by the peptide through the lipid order/disorder [307]. Not only peptide/membrane interaction was studied by MD, but also the interaction between a broad range of bioactive molecules, like drugs or glycolipids with membranes has been assessed by this approach [308,309].

Domain formation has also been simulated from different lipid compositions with CG models, matching the experimentally observed liquid-ordered and liquid-disordered domains [255,310]. Lipid partitioning and biomolecules sorting between several lipid phases as well as the effect of biomolecules on phase formation have also been studied using CG simulations, such as the partition of WALP peptides in the liquid-disordered phase or the promotion of lipid mixing by vitamin E [311–313]. The preference of biomolecules for different lipid phases or compositions can also be assessed by the potential of mean force (PMF) method that consists in them from the bilayer to bulk water. Using this method, it was shown that cholesterol has a strongest preference for more ordered bilayers [314–316] and influences the partitioning of small molecules [317].

Regarding lipid specificity, the interaction between Chol and different membranotropic peptides has been studied by MD. The lipid-destabilizing tilted peptides from α synuclein [244], Aβ peptide [318], and HIV-1 [319], which were also identified as cholesterol binding motives, show a perfect fit with Chol through Van der Waals forces [241,242,320]. A specific role for Chol has also been suggested in the formation of toroidal pores [321] and in the binding to GM1 ganglioside [320] of a Aβ peptide fragment.

4. Case study: lipid specificity of cyclic lipopeptides

Most of the complementary biophysical methods described in this review have been successfully applied to analyze the interactions of cyclic lipopeptides (CLPs) with model membranes in terms of lipid specificity. CLPs are interesting molecules in the scope of this review since they combine a peptidic moiety and a lipidic tail and exert their activity via their interaction with the plasma membrane.

We will focus in this chapter on some CLPs from *Bacillus subtilis* strains, namely surfactin, fengycin and mycosubtilin (Fig. 4). Those molecules were extensively studied in our lab and in the literature in general.

Surfactin (SF), whose structure is depicted on Fig. 4A, is mainly characterized by its antibacterial and antiviral activities [322]. It has notably been shown by Langmuir trough that SF is able to penetrate in different monolayers constituted by phospholipids (PL) differing in their polar heads, i.e. DMPC, DMPE or DMPA. The penetration is more important in DMPC than in DMPE monolayers and is greatly reduced in DMPA monolayers, probably because of charge repulsion; the addition of Chol in PL monolayer does not change SF insertion [323]. In membranes, the presence of Chol attenuates the destabilizing effect of surfactin [324]. This is in accordance with studies showing a preferential lysis effect of surfactin on Chol-free liposomes [325] and a higher impact of SF on red blood cells when Chol content is decreased [326]. The preference of surfactin for membranes with lower Chol content could explain why SF preferentially disrupts bacterial membranes lacking Chol. This sterol effect is specific to Chol: indeed, stigmasterol has no effect on surfactin interaction with LUV [127].

According to calculated interactions energies, the association of SF with DPPC is more favorable than with DPPE or DPPS [327]. PE phospholipids have been shown to attenuate the membrane-perturbing effect of SF whereas the effect of DPPC was to promote surfactin-induced leakage [324], indicating that bilayer sensitivity to surfactin increases with the lipid tendency to form lamellar phases. Eeman et al. [91] have suggested the formation of specific interactions between SF and DPPE. Conformational accommodations between the cone-shape of DPPE and the inverted cone-shape of surfactin as well as the ability of both DPPE and surfactin polar heads to form, respectively, inter- and intra-molecular hydrogen bonds lead to believe that specific interactions such as hydrogen bonding could also exist between PE and SF molecules.

Moreover, the length of PL acyl chain plays a major role for the penetration, which is increased in the presence of DMPC compared to DPPC [323]. Grau et al. [328] suggested the formation of complexes between SF and C14 phospholipids. It has been hypothesized that the interactions between SF and PL alkyl chains are crucial for its insertion into the membrane [329]. According to another study [91], the lipid monolayer thickness and the presence of electrostatic repulsions from the interfacial PL film do not significantly influence SF insertion in terms of kinetics but, in contrast, these parameters strongly modulate the ability of SF to alter the nanoscale organization of the lipid films.

Since surfactin penetration is more important in the presence of PC, the nanometer scale organization of the mixed SF/DPPC monolayers have been investigated [330]. A phase separation between DPPC and SF by the formation of bidimensional domains was observed on AFM images [161,330]. In these assays, DPPC molecules have a vertical orientation, with the polar head groups in contact with the mica surface and the SF peptide ring also laying on the mica [330]. Recent studies have shown that SF/phospholipid interactions depend not only on the chemical structure of phospholipids but also on their physical properties and

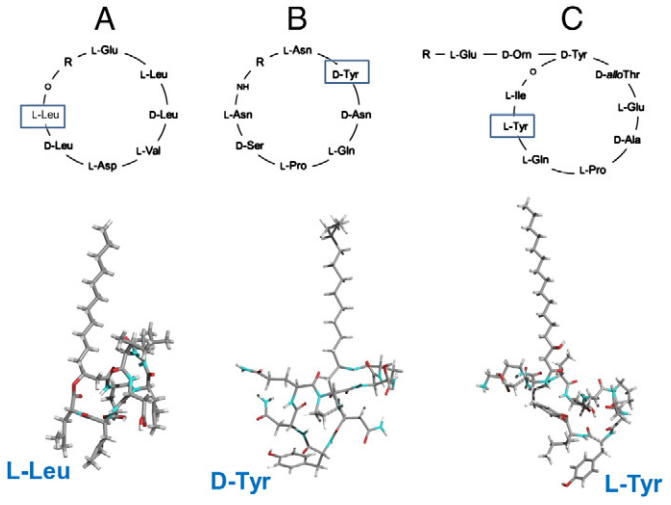


Fig. 4. Sequence (above-R represents the alkyl chain) and 3D structure (below) of CLPs from *B. subtilis*: A. Surfactin (3D structure from NMR). B. Mycosubtilin (3D structure is a model). C. Fengycin (3D structure is a model). For each peptide, a residue is highlighted as landmark.

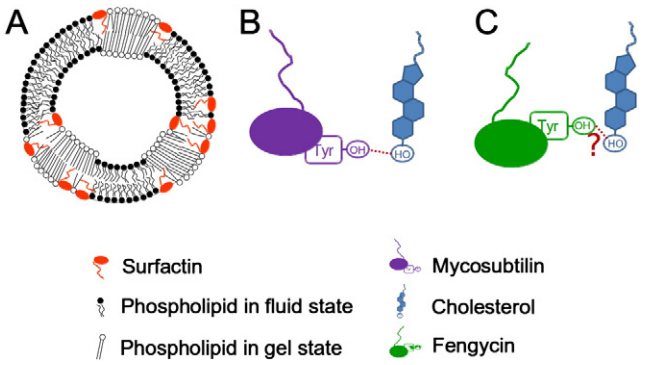


Fig. 5. Schematic models of the lipid preferential interaction of different CLPs: (A) Schematic model of the preferential insertion of surfactin into a bilayer composed of fluid and rigid domains for a SF concentration below the CMC. (B) Illustration of the preferential interaction of mycosubtilin with Chol via the sterol secondary alcohol function and Tyrosine. (C) Illustration of the suggested preferential interaction of fengycin with Chol via the sterol secondary alcohol function and the L-tyrosyl residue.

intrinsic organization [127,326]. It has notably been shown that surfactin exhibits enhanced binding to liposomes containing solid-ordered domains compared to those with liquid-ordered and liquid-disordered domains [127]. The presence of rigid domains can play an essential role in the first step of surfactin insertion [161]. In this recent paper, it was suggested that at concentrations below the CMC, SF inserts at the boundary between gel and fluid lipid domains, inhibits phase separation and stiffens the bilayer without global morphological changes of liposomes (Fig. 5A). At concentrations close to CMC, surfactin solubilizes the fluid PL phase and increases order in the remainder of the lipid bilayer. At higher SF concentrations, both the fluid and the rigid bilayer structures are dissolved into mixed micelles and other structures presenting a wide size distribution.

Mycosubtilin (Fig. 4B), another lipopeptide from *Bacillus* sp. has also been widely investigated; it is the most active member of iturin lipopeptide family known as powerful antifungal agents [204,331]. The initial studies have shown that mycosubtilin binds efficiently to bilayers constituted by DPPC and DPPC and Chol [208]. This was confirmed by the increase of ion permeability of planar membranes induced by mycosubtilin, especially in the presence of Chol [332]. In the same way, mycosubtilin and Chol interact strongly in monolayers in all proportions and form a mycosubtilin–Chol (1:2) complex [332]. In the light of these first results, it has been suggested that the action of mycosubtilin takes place by the formation of ternary mixture constituted by mycosubtilin, phospholipids and sterols [331]. A systematic study of the interactions of mycosubtilin with different lipid monolayers showed that the penetration of the lipopeptide within lipid monolayers was more important in the presence of sterols such as Chol and ergosterol [79,86,92,204]. The importance of the secondary alcohol function of sterols for mycosubtilin penetration was highlighted since its suppression via acetylation drastically decreases the penetration of the lipopeptide [79,92]. Yet, the synergic effect of sphingomyelin, DPPC and Chol on the mycosubtilin penetration was the most interesting results, suggesting a privileged interaction of the lipopeptide with sterol-enriched microdomains of sensitive cells [79,92]. At the molecular level, mycosubtilin interacts with the PL alkyl chains, inducing a fluidizing effect in which the β-amino fatty acyl residue is involved [86,204]. Moreover, a modification of the peptide backbone conformation and the involvement of the Tyr residue have been observed by IR spectroscopy (as mentioned above) when the peptide interacts with sterol-containing liposomes [79]. Taking together, these findings suggest a preferential interaction between the sterol secondary alcohol function and the tyrosyl residue, which seems to be crucial for the biological activity of the lipopeptide [79] (Fig. 5B). This is further assessed by nonlinear optics measurements developed to follow specifically the Tyr residue at the air–water interface [92,203,206]. Indeed, in the presence of sterol monolayers, the orientation of tyrosine changed drastically, contrarily to other residues [207].

Like mycosubtilin, fengycin is an antifungal lipopeptide synthetized by *B. subtilis* strains and is constituted by ten amino acids (eight of them forming a cyclic structure) and by a β-hydroxy fatty acid chain linked to the peptide cycle (Fig. 4C) [333]. Fengycin has two acidic and one basic residues and it is therefore negatively charged [334]. In mixed monolayers, fengycin interacts with DPPC with a fluidizing effect, inducing a significant perturbation of the lipid organization [335]. Partial dissolution of DPPC condensed domains was shown by AFM, underlying the role played by the physical state of the lipid on fengycin-membrane interactions [187]. Moreover, a recent study showed that fengycin is poorly miscible with fluid lipids [336]. MD simulations show that fengycin promotes positive curvature in POPE/POPG, DPPC and POPC bilayer systems. It has a preferential interaction with anionic POPG lipids (as compared to POPC and DPPC), with an attractive behavior despite the expected charge repulsion [173]. Fengycin has the capacity to modulate the formation of 2D domains in skin membrane [146]. This CLP is able to interact with the lipid constituents of the extracellular matrix of *stratum corneum* [152]. In particular, it has been shown that the presence of Chol facilitates fengycin incorporation, as assessed by the partition of fengycin into Chol-rich phases [152]. As for mycosubtilin, the involvement of Tyr residue and the secondary alcohol group of Chol during fengycin/Chol interactions must be confirmed by testing the interactions of this lipopeptide with cholesterylacetate monolayers and by looking for the antifungal activity of the Tyr-methylated or -acetylated fengycin. The potential interaction between Tyr and Chol is illustrated on Fig. 5C.

5. Conclusions and perspectives

The exact manner by which bioactive molecules interact with PM and molecular details of this mechanism is an area of active research, as shown by the numerous examples cited in this review, and also a matter of intensive debate in some cases. The use of model membranes with specific lipid composition is a way to circumvent the high complexity of the natural lipid bilayer and to focus on lipid specificity.

Biophysical tools are a major way to understand these processes and can help solving problems of biological importance. In this review, we have listed some of these techniques, others are available, but it is important to stress the complementarity between all these approaches, giving a detailed picture of what is happening at the molecular level.

The constant evolution of the biophysical techniques, such as mass spectrometry, will provide in near future a better correlation between *in vitro* and *in vivo* studies. Techniques that allow monitoring living cell membranes at molecular (even atomic) resolution are constantly being improved and should help to better understand this complex lipidic structure and the processes occurring at its interface.

As a conclusion, a better understanding of the interaction between biomolecules and the plasma membrane at the molecular level, and more specifically with one particular lipid, is not only important in the elucidation of various biological processes but could also serve to design molecules with tailored functionalities for drug development, especially in the field of health, such as antibiotic and drug delivery developments. Furthermore, the cell membrane is often overlooked in this area since it is not the target of the pharmacological molecule as such. However, the interaction of most of these exogeneous amphiphilic compounds with the plasma membrane can influence its structure and hence its normal function.

On the other hand, fields such as biocontrol for plants could also benefit from advances in the biophysical elucidation of the interaction between natural bioactive molecules and plant lipids. Indeed, understanding how molecules from the rhizosphere help plant to be protected by acting specifically on membranes and eliciting plant defense mechanisms should lead to the design of potent biobased molecules, avoiding an overuse of chemical pesticides.

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