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Recent advances in the use of Langmuir monolayers as cell membrane models

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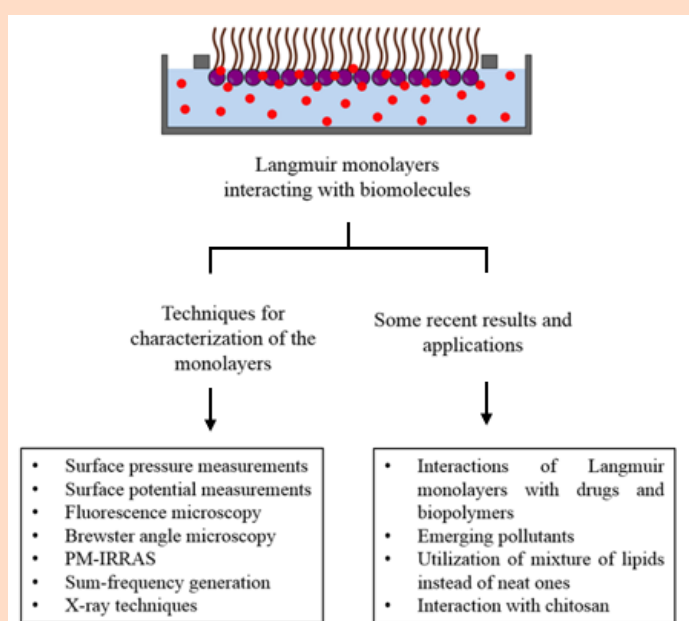
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ABSTRACT: Understanding the role of biomolecules in cells at the molecular level has been the trade of Prof. Marcio Francisco Colombo and Prof. João Ruggiero Neto in their careers, which is why it was found appropriate to address the use of Langmuir monolayers as cell membrane models in this special issue. In the review paper, we elaborate upon the reasons why Langmuir monolayers are good models with the possible control of membrane composition and molecular packing. After describing several experimental methods to characterize the Langmuir monolayers, we discuss selected results from the last five years where monolayers were made to interact with pharmaceutical drugs, emerging pollutants and other biologically-relevant molecules. The challenges to take the field forward are also commented upon.



1. Introduction

Langmuir monolayers¹⁻³ have long been used as simplified membrane models as they mimic half of the cell membranes that are formed by a fluid lipid bilayer containing proteins and polysaccharides^{4,5}. The reason why membrane models are employed is the difficulty to characterize the cells directly, particularly if molecular-level information is required. Since the seminal works by RAYLEIGH⁶, Langmuir⁷ and Nandi and Vollhardt⁸, monolayers have been utilized for *in situ* characterization of surfactant molecules at the air-liquid interface and in the formation of Langmuir-Blodgett (LB) films^{9,10}. Because lateral organization is important and there are specific lipid functions in biomembranes, the Langmuir monolayer model is advantageous, as the area per molecule and lipid composition are controlled precisely. Furthermore, with monolayers, one may investigate biomolecule-membrane interactions by modifying and controlling the lipid charge and structure, and subphase conditions, such as pH and concentration of the biologically-relevant molecules^{11,12}.

Since the 1990s, novel *in situ* methods have been made available to study liquid interfaces with microscopic and molecular resolution, as described in recent reviews^{1-3,13}. In Section 2 in this review paper, we describe several methods to characterize Langmuir monolayers, while selected results from the last five years with monolayers used as membrane models are discussed in Section 3. These results refer to a few topics that have gained prominence, *viz.* interaction with pharmaceutical drugs and emerging pollutants, enzyme activity, use of lipid mixtures to better mimic a real membrane and interaction with chitosans.

2. Methodology

Langmuir monolayers are obtained with instruments referred to as Langmuir troughs, with which a solution of an amphiphilic material is spread at the air-water interface. These Langmuir troughs are made of inert materials, usually Teflon, and contain movable barriers, one pressure sensor and one dipper. For monolayer formation, a small volume of a diluted solution of the selected compound in a volatile organic solvent (usually chloroform) is spread over the water surface. The barriers allow the area occupied to be varied and the dipper permits immersion of substrates for LB film deposition. The pressure sensor measures the surface pressure (π), normally with the Wilhelmy method¹⁴. After solvent evaporation, the barriers

compress the molecules at a constant rate, which makes them oriented in relation to the water surface, with the hydrophobic portions facing air and hydrophilic portions in contact with the water. When the film is closely packed and continues to be compressed, collapse occurs with disordered layers being formed¹⁵. There are several methods to study the properties of a Langmuir monolayer. For various spectroscopic and microscopy methods and other *in situ* characterization techniques, the Langmuir trough is installed to give access to the measuring systems. In this review paper, we shall introduce methods which have been employed in Brazil or by Brazilian researchers in collaboration with international partners.

2.1 Surface pressure measurements

The most popular way to characterize Langmuir monolayers is to obtain the surface pressure-area (π -A) isotherm³. From the isotherms, one may determine the molecular area, monolayer phases, collapse behavior, compressibility and interaction with species in the subphase. The measurement is usually done in a pseudo-equilibrium condition, with a continuous compression of the monolayer while the surface pressure is monitored. Surface pressure is measured by either of two main methods: the Langmuir balance, which is a differential measurement, and the Wilhelmy plate, an absolute method that involves the forces acting on the plate (made of platinum or filter paper) partially immersed in the subphase³. The surface pressure (π) is defined as the difference between the surface tension of one subphase in absence of the compound (γ_o) and in the monolayer presence (γ), as in Eq. 1:

$$\pi = \gamma_o - \gamma \quad (1)$$

The minimum pressure is zero and the maximum is around 73 mN m⁻¹ (at 25 °C), which corresponds to the surface tension of pure water if the monolayer is stable¹⁴. The factors that affect the shape of surface-pressure isotherms include experimental conditions and chemical modification of the molecule's structures, such as their polarity, size and shape¹⁶. The analysis of surface pressure isotherms yields information on the collapse pressure and the compressibility modulus (C_S^{-1})¹⁷. The latter allows one to characterize the state of the monolayers and the phase transitions, and may be calculated using Eq. 2:

$$C_S^{-1} = -A \frac{d\pi}{dA} \quad (2)$$

where A corresponds to the mean molecular area and π is the surface pressure.

2.2 Surface potential measurements

The surface potential (ΔV) technique has been utilized over decades with Langmuir monolayers. It is defined as the potential difference between an aqueous surface in the presence of a Langmuir monolayer and a surface without the monolayer. This measurement provides information about the dipole moments of the film-forming materials, in addition to changes in orientation of water molecules in the subphase and of the double-layer formed in ionized monolayers between the electrolytic subphase and the headgroups³. The measurement of surface potential is usually made with a Kelvin probe in a vibrating capacitor scheme. The surface potential changes when the film is compressed, owing to reorientation of the head or tail groups. Theoretical models have been used to relate the measured potentials with the dipole moments of the molecules. In one of these models¹⁸, the monolayer was considered as a three-layer capacitor, each layer with its “effective” dipole moment and local dielectric constant. As the interactions between the molecules are weak for large areas per molecule, the surface potential of the monolayer is zero. During monolayer compression, there is a critical area in which the potential is no longer null and increases rapidly with the decrease in area per molecule. It is worth mentioning that the surface potential is more sensitive to the organization of the Langmuir monolayers than the surface pressure¹⁴.

2.3 Fluorescence microscopy

Fluorescence microscopy is used to obtain structural information and study the dynamics of possible chemical and structural changes in Langmuir monolayers. The technique requires that molecules in the monolayer contain dyes or chromophores¹⁴, with an incident light (from the air or from the water) exciting the monolayer material or incorporated dyes. As the solubility of the fluorescent probe depends on the monolayer state, a contrast is originated upon changing these states³. Therefore, with fluorescence microscopy one may inspect the molecular aggregates and domains at different film compression stages. The analysis of fluorescence microscopy data may be difficult due to the possible segregation of probe molecules, which can form separate monolayer phases¹⁹. This occurs because a fluorescent amphiphilic molecule can be added to the

monolayer. Since this probe could act as an impurity in the monolayer, its solubility can vary in the coexisting phases. Also, significant are the possible photochemical transformations in the fluorescent probes, which should be accounted for^{20,21}.

2.4 Brewster angle microscopy

Brewster angle microscopy (BAM) was reported in 1991, independently, by two groups: Hénon and Meunier from France and Hönig and Möbius from Germany^{21,22}. Similarly to fluorescence microscopy, BAM allows the observation of the mesoscopic morphology and ordering of condensed phase domains²³. One of the main advantages of BAM compared to fluorescence microscopy is that it does not require external probes, which means that the monolayers may be visualized on a mesoscopic scale without external interference²⁴. Brewster angle microscopy also provides information about monolayer phases, packing of the molecules, phase transitions and chemical modifications²⁵. The principle of BAM is based on reflection spectroscopy. The plane interface reflectivity between two media of refractive index (n_1 and n_2) depends on the polarization (α) of the incident light and on the angle of this incidence (θ). For p polarization (electric field in the plane of incidence) and considering a Fresnel interface, the reflectivity vanishes at the Brewster angle (θ_B), as indicated in Eq. 3:

$$\tan[\theta_B] = n_2/n_1 \quad (3)$$

Condensed monolayer phases affect the refractive index with measurable changes in reflectivity²⁴. Three origins may be identified for the reflectivity: the thickness and roughness of the interface and the anisotropy of the monolayer²¹. For the air-water interface, the Brewster angle is 53° for p polarization light. When a monolayer is introduced, a new interface is formed and α is changed. Thus, the light is reflected and can be observed with a microscope, which allows for monolayer visualization^{26,27}.

2.5 Polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS)

Spectroscopy in the infrared region allows one to investigate vibration modes of the chemical bonds. For Langmuir monolayers, it has become frequent to employ polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS). This method is

sensitive to the component of the perpendicular dipole moment in relation to the substrate¹⁴, thus yielding information on the orientation of film-forming molecules^{28,29}. The importance of polarization modulation is related to the minimization of the absorption of the water vapor and making PM-IRRAS surface specific³⁰. Polarization modulated infrared reflection absorption spectroscopy was developed around 1990 and has been utilized with metallic substrates as well as in Langmuir monolayers. It combines Fourier transform and mid-infrared reflection spectroscopy with polarization modulation of the incident beam with two-channel electronic and mathematical processing of the detected signal³¹. By alternating *s*- and *p*-polarizations in the impinging light at a high frequency using a photoelastic modulator, the reflectivity of both polarizations are detected. The difference between them yields surface specific information while their sum serves as reference. The ratio between the difference and sum is the PM-IRRAS signal, in which the gas phase absorbance is compensated³². With PM-IRRAS one may distinguish between in-plane and out-of-plane vibrations. For instance, positive bands correspond to the vibrations parallel to the water surface, while negative bands are due to vibration modes normal to the water surface³.

2.6 Sum-frequency generation

Sum-frequency generation spectroscopy (SFG) has been developed as a surface-specific technique for interfaces and surfaces^{33,34}. As a second-order nonlinear optical process, SFG is forbidden under the electric-dipole approximation in media with centrosymmetry, and a signal is only measured when the inversion symmetry is broken^{35,36}. Sum-frequency generation spectroscopy utilizes different input/output polarization combinations, which provide a great deal of structural information³⁷, including for different Langmuir monolayer phases. A unique feature of SFG is the possibility to investigate the interfacial water layers, which can be affected by changes in ion concentration and pH^{38,39}. In SFG, two input laser beams overlap to generate an output at a frequency which is the sum of the incoming frequencies. One frequency is visible while the infrared beam is tunable. When this infrared frequency approaches a surface resonance, the SFG output is enhanced and a spectrum of the surface or the interface is obtained⁴⁰, in some cases identifying the chemical groups⁴¹. The vibrational spectrum is thus obtained with the detection of the light with summed frequencies⁴².

In general, infrared spectroscopy has enough sensitivity to detect a surface monolayer. However, the spectrum is usually complicated and the information about the conformation of the monolayer chain is indirect. In SFG, on the other hand, the spectra of the hydrocarbon chains are simplified by the intrinsic selection rules and they are sensitive to chain conformation, which means that they can be used to provide qualitative information about chain conformation⁴⁰. The selection rules for sum frequency involve the asymmetric environment, and the asymmetry must be satisfied in molecular and macroscopic levels. On a macroscopic scale, the sum frequency is inactive, because isotropic distribution of molecules in the bulk phase is centrosymmetric. For interfacial molecules to be active, a net polar orientation is necessary, since no SFG signal is observed when the surface structure is completely disordered^{42,43}.

2.7 X-ray techniques

The structure of Langmuir monolayers started to be investigated in detail in the 1980s when synchrotron light sources became available. With grazing incidence X-ray diffraction (GIXD) measurements, it was possible to determine the in-plane structures for the first time, including tilt directions and tilt angles with Angstrom resolution⁴⁴. This technique is based on the total reflection phenomenon, in which the electromagnetic wave propagates at a critical angle along the boundary between two media and is totally reflected from the medium with the lower refractive index. The GIXD is highly surface sensitive because a monochromatic X-ray beam with a well-defined wavelength is used to focus the water surface at an angle α_i (a value below the critical angle α_c of total external reflection). The evanescent wave propagates along a top layer of only 8 nm. For a crystalline monolayer, the evanescent wave may be scattered from the lattice planes as in a Bragg scattering⁴⁵. The types of information that may be obtained with GIXD include molecular organization in terms of unit cell dimensions and orientation of the molecules in relation to the interface.

In addition to GIXD, two other techniques have been important for the study of Langmuir monolayers: specular X-ray reflectivity (XR) and total reflection X-ray fluorescence (TRXF). With XR, one may probe non-structured liquid monolayers, which is not possible with GIXD. From XR experiments, the vertical structure of the monolayer may be determined, regardless of the phase state. The incident angle α_i can

vary from 0.01 to 0.8 \AA^{-1} of the vertical scattering vector component (Eq. 4).

$$Q_z = (4\pi/\lambda) \sin(\alpha_f) \quad (4)$$

The background scattering from the subphase is measured at $2\theta = 0.7^\circ$ and subtracted from the specular signal measured at $2\theta = 0^\circ$ ⁴⁵.

Total reflection X-ray fluorescence is a simple method to characterize quantitatively the monolayers⁴⁶. For the coupling between electron and X-ray to be efficient, the gap between the energy of the X-ray beam and the edge energy should not be too large, which means that the X-ray energy depends on the type of element to be detected. However, in some cases, the fluorescence process is inefficient and the radiation is much weaker than the primary beam. Hence, light elements cannot be detected because of instrumental limitations and low X-ray fluorescence yields. In TRXF, the measurement does not depend on the structure and monolayer composition, it depends on the experimental conditions, the fluorescence yield for a line and the X-ray absorbance of an element. The fluorescence intensity (I_i^f) of an element I with a concentration profile $c_i(z)$ (in the directional normal to the interface) is given in Eq. 5:

$$I_i^f = b_i \int I_{ex}(z) c_i(z) dz \quad (5)$$

where $I_{ex}(z)$ corresponds to the exciting X-ray intensity at a distance z from the surface and b_i is a constant⁴⁵.

3. Summary of recent results

The use of Langmuir monolayers as cell membrane models has continued as a popular topic, with more than 500 papers in indexed journals (in the Web of Science in July, 2020) over the last five years. For this review paper, we have chosen a few topics associated with drugs and biopolymers, which interactions with cell membranes are essential for their physiological action⁴⁷.

The first topic is associated with pharmaceutical drugs which physiological action may be correlated with the ways they interact with cell membranes. Rodrigues et al.⁴⁸ investigated bacitracin, a drug used for treating minor wounds. Bacitracin is able to disrupt membrane models representing gram-positive and gram-negative bacteria. They observed that bacitracin is incorporated in 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-choline (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DPPG) and 1,2-

dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS) monolayers, and affects the monolayer morphology, as they showed in BAM images. The effects from bacitracin depended on the nature and microenvironment of the monolayer, as well as on the lipid polar head. For DPPC and DPPG, bacitracin expands the lipid monolayer, while for DPPS the drug condenses it. The observation that bacitracin interacted with the phospholipid monolayers at a surface pressure typical of a real membrane model (i.e. 30 – 35 mN/m) was of biological relevance⁴⁸.

Węder et al.⁴⁹ studied 2-hydroxyoleic acid (2OHOA or Minerval) and its interaction with different membrane components, such as cholesterol, sphingomyelin and phosphatidylcholine⁵⁰. They observed that the interactions between the lipids and the 2OHOA in the monolayer are more favorable when the monolayer is more fluid. The effects of 2OHOA depended on the condensation of monolayers mimicking lipid rafts; the ability of 2OHOA to destabilize and modify the morphology of the monolayers was suggested to be linked to its activity. Some classes of peptides are promising for killing bacteria, especially as they can disrupt bacteria membrane. This has motivated a number of studies with Langmuir monolayers⁵¹, including those from Prof. João Ruggiero⁵². In the latter study, the interaction of peptides was studied with Langmuir monolayers of DPPC, which was used to verify the influence of a peptide on lipid packing during the LE-LC coexistence plateau. Their results permitted to confirm that the subphase pH is an important parameter which modulates the peptide surface activity. Also relevant was the conclusion that the mutual lateral interaction could stabilize the peptides in the hydrophobic region of the membrane.

The importance of immobilizing enzymes in Langmuir and Langmuir-Blodgett films arises from the possible monitoring of catalytic activity at the molecular level^{13,53}. Also significant is the finding that the environment provided by the lipid-enzyme architecture could conserve the catalytic activity for a long time^{54,55}, which is interesting for biosensors. Rocha Junior and Caseli⁵⁶ studied the surface activity of the enzyme asparaginase at the air-water interface. Asparaginase is responsible for catalyzing the hydrolysis of asparagine to aspartic acid and ammonia and serves as an antitumorigenic agent⁵⁷. They detected the formation of mixed asparaginase-DPPC monolayers using surface pressure and surface potential measurements and vibrational spectroscopy. Asparaginase decreased the lipid surface elasticity, increased the surface potential and condensed the

monolayer. When asparaginase was immobilized in phospholipid LB films, its activity was better preserved than in a homogeneous medium⁵⁶.

An increased use of emerging pollutants with Langmuir monolayers has been noted for two main reasons. These pollutants have attracted considerable attention and their study in *in vivo* systems is difficult⁵⁸. Alessio et al. investigated the interactions of the pollutants amoxicillin (AMX) and methylene blue (MB) with a simple membrane model consisting of DPPC monolayers⁵⁹. Amoxicillin is an antibiotic of the penicillin family used to treat bacteria-related infections, and MB is a phenothiazine derivative used

as a medicine⁶⁰, among other applications⁶¹. Amoxicillin and MB shifted the surface pressure of DPPC monolayers to larger areas and made these monolayers more compressible, as observed in Fig. 1. Even more important was the observation that a stronger effect occurred when the two pollutants were mixed, which was corroborated with PM-IRRAS data. This synergistic effect is in line with the problems reported about cooperative action of pollutants effects⁵⁹.

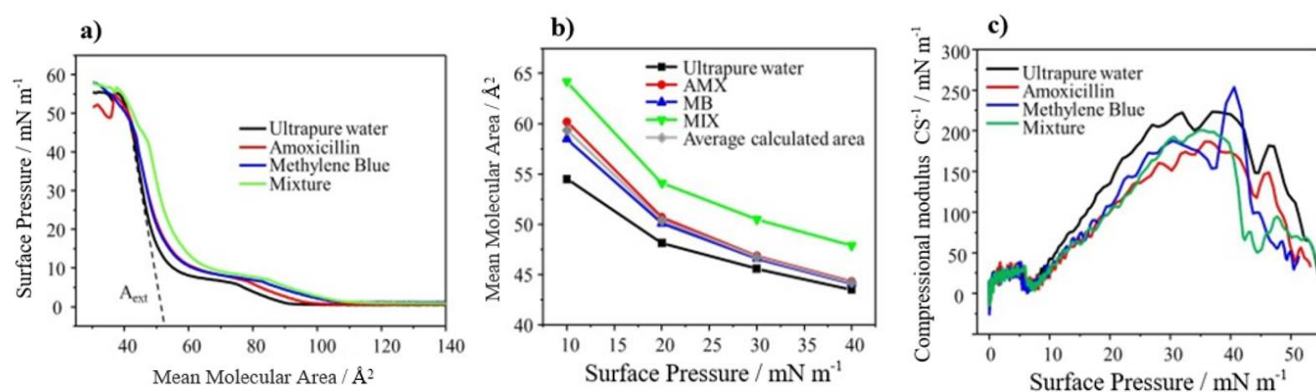


Figure 1. (a) Surface pressure isotherms of DPPC for 10⁻⁴ mol/L subphases with AMX, MB and the mixture at 23 °C. (b) Modifications of the area induced by the subphases of AMX, MB and the mixture. (c) Compressional modulus versus surface pressure for DPPC monolayers obtained from the π -A isotherms. Reproduced from Maximino et al.⁵⁹ with Elsevier permission.

Another work related to pollutants by Węder et al.⁶² involved the persistent organic pollutants polycyclic aromatic hydrocarbons (PAHs)⁶³ that can easily migrate in the environment. One way to eliminate PAHs is through bioremediation by using soil decomposer consortia in bacterial species capable of PAHs degradation^{64,65}. However, the surface of the soil bacteria is hydrophilic, while PAHs are hydrophobic, so the direct contact between them is very limited. With this problem in mind, the authors proposed to study Langmuir monolayers from bacterial phospholipids as model membranes, as the studies in the literature are predominantly based on phosphatidylcholines (PC)⁶⁶, which do not occur in this type of membrane. The lipid mixtures they used contained cardiolipin, phosphatidylglycerol and phosphatidyl ethanolamine. Six PAH molecules were employed, which showed different behaviors in contact with the monolayers. The results do not depend on the kind of the polar headgroup of the lipids. Polycyclic aromatic hydrocarbon molecules do not have any polar

groups and, therefore, they are incorporated between the hydrophobic chains of the phospholipid and interact with them, avoiding the hydrated regions of the monolayers. Based on the results for the various monolayers, Węder et al.⁶² suggested that the toxicity of PAH molecules is directly related to their interactions with the membrane phospholipids.

One of the major challenges in the use of Langmuir monolayers as cell membrane models is to mimic the rich variety of membrane composition. For decades, most studies employed only neat phospholipids in the monolayers in order to make it simple. In recent years, attempts have been made to better mimic the membrane composition by using mixtures of lipids. Herein, we mention some contributions from the last four years in this topic. Sun et al.⁶⁷ used the mixtures of POPC/DPPC and POPC/DPPC/Chol (POPC is 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and Chol is cholesterol) to investigate interactions with *Lycium barbarum* polysaccharides (LBP), natural biopolymers used in medicine and in the food industry⁶⁸. A few number of LBP molecules

interacted with POPC/DPPC/Chol monolayers in comparison to POPC/DPPC monolayers, and $C_S^{-1}_{\max}$ was higher for the ternary mixture. These results indicated that the presence of cholesterol led to a more rigid, more stable membrane at the air-water interface. They were interpreted as considering that cholesterol protected the cell membrane from the effects of LBP. Another example of lipid mixtures in Langmuir monolayers included different proportions of Chol and sphingomyelin (SM) in a study of effects from the antimalarial drug cyclosporine A⁶⁹. Cholesterol is known to affect membrane fluidity^{70,71} and forms the so-called lipid rafts along with SM^{72,73}. Wnętrzak et al.⁶⁹ observed that cyclosporine A was distributed on the monolayers in different ways, depending on the Chol-SM proportion. Cyclosporine A induces modifications in SM-Chol model membranes, especially in their mechanical properties. These modifications could affect the antimalarial activity of cyclosporine A, since this drug may disorganize SM-rich domains, which destabilizes the vacuolar membrane, preventing the development of parasites.

As already mentioned, Chol affects the conformational order and membrane permeability, thus regulating the lateral organization of membrane components^{13,70,72,74}. This has been studied in detail on aminophospholipid membranes⁷⁵. The isotherms and compressibility modulus depend on the Chol concentration, and the same applies to the molecular organization inferred from X-ray reflectivity measurements (XRR).

The third type of representative study of cell membrane models involves chitosans, which applications in medicine depend on their interactions with biomembranes. Owing to their biocompatibility and biodegradability, chitosans have been used in drug delivery⁷⁶ and as bactericidal agents⁷⁷. Pavinatto et al.⁷⁸ investigated the interaction between two samples of chitosan with different molecular weights and zwitterionic (DPPC) and negatively charged (DPPG) phospholipids. The action of chitosan depends on three factors: degree of acetylation^{79,80}, molecular weight⁸¹ and functionalization⁸². The goal of this study was to show that smaller chitosan chains are more capable of penetrating into the membranes⁸³. Both chitosans expanded the DPPC and DPPG monolayers and reduced their compressibility modulus, but the effects were more pronounced for the low molecular weight sample, as shown in Fig. 2. Furthermore, interaction was stronger with DPPG, owing to its negative charge. The reason why chitosans with lower molecular weight had more access to the monolayer was identified by dynamic light scattering measurements with the

chitosan solutions. Larger aggregates were observed for the high molecular weight chitosan, which hampered the access to the phospholipid hydrophobic tails⁷⁸.

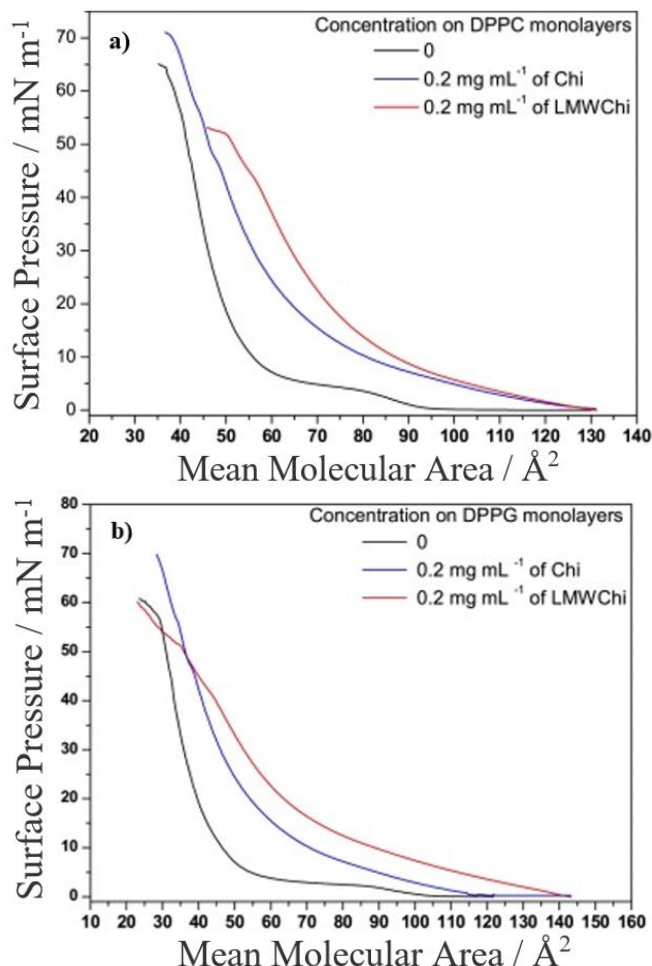


Figure 2. Surface pressure-area isotherms for (a) DPPC and (b) DPPG monolayers. Subphase: TS buffer pH 3.0 and 0.2 mg mL⁻¹ commercial chitosan (Chi) and lower molecular weight chitosan (LMWChi). Reproduced from Pavinatto et al.⁷⁸ with Elsevier permission.

Until recently, all the experiments with Langmuir monolayers were made with chitosans that were only soluble at acidic subphases. This has changed with the development of a novel strategy to produce chitosans soluble at a wide pH range with controllable degree of acetylation⁸⁴. A chitosan with 35% acetylation degree (Ch35%) was made to interact with Langmuir monolayers of equimolar mixtures of (SM/DPPC/Chol) to mimic lipid rafts in cell membranes⁸⁵. The most important observation was that interaction with such lipid rafts occurred at chitosan concentrations much

lower than reported in the literature, as shown in Fig. 3 for a subphase of phosphate buffer saline (PBS) solution. In fact, interaction with the SM/DPPC/Chol was always stronger than for the pure DPPC, and this applied not only to the high molecular weight Ch35%, but also for other types of chitosan. It is also worth noting that the interactions with SM/DPPC/Chol monolayers are even more pronounced for acidic subphases.

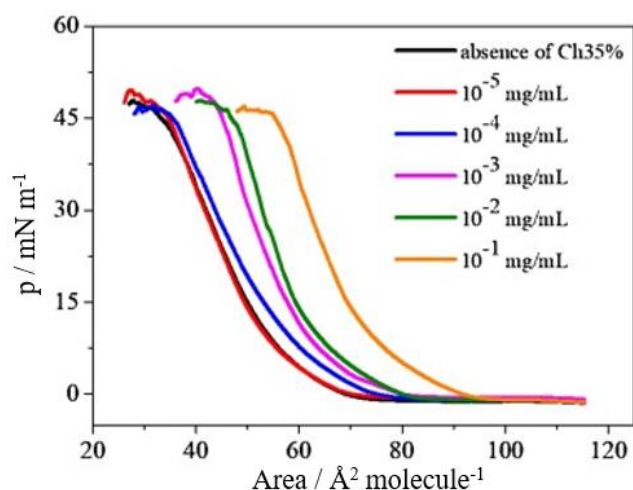


Figure 3. Surface pressure-area isotherms for monolayers of SM-DPPC-chol (1:1:1). Subphase: PBS (pH 7.4) containing Ch35% at different concentrations. Reproduced from Pereira et al.⁸⁵ with Elsevier permission.

4. Final Remarks

The mimicking of cell membranes is, today, one of the most noble uses for Langmuir monolayers. In spite of the simplifications in the modeling, these monolayers are useful to obtain molecular-level information, which is virtually impossible with any other method. One may, for instance, identify the reasons why a peptide is effective against gram-positive bacteria, but not for gram-negative bacteria by simply verifying that the peptide cannot disrupt a Langmuir monolayer of lipopolysaccharide⁵¹. With the *in situ* vibrational spectroscopy techniques, on the other hand, it is possible to determine the chemical groups involved in the interactions between biologically-relevant molecules and the model membranes. While recent achievements in modeling are promising, some of which were discussed in this review paper, there are important challenges to move the field forward. In our view, the two most relevant hurdles are the need to use more complex mixtures of

lipids and other compounds to better mimic a real cell membrane and the need to understand the molecular-level interactions based on theoretical models. The latter has been addressed with molecular dynamics simulations⁸⁶, but the results are still limited, owing to computational resources and to phenomena involving charge transfer that is difficult to simulate with classical methods.

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