Biophysical Chemistry 246 (2019) 1–7

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: www.elsevier.com/locate/biophyschem

Interfacial vibrational spectroscopy and Brewster angle microscopy distinguishing the interaction of terpineol in cell membrane models at the air-water interface

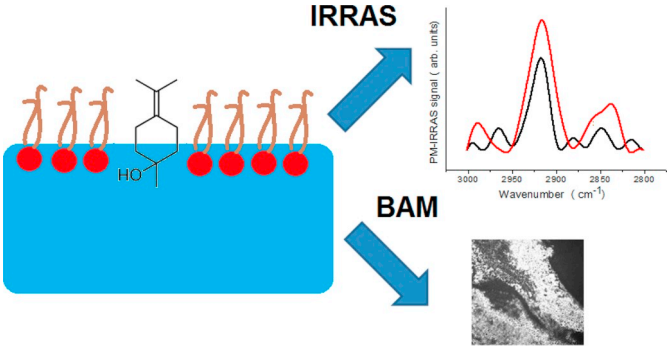
Guilherme Nuñez Jaroque, Patrícia Sartorelli, Luciano Caseli*

Institute of Envinonmental, Chemical and Pharmaceutical Sciences, Federal University of São Paulo, Diadema, SP, Brazil

HIGHLIGHTS

- Gamma-terpineol is isolated.
- Gamma-terpineol is incorporated in biointerface models.
- Gamma-terpineol changed the thermodynamic and structural properties of DPPC and DPPE monolayers.
- BAM and PM-IRRAS reveal that the lipid modulate the interaction with gamma-terpineol.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
γ-terpineol
Langmuir monolayers
DPPC
DPPE
Air-water interface

ABSTRACT

In this paper, γ-terpineol, a known monoterpene with biological activity, including in lipidic interfaces, was incorporated in Langmuir monolayers of selected phospholipids as a model for cellular membranes. Surface pressure-area isotherms showed that selected amounts of γ-terpineol expand DPPC and DPPE monolayers and decreased the monolayer elasticity, confirming the lipid/compound interaction. Characterization with vibrational spectroscopy and Brewster angle microscopy pointed that γ-terpineol adsorbs on the polar heads of the phospholipids, affecting the gauche conformations of the aliphatic chains, and different patterns were observed for DPPC and DPPE monolayers, indicating a characteristic molecular accommodation of γ-terpineol along the polar head of the phospholipids. Therefore, distinctive interactions with DPPC and DPPE could be observed regarding the incorporation of γ-terpineol with each lipid, leading to particular molecular arrangements at the air-water interface and pointing the modulation of the interaction according to the chemical composition of the monolayer.

1. Introduction

Terpineols are monocyclic monoterpene tertiary alcohols present in several plant species [1–3]. Between the isomers of terpineols, γ-terpineol may play an important role in the pharmaceutical and agricultural industries because of the biological applications of essential oils containing this compound as an antioxidant, antinociceptive, microbicide, antinematodal, anticancer, and an insecticidal compound

* Corresponding author.
E-mail address: lcaseli@unifesp.br (L. Caseli).

https://doi.org/10.1016/j.bpc.2018.12.003
Received 6 December 2018; Received in revised form 18 December 2018; Accepted 18 December 2018
Available online 20 December 2018

[4–9].

Particularly, the isomers of terpineol exhibit a potential activity against bacteria [10], encouraging researches on the related mechanism of action, especially when interactions with cellular membranes are involved. For that, it of interest to use simplified models for biological membranes since details at the molecular scale can be achieved. These models involve, for instance, liposomes [11] or Langmuir monolayers [12], which are complementary systems. Particularly, Langmuir monolayers are floating films formed by insoluble amphiphiles at liquid-gas interfaces. The recent employment of membrane lipids as Langmuir monolayers has motivated the investigation of these films as models for half a membrane [13] and interactions with microbicide and anticancer drugs have been studied [14–16].

Considering the lack of information about the mechanism of action of γ-terpineol in membrane models, especially in Langmuir monolayers, this paper aims to investigate the action of this compound in two types of lipids: one that approximates the more common polar head of the external monolayers of biomembranes: phosphatydilcholines [17]; and the other one representing models for cell membrane of bacteria: a phosphadylethanolamine [18]. Also, phosphadylethanolamines present as a high percentage in the external layer of cancerous membrane cells, representing therefore a model for tumorigenic cells [19].

The present study focuses therefore on the evaluation of the biophysical consequences on the phospholipids present on microbial membranes upon their interaction with γ-terpineol. For this purpose, lipid Langmuir monolayers of saturated phospholipids were employed with the objective to encompass the study of γ-terpineol effect in different lipid phases that the cellular membrane encompasses, including expanded and condensed ones. Dipalmitoylphosphatidylcholine has also been used to study the gastric toxicity of nonsteroidal anti-inflammatory drugs [20], which facilitates the access of data for comparison.

2. Materials and methods

The phospholipids dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE) were acquired from Sigma-Aldrich and dissolved in chloroform (Synth) to result in solutions with concentrations of 0.5 mg/mL.

In order to isolate γ-terpineol, leaves of *Chenopodium ambrosioides* (46 g), collected in the city of Rio Claro, SP, Brazil, were extracted by steam distillation in a Clevenger type apparatus. After continuous distillation during 4 h, 0.350 g of crude essential oil (yield 0.76%) were obtained after drying with anhydrous Na_2SO_4. Part of this oil (300 mg) was subjected to flash chromatography on Si-gel column coated with $AgNO_3$ (15%) eluted with a gradient of CH_2Cl_2:MeOH to give 8 fractions which were individually analyzed using gas chromatography (GC). Fraction 2 showed to be composed by pure γ-terpineol (99% by GC) (120 mg), whose structure was identified by NMR and MS spectral analysis and comparison with literature data [19,20].

The compound γ-terpineol was dissolved in chloroform resulting in a solution with concentration of approximately 0.5 mg/mL. Water employed as subphase for the lipid monolayers was purified by the Milli-Q® system (resistivity 18.2 MΩ.cm; pH 5.6; surface tension of 72.8 mN/m at 20 °C).

Aliquots of γ-terpineol solution were first spread on then the air-water interface to test its surface activity. For the lipid monolayers, DPPC or DPPE solution was spread on the air-water interface. For mixed monolayers with γ-terpineol, the compounds were co-spread from a previous solution containing the mixture in selected proportions in mol (5 or 10% of γ-terpineol).

A Langmuir trough from KSV-Nima Instruments (mini-trough model with total capacity of 200 mL) was employed for the formation of the Langmuir monolayer. After 20 min previously waited for solvent evaporation, the air-water interface was compressed at a speed of 10 mm/min and the surface pressure measured by the Wilhelmy's method.

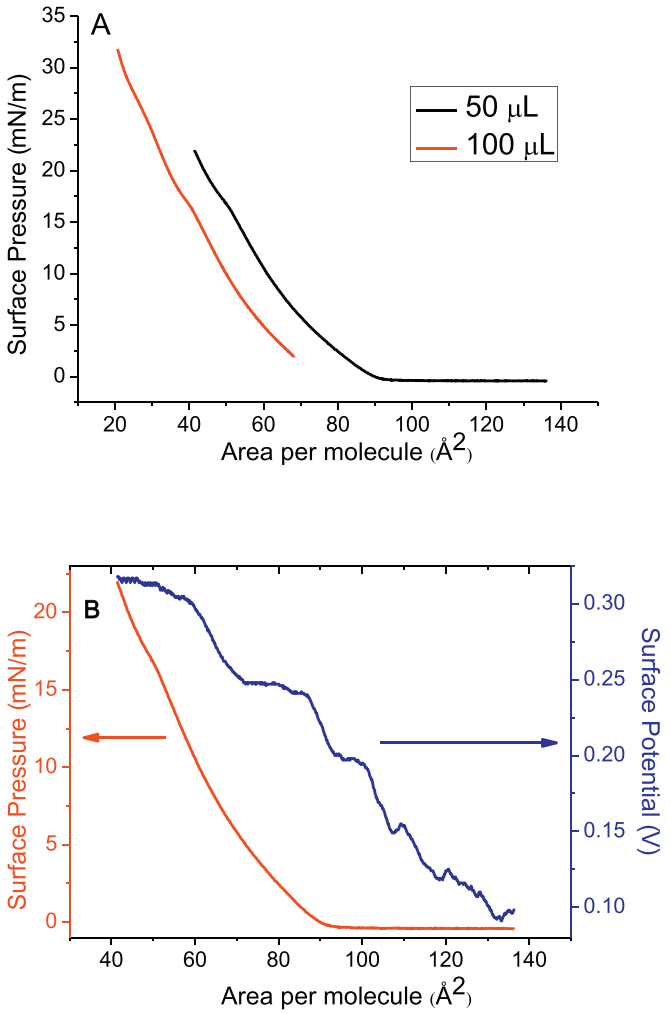


Fig. 1. (A) Surface Pressure-Area Isotherms for γ-terpineol in two different volumes of spreading from a 0.5 mg/mL solution in chloroform. (B) Surface Pressure and Surface Potential-Area isotherms for γ-terpineol in 50 μL of spreading volume from a 0.5 mg/mL solution in chloroform.

Surface potential was measured with a Kelvin probe. For polarization-modulation infrared reflection-absorption spectroscopy (PM-IRRAS) spectra or Brewster angle microscopy (BAM) measurements, the films were compressed up to the pressure of 30 mN/m. PM-IRRAS spectra were obtained through the KSV PMI 550 (KSV-Nima Instruments) spectrophotometer at an angle of incidence of 75°, and BAM images with a microscopy from KSV-Nima.

All the experiments were carried out at a temperature of 25 ± 1 °C, and repeated at least three times to ensure reproducibility. Representative curves or images are shown.

3. Results and discussion

The monoterpene γ-terpineol, isolated from esssential oil of *Chenopodium ambrosioides*, had its structure defined by analysis with ^{13}C NMR and EIMS data and compared with data previously described in the literature [21,22].

Fig. 1 shows the surface activity of γ-terpineol spread in two different aliquots, as indicated. For 100 μL, the initial surface pressure is about 3 mN/m and increases sharply upon compression, reaching surface pressure values as high as 32 mN/m. With 50 μL, however, the isotherm shows a more expanded film since the surface pressure start increasing at higher areas. This indicates a probable aggregation of the compound when excessive amounts are spread. Panel B shows the

surface potential-area isotherms using a volume of 50 μL. The surface potential increases continually with compression reaching a maximum value of 305 mV. Also, we performed cycles of compression-decompression, and they did not show significant hysteresis pointing to stable films. However, although the results point to the surface activity of γ-terpineol, this molecule, although of amphiphilic structure, cannot form stable Langmuir monolayers at the free water surface due to its high water solubility (estimated to be as high as 3.68 g/L). This is proved in Fig. 1 by a shift towards low molecular areas with larger amount of molecules spread at the surface and non-zero value of surface pressure. Moreover, the shape of the isotherm resembles an untrue monolayer, i.e. surface pressure rises due to the compression of aggregates formed at the surface. Furthermore, the collapse of the monolayer could not be determined even with higher amounts of spread solution, confirming the relative solubility of γ-terpineol when subjected to compression. Such structures of molecules dissolving into bulk water cannot be subjected to precise quantitative analysis, and the interaction with lipids at the air-water interface must consider the action of the compound that is subjected to be partially dissolved in the aqueous subphase. This resembles cases in vivo in which drugs attack lipidic surfaces of biological interest, such as cellular membranes or liposomes, with the drug coming from aqueous media. It is also important to note that the use of different solvent species of spreading solvents (hexane, for instance) and longer waiting times after spreading resulted in similar results, confirming the intrinsic instability of terpineol.

Fig. 2 shows the surface pressure-area isotherms for DPPC, a known lipid as model for cells of erythrocytes [23,24]. Its isotherm is typical [25] with a first order transition between the 2D states liquid-expanded and liquid-condensed represented by a plateau region. In the conditions employed in this work, this plateau occurs approximately at 8 mN/m between the DPPC molecular areas of 80 and 60 Å^2. With γ-terpineol incorporated in the DPPC monolayer, the curves are progressively shifted to higher DPPC molecular areas, as an indicative of the compound incorporation. It is important to emphasize that as γ-terpineol is partially soluble in the aqueous subphase, we show the area per molecule related only to the lipid molecules, insoluble in the water and present entirely at the air-water interface. As the isotherms are shifted to higher areas per lipid molecule, γ-terpineol is incorporated at the interface, contributing to the area per molecule. The shift is more prominent at lower DPPC molecular surface densities, but less prominent in higher lipid densities. At the surface pressure of 30 mN/m, value that approximates the properties of a natural cellular membrane [26], the curves are shifted from 53 Å^2/molecule to 59 and to 65 Å^2/lipid molecule, for 5 and 10% in mol of γ-terpineol, respectively. The shift values of 5 and 12 Å^2 are relatively high considering the percentage of γ-terpineol inserted in the monolayer, which would represent an area per γ-terpineol molecule of 100 and 120 Å^2, respectively, if all its molecules are presented at the air-water interface. So these higher values compared to that for Fig. 1 may be an effect of the molecular accommodation of the supramolecular system formed at the air-water interface, or, more specifically, an effect of lateral repulsive interactions between the lipid and the compound. However, for higher surface pressures, these values decrease progressively as we can be note from the isotherms. For instance, at 50 mN/m, the isotherms shift from 49 Å^2, for pure DPPC, to 52 and to 56 Å^2/lipid molecule, for 5 and 10% in mol of γ-terpineol, respectively. This would correspond to γ-terpineol area values of 60 and 70 Å^2, respectively, which is closer to the molecular areas expected to γ-terpineol. This indicates therefore a denser packed molecular accommodation between the film components with interface compression.

It is also interesting to note that the profile of the curve changes noticeably, indicating that the effect underwent by the monolayer due to the lateral compression may pass not only by an elastic response, but also by viscoelastic phenomena, such as aggregation, desorption-adsorption, and lateral molecular accommodation. Such rheological properties can be measured by taking the so-called surface elasticity,

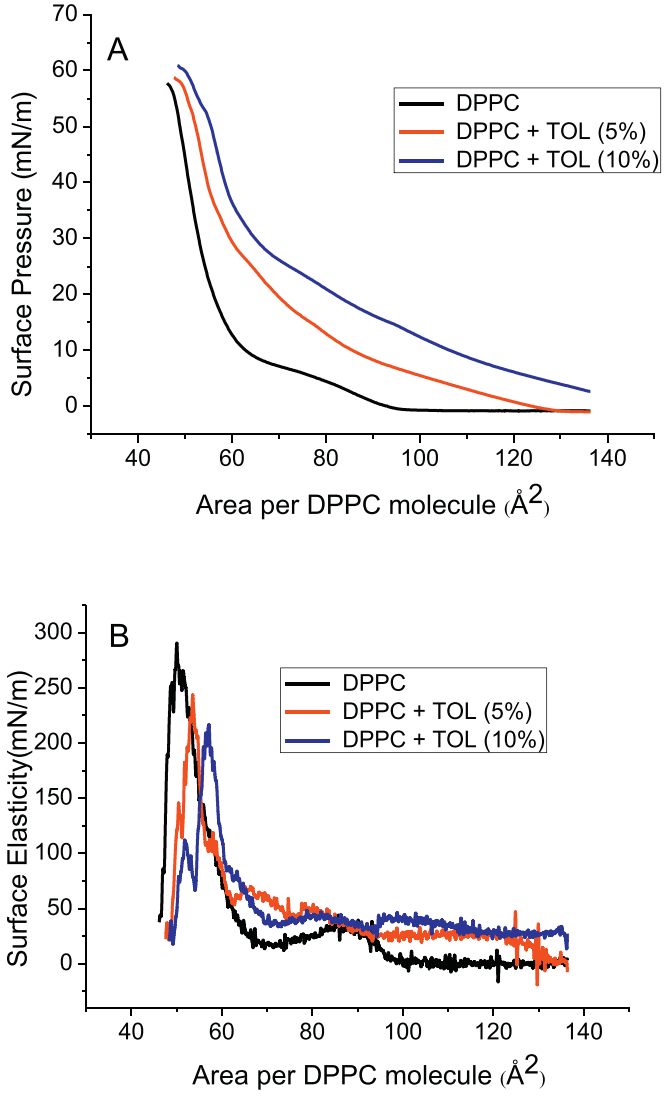


Fig. 2. (A) Surface Pressure and (B) Surface Elasticity-Area isotherms for DPPC, alone or mixed with γ-terpineol (TOL) at the molar proportions indicated in the insets.

which represents the compressional modulus of the floating monolayer at a given temperature, T, and defined as $-A\left(\frac{\partial\pi}{\partial A}\right)_T$. This quantity may be calculated directly from the surface pressure (π)-area (A) isotherm, as presented in Fig. 2B. As a result, it is clear that the first order transition for pure DPPC, seen in the plateau region for the π-A isotherm, corresponds to the region in Fig. 2B where the elasticity decays to values close to zero, between 80 and 60 Å^2. In the case of mixed monolayers, there is no LE-LC phase transition as confirmed by the compressibility modulus dependency. Also, the maximum value of surface elasticity attained by the monolayer is lower when the lipid film contains γ-terpineol. This is common for drugs or other compounds when inserted in rigid lipid monolayers [27,28]. Usually the introduction of a new compound makes the 2D film more flexible to compression. In other words, the monolayer can achieve more possible molecular rearrangements as a response to the increasing surface molecular densities.

Fig. 3 shows the surface pressure-area isotherms for DPPE, a known lipid as model for bacterial [29], tumorigenic [30] and protozoal [18] cells. Its isotherms are typical [31,32], with an abrupt increase of surface pressure with compression due to the transition between the gaseous and the liquid-condensed states. With γ-terpineol, we also observe a shift of the isotherms to higher lipid molecular areas as a consequence

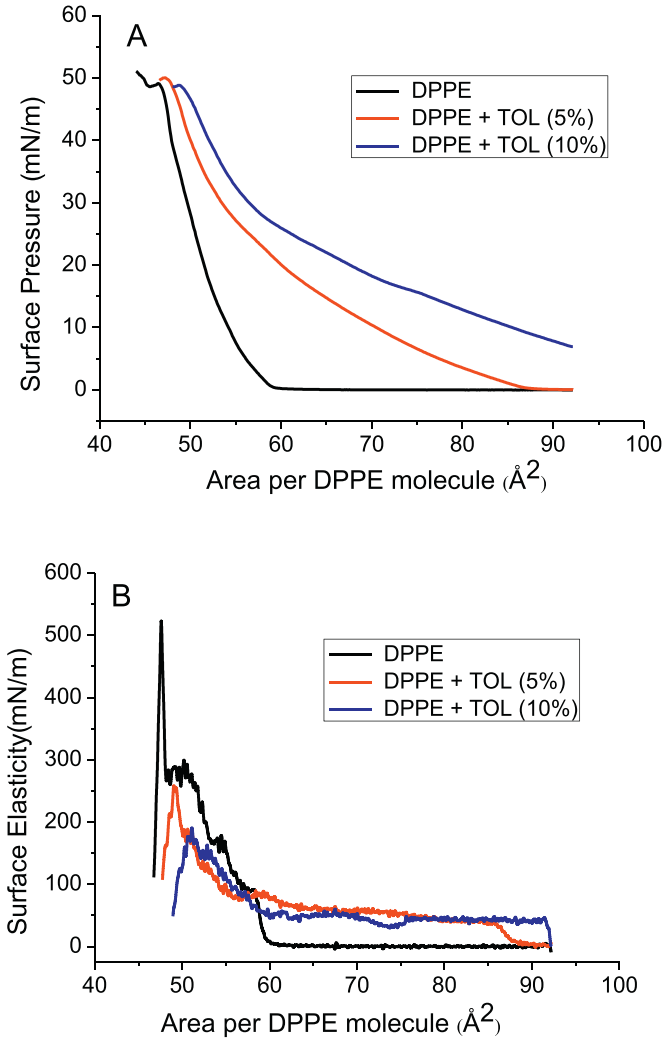


Fig. 3. (A) Surface Pressure and (B) Surface Elasticity-Area isotherms for DPPE, alone or mixed with γ-terpineol (TOL) at the molar proportions indicated in the inset.

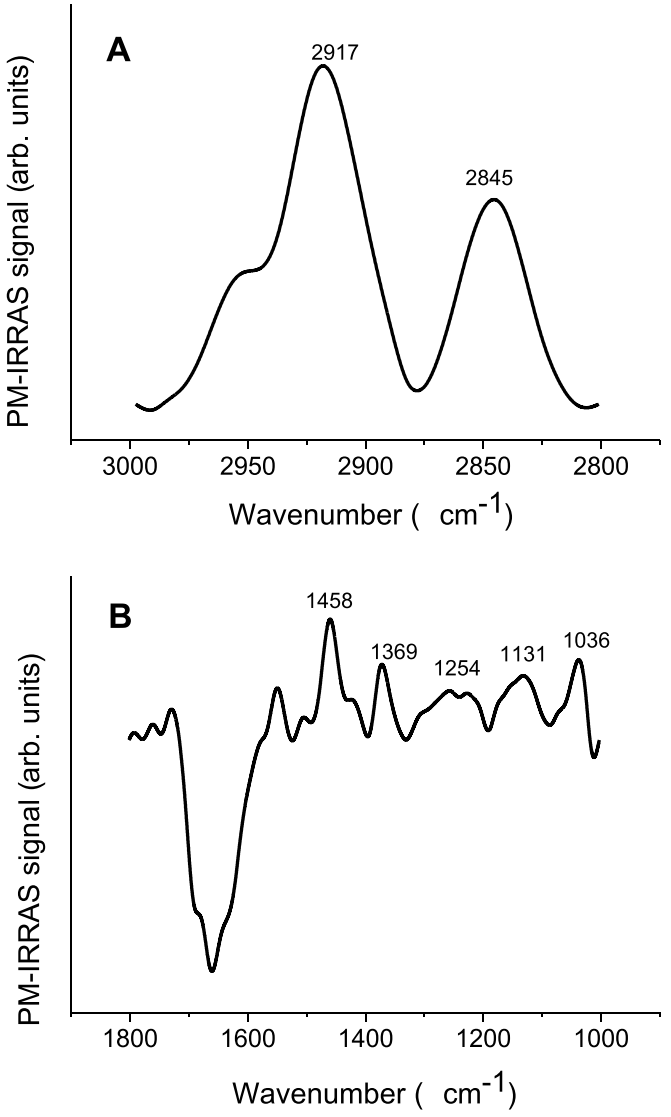


Fig. 4. PM-IRRAS spectra for γ-terpineol spread at the air-water interface at 20 mN/m.

of the monolayer expansion. At 30 mN/m, the isotherm shifts from 50 $Å^2$/lipid molecule, for pure DPPE, to 53 and to 57 $Å^2$/lipid molecule, for 5 and 10% in mol of γ-terpineol, respectively. These values correspond to a γ-terpineol molecular areas of 60 and 70 $Å^2$/molecule, respectively. At 50 mN/m, these values decrease as observed in the profile of the isotherms. At 45 mN/m, the isotherm shifts from 47 $Å^2$/lipid molecule, for pure DPPE, to 49 and to 51 $Å^2$/lipid molecule, for 5 and 10% in mol of γ-terpineol, respectively. These values correspond to γ-terpineol molecular areas of 40 $Å^2$/molecule for both percentages. Although for lower surface pressures the mixture may represent positive excess areas due to repulsive interactions or to molecular accommodation restrictions, for higher surface pressures, even at 30 mN/m, the mixture approximates better the thermodynamic behavior of ideal mixtures.

The compressional data (Fig. 3B) show two kinds of tendency according to the molecular density to be analyzed. For lower molecular densities, the mixed monolayer present higher values of surface elasticity since the values of surface pressure increases continually with compression even at high molecular areas. For DPPE molecular areas as low as 57 $Å^2$, the behavior is the opposite since the mixed monolayer present higher values of surface elasticity when compared to the values calculated for pure DPPE monolayers. However, for molecular areas higher than 57 $Å^2$, pure DPPE monolayers attain a high degree of elasticity attaining higher values than the mixed γ-terpineol/DPPE monolayers. As inferred for DPPC, mixed monolayers are more flexible to molecular rearrangements when compressed and consequently present more viscoelastic features.

Fig. 4 shows the PM-IRRAS spectra for γ-terpineol alone at the interface. The bands centered at 2845 and 2917 cm^{-1} are attributed to the CH_2 stretching mode, symmetric and antisymmetric, respectively. The well-defined bands suggest a preferential orientation of the bands, which may be related to the orientation of the molecule at the air-water interface, with the hydroxyl groups turned towards the aqueous subphase, and the ring aliphatic group oriented perpendicular to the air-water interface plane. The negative band to the baseline around 1680 cm^{-1} is related to the surface water bends and, considering that a background with pure air-water interface was subtracted from the spectrum, this should be an effect of the difference of reflectivity between the interface covered and uncovered by the monolayer [33], and may indicate alteration of the water molecules orientation due to the presence of the amphiphile at the interface. The band at 1458 cm^{-1} is attributed to the CH_2 bending mode, and that at 1369, 1254, 1131, and 1036 cm^{-1} are attributed to CH_3 deformation in the isopropyl group, stretching mode of C=C, C−O stretch in tertiary hydroxyl groups, and ring breathing mode in the aliphatic cycle, respectively.

Fig. 5 shows the effect of the monoterpene on the PM-IRRAS spectra

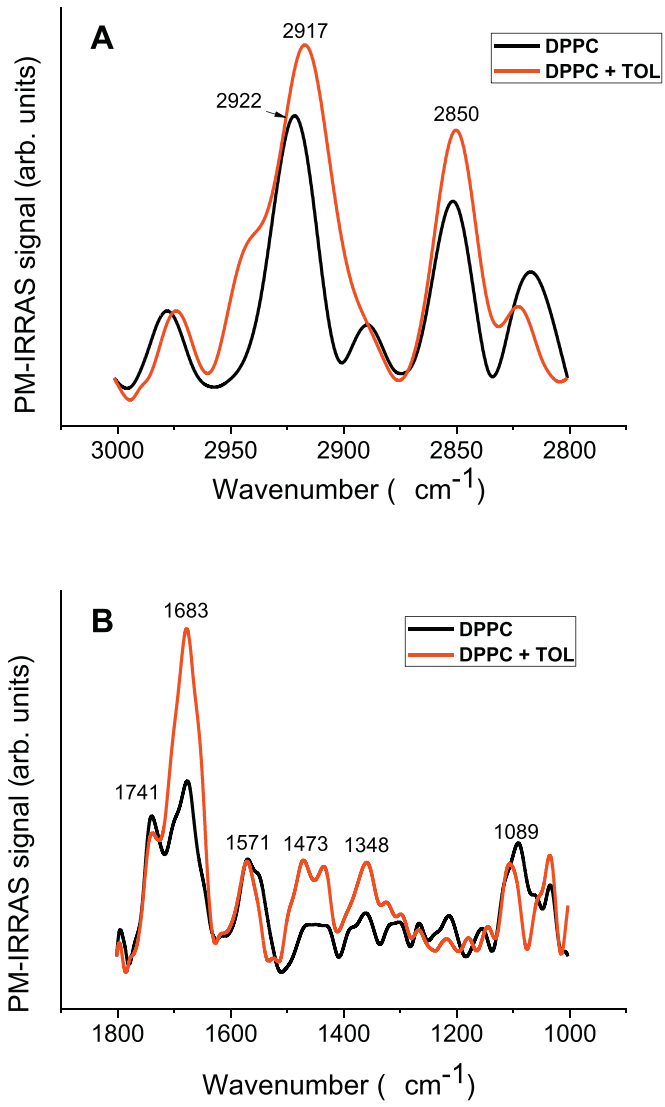


Fig. 5. PM-IRRAS spectra for DPPC, alone or mixed with γ-terpineol (TOL) in the molar proportions indicated in the insets.

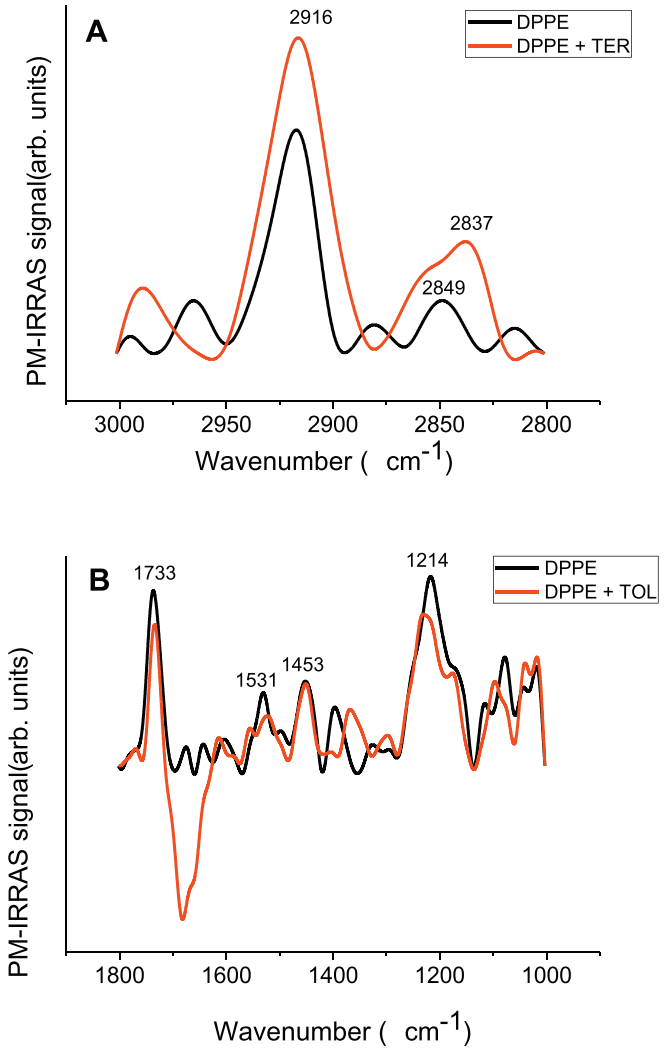


Fig. 6. PM-IRRAS spectra for DPPE, alone or mixed with γ-terpineol (TOL) in the molar proportions indicated in the insets.

for DPPC monolayers. The bands CH_2 symmetric and antisymmetric bands are centered at 2850 and 2922 cm^{-1}. The intensity of antisymmetric CH_2 stretching mode is more sensitive to the conformational ordering [34]. Also, frequencies of the CH_2 stretching absorption bands of alkyl chains are extremely sensitive to the conformational changes of the chains [35–38] and only when the chains are highly ordered (all-trans conformation), the narrow absorption bands appear around in the infrared spectrum [35]. As a result the shift of the peak from 2922 to 2917 cm^{-1} may indicate the decrease of gauche conformers and therefore the increase of the order of the monolayer. Another fact that corroborates with this fact is the increase of both bands (symmetric and antisymmetric) after γ-terpineol incorporation. As both spectra were taken with the same background (same aqueous subphase), these relative values can be compared.

Also we observe roughly changes in the hydrophilic region. The band centered at 1741 cm^{-1} is attributed to carbonyl stretching mode and is little affected after γ-terpineol incorporation. The bands centered at 1580 and 1683 cm^{-1} is attributed to water bending mode. The band centered at 1473 cm^{-1}, attributed to CH_2 bending mode, is split in two bands for the mixed monolayer. The bands in region between 1000 and 1300 cm^{-1} can be attributed to phosphate stretches. In general, this region is affected not only because of this interaction of the compound with the phosphate group, but also because γ-terpineol presents some typical bands in this region. On the whole, these results say that the incorporation of the compound in the DPPC monolayer affects the hydrophilic region of the phospholipid, especially in the region of phosphate groups, suggesting the interaction of γ-terpineol with these groups.

Fig. 6 shows the effect of the monoterpene on the PM-IRRAS spectra for DPPE monolayers. The bands CH_2 symmetric and antisymmetric bands are centered at 2849 and 2916 cm^{-1}. The peak from 2837 to 2949 may indicate increase of gauche conformers and therefore increase of the disorder of the monolayer. Another fact that corroborates with this fact is the shoulder observed for the symmetric band, which also appears wider.

Furthermore we observe some modifications in the hydrophilic region. The band centered at 1733 cm^{-1} is attributed to carbonyl stretching mode and is little affect after γ-terpineol incorporation. The negative band centered at 1680 cm^{-1} is attributed to water bending mode and disappears with γ-terpineol, and that band in 1531 cm^{-1} is attributed to NH deformation. The band centered at 1453 cm^{-1}, attributed to CH_2 bending mode is little affected, and the bands in region between 1000 and 1300 cm^{-1}, attributed to phosphate stretches, is, as a whole, little altered with γ-terpineol. These results suggest that the compound causes more considerable effects in the disorder of the monolayer and less in the phosphate region rather than in hydrophobic

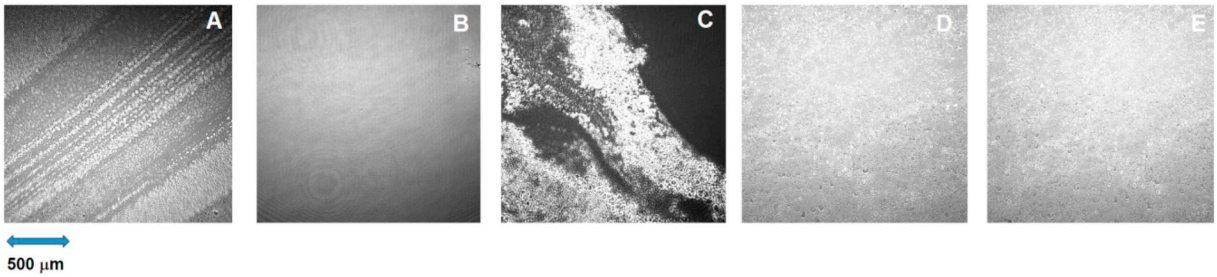


Fig. 7. BAM images for γ-terpineol (A: 20 mN/m); DPPC (B: 30 mN/m), DPPC + γ-terpineol 5% in mol (C: 30 mN/m); DPPE (D: 30 mN/m), DPPE + γ-terpineol 5% in mol (E: 30 mN/m).

tails of the films.

Fig. 7 shows the BAM images for the floating monolayers investigated in this present work. As suggested in the studies about the surface activity of pure γ-terpineol at the air-water interface, this compound tends to aggregate at the air-water interface. Panel A shows striations marking two types of long domains that shear across the interface. This fact points to the formation of aggregates at the interface, characterized by an edge of parallel plates that slid past each other causing tensional stress. This confirms the isotherms for the compound, in which γ-terpineol does not form true Langmuir monolayer since the images show non-homogeneous structures of the compound adsorbed at the surface. For DPPC (Panel B) the image shows an homogenous pattern, in agreement with the literature [39], but the insertion of γ-terpineol (Panel C) cause the formation of aggregates probably because of the repulsion between adjacent molecules, inducing the formation of defects, causing strike-slip faults, which leads to concentrated molecular protuberances. Although this technique is not able to detect the species that are forming the aggregates, considering that phospholipids are in higher molar proportion at the interface, it is probable the supramolecular system containing the phospholipid and γ-terpineol must cause the formation of such morphology. For DPPE, however, the image also presents a relatively homogeneous pattern (Panel D), but with the identification of small bright spots distributed along the image. This could be related to the degree of condensed monolayer formed. Consequently, when submitted to compression up to high values of surface pressure and allowed to relax, the formation of aggregates must be formed as a consequence of defects associated to the monolayer compression rate, which even being with a low value, is not slow enough to occur in equilibrium. Interestingly, after the introduction of γ-terpineol, this pattern is not changed significantly (Panel E), reveling distinctive effects of γ-terpineol for DPPC and DPPE monolayer. In general, the BAM images are remarkable because reveals that such interaction is modulated by the lipid composition at the interface.

4. Conclusions

Distinctive effects for DPPC and DPPE monolayers at the air-water interface were observed upon incorporation of γ-terpineol. For both lipids, the monolayers were expanded, indicating γ-terpineol incorporation, and also a decrease in the in-plane elasticity, indicating viscoelastic effects for the mixed structure. However, for DPPC, an increase of the order of the monolayer was detected with PM-IRRAS, while for DPPE, the mixed monolayers presented an increase of the gauche conformers. More significant changes in vibrational bands for DPPC phosphates, when compared with DPPE ones, suggests a positioning of the compound closer to this group. This distinctive effect preserves γ-terpineol closer to the shear layer between the DPPE polar head and the aqueous subphase. This characteristic molecular architecture between the two phospholipids may induce to different patterns of topographical aggregation, as observed with BAM, and confirms the modulating of γ-terpineol / lipid interface interaction by the monolayer composition. We believe these data can be important to correlate the possible biological activity of this compound with the molecular mechanism of action in biological interfaces such as cellular membranes or even liposomes for drug delivery.

Acknowledgments

We are obliged to Fundação de Amparo à Pesquisa do Estado de São Paulo, Nacional FAPESP, Brazil (grant numbers: 2015/23446-0; 2013/14262-7) and Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Brazil (grant number: 400896/2016-8) for the financial support. GNJ had a CNPq fellowship.

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