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# Antiradical activity of gallic acid included in lipid interphases

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ABSTRACT

Polyphenols are well known as antioxidant agents and by their effects on the hydration layers of lipid interphases. Among them, gallic acid and its derivatives are able to decrease the dipole potential and to act in water as a strong antioxidant. In this work we have studied both effects on lipid interphases in monolayers and bilayers of dimyristoylphosphatidylcholine. The results show that gallic acid (GA) increases the negative surface charges of large unilamellar vesicles (LUVs) and decreases the dipole potential of the lipid interphase. As a result, positively charged radical species such as $ABTS^{\bullet+}$ are able to penetrate the membrane forming an association with GA. These results allow discussing the antiradical activity (ARA) of GA at the membrane phase which may be taking place in water spaces between the lipids.



## 1. Introduction

It is well known that gallic acid (GA), 3,4,5-trihydroxybenzoic acid (Fig. 1A) and its derivatives are biologically active compounds widely present in plants [1–3] and beverages such as tea and wine and is one of the anticarcinogenic polyphenols present in green tea [4,5].

GA is a strong chelating agent that forms complexes of high stability with iron(III) [6,7]. It has shown phytotoxicity and antifungal activity against *Fusarium semitectum, Fusarium fusiformis* and *Alternaria alternata* [8].

Free radicals occur as a natural consequence of cell metabolism and have been implicated in the etiology and pathogenesis of numerous disease states including cardiovascular disease, cancer and diabetes [9–11]. They are also produced as a result of oxidative stress [12–14]. Gallate esters present antioxidant capacity against hydroxyl, azide, and superoxide radicals [15–19] and they are able to scavenge hypochlorous acid at a rate sufficient to protect α-1-antiproteinase against inactivation [20–23]. Also, GA is of great interest in arteriosclerosis prevention [24].

One of the targets of free radicals is the lipid molecule in cell membranes [25]. In this regard, it has been shown that GA decreases the peroxidation of ox brain phospholipids [26]. It is known that the association of GA with phospholipid in organic solvents improves its antioxidant potential of GA by enhancing its bioavailability [27]. However, to our knowledge, no data about the activity of GA included in lipid membranes has been reported. Thus, it is of interest to study the interaction of this compound with lipid membranes and its chemical activity in the presence of lipids.

In terms of membrane structure, polyphenols are able to interact with lipid membranes causing a collapse of the water space between bilayers. This has been ascribed to the decrease of the membrane dipole potential induced by polyols and polyphenols. In particular, it has been shown that GA reduces the dipole potential of lipid monolayers spread on an air–water interface in around 30–40 mV [28].

However, there are no systematic studies correlating the antioxidant properties of GA with its interaction with membrane particles. For this reason, the aim of this paper is to analyze the surface changes promoted by GA on lipid interphases of neutral lipids such as phosphatidylcholines (DMPC) and its influence on antiradical activity (ARA).

The radical cation derived from 2,2′-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) (Fig. 1B) is commonly used to evaluate the antioxidant effectivity of pure compounds and complex mixtures (A. M. Osman et al. and references there in [29]). These radical cations can be generated by enzymatic, chemical, and electrochemical means. Several studies have shown that the kinetics of the reaction between the $ABTS^{\bullet+}$ radical cations and polyphenols in solution is rather complex and the lack of relationship between the rate law and stoichiometric

*Abbreviations:* GA, gallic acid; DMPC, 1,2-dimyristoylphosphatidylcholine; ABTS, 2,2′-azino-bis-3-ethylbenzo thiazoline-6-sulfonic acid; ARA, antiradical activity

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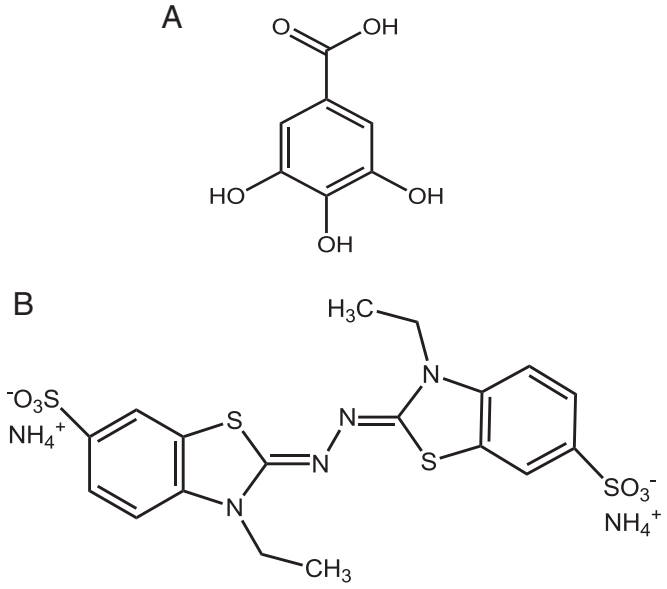


**Fig. 1.** Molecular structure of (A) GA and (B) $ABTS^{\bullet+}$.

factors has been also reported [28,29,32]. In this paper, we have compared the $ABTS^{\bullet+}$ reaction with GA in solution and in lipid membranes to infer the influence of lipid matrix on the ARA.

Thus, we have characterized the association of GA to DMPC LUVs measuring the zeta potential, the dipole potential and surface pressure and its effects on the kinetics of the antioxidant reaction with $ABTS^{\bullet+}$ of GA adsorbed on lipid surfaces.

## 2. Methodology

### 2.1. Materials

1,2-Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and its purity was checked by thin layer chromatography using a chloroform/methanol/water mixture. A single spot was found; therefore, it was used without further purification (>99% pure). Chloroform and KCl were of analytical grade. 2,2′-azino-bis-3-ethylbenzo thiazoline-6-sulfonic acid (ABTS) (Fig. 1B) and GA were purchased from Fluka and Anedra, respectively and potassium persulfate was from SIGMA (Saint Louis, MO). Water was of ultrapure quality (0.009 μS/cm) obtained in an OSMOION 10.2 equipment.

### 2.2. Liposome preparation

DMPC multilamellar liposomes (MLVs) were prepared by dispersing the lipids by vortexing in 1 mM KCl at temperatures higher than that of the phase transition for 60 min. Large unilamellar vesicles (LUVs) were prepared by extruding the liposome dispersions through a polycarbonate membrane (pore size 1000 nm) above the transition temperature of the lipids. Then, the samples were cooled down to working temperature. After LUV preparation, solutions of similar ratios of GA and DMPC were prepared to determine the zeta potential (ζ) and antiradical activity (ARA).

### 2.3. Zeta potential

The zeta potentials (ζ) of DMPC LUVs were determined in Zeta-Meter System 3.0 equipment. All measurements were done at 22 ± 2 °C and pH = 4–4.5.

The voltage was fixed at 75 V. In this method, individual particles are visualized under the microscope and the mobility is determined automatically particle by particle. The total lipid concentration in all cases was 33 μM and the GA concentration was increased from 0 up to 0.83 mM. A total of 20 measurements were carried out focusing different particles for each sample. Data reported are the average of measurements done for each condition with, at least, three different batches of liposomes.

The size and number of vesicles in each sample were determined using the software provided in an Olympus CKX 41 inverted fluorescence microscope with a magnification of 40×.

### 2.4. Dipole potential

Dipole potential ($\Psi_D$) was determined in monolayers formed on an air–water interface by spreading chloroform solutions of lipids on an aqueous subphase (KCl 1 mM) as described before [30,31].

The values of the interfacial potential were determined through a circuit of high impedance, connecting a vibrating electrode above the monolayer and a reference Ag/AgCl electrode in the aqueous subphase. The zero of the potential was achieved with the aqueous solution after extensive cleaning by vacuum.

Lipids were added in carefully measured aliquots of a solution in chloroform of known concentration. After each addition the potential was allowed to stabilize. The dipole potential reached a saturation value after subsequent additions.

The values of areas with this method were obtained following a procedure previously described [31]. Temperature was set at 22 ± 2 °C

### 2.5. Formation of lipid monolayers. Measure of surface pressure and area per lipid calculation

The saturation point of monolayers with and without GA was monitored by measurements of the surface pressure of the lipid monolayers in a Kibron μtrough S equipment, at constant temperature and area.

Aliquots of a chloroform solution of lipids were spread on a clean surface of 1 mM KCl or aqueous solutions with 10 mM GA and left to reach constant surface pressures, until no changes were observed with further additions of lipids (saturation). Results of surface pressure were expressed in mN/m. With these criteria, areas per lipid were calculated with the first point of the saturation plateau of a curve of monolayer surface pressure vs nmol of lipids added to a constant area of the trough.

### 2.6. Antiradical activity determination (ARA)

The method used to monitor the antiradical activity (ARA) was the radical cation method ($ABTS^{\bullet+}$) (Fig. 1B). It consists of monitoring by spectrophotometry the $ABTS^{\bullet+}$ absorbance reduction at 734 nm after antioxidant addition. $ABTS^{\bullet+}$ solution (initial absorbance = 1.00) was placed into a cuvette and mixed with aliquots of different GA/DMPC ratios to a final volume of 1 mL. The radical inhibition percent by GA was calculated, applying Eq. (1), [32,35]

$$\%\mathrm{ARA} = 100 \times \left[1 - \frac{A_{ss}}{A_0}\right] \quad (1)$$

where $A_0$ is the absorbance of $ABTS^{\bullet+}$ solution before adding the antioxidant and $A_{SS}$ is the absorbance of the solution at the steady state. Origin 8.0 software was used to estimate the $A_{SS}$ values by mathematical fitting of kinetic curves according to Eq. (2).

$$A_{(t)} = A_{ss} + A_1 e^{-k_1 t} + A_2 e^{-k_2 t} \quad (2)$$

where $A_{(t)}$ is the absorbance in time (t); $A_1$ is the maximum absorbance at the first step; $k_1$ is the kinetic constant at the first step and $k_2$ is the kinetic constant at the second step.

In order to express ARA in equivalent μmol of this compound, calibration curve was prepared with GA.

$ABTS^{\bullet+}$ solution was obtained by reaction of 7 mM ABTS solution with 2.45 mM potassium persulfate in a 1:0.5 ratio, incubated in darkness for 16 h at room temperature. The obtained radical was diluted with ultra-pure water to absorbance 1.00 at 734 nm ($\varepsilon_{734nm} = 1.5 \times 10^4\ M^{-1}\ cm^{-1}$). The initial concentration of the radical $ABTS^{\bullet+}$ in the cuvette was 66.7 μM and the final concentration of GA in the cuvette was between 0 and 25 μM (pH between 4 and 4.5).

The antiradical activity was determined using an UV–Vis Spectrophotometer JASCOV-630 with a detector of silicon photodiode (S1337). Temperature was set at 22 ± 2 °C.

## 3. Results

### 3.1. ARA in LUVs

The antiradical activity at different GA concentrations in the absence and the presence of DMPC LUVs is shown in Fig. 2A. In the presence of LUVs the ARA at GA concentrations higher than 2.5 μM decreases with respect to the control without lipids, showing a nonlinear behavior. In addition, the decrease of the absorbance as a function of time (insert graph in Fig. 2B) has a biexponential behavior, suggesting that there are two steps in the kinetic process, a fast step and a slow one in both cases. This is better observed in the logarithm plot of absorbance vs time (Fig. 2B). The fast step seems not to be affected by the presence of lipids. On the contrary, the slow kinetic phase is relatively slower in the presence of LUVs than in its absence. These results may be ascribed

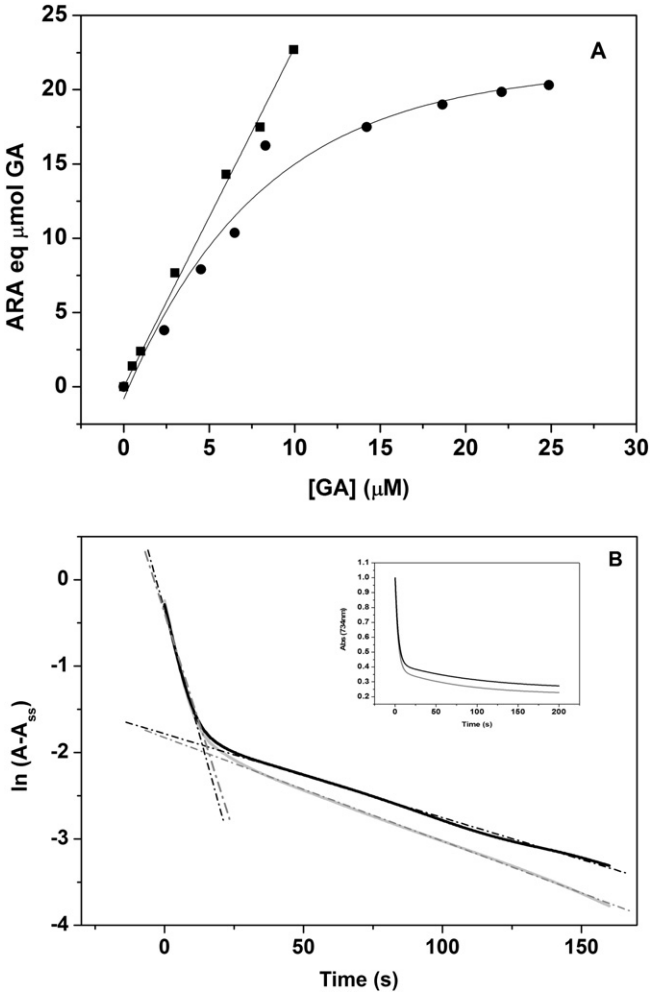


**Fig. 2.** (A) ARA vs GA concentration in the absence (■) and the presence (●) of DMPC LUVs. Lipid concentration as LUVs 1.66 μM. (B) Kinetics of ARA for 8 μM GA with (black solid line) and without LUVs (gray solid line).

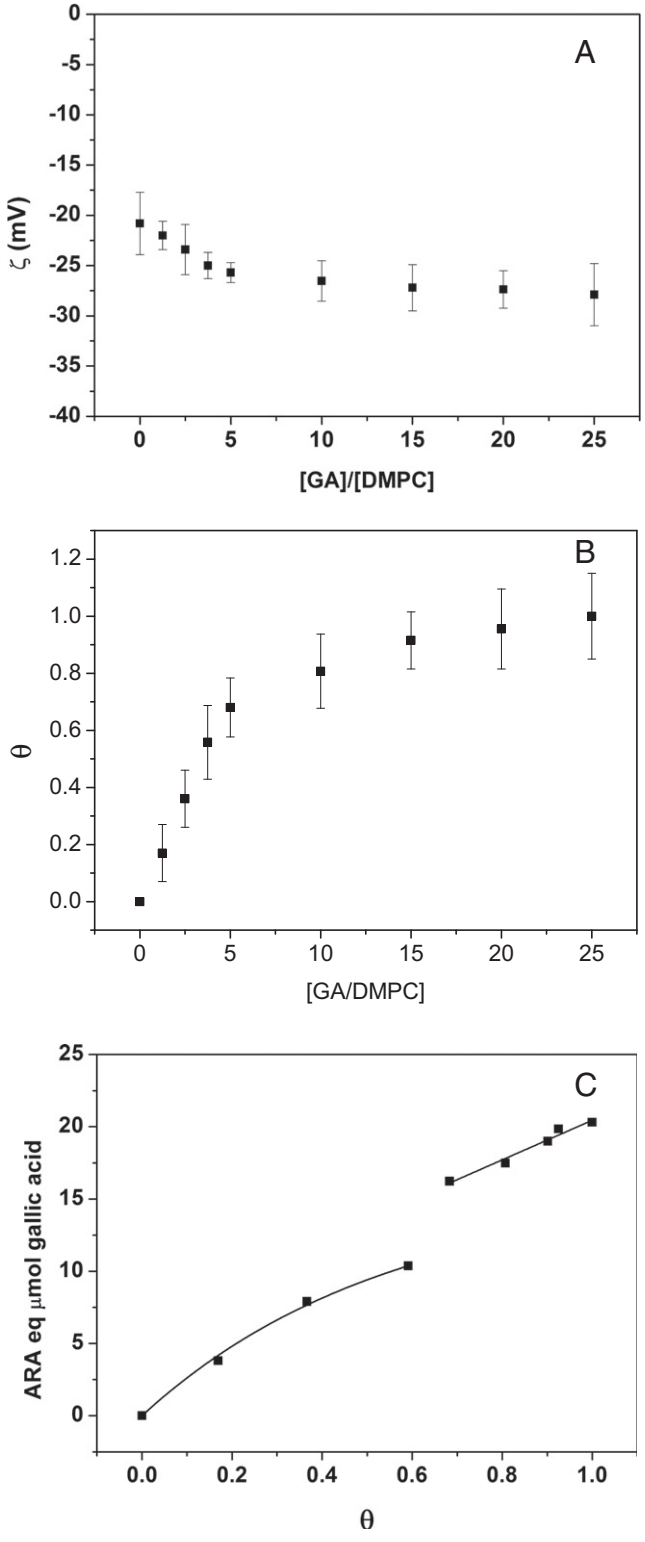


**Fig. 3.** (A) Variation of the zeta potential (ζ) of DMPC LUV liposomes for different GA/DMPC ratios at 22 ± 2 °C. (B) Degree of coverage (θ) using the zeta potential data according to Eq. (3) (see text). (C) Antiradical activity (ARA) plotted as a function of the degree of coverage of DMPC LUVs.

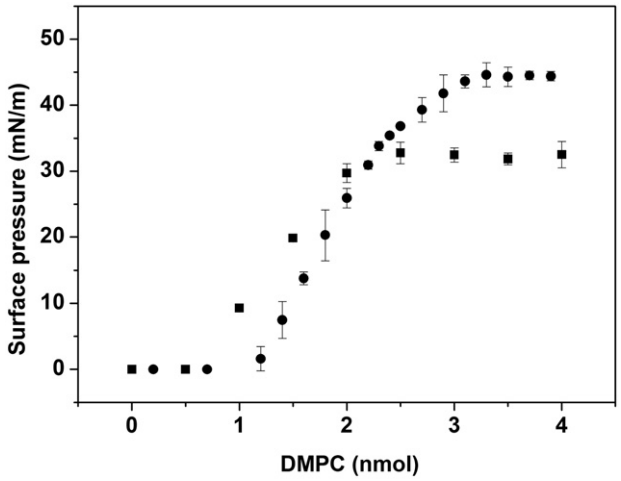


**Fig. 4.** Surface pressure as a function of the excess of surface concentration of DMPC spread on 1 mM KCl solution (pH = 2) (●) and on solution containing 10 mM GA solution (pH = 2) (■) at 22 ± 2 °C.

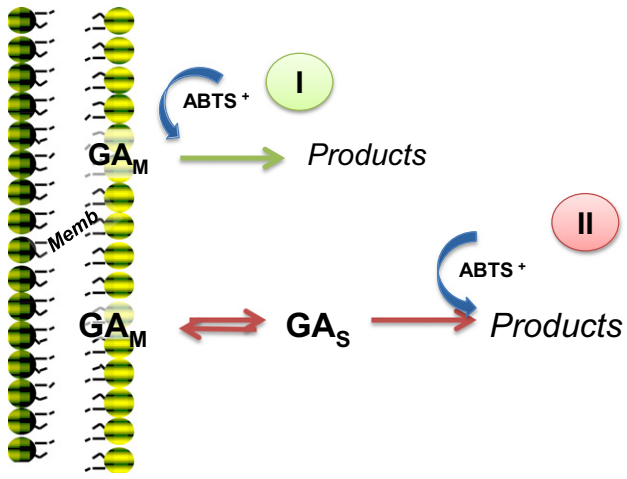


**Fig. 6.** Schematic description of ABTS with gallic acid (GA) inserted in the bilayer (Scheme I) and in solution (Scheme II).

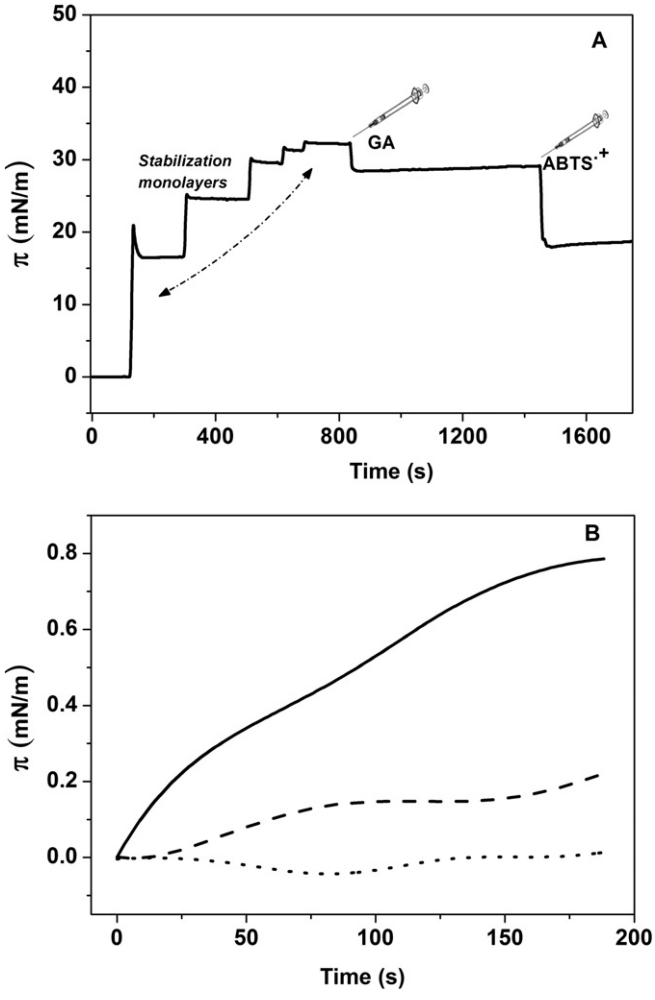


**Fig. 5.** (A) Addition of GA to a final concentration of 1.11 mM to a preformed monolayer of DMPC at an initial surface pressure of 32 mN/m. In the same figure is observed the effect of the addition of $ABTS^{\bullet+}$ (final concentration of 0.016 mM) after stabilization of GA with the DMPC. (B) Effect on surface pressure of the addition of GA (dotted line), $ABTS^{\bullet+}$ (dashed line) to stabilized DMPC monolayer and of $ABTS^{\bullet+}$ monolayer previously stabilized in GA (solid line).

to a partial adsorption of GA to the LUV external surface, which affects the effective concentration of GA in the solution.

If the adsorption process of GA to the lipid membrane would be reversible, all adsorbed GA would be displaced to the aqueous solution and a similar ARA than in water should be obtained at long times (Scheme II in Fig. 6).

This is contrary to the observation since GA reaction is lower in the presence of lipids above 2.5 μM. Thus, it is possible that part of GA added to the solution becomes irreversibly adsorbed to the lipid phase. The irreversible GA adsorption was tested in assays in which DMPC LUVs incubated previously with GA were washed extensively with free-GA solution. The mixture was centrifuged for 10 min at 6000 rpm and the pellet was resuspended in KCl solution without GA. This procedure was repeated three times. Then, aliquots were taken and ARA was measured again.

It was observed that the difference in the ARA between unwashed and washed vesicles was less than 5%, suggesting that the difference was within the experimental error. Thus, it is concluded that GA in the membrane is reactive to $ABTS^{\bullet+}$ (Scheme 1 in Fig. 6).

In order to have a direct evidence of the adsorption of GA to the lipid interphase zeta potential measures were done next.

### 3.2. Zeta potential and GA affinity

The titration of DMPC vesicles with GA promotes a shift to more negative values of the zeta potential with respect to pure lipids (Fig. 3A). This decrease indicates that GA adds a net negative charge to the membrane surface, which is congruent with the fact that the $pK_a$ of the COOH group is 4.5.

Based on zeta potential measures, the degree of coverage of LUVs can be related with the GA concentration by means of the isotherm described by Eq. (3) [33]:

$$\theta = \frac{[GA/DMPC]^n}{K + [GA/DMPC]^n} \quad (3)$$

The standard deviations of the zeta potential values used for the $\theta$ calculation were calculated from the average of 20 significant measures in three different batches of LUV preparation. In all the cases, it was around 10% (Fig. 3B). The best fit of data is for a binding constant K = 0.305 ± 0.010 and an "n" coefficient equal to 1.57 ± 0.09, suggesting a non-Langmuir adsorption. The saturation concentration according to these data is around 15 mol GA/mol lipid.

In Fig. 3C, the ARA data are related to the degree of coverage of the vesicles by GA for similar GA/DMPC ratios. It is observed that at 60% coverage, the curve suggests saturation. At this point, a jump in the ARA is observed, from which a linear increase is apparent. These results can reflect that, at low GA concentration, it is adsorbed to vesicles and the ARA takes place along mechanism I in Fig. 6. At around 60% coverage the vesicles would be saturated and the GA excess remains in the solution, giving the straight line from 0.6 to 1.0.

### 3.3. Interaction of GA with lipid monolayers

The results of Fig. 3B showing a non-Langmuir adsorption behavior, suggest that GA affects the structure of the lipid interphase when it is adsorbed from the aqueous solution. In order to have a direct evidence of this structural change, we have determined the area per lipid in monolayers in the presence of GA.

In Fig. 4, the changes in the surface pressure as a function of the surface concentration of DMPC spread at 22 ± 2 °C on 1 mM KCl (pH = 2) and on a solution of 10 mM GA (pH = 2) are shown. It is observed that the excess of lipids on GA solution is lower than on water, indicating that the area per lipid is larger in the presence of the polyphenol. The area at the saturation point is reached with the addition of 3.5 nmol of DMPC on water which gives a limit area of 63.3 ± 2 $Å^2$/molecule, as reported previously for this phospholipid [31]. In contrast, when the monolayer is formed on 10 mM GA, the surface saturates with 2.5 nmol of DMPC giving an area per molecule of 88.6 ± 2 $Å^2$/molecule (Table 1). This is not an effect of the hydrolytic pH of the GA solution since similar values of area and surface pressure were obtained in control experiments with pure DMPC at pH 2 and pH 4. Therefore, it may be concluded that GA acts as a spacer between the lipid head groups, which would explain the decrease in the dipole potential, as shown in Table 1.

Moreover, the surface pressure at saturation is around 44.6 ± 1.8 mN/m and 32 ± 0.4 mN/m when the lipids saturate on GA solution. In Table 1, it is shown that the injection of GA underneath a monolayer of DMPC formed on water and saturated at a surface pressure of 44.6 ± 1.8 mN/m (Fig. 4), causes no perturbation. In contrast, the surface pressure changes to 26.1 ± 0.6 with the injection of GA to a final concentration of 1.11 mM when the initial surface pressure is 32.7 ± 0.4 mN/m (Fig. 5A). This surface pressure decreases contrary to what would be expected for a deep penetration of the molecule into the lipid monolayer. However, this decrease in surface pressure denotes an increase in the surface tension to values corresponding to water and it is congruent with the area increase calculated by the excess concentration in Fig. 4. This result gives support to the dipole potential decrease. In this condition the subsequent addition of $ABTS^{\bullet+}$, which triggers the ARA reaction, produces a further decrease in the surface pressure and a recovery of the dipole potential to a value above that of pure DMPC (Fig. 5A and Table 1).

The kinetics of the effect on surface pressure of $ABTS^{\bullet+}$ on DMPC monolayer without and with GA is shown in Fig. 5B. The addition of GA to DMPC monolayers (dotted line) does not show a significant evolution in time as well as DMPC monolayers with $ABTS^{\bullet+}$ alone (dashed line) once it is stabilized in the membrane. However it is noticeable that the changes in time observed in DMPC monolayer stabilized with GA upon the injection of $ABTS^{\bullet+}$ (solid line) producing a drastic increase in surface pressure, which evolves during several minutes.

**Table 1**
Changes in the initial surface pressure and molecular area produced by the injection of GA in the subphase of DMPC monolayers at different initial surface pressures.

| | DMPC | DMPC–GA | DMPC–GA–$ABTS^{\bullet+}$ |
|---|---|---|---|
| Area per molecule at saturation ($Å^2$) | 63.3 ± 2 | 88.6 ± 2 | – |
| π (mN/m) | 44.6 ± 1.8 | 44.6 ± 1.8 | 44.6 ± 1.8 |
| π (mN/m) | 32.7 ± 0.4 | 26.1 ± 0.6 | 18.8 ± 0.7 |
| Dipole potential (mV) | 410 ± 10 | 390 ± 10 | 435 ± 10 |

## 4. Discussion

In previous works, it has been demonstrated that polyphenols such as tannic acid, composed by several GA residues, are able to collapse the water space between bilayers and to decrease the dipole potential. These results have been explained by the interaction of the PC groups with GA residues [28].

In our work, we studied the direct effect of GA molecules with DMPC membranes and its effects on membrane structure and ARA.

The non-Langmuir isotherm indicates that GA adsorption to DMPC LUVs is produced in non-independent sites. That is, GA may produce a rearrangement of the lipid membrane surface in order to locate more GAs.

The monolayer experiments denote that GA affects the membrane structure by increasing the area per lipid and decreasing the surface pressure. As observed in control experiments, the zeta potential of DMPC LUVs is negative. The addition of GA promotes a more negative zeta potential, which can be ascribed to the insertion of negatively charged molecules of GA to the interphase. The presence of these charges would promote an area expansion due to the repulsion in the head group region.

The point that GA, which is negative, can insert into a negatively charged surface denotes that other forces, besides electrostatic ones, drive the GA interaction with the membrane. This can be ascribed to the property of the molecule to concert hydrogen bonds between the OH groups and hydrogen bonding residues of the lipids, such as carbonyl and phosphate groups. This would be sustained by the observation that GA decreases the dipole potential of monolayers, as reported previously [28] (Table 1).

The decrease of dipole potential can be due to different reasons. One is because the net dipole of the GA inserts in an opposite direction to that of the dipoles contributing to the DMPC lipid membrane dipole potential such as carbonyl groups and polarized water molecules [30–34]. According to our results, the area per lipid increases with GA, which would explain the dipole potential decrease as a consequence of the dipole density decrease with expansion.

Taking into account the final value of the surface pressure of lipid stabilized with GA, attained in two different experiments: one by forming the DMPC monolayer on the GA solution and the other by injecting a GA solution underneath a stabilized monolayer of pure lipids (Fig. 4 and 5A), it is possible to infer that GA increases the exposure of water between the lipid head groups. This conclusion is reached considering that the decrease in surface pressure would correspond to the increase of surface tension to values obtained without lipids.

According to the kinetic profiles (Fig. 5A–B) of the surface pressure, GA and $ABTS^{\bullet+}$ not only interact with the monolayer, but also form a chemical association. This interpretation is based on the subsequent increase in surface pressure during time, when $ABTS^{\bullet+}$ was added to a monolayer stabilized in GA in comparison to the negligible effects produced by GA and $ABTS^{\bullet+}$ when they were added alone. Upon addition of GA, the surface pressure decreases and remains unchanged during the first minutes. This would suggest that GA does not desorb from the membrane once it has interacted, confirming the results of the experiments with washed LUVs. This is congruent with the data in Fig. 2A in which the ARA is lower in the presence of LUVs. If the ARA would displace the equilibrium of the reversible adsorption of GA, similar values to that obtained in the absence of liposomes should be obtained at long times. However, there is a direct correlation of ARA with the GA adsorbed to the membrane as derived from the data in Fig. 3C.

As $ABTS^{\bullet+}$ causes an important increase in the dipole potential opposite to that caused by GA (c. a. 490 mV), it is reasonable to think that the

dipole potential recovery is due to a partial neutralization of the GA dipole by the $ABTS^{\bullet+}$ dipole (Table 1).

We can conclude that GA is able to interact with both monolayers and phospholipid bilayers. Adsorption is accompanied by changes in the packing of the membrane and in electrical properties such as: i) reduction in the surface potential, and ii) decrease in the dipole potential in these membrane systems. In addition, $ABTS^{\bullet+}$ reacts with GA inserted in the membrane, suggesting that it is accessible to the radical species. Moreover, since the kinetics in LUVs does not differ significantly from that obtained in water, it is likely that the GA/$ABTS^{\bullet+}$ association denoted by the drastic increase in surface pressure in Fig. 5B is a previous step in the ARA, which would take place in water spaces between the lipids. The observation that GA expands the monolayer decreasing the surface pressure is congruent with the interpretation that GA may form water areas in which the ARA can occur.

The observation of the kinetic curves shown in Fig. 2 indicates that at long times ABTS concentration represented by the optical density tends asymptotically to a value of 0.2, indicating that the reaction with GA is not complete, i. e. it reaches an equilibrium. Similarly, in the presence of LUVs the asymptotical values is higher, suggesting that the equilibrium between GA and ABTS is achieved at a higher ABTS concentration. That is, the ABTS consumption is lower in LUVs. Thus, at equilibrium, there may be a population of not reactive GA present in the membrane.

As the rate of conversion of GA in LUV seems to be lower, it is likely that the reaction in GA-membrane is partially hindered, probably by a deficient access of ABTS to GA inserted in the membrane. This slow down of the direct reaction GA with ABTS in the membrane phase would denote that the kinetics of conversion changes. This might be related with the concomitant cooperative change denoted in the isotherms of Fig. 3, showing a deviation for a Langmuir behavior.

These effects may be related to its action as an antioxidant in water-lipid interfaces, which should be taken into account before a potential biological and/ or pharmacological application of this type of compounds.

Oxidative stress, which often arises as a result of the imbalance in the human antioxidant status, has been implicated in aging and in a number of human diseases such as cancer, atherosclerosis, malaria and in rheumatoid arthritis. The current status of free radicals in nutrition and dietary antioxidants considers the possibility of the use of a range of antioxidants, combined with methods for measuring oxidant generation [36]. The possibility of inserting GA in lipid vesicles may favor the modulation of its activity by an adequate lipid composition, which would help to delineate the modulation of free radicals in the human body. The comparative biomimetic experiments permit a deeper understanding of the interaction and penetration of biological membranes by biophenols.

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