# *Electrochemical Characterization of a Mixed Lipid Monolayer Supported on Au(111) Electrodes with Implications for Doxorubicin Delivery*

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## ABSTRACT

The cationic lipid didodecyldimethylammonium bromide (DDAB) is one of the agent that is included in formulations for liposomes with anchored gold nanoparticles as drugs carriers because its positive charge facilitates the anchoring of the negatively charged stabilized gold nanoparticles to the lipid components of the liposomes. In this paper a thermodynamic analysis of Langmuir isotherms was performed, as a first step in the preparation of liposomes including DDAB, the phospholipid 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and cholesterol(Ch),to decide about the most stable combination. Monolayers of DMPC:Ch:DDAB with the most energetically favourable composition, were transferred to Au(111) electrodes by the Langmuir-Schaefer technique in the electrochemical cell and characterised by impedance spectroscopy. The results were compared with those obtained with electrodes coated with DMPC:Ch films

that did not contain DDAB. In both cases the frequency dispersion of impedance data indicates high homogeneity of the films in a wide potential range around the capacitance minimum.

The inclusion of the anticancer drug doxorubicin (DOX) into the mixed lipid monolayers and its electrochemical reduction at pH 4.5 were studied by voltammetry and by impedance spectroscopy. At potentials out of the faradaic region the inclusion of DOX does not affect significantly the frequency dispersion of the impedance but decreases the capacitance. However, at negative potentials the analysis of the high frequency dispersion of the impedance and the influence of the scan rate on the voltammograms indicate a reduction process with contribution of adsorption and diffusion of DOX.

Diffusion was avoided by transferring the electrodes coated in the Langmuir trough to the electrochemical cells that do not contain the drug. Under these conditions the reduction of the adsorbed DOX was found to obey the model of a surface confined electrode and the charge transfer resistance, $R_a$, and adsorption capacitance, $C_a$, were obtained at potentials of the faradaic region. The combination of both parameters provides the rate constant for the reduction in a wide potential range that allows us to propose a sequential mechanism with two determining steps.

*Dedicated to Professor Renata Bilewicz for her 65th birthday and in recognition of her contribution to electrochemistry*

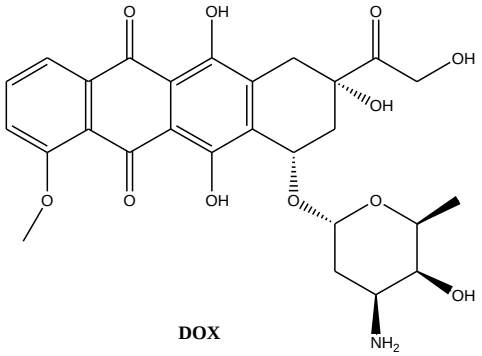
Keywords: lipid monolayers, DMPC, DDAB, cholesterol, Au(111) electrodes, voltammetry, impedance spectroscopy.

## 1.-INTRODUCTION

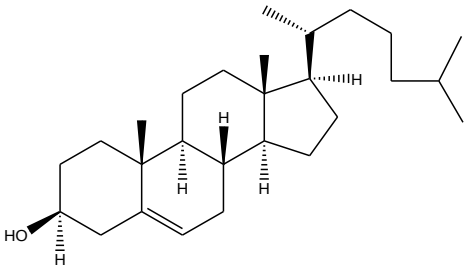
New strategies in drug delivery involve the use of complex platforms formed by functionalized liposomes as drug carriers. Liposomes with anchored gold nanoparticles are nontoxic and non-immunogenic drugs vehicles that are being investigated,

especially for anticancer drugs that show strong side effects, because of their easy targeting and cell uptake of the drug that reduce the necessary dosage [1–5].

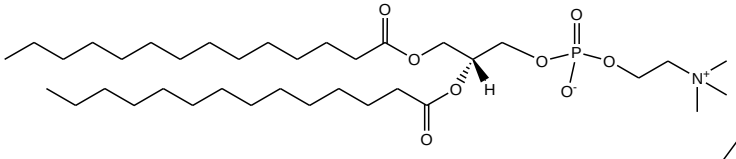
The stability of the liposome and the interactions with the metal nanoparticle strongly depend on the layer components. Phospholipid monolayers have been frequently usedas model biomembranes and their characterizations at the gas/aqueous interphase of the Langmuir trough have been useful in the interpretation of drug interactions with tumor cell membranes and with drug delivery liposomes [6–9]. Phospholipid liposomes are known to be excellent drugs carrier but the anchor of gold nanoparticles to their surface requires the inclusion of positively charged surfactants in their formulation. With this purpose, the amphiphilic didodecyl-dimethylammonium bromide (DDAB) (See Fig 1) has been assayed in pharmaceutical formulations[10]. On the other hand, the inclusion of cholesterol (Fig. 1) in phospholipid layers is known to have significant effects on the stability and fluidity of the layers [11]. One of the objectives of this paper is to take advantage of the Langmuir trough facilities for preparation and characterization of phospholipid monolayers to decide about the most stable combination of phospholipids, DDAB and cholesterol for pharmaceutical formulations.



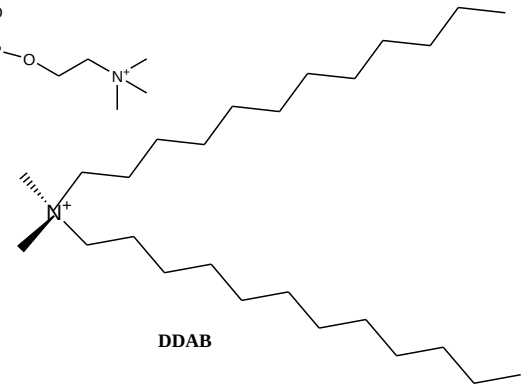

**DOX**




**Cholesterol**




**DMPC**




**DDAB**

Figure 1.- Chemical structures of1,2-dimirystoyl-sn-glycero-3-phosphocholine (DMPC), doxorubicin (DOX), cholesterol and didodecyldimethylammonium bromide (DDAB).

The composition of the liposomes can affect not only the interactions with the gold nanoparticles but also the interactions with the drug and hence the drug delivery properties of the lipid layers. Therefore, the inclusion of the drug in the lipid layer has to be essayed. In this paper doxorubicin (DOX), an anthracycline anticancer drug has been selected, as it is a widely used drug in the treatment of several kind of cancers but presents high toxicity and low remaining time in blood [12], so new strategies are being investigated to diminish both inconveniences, including the use of bioconjugated gold nanoparticles (AuNPs) as DOX carriers[12].

The transfer of the monolayers formed at the gas/aqueous phase to metal electrodes allows the application of electrochemical techniques to characterize the behaviour of the lipid structures under the high electric fields of the electrode interface, of the same order than those reached in the biological membranes ($10^7$ to $10^8$ V $m^{-1}$)[11,13–15]. Among these techniques, the Electrochemical Impedance Spectroscopy (EIS) is particularly interesting as it provides simultaneous insight about the dielectric and blocking properties of the supported lipid layer and about the kinetics of the possible electron transfers taking place at the electrode interface[16]. This information can be useful to select well-orderedwith minimum defect sites liposome films and to determine the inclusion of the drug.

Doxorubicin is a redox-active molecule, due to their quinone and hydroquinone groups (Fig 1) that can be respectively reduced and oxidized.The interactions of DOX with lipid structures and its redox properties in there has been previously studied[17–24]. At lipid monolayers modified gold electrodes DOX undergoes a $2e^-/2H^+$ reduction by penetrating the monolayer or at the defect sites of the monolayers. However, the electrodes coated with lipid bilayers seems to have mainly a blocking effect to the redox DOX processes that was interpreted as due to the low hydrophilic interactions of DOX with the headgroups of the lipid[24].

In this paper, the optimal component ratios of mixed phospholipid-cholesterol-DDAB films has been screened out according to their Langmuir monolayer behaviour to decide about the most thermodynamically favourable formulation for DOX delivery. One of

the phospholipids commonly occurring in natural cell membranes, 1,2-dimirystoyl-sn-glycero-3-phosphocholine (DMPC) (Fig. 1), has been selected, as it has been frequently included in many drug delivery formulations. The most stable monolayer composition has then been used to create monolayers at the electrolyte/inert gas interface of an electrochemical cell that have been transferred to Au(111) electrodes by the Langmuir-Schaefer technique. In this way, the electrochemical methodology could be applied to analyze the stability and electrical properties of the supported film as a function of the potential applied to the electrode. On the other hand, the electrochemical reduction of DOX on the monolayer modified Au(111) electrodes has been studied by cyclic voltammetry and EIS in order to decide about the inclusion of DOX in the film and its interaction with gold. Similar electrochemical experiments have been performed with Au(111) electrodes that were coatedby the Langmuir-Shaefer method in the presence of DOX at the subphase of the Langmuir trough and after that transferred to the electrochemical cell filled only with the supporting electrolyte. The influence of DDAB on the monolayer characteristics and on DOX inclusion was investigated by performing the same kind of experiments with monolayers containing the same DMPC/cholesterol composition but not including DDAB.

## 2.- EXPERIMENTAL.

*Reagents and solutions.*

Stock solutions of DMPC (Avanti Polar Lipids), DDAB (Sigma-Aldrich) and Cholesterol (Sigma-Aldrich) were prepared in Chloroform (Sigma-Aldrich, analytical degree) with respective concentrations of 0.80, 0.77 and 0.43 mg/ml. They were kept at -20 ºC. The lipid mixes were prepared just before used from these stock solutions by adding the required volumes of each solution to reach the desired molar fractions. Supporting electrolyte solutions for the electrochemical experiments were made up of 0.1 M $KClO_4$ (Merck, analytical grade) and a 0.01M acetic acid (Merck, analytical grade)/0.01M sodium acetate (Merck, analyticalgrade) buffer of pH 4.5. Stock solutions of Doxorubicin hydrochloride (Sigma Aldrich) of 2.41 mM concentration were prepared in water and were kept at 4 ºC under light protection. They were renovated after 2 weeks to guaranty their stability. Ultrapure water, freshly purified with a Millipore

Direct-Q or a Millipore Milli-Q systems, was used. The pH of the solutions was measured with a SensoDirect pH-meter equipped with a combined electrode type 225.

*Electrochemical cell and electrodes*

For the electrochemical experiments a three electrodes cell was used. The working electrode was an Au(111) single crystal, prepared according to Clavilier method [25]. It was flame annealed before each experiment and transferred to the cell to get dried in the inert gas atmosphere. The counter electrode was a flame annealed gold wire. As reference electrode a $Hg/Hg_2SO_4(s)/K_2SO_{4(sat)}$ electrode was used, connected to the cell via a salt bridge containing the same supporting electrolyte as in the electrochemical cell. However, the reported potentials will be referred to the saturated calomel electrode (SCE). In the experiments with DOX the cell was light protected with aluminum foil.

*Monolayer formation at the Langmuir trough and transfer to the gold electrodes*.

A two movable barrier NIMA 611D trough of 150 ml and surface area of 270 $cm^2$was used under computer control by the NIMA516 software. It was equipped with a Wilhelmy plate made with Whatman 1CHR paper (1cm wide) that was renovated after every experiment. After cleaning the subphase vessel (with ultra-pure water and methanol), it was added the subphase (c.a. 150 $cm^3$) and a small volume (between 20 and 40 μl) of the lipid solutions in chloroform was spread over the subphase surface and allowed to evaporate the organic solvent for approximately 20 min. Compression was then performed at a speed of 30 $cm^2$/min. In the experiments for the thermodynamic study with different lipid mixtures compositions, the subphase was ultra-pure water. However, the monolayers transferred to the gold electrodes were prepared in the trough using a subphase of supporting electrolyte at pH 4.5 and 10mM DOX concentration. The Langmuir-Schaefer method was used to transfer the monolayer to the Au(111) electrodes, at a constant pressure of 30 mN/m. Previously, the monolayer was allowed to preconcentrate DOX at this pressure during about 20 min.

The Langmuir trough was also used to determine the equilibrium pressure of the different monolayers by successive additions of the lipid mixtures at constant area of 35 $cm^2$ and registration of the surface pressure as a function of time. All the experiments

were performed at 27 ºC. The trough was under a cabin that was light protected in the experiments with DOX.

*Monolayer formation at the electrolyte/inert gas interface in the electrochemical cell and transfer to the gold electrodes.*

In the electrochemical cell the monolayers were formed by spreading an excess of lipid mixture in chloroform, once the cell with the electrolyte solution and the electrodes was deaerated by bubbling argon for at least 20 min. The chloroform was allowed to evaporate and the monolayer to attain the respective surface pressure by waiting at least 30 min. The working electrode was then slowly lowered with a micrometer screw to touch the monolayer at a controlled potential of - 0.100 V (at which the phospholipid monolayers are known to be well ordered and stable.[16,26–28]

*Electrochemical experiments.*

Cyclic voltammetry experiments were performed with an IviumStat multipurpose electrochemical system (Ivium Technologies). The scan rate has been varied in the range 10mV/s to 20 V/s. Before every scan the electrode was kept at -0.100 V for at least 1 min in order to get the monolayer reorganization. For the impedance measurements an Autolab multifunctional electrochemical system (PGSTAT30) equipped with a frequency response analyzer module (FRA) was used. In order to collect data in a wide frequency spectrum the multi-sine Fourier transformation mode of the instrument was used and two pulses of 15 frequencies each were applied in the range 10 Hz to 10 kHz at every potential.

The stability of the coated electrode was checked by scanning the potential in a small potential range around the potential of the capacitance minimum while the impedance is registered. A well-coated and stable electrode is considered when almost flat capacitance-potential curves are obtained that do not change in successive scans. However, for the impedance analysis a *step by step* procedure was applied in which before every sample potential ($E_{sample}$) and pulse of frequencies the monolayer coating the electrode was allowed to reorganize at -0.100 V vs SCE. The potential $E_{sample}$ was then applied during 2s. In order to check the reorganization of the monolayer coated electrode the experiments were programmed in several series of intercalated potentials, each series with step intervals ($\Delta E$) of ±20 mV or -40 mV.

## 3.- RESULTS AND DISCUSSION

### 3.1.- Formulation Optimization Based on the Langmuir Isotherms.

The Langmuir trough technique can provide valuable information as a first step in the evaluation of the properties of the amphiphiles mixes of the three components for their use as new delivery systems. The cationic species DDAB provides to the DMPC liposomes the necessary positive charge to anchor stabilized negative gold nanoparticles. Liposomes formed with the 3:1 DMPC:DDAB composition are known to facilitate the anchoring [10], so in this study this ratio has been maintained and the effect of cholesterol on the two lipid components has been studied by registering the isotherms containing different cholesterol molar fractions. In Figure 2 the isotherms (surface pressure ($\pi$) vs area per molecule (A)) of some of the three- components monolayers are compared to the isotherms of the individual components monolayer, DMPC and Cholesterol. The isotherms of the lipids are representative of liquid like phases. The effect of DDAB on the DMPC isotherm is to shift the isotherm towards lower area per molecule and to increase the compressibility of the monolayer, as lower values of the compression modulus, defined as $Cs^{-1} = -A\ (d\pi/dA)$[29] are obtained (not shown). On the other hand, cholesterol presents a more rigid isotherm and introduces some rigidity in the three- components monolayers. The collapse pressure remains practically the same in the DMPC isotherm and in the 3:1 DMPC: DDAB isotherm (50 mN $m^{-1}$) but is lower in the mixed monolayers with high cholesterol molar fractions (45mN $m^{-1}$).

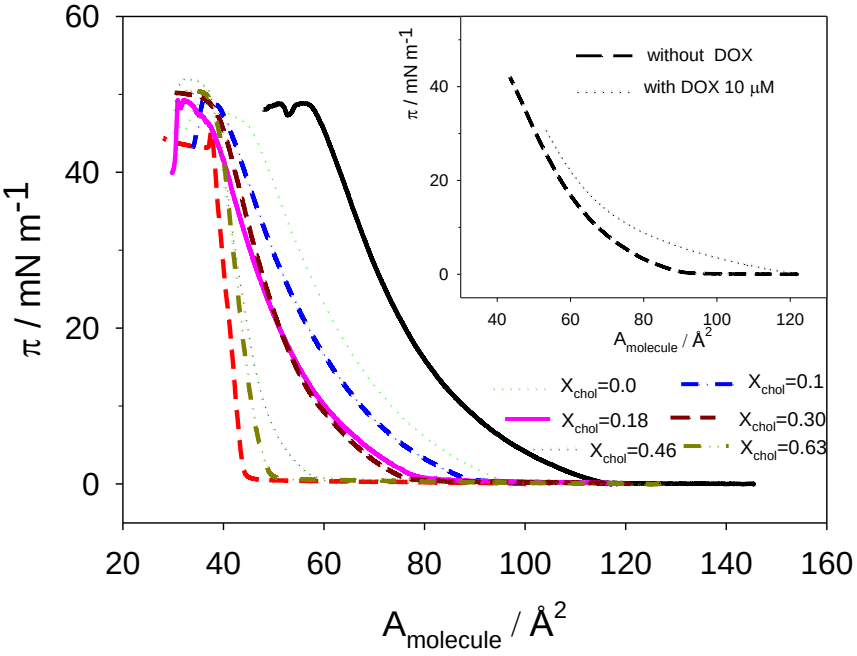



Figure 2. Isotherms measured at 27°C in the Langmuir trough with $H_2O$ in the subphase for films containing pure components DMPC (solid black line) and cholesterol (dashed red line) and films containing mixtures of DMPC:DDAB (3:1 molar) with different molar fractions of cholesterol indicated in the figure ($x_{chol}$). The inset shows the isotherms obtained for the films formed by mixtures of DMPC:DDAB (3:1) with $x_{chol}$= 0.10 in the absence and in the presence of DOX 10 μM in the subphase.

A more quantitative information can be obtained by the analysis of the excess area, $\Delta A_{exc}$ and the excess free energy of mixing, $\Delta G_{exc}$, at fixed surface pressures as a function of the cholesterol molar fraction. These magnitudes are defined in equations (1) and (2), respectively, as deviations from the ideal behavior, which is understood as if each component behaves independently of the others.[30]

$$\Delta Aexc = A_{exp} - A_{ideal} = A_{exp} - [A_{xCh=0} + (A_{xCh=1} - A_{xCh=0})\, x_{Ch}] \quad (1)$$

$$\Delta G_{exc} = \int_0^{\pi} A_{exp}\, d\pi - x_{Ch} \int_0^{\pi} A_{xCh=1}\, d\pi - (1 - x_{Ch}) \int_0^{\pi} A_{xCh=0}\, d\pi \quad (2)$$

According to these definitions, negative $\Delta A_{exc}$ and $\Delta G_{exc}$ values are indicative of attractive interactions between the molecules of the different components. This seems to be the case in the three- components monolayers. In Figures 3 and 4 $\Delta A_{exc}$ and $\Delta G_{exc}$are respectively represented as a function of cholesterol molar fraction, at three surface pressure values (5, 15 and 30 mN $m^{-1}$). Two minima are detected in both Figures

corresponding to 0.10 and 0.43 cholesterol molar fraction, which represent the most energetically favorable mixed monolayers possessing the maximum miscibility.

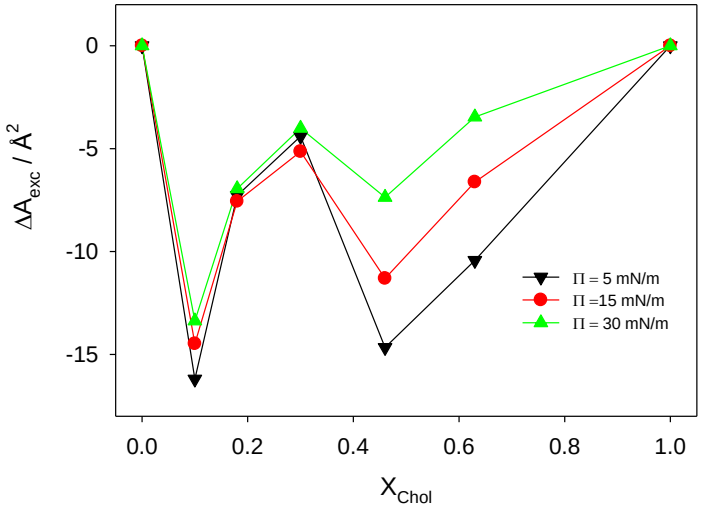



Figure 3.-Excess molecular area as a function of the molar fraction of cholesterol in the mixed lipid films at the constant surface pressures indicated in the figure, obtained from the isotherms in Figure 2.

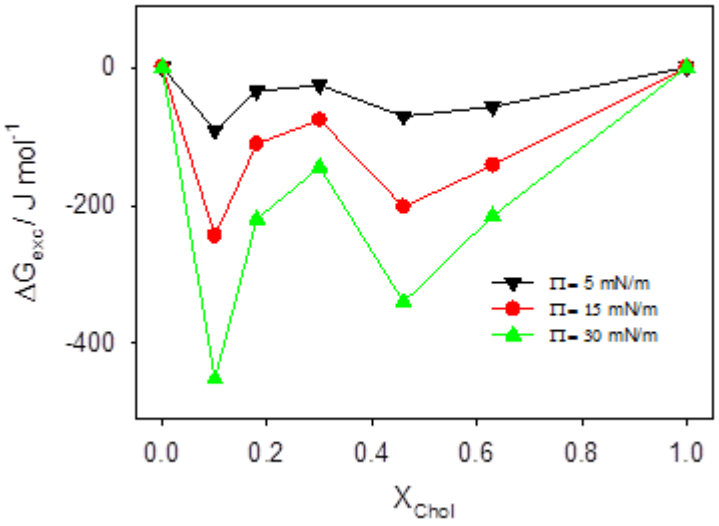



Figure 4.-Excess Gibbs free energy as a function of the molar fraction of cholesterol at the constant surface pressures indicated in the figure, obtained from the isotherms in Figure 2.

The monolayer with 0.1 cholesterol molar fraction has then been selected for the electrochemical characterization and liposomes preparation as it presents closer

similarities with the DMPC monolayers. To infer about the DDAB effect, the electrochemical studies have also been performed using the DMPC:Ch mixtures with 0.1 cholesterol molar fraction. In the insert of Figure 2 are shown the isotherms of the three- components films obtained with subphases of the supporting electrolyte and with subphases that also contain DOX at the working concentration of 10 μM. It can be observed that the addition of DOX to the subphase produces a somewhat higher minimum area per molecule and a lowering of the compression modulus (not shown), indicating the inclusion of DOX into the films and its fluidizing effect on the films.

### 3.2.- Electrochemical Characterization of the Mixed LipidsCoated Gold Electrodes.

The Au(111) electrodes, coated by the Langmuir-Schaefer method in the electrochemical cell at their respective equilibrium surface pressure were characterized by the impedance technique. The equilibrium surface pressures, measured in the Langmuir trough, are somewhat higher in the presence of DOX in the subphase (45 and 44 mN/m for the three-components and two- components films, respectively) than in the absence of DOX (40 and 38 mN/m for the three-components and two- components films, respectively). The pseudocapacitance values, defined as $C_{ps} = (\omega \cdot Z'')^{-1}$ with $Z''$ being the imaginary impedance component(measured at 1 kHz) are plotted as a function of the applied potential in Figure 5, for electrodes coated with the three-components and with the two-components monolayers. The data were obtained by the *step by step* procedure and it can be observed in Figure 5 that there is almost no hysteresis between the negative going and the positive going potential scans, even after the electrode have reached the more negative potential limit (-0.600 V). The curves show a minimum capacitance region around -0.050 V for the DMPC:DDAB:Ch coated electrode and at somewhat more negative potentials for the DMPC: Ch coated electrode (around -0.150V). The effect of DDAB is to lower the value of the capacitance minimum, indicating an electrode coated by a more organized film.

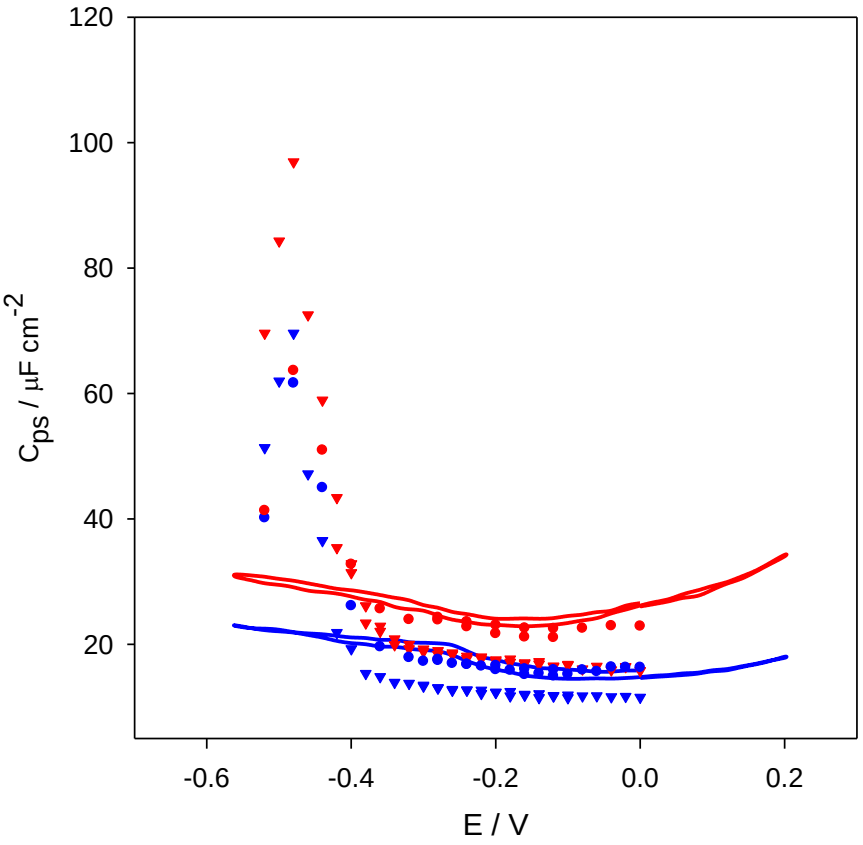



Figure 5.- $C_{ps}$ vs E plots measured with the *step by step* procedure at 1 kHz with Au(111) electrodes modified with monolayers formed in the electrochemical cell with mixtures of DMPC:Cholesterol (blue lines) and DMPC:DDAB (3:1) -Cholesterol (red lines). $C_{ps}$ vs E plots in the presence of DOX in the electrochemical cell: without DDAB (blue triangles) and with DDAB (red triangles). $C_{ps}$ vs E plots obtained with Au(111) electrodes covered by Langmuir-Shaefer method without DDAB (blue circles) and with DDAB (red circles) in the presence of 10 µM DOX in the subphase of Langmuir trough.

The impedance data at every potential were analysed as a function of frequency according to the model of a series combination of a resistance (representative of the Ohmic drop in the cell) and a constant phase element (CPE) (representative of the charging of the electrode interface). The impedance of this element is described as $Z_{CPE} = (Q\ i\ \omega)^{-\alpha}$, where Q is a frequency independent parameter and $\alpha$ is a coefficient ranging from 0 to 1. For $\alpha$= 1 the element becomes a pure capacitance, so the cell impedance can be modeled as a series RC circuit. Values of $\alpha$ lower than 1 are usually interpreted as surface roughness or heterogeneities [31,32]. The electrodes either coated with the three-components films or with the two-components films provides

values of $\alpha$ between 0.96 and 0.91 in a wide potential region ranging from 0.200 to -0.500 V. According to the interpretation of $\alpha$, based on the existence of heterogeneities these values indicate that the coatings are uniform on the whole surface, i.e., no separation of domains takes place. The frequency dispersion of the impedance data is the same in the case of the two kinds of mixed lipid films. However, it does not mean that the lipid films are well organized, on the contrary, the pseudocapacitance values are higher than expected for a compact organised lipid tails of low dielectric constant ε (adopting typical values for ε and the length of the tails) [33]. In fact, it seems that the theoretical value of capacitance for a well organised membrane is only fulfilled by tethered bilayers [33–35]. The monolayers studied in this work, formed by the Langmuir-Shaefer technique, have a more fluid behaviour as they are physisorbed and they may include in the proximity of the metal surface in addition to hydrophobic tails the hydrophobic part of the other components and even some hydrophilic head group and water molecules cannot be excluded. Structural studies by other techniques are planned. The comparison between the pseudocapacitance curves obtained for the two kinds of monolayers suggests that the DDAB component favours the organization of the tails in the monolayer.

### 3.3.- Electrochemical Characterization of the Mixed Lipids Coated Electrodes in the Presence of Doxorubicin.

The pseudocapacitance vs potential curves (at 1 kHz) of the electrodes coated with mixed lipids in electrochemical cells containing doxorubicin have also been plotted in Figure 5. They show also a flat region with lower values of the pseudocapacitance in comparison with the plots of the electrodes coated with the respective films in the absence of DOX. This observation indicates that DOX is incorporated into the films, interacting either with the tails or with the polar head of the phospholipids, but inducing a tighter organization of the film. This effect is opposite to the observed effect of DOX on the lipids films at the interface of the Langmuir trough, so the interaction of DOX with gold may play also some role in the organization of the films on the electrode. The decrease is more significant in the case of the electrodes coated by the mixed DMPC: Ch films (about 8 μF $cm^{-2}$) than in the case of the mixed phospholipid containing also DDAB (decrease about 3 μF $cm^{-2}$). On the other hand, the inclusion of DOX in the

films is clearly inferred by the large increase of pseudocapacitance at potentials coincident with DOX reduction. The onset of the reduction is clearly detected at about -0.350 V and the maximum pseudocapacitance values are raised at -0.480 V, in the two kind of coated electrodes. The impedance analysis of the reduction presented below confirms the inclusion of DOX into the film structure. Moreover, it has been previously stated in the literature [21, 22, 24] that incorporation of the drug and that of the antracyclic analog, daunorubicin, into lipids or lipid-like films is mainly determined by the hydrophobic interactions. Thus, the reduction of the drugs on Langmuir-Blodgett monolayers could be detected but tethered bilayers exert a blocking effect on their reductions [22,24]. The analysis of the impedance data as a function of the frequency at potentials of the flat pseudocapacitance region (out of the faradaic potential region) has shown a good agreement with the series combination of a resistance and a CPE model with $\alpha$ values ranging between 0.96 and 0.94, indicating the uniformity of the films.

### 3.4.-Electrochemical Reduction of Doxorubicin on the Mixed Lipid Coated Gold Electrodes.

#### 3.4.1-Reduction of doxorubicin on modified electrodes in the electrochemical cell

The reduction of DOX on the electrodes modified in the same electrochemical cells with the two kinds of mixed lipid films have been studied by cyclic voltammetry (CV) and by electrochemical impedance spectroscopy (EIS). Some voltammograms obtained at 1 V/scan be observed in Figure 6 in comparison with the one obtained on the bare gold electrode at the same conditions. The voltammogram on the bare electrode was also registered after preconcentration of DOX at a potential of -0.100 V, at which DOX get adsorbed on gold. Curiously, the reduction peaks take place at practically the same potentials on the modified and on the bare electrodes. It seems that the interactions in the lipid monolayers do not affect the reduction, so the orientation of adsorbed DOX should be the same as on the bare electrode. The phospholipid films do not seem to exert a significant blocking effect as the peak intensities are of the same order of magnitude in all cases, suggesting that the inclusion of DOX in the films is controlled by the potential.

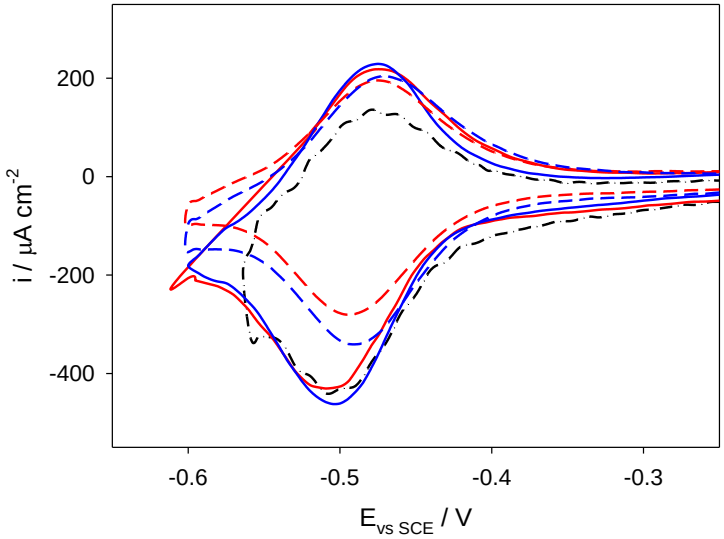



Figure 6.- Cyclic voltammograms obtained at 1 V $s^{-1}$ for the reduction of DOX with Au(111) electrodes modified in the electrochemical cell with a monolayer containing DMPC-Cholesterol (blue solid line) and DMPC:DDAB (3:1) - Cholesterol, (red solid line). Black dot-dash line represents the voltammogram obtained with the bare Au(111) electrode. Dashed lines represent the voltammograms obtained with the Au(111) electrode modified by Langmuir-Shaefer method with films containing DMPC-Cholesterol (blue) and DMPC:DDAB (3:1) - Cholesterol (red).

The analysis of the influence of the scan rate on the peak intensities of the reduction ($I_{pc}$) and subsequent oxidation in the reversed scan ($I_{pa}$), shows better linear behaviour when peak intensities are plotted versus the scan rate, v, than when represented versus the square root of the scan rate, $v^{1/2}$, in accordance with surface confined redox processes. However, the linear regression is not completely satisfactory (see Figs 7a and 7b). The linear dependence of peak intensities is an indication of a surface confined reaction.

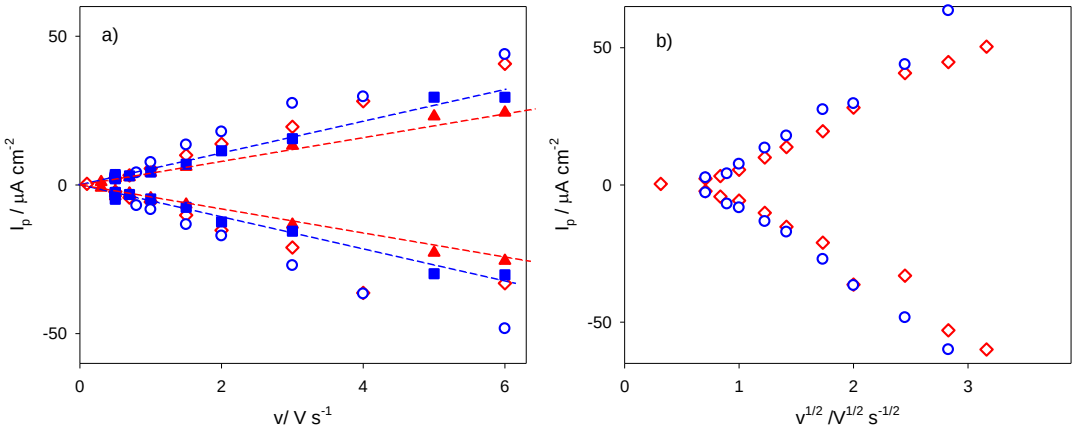



Figure 7.- $I_p$ vs v plots (a) and $I_p$vs $v^{1/2}$ plots (b) obtained: i) with Au(111) electrode modified in the electrochemical cell with DMPC:Cholesterol (blue open circles) or DMPC:DDAB (3:1) – Cholesterol (open red diamonds). ii) with the Au(111) electrode modified by Langmuir-Shaefer method with DMPC:Cholesterol (blue solid squares) or DMPC:DDAB (3:1) - Cholesterol (red solid triangles). The lines represent the linear regressions of the plots with solid symbols.

The EIS data may help to clarify if the reduction process of DOX on the lipid-modified electrodes is a surface confined reaction. Laviron et al. derived the impedance equations for this kind of processes.[36] According to them, the description of the faradaic process involves a series connection of two frequency independent parameters, the charge transfer resistance, $R_a$, and the adsorption capacitance, $C_a$, due to the redox process of the adsorbed reactant. The electrode impedance and admittance include also the charging of the interface, represented by a capacitative element, the high frequency capacitance, $C_{HF}$, which is in parallel with the faradaic process. According to this model, the Nyquist impedance plots should exhibit a circular arc characteristic of a faradaic process and a vertical line at lower frequencies corresponding to a pure capacitative behaviour. In Figure 8 the Nyquist plot are shown for the impedance at a potential of the faradaic region (-0.480V) obtained with the electrode coated by the lipid film that contains DDAB, in comparison with the impedance at a potential out of the faradaic region (-0.150V). The vertical line at low frequencies, corresponding to a pure capacitance, is not observed in any of the plots, but straight lines with a slight inclination angle of about 10 degrees with respect to the vertical line, very different from the 45 degrees expected for diffusion controlled faradaic process. Moreover, the angle is practically the same for the plots obtained at the two potential values.

Therefore, the deviation from the vertical line seems to be related to heterogeneities of the films. For this reason the model for the surface confined reaction was modified by changing the $C_{HF}$ capacitance for a constant phase element (CPE), $Q_{HF}$with an $\alpha$ value identical to the value obtained at potentials out of the faradaic region (between 0.96 and 0.91). However, the experimental impedance data at potentials of the faradaic region could not be fitted well to the model. The reason for this must be related to the contribution of DOX diffusion to the electrode to the global electrode reaction.

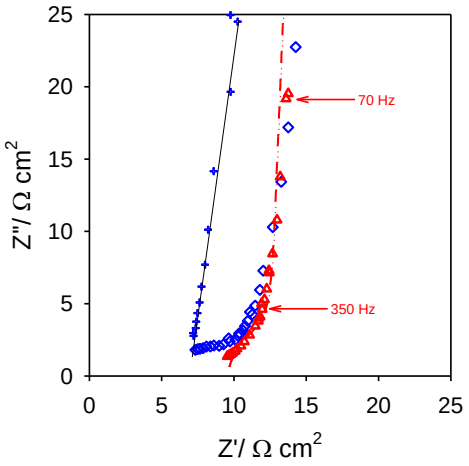



Figure 8.- Nyquist plots obtained for solutions of 10 µM DOX at pH 4.5 withan Au(111) electrode modified by a monolayer of DMPC:DDAB (3:1) - Cholesterol formed in the electrochemical cell at E =-0.48 V (blue diamonds) and at E =-0.150 V (bluecrosses). The solid black line represents the theoretical Nyquist plot for a series R-CPE circuit with $\alpha$= 0.95. Nyquist plots obtained with the electrode modified by Langmuir-Shaefer method with films of DMPC:DDAB (3:1) - Cholesterol (red triangles). Red dot-dot-dashed line is the theoretical curve obtained from the fitting to the impedance model for a surface confined reaction with $C_a$ = 73 µF $cm^{-2}$, $R_a$ = 8.1 Ω $cm^2$ and $\alpha$= 0.95.

### 3.4.2-Reduction of doxorubicin on modified electrodes in the Langmuir trough

In order to avoid the diffusion of DOX and to analyse only the DOX adsorbed on the electrodes, the electrodes were coated at 30 mN/m by the Langmuir-Schaefer method at the interface of the Langmuir trough with a 10 µM DOX containing electrolyte subphase. After that, the coated electrodes were transferred to the electrochemical cell filled only with the supporting electrolyte.

Some CV curves have been included in Figure 6. They show lower $I_{pc}$ values than the CV curves obtained with electrodes coated in the electrochemical cell, and they are

shifted to somewhat higher potentials. The peak intensities show good linear relationship with the scan rate, as can be observed in Figure 7a, for DOX reduction on the electrodes coated in this way, either with the three- component mixes or with the two-component mixes, indicating the absence of diffusion. Therefore, the rate of the redox reaction can be formulated as:

$$v = k_f \Gamma_{Ox} - k_b \Gamma_{Red} \quad (3)$$

Where $k_f$ and $k_b$ are the forwards and backwards rate constants of the surface reaction and $\Gamma_{Ox}$ and $\Gamma_{Red}$ are the surface concentrations of oxidized and reduced species respectively. For this kind of process the value of initial surface concentration of the reactant at the pre-concentration potential, Γ, can be obtained from the slopes of the $I_{pc}$ vs v plots according to:

$$I_{pc} = -\frac{n^2 * F^2}{4RT} * v * A * \Gamma \quad (4)$$

Where n is the number of electrons, F the Faraday constant and A the electrode area.The values obtained for the coated electrodes by DDAB containing film and by the DMPC-Ch film, are $3.3 \cdot 10^{-11}$ mol $cm^{-2}$ and $4.2 \cdot 10^{-1}$ mol $cm^{-2}$, respectively. The same values are obtained from the area of the reduction peaks according to:

$$\Gamma = \frac{Q}{nFA} \quad (5)$$

The surface concentration attained are of the same order of magnitude than the values reported for doxorubicin and its analogous antracyclic drug, daunorubicin, on electrodes modified with phospholipid and tiolipid monolayers and bilayers self-assembled or transferred by the Langmuir-Blodgett method, depending on the lipid composition[18,19,21,22,24].The somewhat lower value obtained for the electrode coated with the DDAB containing monolayer is in agreement with previous observation that the adsorption of DOX is less favourable in well- ordered lipid films, like self-assembled or cholesterol containing phospholipid films.[21]

The pseudocapacitance vs. potential plots, at 1kHz,for coated electrodes in this way, using the two types of lipid mixtures have been included in Figure 5. At potentials out of the faradaic region the curves show similar values than the electrodes coated in the electrochemical cell by the respective lipid mixes in the absence of DOX. However, the

inclusion of DOX is clear at potentials of the faradaic region at which high pseudocapacitance values are observed (see Fig 5). The frequency dispersion at potentials out of the faradaic region provides also values of αin the range 0.96 to 0.91 and the Nyquist plots at potentials of the faradaic region could well be fitted to the model for a surface confined reaction with a constant phase element describing the charging of the double layer, $Q_{HF}$, that includes α values between 0.93 and 0.9. In Figure 8 a representative Nyquist plot is presented at a potential of -0.480 V for the electrode coated with the film that contains DDAB and the curve obtained from the fitting to the model.

On the other hand, the frequency dependence of the real and imaginary components of the electrode admittance, $Y'_{el}$ and $Y''_{el}$, (obtained as the reciprocal of the cell impedance after correction of the ohmic resistance) are described by the equations:

$$Y'_{el} = \frac{R_a\, C_a^2\, \omega^2}{1 + R_a^2\, C_a^2\, \omega^2} \qquad (6a)$$

$$Y''_{el} = \frac{C_a\, \omega}{1 + R_a^2\, C_a^2\, \omega^2} + C_{HF}\, \omega \qquad (6b)$$

Were ω is the angular frequency. According to equation (6a) the plots of the inverse of the real electrode admittance components, $1/Y'_{el}$ versus $1/\omega^2$ should be straight lines, so the two parameters representative of the faradaic process at every potential, $R_a$ and $C_a$, can be obtained just by linear regression fittings. In Figure 9 some of these plots are shown (at a potential of -0.480 V) for DOX reduction on the coated electrodes in the Langmuir trough. They are good straight lines if not too low frequencies are considered, because at low frequencies the CPE representing the charging of the double layer has a higher contribution to the electrode admittance so the plots deviates from linearity. The plots corresponding to the reduction on the electrodes coated in the electrochemical cell (at the same potential of -0.480) have also been included in Figure 9, for comparison. They clearly deviate from linearity in the whole frequency range, confirming the failure of the reduction model that does not include diffusion in these cases.

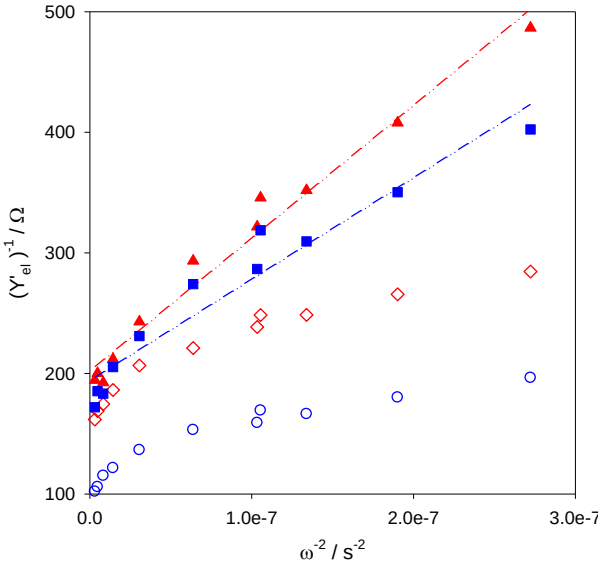



Figure 9.- $1/Y_{el}^{'}$ versus $1/\omega^2$ plots obtained at -0.48 V i) with the Au(111) electrode modified in the electrochemical cell with DMPC-Cholesterol (blue open circles) or DMPC/DDAB (3:1) – Cholesterol (open red diamonds) and ii) with the Au(111) electrode modified by Langmuir-Schaefer method with DMPC – Cholesterol (blue solid squares) or DMPC:DDAB (3:1)-Cholesterol (red solid triangles). The lines represent the linear regressions of each plot.

The frequency independent parameters $R_a$, $C_a$ and $C_{HF}$ were obtained by fitting the impedance data in the whole experimental frequency range to the model that includes a CPE representing the charging of the double layer, so the values for α were also obtained and found to be in the range 0.96 to 0.90. In Figures10a and 10bthe $R_a$and the $C_a$ values are respectively represented as a function of potential. The $R_a$-E data show a minimum and the $C_a$-E data a maximum at a potential close to the reduction peak potential. It was observed that the amount of DOX that is adsorbed on the electrode decreases after exploring the most negative potentials, so the values of the parameters $R_a$ and $C_a$ obtained in successive series of potentials are increasing and decreasing respectively, as their dependences with the surface excess of the reactant are inverses. Nevertheless, the forwards rate constant for the redox reaction, $k_f$, could be obtained at every potential from the (Ca Ra) product, which is independent of the surface concentration of reactant, by the equation [37]:

$$\frac{1}{k_f} = \frac{1}{R_a C_a [1 + \exp(\varphi)]} \qquad (7)$$

where φ is defined as φ = (nF/RT) (E-$E_{eq}$), with $E_{eq}$ being the equilibrium potential that was obtained from the extrapolation of the peak potentials at zero scan rate and was found to be -0.480 V.

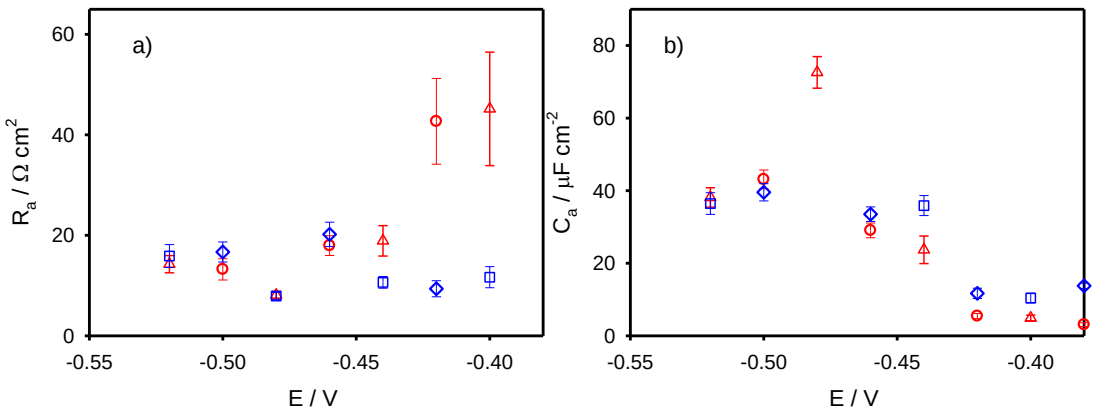



Figure 10.- a) $R_a$ vs E and b) $C_a$ vs E plots obtained from the frequency analysis of EIS data to the model for a surface confined reaction with: DMPC – Cholesterol. First negative going series of potentials (blue squares) and second negative going series (blue diamonds).DMPC:DDAB (3:1) - Cholesterol first negative going series of potential (red triangles) and second negative going series (red circles).

In Figure 11 are shown, the ln($k_f$) vs E plots for the reduction on the two kind of electrodes coated in the Langmuir trough. They deviate from the linear plots that are expected for single elementary Butler-Volmer steps with constant charge transfer coefficients. On the contrary, they are curved lines with decreasing slopes as the potentials are getting more negative.

For a redox reaction involving the exchange of two electrons and two protons, the number of intermediates and sequential or/and parallel steps to be considered is high. However, if only sequential steps are taken into account, in view of the slight deviation from linearity observed, and the intermediates are considered to be unstable and in their stationary states, the kinetic steady state approach can be applied and a general equation can be derived for a CECEC mechanism in which all the steps contribute to the global rate constant, $k_f$ [38]:

$$\frac{1}{k_f} = \frac{1}{B_0} + \frac{\exp(\frac{1}{4}\varphi)}{A_1} + \frac{\exp(\frac{1}{2}\varphi)}{B_1} + \frac{\exp(\frac{3}{4}\varphi)}{A_2} + \frac{\exp(\varphi)}{B_2} \qquad (8)$$

In the derivation, a value of 0.5 for the charge transfer coefficient of an elementary electron transfer step was consider. The kinetics coefficients $B_i$ and $A_i$ of the chemical steps and the electron transfer steps respectively, include the product of the rate constant of the corresponding step by the equilibrium constants of all the preceding steps.

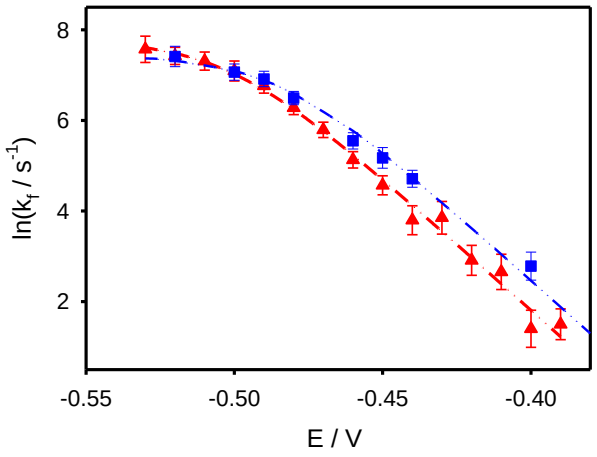



Figure 11.- ln($k_f$) vs E plots calculated from the values of $C_a$ and $R_a$in figure 10 with: DMPC:Cholesterol (blue solid squares) or DMPC:DDAB (3:1) - Cholesterol (red solid triangles). The lines represent the fitting to equation (9).

The fitting of the experimental ln $k_f$ vs E data to equation (8) has shown that in fact only two summations, the first and the forth ones, contribute to the value of $k_f$, in the whole examined potential range, so it can be written that:

$$\frac{1}{k_f} = \frac{1}{B_0} + \frac{\exp(\frac{3}{4}\varphi)}{A_2} \qquad (9)$$

| Monolayer | $B_0$ / $s^{-1}$ | $A_2$ / $s^{-1}$ |
|---|---|---|
| DMPC:DDAB (3:1) - Chol | 2400±370 | 655±50 |
| DMPC: - Chol | 1700±190 | 1260±90 |

Table 1.- Kinetics parameters obtained from the analysis of ln($k_f$) vs E in figure 11 according to equation (9).

The first summation represents the contribution of a chemical step preceding the first electron transfer that can be identified as a protonation step, so $B_0$ contains the rate constant for this protonation step. On the other hand, $A_2$ contains the product of the equilibrium constant of this initial protonation step and any other step that can precede the second electron transfer and the rate constant for this step. The $B_0$ and $A_2$ values obtained with the coated electrodes with the two kinds of lipid films are given in Table 1, while the theoretical ln(kf) vs E curves generated with these values and equation (9) can be observed in Figure 11 to fit well to the experimental values. The $B_0$values obtained for DOX reduction on the electrodes coated with three- or two-components are within the experimental errors. However, the $A_2$ value is somewhat lower for the reduction on electrodes coated by the DDAB containing lipid mixture, so it seems the DDAB exert some kind of inhibitory effect on the second electron transfer or the equilibrium constants of the preceding steps in the mechanism.

## 4.-CONCLUSIONS

The combination of the Langmuir trough and the electrochemical methodology has allowed us to reach interesting conclusions about the combination of components for the preparation and characterization of liposomes for drug delivery. Lipid films coated gold electrodes have been prepared and they have been used to study the characteristics of the films and their possibilities of interactions with gold to form liposomes with anchored gold nanoparticles. In addition, the study has provided also a detailed picture of the inclusion of the anticancer drug doxorubicin in the lipid films, its interaction with gold and its electrochemical reduction mechanism.

Thus, the thermodynamic analysis of the isotherms of the mixes of the lipid DMPC, frequent component of cell membranes, the cationic amphiphilic DDAB, that facilitates the anchoring of gold nanoparticles to liposomes, and cholesterol, has allowed us to optimize the composition for the preparation of stable films. The transfer to Au(111) electrodes of the films with the most stable formulation and the analysis of the impedance data as a function of the frequency indicates that the electrode is coated by a homogeneous film in a wide potential range. The comparison of these impedance results with those obtained with electrodes coated with films that do not contain the DDAB suggests that the effect of DDAB is to induce a better organization of the lipid tails.

The reduction of DOX on electrodes coated in the electrochemical cell in the presence of DOX at a concentration of $10^{-5}$ M by voltammetry and impedance spectroscopy indicates contribution of adsorption of DOX on the electrode and diffusion from the reactant in solution.

The preparation of coated electrodes in the Langmuir trough in the presence of DOX and their transfer to electrochemical cells that do not contains DOX has allowed us to conclude that the amount of DOX that is adsorbed is somewhat lower in the electrodes coated with lipid mixes that contain DDAB than in the electrodes coated only with DMPC:cholesterol.

The mechanism of DOX reduction on the gold electrodes coated in the Langmuir trough was deduced from the impedance data as a function of the frequency and of the potential within the faradaic potential range. It was concluded that the reduction obeys the model of a surface confined electrode reaction with a forwards reduction rate constant that is determined by two steps: a chemical reaction, presumable a protonation reaction, preceding the first electron transfer, and a second electron transfer.

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