Surface chemistry and spectroscopy studies on 1,4-naphthoquinone in cell membrane models using Langmuir monolayers

Nadia Hussein, Carla C. Lopes, Paulo Castanho A. Pernambuco Filho, Bruna R. Carneiro, Luciano Caseli * 
Federal University of São Paulo, Diadema, SP, Brazil

ABSTRACT

Investigating the role of drugs whose pharmaceutical activity is associated with cell membranes is fundamental to comprehending the biochemical processes that occur on membrane surfaces. In this work, we examined the action of 1,4-naphthoquinone in lipid Langmuir monolayers at the air–water interface, which served as a model for half of a membrane, and investigated the molecular interactions involved with tensiometry and vibrational spectroscopy. The surface pressure–area isotherms exhibited a noticeable shift to a lower area in relation to 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dihexadecanoyl-sn-glycero-3-phospho-L-serine (DPPS) lipid monolayers, which indicated a disruption of the monolayer structure and solubilisation of the lipids towards the aqueous subphase. To better correlate to the action of this drug in biological membrane events, cell cultures that represented tumorigenic and non-tumorigenic cells were spread onto the air–water interface, and 1,4-naphthoquinone was then incorporated. While only slight changes were observed in the non-tumorigenic cells upon drug incorporation, significant changes were observed in the tumorigenic cells, on which the organisation of the Langmuir monolayers was disrupted as evidenced by tensiometry and vibrational spectroscopy. This work then shows that this drug interacts preferentially for specific surfaces. In simplified models, it has a higher effect for the negative charged DPPS rather than the zwitterionic DPPC; and for complex cell cultures, 1,4-naphthoquinone presents a more significant effect for that representing tumorigenic cells.

1. Introduction

The actions of drugs in cell membranes involve mechanisms that include interactions at a molecular level, which are not well understood. Among these drugs, naphthoquinones are organic molecules that form the main chemical structures of several natural compounds such as vitamin K. Some naphthoquinone derivatives reportedly have pharmacological properties presenting cytotoxicity. Many are considered antimicrobial [1,2] (antibacterial, antiviral, antifungal) and insecticidal [3], and others present antipyretic and anti-inflammatory properties [4]. The action of naphthoquinones as anti-cancer agents has been reported [5,6], with potential activity as cell membrane (tumorigenic or healthy) disruptors. In particular, 1,4-naphthoquinone is present in various chemotherapeutic mixtures [5]. This drug is a potent cell growth and tumour angiogenesis inhibitor [7], but little is currently known about the molecular mechanism of action of this drug when it interacts with cell membranes.

Given this lack of knowledge, the present work aims to understand the drug–cell interactions at the molecular and cellular levels using Langmuir monolayers as cell membrane models. The Langmuir monolayer is a system recognised as able to mimic half of a membrane [8], and basically consists of stable monomolecular-thick films formed when an amphiphile is spread on the top of the air–water interface [9]. Lipids are frequently employed as primary material used to produce such films and are subsequently employed for detailed investigations of the interactions between membrane proteins [10,11], enzymes [12], ions [13], polysaccharides [14], nucleic acid based-compounds [15] and synthetic polymers [16]. Although the use of proteins that interact with only one side of the membrane, such as GPIs, has already been investigated [10], interest is growing in the study of proteins that interact with both sides of the membrane, such as transmembrane proteins [17].

In this paper, the action of an antineoplastic drug, 1,4-naphthoquinone, is investigated at the molecular level in model membrane systems consisting of lipid monolayers. This technique has been demonstrated as a potent tool for detailed investigations of molecular mechanisms in simple model systems in which the composition, surface pressure and surface rheological properties can be highly controlled. Some additional experiments have been carried out with cell cultures—tumorigenic or healthy—to better understand the action of this drug as an anticancer agent.
2. Materials and methods

2.1. Materials

The 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dihexadecanoyl-sn-glycero-3-phospho-L-serine (DPPS) sodium salt were purchased from Sigma–Aldrich (St. Louis, MO, USA) and dissolved in chloroform (Synth, Diadema, Brazil) to a concentration of 0.5 mg/mL. The monolayer subphase approximated physiological conditions and consisted of a 50 mM phosphate buffer and 150 mM NaCl at a pH of 7.4. The water employed was purified using a MilliQ-Plus System (resistivity 18.2 MΩ cm, pH 5.5).

2.2. Cell culture

An endothelial cell line derived from rabbit aorta (EC) [18] transfected with an EJ-ras oncogene (EJ-ras EC) [19] was grown in F-12 medium (Gibco BRL, Grand Island, USA) supplemented with 10% fetal calf serum (FCS) (Cultilab, Campinas, SP, Brazil) in the presence of penicillin (100 U/mL) and streptomycin (100 μg/mL). The cell lines were grown at 37 °C in a humidified, 2.5% CO2 atmosphere and sub-cultured every week with pancreatin (Sigma–Aldrich, St. Louis, MO, USA).

2.3. Cell extract

The cells were maintained in culture at 37 °C in a humidified, 2.5% CO2 atmosphere for 1 week. Then, the culture medium was removed, and the cells were washed twice with F-12 medium and removed from the dish with 3.5 M urea in 25 mM Tris–HCl (pH 7.8) to obtain the cell extract.

2.4. Langmuir monolayers

The Langmuir monolayers were obtained by spreading a chloroform solution of DPPC, DPPS, or cell cultures on the surface of an aqueous buffer solution. The surface pressure–area (π–A) isotherms were obtained in a mini-KSV Langmuir trough equipped with a surface pressure sensor (the Wilhelmy method), with an interface compression rate of 5 Å2/molecule−1 min−1. For mixed drug–lipid monolayers, first 1,4-naphthoquinone, dissolved in chloroform in a concentration of 1.04 mg/mL, was deposited on the air–water interface. DPPC or DPPS, dissolved in chloroform, was further spread. After allowing the chloroform to evaporate for 20 min. (and absence of this solvent attested with vibrational spectroscopy), the DPPC or DPPS monolayer was compressed to a surface pressure of 30 mN/m. This surface pressure was chosen because it corresponds to the lateral surface pressure of the membrane [20]. For the aqueous suspension extracts, they were spread after drug deposition and chloroform evaporation. It is important to emphasise that by using these procedures there was no contact of the cell extract with the solvent. The system was then allowed to stabilise for 1 h after the surface pressure of 30 mN/m has been reached. The stabilisation was confirmed using Polarisation-Modulation Infrared Reflection–Absorption Spectroscopy (PM-IRRAS) spectroscopy until no variation in the signal was detected. The surface pressure was maintained at 30 mN/m by moving the barriers, and the stabilisation of the monolayer was monitored until no additional movement of the barriers was needed. The PM-IRRAS measurements were taken using a KSV PMI 550 instrument (KSV Instruments, Ltd., Helsinki, Finland) at a fixed incidence angle of 75°. The surface pressure–area isotherms were obtained to evaluate the manner in which the drug shifts the monolayer to large areas and to investigate the mixed monolayer in the 2-D states achievable by the monolayer from the expanded phases to the collapse. Thus, the monolayer was expanded to the maximum area allowed for the Langmuir trough and then compressed until its collapse. All experiments were carried out at a controlled room temperature (25 °C).

3. Results and discussion

3.1. DPPC monolayers

First, it is important to emphasise that pure 1,4-naphthoquinone when spread alone at the air–water interface does not form regular Langmuir monolayers, forming spots that are not able to spread uniformly along the surface. Therefore, all the studies exposed here show the interaction of this compound mixed with lipids or cell cultures. In fact, this is the first evidence that 1,4-naphthoquinone has surface activity enhanced with the presence of cell membrane lipids.

The effect of 1,4-naphthoquinone on a DPPC monolayer was investigated using surface pressure–area isotherms (Fig. 1). Pure DPPC presented a typical curve with a transition between the liquid-expanded and liquid-condensed states as represented by a plateau at approximately 95 and 70 Å2 molecule−1 and at a surface pressure of approximately 5 mN/m.

First of all, it is important to emphasise that by mixing lipids and drug in the same solution, prior to the spreading on the air–water interface, the same results are obtained, indicating that we are working in the equilibrium regime. In view of that, to obtain the sequential surface pressure–area isotherms, first the drug was deposited, and after was the lipid solution. This kind of procedure was carried out because we intended to compare with the sequential results obtained when the cell cultures were spread, instead of the pure lipids. As for the last case we did not wanted the presence of chloroform, it was necessary to spread first the drug and then wait for chloroform evaporation.

The incorporation of small quantities of 1,4-naphthoquinone shifted the curve to lower DPPC molecular areas. This effect is usually attributed to the condensation of the lipid monolayer due to the stabilisation of the lateral repulsion between the chains. This phenomenon is common, especially when treating charged lipids with ions [13]. However, in this case, monolayer condensation is unlikely, not only because the DPPC is zwitterionic and the charge of ions is weak, but also because the shift to the lower areas was too large to simply be attributed to a condensation effect. For example, the isotherm shifted from 51 Å2 for pure DPPC...
to 36 Å² for the monolayer with a reduced quantity of the incorporated drug. This effect could be therefore attributed to the destabilisation of the drug caused by the drug itself, which led to either the solubilisation of the drug in the aqueous subphase or the formation of collapsed-like structures. Such effect is corroborated by the kinetic adsorption data provided in Fig. 2, which shows the increase in the surface area of a pre-formed and compressed monolayer after drug incorporation. However, we must to emphasise that the experiments are different. For the surface pressure–area isotherms, the drug is incorporated as a mixed monolayer at low surface pressures and compressed together the lipids until the desired surface pressure. For the kinetic curves, the drug is tried to be incorporated in a pre-formed and well-packed lipid monolayer, already in a pre-determined surface pressure. Consequently the effects should be different. For instance, in the surface pressure of 30 mN/m, since the surface packing is higher, the drug has more difficult to be incorporated, and the increase in surface area should be lower. This result is significant because it showed that even for this case, the drug is able to destabilise the membrane and cause some decrease in the surface pressure, corroborating to the effect observed when the drug is incorporated at lower surface pressures.

With increasing quantities of 1,4-naphthoquinone incorporated, the isotherms (Fig. 1) are shifted to lower areas as predicted. It is important to emphasise that the X-axis considered is the average area available for all molecules present at the interface (lipid and the drug). Lower quantities of the drug were also tested, and up to 0.5%, and the effect was similar to that for 5% of the drug. At even lower quantities, no effect was observed in the isotherm. Therefore, the range of tractability for the destabilising effect was as low as 0.5%.

Again, one must emphasise that when only the drug is spread at the air–water interface and is compressed to the end, no observable increase occurs in the surface pressure. This fact is associated with the fact that the drug could not be dispersed uniformly on the air–water interface after the spreading of its solution on the water surface, which reflects the low spreading capability. Therefore, the presence of DPPC at the air–water interface facilitates the homogeneous dispersion of the drug along the surface.

Furthermore, one must emphasise that the plateau, which characterises the phase transition at approximately 5 mN/m, becomes less evident as higher concentrations of the 1,4-naphthoquinone are inserted. In addition, the slope for increased surface pressures (above 10 mN/m) becomes progressively less angled. Both phenomena are consequences of the component mixture that affects the rheological properties of the lipid monolayer. The slope is altered as the compressibility of the monolayer surface changes, i.e., the monolayer becomes more compressible with the addition of the drug. This is commonly attributed in the literature to an effect of the lateral fluidisation of the monolayer as a new component prevents the lipids from attaining a well-packed structure [21]. The so-called in-plane elasticity (E) can be calculated as \( -\Delta A / (\partial n / \partial A) \), in which A is the molecular area, and \( \partial n / \partial A \) is the surface pressure. Table 1 summarises these data and indicates that increasing the quantity of 1,4-naphthoquinone progressively decreases the monolayer’s in-plane elasticity. The data are shown for 30 mN/m, which corresponds to the lateral pressure of a biological membrane [20]. The reduction of in the in-plane elasticity is interesting because it indicates that the drug at the interface causes a disruption of the order of the packed lipid monolayer. This fact can be then related to the introduction of a new component interacting with the hydrophobic and hydrophilic parts of the lipid.

Table 1

<table>
<thead>
<tr>
<th>Monolayer</th>
<th>E (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>235</td>
</tr>
<tr>
<td>DPPC + 5% 1,4-naphthoquinone</td>
<td>106</td>
</tr>
<tr>
<td>DPPC + 10% 1,4-naphthoquinone</td>
<td>83</td>
</tr>
<tr>
<td>DPPC + 20% 1,4-naphthoquinone</td>
<td>73</td>
</tr>
<tr>
<td>DPPC + 30% 1,4-naphthoquinone</td>
<td>61</td>
</tr>
<tr>
<td>DPPC + 50% 1,4-naphthoquinone</td>
<td>59</td>
</tr>
</tbody>
</table>

Then, we can presume a model of interaction between the drug and the phospholipid in which the carbonyl groups of 1,4-naphthoquinone may interact preferentially with the polar head of the phospholipid, while some of the hydrophobic groups of the aromatic ring of the drug may interact with the alkyl chains of the lipid. More details about such interaction could be obtained if vibrational spectroscopy is employed, as shown in the next paragraphs.

Fig. 3 provides the PM-IRRAS spectra for the monolayer. Panel A presents the region of C=H stretches in CH₂. The band at 2852 cm⁻¹ is ascribed to the symmetric stretching, and the one at 2913 cm⁻¹ is ascribed to the asymmetric stretching. With drug incorporation, an inversion of the relative intensities occurs between the symmetric and asymmetric bands, shifting from 0.85 for pure DPPC to 1.16, for drug-incorporated DPPC. This effect may be due to the disorder caused by the drug. Panel B primarily shows the region of the hydrophilic moiety in the DPPC. The band at 1741 cm⁻¹ is attributed to the C=O stretch from the phospholipid and does not significantly change upon drug incorporation. The three bands whose central band is at 1676 cm⁻¹ are attributed to the surface water bending, and with drug incorporation, may overlap the carboxyl stretching in the 1,4-naphthoquinone, which occurs at ~1690 cm⁻¹ [22]. The primary change occurs in the band at 1268 cm⁻¹, which is attributed to phosphate stretching, which disappears with drug incorporation. In addition, a small band at 1062 cm⁻¹ appears with 1,4-naphthoquinone incorporation and is attributed to the C–H in-plane bands in the aromatic drug compounds, indicating their likely presence at the interface.

### 3.2. DPPS

Many cancerous cells reportedly contain excess lipids with serine heads [23]. Several studies have revealed a high frequency of apoptosis in spontaneously regressing tumours and tumours treated with cytotoxic anticancer agents [24]. Changes on the surfaces of apoptotic cells, such as exposure to phosphatidylserine, have been identified [25]. Phosphatidylserine lipids are negatively charged and normally dominate the membrane leaflets facing the cytosol [26]. The surface exposure of the PS lipids has been...
reported for activated platelets and senescent erythrocytes [27], and the cells have been shown to undergo apoptosis to break up the phospholipid asymmetry of their plasma membranes [25] and expose the PS. Thus, we employed DPPS as model for tumorigenic cells. Fig. 4 provides the surface pressure–area isotherms. Basically, the presence of 1,4-naphthoquinone shifts the isotherm to lower areas. However, with increasing drug concentrations, the curves progressively shift to lower areas. The mechanism of the monolayer collapse also changes significantly. With the DPPS, the increase in the compressibility at approximately 50 mN/m initially signals the formation of the disordered multilayer structures, and a constant surface pressure begins only at 60 mN/m. With the drug, possibly due to the likely solubilisation of the lipid in the aqueous subphase, the monolayer is in a less compressible state until the surface pressure reaches approximately 60 mN/m, which may be associated with the fact that in the presence of the drug with its destabilising effect on the film, the phospholipid molecules have another way to molecularly readapt under compression; instead of collapsing, the monolayers move toward the aqueous subphase that is subjected to solubilisation with the drug.

This fact is corroborated by the data in Fig. 5, which demonstrates that the surface pressure decreases after the introduction of the drug in a constant area regime. The data on (Table 2) indicate that the surface elasticity values decrease only slightly with up to 30% 1,4-naphthoquinone, but with 50% of the drug, the monolayer becomes significantly more compressible. The PM-IRRAS spectra (Fig. 6) exhibit no significant change in the hydrophobic region. However, substantial changes are noted in the hydrophilic region. A significant negative band is observed at 1678 cm$^{-1}$ indicating surface water vibrations. Another band at 1527 cm$^{-1}$ [28] is related to interfacial water, which is reportedly to be orientated in such a way that the oxygen is exposed to the air phase. The dipole is then disposed so that the largest positive charge is orientated toward the aqueous phase. This fact is usually corroborated with the surface potential measurements [29], which exhibit a negative surface potential for the surface of pure water. Upon introduction of the DPPS, the water molecules on the surface may contact a negative charge from the phospholipid, which inverts the position of the dipole in the water molecules and is associated with the negative band in the PM-IRRAS [28]. Additionally, the absence of water molecules provides the difference in reflectivity between the surface covered with the lipid monolayer and the surface that is not covered to yield the given spectra [28].

Interestingly, after 1 h with naphthoquinone, these bands disappear. The phosphate stretching band at 1250 cm$^{-1}$ also disappears, and the bands at 1120 and 1211 cm$^{-1}$, attributed to the drug, are evident in the spectra. The band in 1461 cm$^{-1}$ and in 1653 cm$^{-1}$ indicates the vibration of C–H and carbonyl from 1,4-naphthoquinone. In addition the band characteristic of carbonyl stretching becomes more evident at ~1700 cm$^{-1}$. These results provide clear evidence that the drug acts strongly with the polar heads of the negatively charged lipids with little effect on the hydrophobic chains. This could indicate that the drug acts
preferentially on the surface of the negatively charged lipids, and this fact could be related to the destabilising effect on the membranes. Thus far, the 1,4-naphthoquinone appears to act in both DPPC, used herein as a model for healthy membranes, and DPPS, which may serve as a model for cancerous cells. However, the effects with DPPS are more evident, especially if we analyse the PM-IRRAS spectra. Drugs that act on cells typically first attack the external surfaces, which may expose the hydrophilic heads. Several cells present primarily zwitterionic heads, but in tumorigenic cells, the quantity of negatively charged lipids increases disproportionally [23].

To better correlate the differences in the actions of such drugs in healthy and sick cells, we used cell cultures from tumorigenic and normal cells.

3.3. Cell cultures

Because a cell culture may present multiple components, distinguishing the role of each one may prove difficult. For this distinction, a correlation using a simplified model for the cell membrane presented in the previous sections is necessary and can provide relevant information. Wild-type cells, representing non-tumorigenic cells, and cells transfected with the EJ-ras oncogene (tumorigenic cells) were utilised. Then it is important to emphasise that as we are treating with lipids at the surface, we are here not focusing on the living cell, but obtaining information from a cell membrane model, represented by a complex Langmuir monolayer, which contains not only lipids, but also all other components present in the culture. Considering the complexity to analyse results from systems like that, they were then compared with simpler models for cell membranes, represented by pure lipid (DPPC or DPPS) monolayers. In this strategy, it was possible to connect a simple model, in which specific interactions can be detected, with a complex, but more realistic, model. By linking the results obtained from these models, broader insights may be then acquired.

Fig. 7 indicates that for the culture considered normal, the drug has little effect on the isotherm. Apparently the drugs decrease the surface pressure for the large areas, and for the relevant surface pressure (30 mN/m), the isotherms appear nearly coincident. The curve does not exhibit any noticeable phase transition due to the high fluidity of the layer, which may consist of a mixture of components, proteins and glycoproteins that can fluidise the monolayer.

The PM-IRRAS spectra (Fig. 8) also exhibit few changes. In particular, a strong positive band for water can be found in the hydrophilic region (Panel B), indicating that the absorption of water molecules with the cell culture could be due to the presence of glycidic chains, which have a high affinity for water. Bands for the glycidic chains appear at 1223 and 1122 cm$^{-1}$, and a phosphate band appears at 1266 cm$^{-1}$. These bands are little affected by the drug.

For the tumorigenic cells, however, the effect of the drug is more pronounced. The isotherms (Fig. 9) exhibit a significant decrease in the surface pressure, as observed for the pure lipid monolayers. The PM-IRRAS spectra also exhibit significant changes, even in the C–H stretching (Fig. 10). The region of 1400–1700 cm$^{-1}$, for example, changes significantly, which corroborates the allegation that this drug has a more significant effect on

### Table 2

<table>
<thead>
<tr>
<th>Monolayer</th>
<th>$E$ (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPS</td>
<td>195</td>
</tr>
<tr>
<td>DPPS + 5% 1,4-naphthoquinone</td>
<td>181</td>
</tr>
<tr>
<td>DPPS + 10% 1,4-naphthoquinone</td>
<td>173</td>
</tr>
<tr>
<td>DPPS + 20% 1,4-naphthoquinone</td>
<td>167</td>
</tr>
<tr>
<td>DPPS + 30% 1,4-naphthoquinone</td>
<td>165</td>
</tr>
<tr>
<td>DPPS + 50% 1,4-naphthoquinone</td>
<td>89</td>
</tr>
</tbody>
</table>

Fig. 6. PM-IRRAS spectra for the DPPS, pure or mixed with 1,4-naphthoquinone (5% in mol). Panel A: C–H stretching regions; Panel B: phosphate regions.

Fig. 7. Surface pressure–area isotherms for cultures of non-tumorigenic cells, pure or mixed with 1,4-naphthoquinone (5% in mol). For comparison, the molecular area displayed is that on which the DPPC or DPPG was spread.
The density of bands in the wavenumber range in these spectra is high since the cell cultures present a high amount of substances, and they must provide a non-ordered structure at the interface. Many chemical groups can contribute for the bands shown in the spectra. However we can observe some remarkable changes with drug incorporation. The band in 2869 cm\(^{-1}\) is split in two bands: one in 2893 cm\(^{-1}\) and the other one in 2862 cm\(^{-1}\). Also, the band in 2893 cm\(^{-1}\) becomes a shoulder after incorporation of 1,4-naphthoquinone. Also the ratio between the intensities of the bands in 2937 and 2962 cm\(^{-1}\) changes from 1.7 to 0.85. In the region of 1000–1800 cm\(^{-1}\) the spectra seem quite noisy, which can be ascribed to the presence of a great amount of proteins and polysaccharides, whose main vibration groups are active in this region. For instance, the band in 1243 cm\(^{-1}\) is not altered, but the band in 1106 cm\(^{-1}\) increases relatively its intensity in relation to the previous one. Both bands are probably attributed to ether vibrations present in polysaccharides. Also the band in 1656 cm\(^{-1}\), attributed to amide I vibration in proteins, becomes better defined with 1,4-naphthoquinone incorporation. Comparing the spectrum from Panel 10A with that from Panel 10B, it seems that the effect is more pronounced for the alkyl vibrations region, which may be associated to high hydrophobicity of this drug. One of the main actions of 1,4-naphthoquinone may be associated with its interaction with the molecules from the cell culture that remains at the air–water interface, on which may alters the organisation of the lipids from the cell culture present at the surface. These molecules are expected therefore to have a more hydrophobic characteristic since they present the highest surface activities from the molecules present the extracts.
The results therefore indicate that in the presence of the drug, the properties of the healthy cell monolayer model were not significantly altered; therefore, the healthy cells appeared resistant to the action of the 1,4-naphthoquinone. However, for the tumorigenic cell monolayer model, the monolayer collapsed as the isotherms shifted to lower regions, and the vibrational spectroscopy indicated molecular disorganisation at the surface. This result could be related to the fact that cancerous cells are reportedly resistant to apoptosis [30]. Therefore, the discovery of drugs that can induce a disruption in the cell membrane and cellular death is of great interest.

4. Conclusions

This paper shows that 1,4-naphthoquinone can be incorporated into a simplified model of the cell membrane outer layer at an air–water interface in a proof-of-concept experiment. The effects of the drug were observed on tumorigenic cells, and zwitterionic and negatively charged lipids, on which part of the monolayer was fluidised due to the molecular disorganisation caused by the drug. A smaller effect was observed in cell models considered normal, indicating that the drug could differentiate between films with specific compositions at the air–water interface. In conclusion, we believe that these results could have a significant impact on the understanding of the interaction between 1,4-naphthoquinone and cell membrane surfaces during biochemical processes.

Acknowledgments

FAPESP, CAPES (nBioNet: Films and Sensors), CNPq (INEO).

References