Interactions of anticancer drugs doxorubicin and idarubicin with lipid monolayers: New insight into the composition, structure and morphology

DOI:
10.1016/j.jcis.2020.07.092

Document Version
Final published version

Citation for published version (APA):
Matyszewska, D., Nazaruk, E., & Campbell, R. A. (2021). Interactions of anticancer drugs doxorubicin and idarubicin with lipid monolayers: New insight into the composition, structure and morphology. Journal of Colloid and Interface Science, 581, 403-416. https://doi.org/10.1016/j.jcis.2020.07.092

Published in:
Journal of Colloid and Interface Science

Citing this paper
Please note that where the full-text provided on Manchester Research Explorer is the Author Accepted Manuscript or Proof version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version.

General rights
Copyright and moral rights for the publications made accessible in the Research Explorer are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Takedown policy
If you believe that this document breaches copyright please refer to the University of Manchester’s Takedown Procedures [http://man.ac.uk/04Y6Bo] or contact uml.scholarlycommunications@manchester.ac.uk providing relevant details, so we can investigate your claim.
Interactions of anticancer drugs doxorubicin and idarubicin with lipid monolayers: New insight into the composition, structure and morphology

Dorota Matyszewska a,⇑, Ewa Nazaruk b, Richard A. Campbell c,⇑,d,*

a Faculty of Chemistry, Biological and Chemical Research Centre, University of Warsaw, Zwirki i Wigury 101, 02-089 Warsaw, Poland
b Faculty of Chemistry, University of Warsaw, Pasteura 1, 02-093 Warsaw, Poland
c Institut Laue-Langevin, 71 avenue des Martyrs, CS20156, 38042 Grenoble, France
d Division of Pharmacy and Optometry, University of Manchester, Manchester M13 9PT, United Kingdom

ABSTRACT

We quantify directly here for the first time the extents of interactions of two different anthracycline drugs with pure and mixed lipid monolayers with respect to the surface pressure and elucidate differences in the resulting interaction mechanisms. The work concerns interactions of doxorubicin (DOx) and idarubicin (IDA) with monolayers of the zwitterionic DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) and negatively charged DMPS (1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (sodium salt)) as well as a 7:3 mixture of the two lipids. These drugs are used in current cancer treatments, while the lipid systems were chosen as phosphocholines are the major lipid component of healthy cell membranes, and phosphoserines are the major lipid component that is externalized into the outer leaflet of cancerous cell membranes. It is shown that DOx interacts with DMPS monolayers to a greater extent than with DMPC monolayers by lower limits of a factor of 5 at a surface pressure of 10 mN/m and a factor of 12 at 30 mN/m. With increasing surface pressure, the small amount of drug (~0.3 mmol/m2) bound to DMPC monolayers is excluded from the interface, yet its interaction with DMPS monolayers is enhanced until there is even more drug (~3.2 mmol/m2) than lipid (~2.6 mmol/m2) at the interface. Direct evidence is presented for all systems studied that upon surface area compression lipid is reproducibly expelled from the

https://doi.org/10.1016/j.jcis.2020.07.092

0021-9797/© 2020 The Authors. Published by Elsevier Inc.
This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
1. Introduction

A better understanding of the mechanisms of drug-biomembrane interactions is of great importance for the identification of potential new therapeutic strategies. As a complementary approach to investigations of drug efficacy with cell membranes in vitro and in vivo [1,2], many mimetic model systems based on lipid monolayers at the air/liquid interface [3–5] and supported lipid bilayers at the solid/liquid interface [6,7] have been developed to provide an understanding of drug-biomembrane interactions on a molecular level. Even though monolayers have only one lipid leaflet, experiments performed using a Langmuir trough have the advantages that the fluidity of cell membranes is retained, and the surface pressure can be tuned [8]. Furthermore, complementary information can be provided by the application of various techniques including spectroscopy [9], electrochemistry [10], rheology [11] or scattering [12]. As such, interactions of drugs with model biomembranes can be studied as a function of temperature [13], concentration [14], pH [15] and composition [16,17]. Understanding such interactions on a molecular level can aid the design of more effective drugs formulations for the treatment of diseases.

Anthracyclines are one of the most potent drugs in cancer treatment. They exhibit cytotoxic activity mainly through the interaction of DNA and topoisomerase II [18,19]. They are amphiphilic molecules possessing three functional domains: (1) dihydroxyanthraquinone ring, (2) daunosamine, amino sugar group, and (3) substituents on the anthraquinone ring [20]. The lipophilicity of anthracyclines varies due to the presence of different substituents. The most common anthracyclines are doxorubicin, daunorubicin, epirubicin and idarubicin. Doxorubicin belongs to the most effective types of anticancer drugs currently used. Idarubicin, derivative of daunorubicin, is more lipophilic and exhibits a higher cellular uptake than daunorubicin [21]. Idarubicin is a useful alternative to other anthracyclines and there is some evidence that it is less cardiotoxic.

There are different mechanisms of drug transport into the cell. In cancer treatment, passive diffusion is regarded as one of the key methods of transport of anthracyclines across the plasma membrane into the tumour [22]. The degree of lipophilicity determines the ability of anthracyclines to be incorporated within the lipid bilayer and, consequently, their passive diffusion. It has been postulated that the interactions of anthracyclines with negatively charged lipids may occur via (1) electrostatic interactions between positively charged drug and negatively charged lipid and/or (2) hydrophobic interactions that involve penetration of the dihydro anthraquinone moiety of the drugs within the lipid bilayer [23]. Positively charged doxorubicin interacts strongly with negatively charged phosphatidic acid or phosphatidylserines, the main anionic phospholipids of plasma membrane. Langmuir studies in combination with the imaging technique Brewster angle microscopy (BAM) have shown that due to the increased lipophilicity of idarubicin, the drug is more effective than daunorubicin in the penetration of lipid monolayers [24]. Moreover, the interactions of anthracyclines were more pronounced with negatively charged monolayers of 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DMPS) than with zwitterionic monolayers of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC). Also, more favorable interactions and stronger penetration of anthracyclines with zwitterionic lipid monolayers have previously been observed at lower surface pressure [25]. The orientation of daunorubicin and idarubicin in lipid monolayers has also been studied with surface-enhanced resonance Raman scattering [26]. The adsorption of the anthracyclines in zwitterionic lipid monolayers increased with the anthracycline lipophilicity, while in the presence of negatively charged lipids, the drugs remained adsorbed on the polar headgroups with limited penetration of other drug molecules. Nevertheless, there is a lack of direct quantitative data on the extent of drug-lipid interactions with different model biomembrane systems available to rationalize different possible structural binding mechanisms.

Knowledge of the location of the drug within a biomembrane is important not only in terms of the efficiency of the passive transport but also the elimination of cytotoxic drugs out of cells related to multidrug resistance (MDR) [27,28]. This information allows one to infer the mechanism of the interactions with cell membranes and the forces that determine the drug transport. While such information is not readily accessible for lipid monolayers, neutron reflectometry (NR) is a powerful technique that can be used to resolve the interfacial structure and composition of different model systems [12,29] through the exploitation of isotopic substitution and in particular custom-deuterium of molecules [30,31]. Based on the comparison of neutron reflectivity data recorded in multiple isotopic contrasts with different structural models, it is possible to infer if a drug penetrates the hydrophobic chains, is located in the polar headgroup region, or is positioned underneath the lipid monolayer. This approach has been used, for example, to elucidate differences in the mechanisms of interactions of short antimicrobial peptides with lipid monolayers of different charge [16], and reveal the location of an anaesthetic in lipid monolayers of different chain lengths [17]. Imaging of the lateral morphology of drug-lipid interactions using BAM has also been used to complement the structural information accessible using NR in a number of recent studies [16,32–34].

The present study focuses on drug-lipid interactions involving monolayers of DMPC and DMPS (Fig. 1). The former lipid was chosen because PC lipids are the most abundant type of phospholipids present in healthy cell membranes [35]. DMPC provides better monolayer fluidity than lipids with longer chains [17,36,37], and it forms more stable monolayers that are less susceptible to oxidation than lipids with unsaturated chains [38–40]. Indeed, DMPC monolayer, which we infer to be in the form of drug-lipid aggregates, yet the nature of adsorption of material back to the monolayer upon expansion is system-dependent. At 30 mN/m, most relevant to human physiology, the interactions of DOx and IDA are starkly different. For DOx, there is a conformational change in the interfacial layer driven by aggregation, resulting in the formation of lateral domains that have extended layers of drug. For the more lipophilic IDA, there is penetration of the drug into the hydrophobic acyl chain region of the monolayer and no indication of lateral segregation. In addition to the Langmuir technique, these advances were made as a result of direct measurements of the interfacial composition, structure and morphology using two different implementations of neutron reflectometry and Brewster angle microscopy. The results provide new insight into key processes that determine the uptake of drugs such as limited drug penetration through cell membranes by passive diffusion as well as activation of drug removal mechanisms related to multidrug resistance.

© 2020 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
The lipids used in the experiments – DMPC, DMPS, 1,2-dimyristoyl-d54-sn-glycero-3-phosphocholine (d54-DMPC), 1,2-dimyristoyl-d54-sn-glycero-3-phospho-L-serine (sodium salt) (d54-DMPS) – were of high purity (>99%) and were purchased from Avanti Polar Lipids. The deuterated lipids contained 54 deuterium atoms on the chains only. Stock solutions were prepared daily by dissolving either DMPC in chloroform or DMPS in a chloroform: methanol 4:1 v/v mixture. Organic solvents were purchased from Sigma Aldrich and were of HPLC grade. The subphase used in Langmuir experiments was either water or water with doxorubicin hydrochloride (AK Scientific) or idarubicin hydrochloride (MedChem) at a concentration of $10^{-5}$ mol/L, which corresponds to the pharmacologically relevant concentrations used in in vivo studies [50,51]. Ultrapure MilliQ water (resistivity 18.2 MΩ cm) was used throughout all the experiments, and D2O (Sigma Aldrich, 99% D) was also used for NR.

2.2. Langmuir technique

The Langmuir trough used to record surface pressure/area isotherms was a KSV-Nima medium trough (Bilin Scientific, Sweden) of dimensions $36 \times 7.5$ cm (270 cm²) equipped with hydrophilic barriers and controlled with KSV Nima LB software version 3.7. A Wilhelmy plate made of a filter paper (changed after each experiment) was used as a surface pressure sensor. After cleaning the subphase, a few drops of lipid solution were spread at the air-water interface using a Hamilton microsyringe and the solution was left for 10 min for solvent evaporation. The barrier speed during compression was 10 mm/min (7.5 cm²/min).

Changes in the phase and thus in the orientation of phospholipid molecules as well as the presence of any phase transitions can be followed by the changes in the values of the reciprocal of compression modulus ($C_s^{-1}$), which is defined as [52]:

$$ C_s^{-1} = -\frac{A}{\alpha \Delta A} $$  \hspace{1cm} (1)

where $A$ is area per lipid molecule in the monolayer, and $\pi$ is surface pressure. This parameter gives information on the phase of the monolayer at a given surface pressure, while any minimum in a $C_s^{-1}$ vs surface pressure plot corresponds to a phase transition occurring in the monolayer. The values should be taken as lower limits due to systematic shifts in the area per lipid molecule scale of the isotherms due to drug-induced lipid loss from the monolayer (see discussion in Section 3.2).

The Langmuir trough used to record compression-expansion cycles of the surface area for NR was a Nima trough with a custom-made insert of dimensions $30 \times 10$ cm equipped with hydrophobic barriers. The only difference to the details above is...
that the barrier speed during compression and expansion was 5.4 mm/min (5.4 cm²/min). Experiments were started within 10 min after spreading the phospholipid in order to capture all accessible features of the dynamic interaction.

All experiments were performed at 21 ± 1 °C.

2.3. Neutron reflectometry

NR experiments were performed using the horizontal neutron reflectometer FIGARO at the Institut Laue-Langevin in Grenoble, France [53]. The technique allows one to measure the ratio of neutrons in the specular reflection to those in the incident beam with respect to the momentum transfer \( Q_z \):

\[
Q_z = \frac{4\pi \sin \theta}{\lambda}
\]

where \( \theta \) is the angle of incidence and \( \lambda \) is the neutron wavelength. Two different implementations of the technique were used.

First, the very high flux at low-\( Q_z \) of the instrument, which has been exploited in recording kinetic data as fast as 1 s [54], and has even led to the first ever measurements at bulk liquid/liquid interfaces [55], was used to resolve the dynamic interfacial composition. Data were recorded in 1-min slices during two dynamic compression-expansion cycles of a Langmuir trough at \( \theta = 0.62° \) using the isotopic contrasts d-lipid/ACMW and h-lipid/ACMW, where ACMW is air contrast matched water (8.1% v/v D₂O in H₂O). The experiments were conducted for phospholipid monolayers formed on subphases containing either 10⁻⁵ mol/L DOx or 10⁻⁵ mol/L IDA. The recently-developed low-\( Q_z \) analysis method of NR [29,56] allows resolution of the interfacial composition of a binary mixture (i.e. the surface excess of each component) in situ during compression-expansion cycles much faster than the traditional implementation of the technique [57,58], even though it is deliberately insensitive to the interfacial structure [59]. The data were analyzed using the batch fit function of Motofit [60]. The uncertainties in the surface excesses are ±0.1 μmol/m² for the lipids and ±0.2 μmol/m² for the drugs, the former being lower because of the exploitation of isotopic substitution. Time-dependent effects were observed in the first minutes of the first cycle where the phospholipid surface excess decreased slightly at the start of the experiments. This loss of lipid from the monolayer can be attributed to the drug-lipid interactions, the effect of which has been observed also in another recent study [16]. While it was our intention to resolve all of the dynamic effects of the system exhibited, in order to eliminate this particular effect from the surface excess analysis, only data for the second cycle are shown for each system in the main text.

Second, the full dynamic range of the instrument was used to resolve the equilibrium interfacial structure. Data were recorded over longer acquisition times of samples that had equilibrated at over longer acquisition times of samples that had equilibrated at

\[ 10 \text{ min after spreading the phospholipid in order to capture all accessible features of the dynamic interaction.} \]

\[ \text{All experiments were performed at 21 ± 1 °C.} \]

2.3. Neutron reflectometry

NR experiments were performed using the horizontal neutron reflectometer FIGARO at the Institut Laue-Langevin in Grenoble, France [53]. The technique allows one to measure the ratio of neutrons in the specular reflection to those in the incident beam with respect to the momentum transfer \( Q_z \):

\[
Q_z = \frac{4\pi \sin \theta}{\lambda}
\]

where \( \theta \) is the angle of incidence and \( \lambda \) is the neutron wavelength. Two different implementations of the technique were used.

First, the very high flux at low-\( Q_z \) of the instrument, which has been exploited in recording kinetic data as fast as 1 s [54], and has even led to the first ever measurements at bulk liquid/liquid interfaces [55], was used to resolve the dynamic interfacial composition. Data were recorded in 1-min slices during two dynamic compression-expansion cycles of a Langmuir trough at \( \theta = 0.62° \) using the isotopic contrasts d-lipid/ACMW and h-lipid/ACMW, where ACMW is air contrast matched water (8.1% v/v D₂O in H₂O). The experiments were conducted for phospholipid monolayers formed on subphases containing either 10⁻⁵ mol/L DOx or 10⁻⁵ mol/L IDA. The recently-developed low-\( Q_z \) analysis method of NR [29,56] allows resolution of the interfacial composition of a binary mixture (i.e. the surface excess of each component) in situ during compression-expansion cycles much faster than the traditional implementation of the technique [57,58], even though it is deliberately insensitive to the interfacial structure [59]. The data were analyzed using the batch fit function of Motofit [60]. The uncertainties in the surface excesses are ±0.1 μmol/m² for the lipids and ±0.2 μmol/m² for the drugs, the former being lower because of the exploitation of isotopic substitution. Time-dependent effects were observed in the first minutes of the first cycle where the phospholipid surface excess decreased slightly at the start of the experiments. This loss of lipid from the monolayer can be attributed to the drug-lipid interactions, the effect of which has been observed also in another recent study [16]. While it was our intention to resolve all of the dynamic effects of the system exhibited, in order to eliminate this particular effect from the surface excess analysis, only data for the second cycle are shown for each system in the main text.

Second, the full dynamic range of the instrument was used to resolve the equilibrium interfacial structure. Data were recorded over longer acquisition times of samples that had equilibrated at a given surface pressure for at least 10 min at \( \theta = 0.62° \) and 3.8° in 4 isotopic contrasts: d-lipid/D₂O and h-lipid/D₂O as well as the two monolayers mentioned above. With reference data on pure DMPC and DMPS monolayers formed on pure water included in our recent publication on modeling NR data of phospholipid monolayers [61], measurements were performed on the same monolayers formed on subphases containing either 10⁻⁵ mol/L DOx or 10⁻⁵ mol/L IDA at surface pressures of 10 and 30 mN/m. The data were analyzed using the co-refinement function of Motofit [60]. Different models of homogeneous stratified layers parallel with the interface were constructed in order to resolve the thickness, density and composition of each layer that minimized the global \( \chi^2 \) value representing the overall difference between the model and experimental data for all 4 contrasts [61]. For each system, at least two structural models were attempted: (1) drug in the acyl chains layer and (2) drug both in the headgroups layer and in an additional thin, hydrated layer underneath the lipid; note that a model with drug present only in the headgroups layer was not attempted as the smallest physical dimension of the drug exceeded that of the headgroups [62]. In the case of DOx interactions with DMPS monolayers at 30 mN/m, where the Kiessig fringes in the experimental data suggested the presence of a more extended interfacial structure, a further model was attempted with drug in two more extended hydrated layers underneath the lipid. In the case of IDA interactions with DMPS monolayers at 30 mN/m, there was no suggestion from the data of an extended layer but where the global \( \chi^2 \) value remained rather high, a further model was attempted with drug present in both the acyl chains layer and the region of the headgroups and underneath the lipid. The following principles described in our modeling paper were included in the models: application of capillary wave roughness damped by bending rigidity according to the phase of the lipid, constraint of the surface excess of acyl chains and headgroups to respect the molecular stoichiometry, and fitting of the extent of acyl chain compaction to take into account the phase of the lipid [61]. Indeed, the extent of acyl chain compaction matched that of the expected monolayer phase, which is higher for DMPS than DMPC as the chains are more condensed [37]. The uncertainty in the surface excesses is reduced to ±0.07 μmol/m² for the drugs as a result of the longer acquisition time, in particular for the data of the h-lipid/ACMW contrast, which is most sensitive to the drug surface excess. A list of parameters used in the modeling can be found in Table S1.

2.4. Brewster angle microscopy

BAM images obtained using a Nanofilm EP3 setup were used to examine the lateral morphology of the air-water interface following the drug-lipid interactions [32]. The instrument was equipped with an UltraBAM objective (Accurion, Germany) with a lateral resolution of 2 μm and a field-of-view of 800 μm × 430 μm. Images were captured during the simultaneous compression of the monolayers at the air-water interface.

3. Results and discussion

3.1. Surface pressure (Langmuir technique)

Effects of the anthracycline drugs DOx and IDA at subphase concentrations of 10⁻³ mol/L on pure DMPC and DMPS monolayers were first examined using the Langmuir technique (Fig. 2 and Table 1). For reference, measurements of the pure lipid monolayers in the absence of drug were conducted. DMPC monolayers have an uplift of the surface pressure at ~90 Å² and upon further compression the acyl chains remain in the liquid-expanded (LE) phase [24,63], which is characterized by molecules that are not tightly packed and have their acyl chains tilted with respect to the normal to the interface (Fig. 2A). This packing is consistent with a modest maximum value of the reciprocal of compression modulus of Cs⁻¹ = 110 mN/m, in agreement with literature data [3,24,63,64], and close to the border of typical ranges of values for the LE (12–100 mN/m) and liquid-condensed (LC; 100–250 mN/m) phases [65]. DMPS monolayers have a similar uplift, but they exhibit a well-defined plateau at a surface pressure of ~4 mN/m, which corresponds to a phase transition from LE to LC chains (Fig. 2B). This phase change is manifested by a minimum in the corresponding Cs⁻¹ versus surface pressure plot. Further compression leads to the formation of solid DMPS monolayers with lipid molecules tightly packed at the air-water interface and a high value of Cs⁻¹ [61,63].
The surface pressure influence of the two drugs on the DMPC monolayers is comparable (Fig. 2A). Their presence leads to a shift of the surface pressure uplift towards larger areas per molecule (~180–200 Å²). Indeed, the areas per molecule corresponding to a well-organized monolayer (~120 Å²) are 42.8 ± 1.0 46.5 ± 1.4 40.2 ± 0.7 110 ± 8 for H2O 67.6 ± 0.5 70.0 ± 0.5 52.7 ± 0.5 85 ± 8 for 10 mol/L IDA 86.7 ± 0.1 86.7 ± 0.5 59.6 ± 0.5 75 ± 9 for 5 mol/L DOx 86.7 ± 0.1 86.7 ± 0.5 59.6 ± 0.5 75 ± 9 for 5 mol/L IDA 86.7 ± 0.1 86.7 ± 0.5 59.6 ± 0.5 75 ± 9 for 5 mol/L IDA (green). Insets: reciprocal of the compression modulus vs surface pressure plots. Horizontal lines denote the surface pressures at which structural NR and BAM measurements are presented below.

Similar observations regarding a modest increase in biomembrane fluidity were made for the interactions of another anticancer drug, topotecan, with DMPC:DMPS liposomes [43], and for dextran, a polysaccharide widely used in different biomedical applications, which also led to a decrease in the condensation and ordering of negatively charged lipids [66]. Additionally, such a shift of the isotherm towards larger areas per molecule suggests an expansion of the monolayer due to possible insertion/penetration of the drug in the monolayer, as was also observed for other drugs such as metronidazole [67] and rifabutin and its analogs [68].

The surface pressure influence of the two drugs on the DMPS monolayers is much more pronounced (Fig. 2B). The surface pressure uplift occurs at much larger areas per molecule (~180–200 Å²), and the shapes of the isotherms are very different. The interactions of IDA result in DMPS monolayers in the LE phase, as the surface pressure plateau from the LE-to-LC phase transition is absent from the isotherm and the value of $C_{max}$ is just 76 mN/m (Table 1). The interactions of DOx also result in DMPS monolayers in the LE phase, but interestingly there is a quasi-plateau at ~25–35 mN/m, and the $C_{max}$ value of 132 mN/m occurs at a higher surface pressure. Together these observations suggest that a conformational change takes place within the monolayer upon compression as a result of the DOx interaction. Furthermore, at high surface pressure (~40 mN/m), the isotherm for DOx intersects that of the pure lipid, resulting in a lower area per lipid molecule in the presence than in the absence of drug. This anomalous result indicates that the area per lipid molecule may have been systematically shifted for the mixed system as a result of a loss of lipid from the monolayer upon compression, which may be attributed to the formation of drug-lipid aggregates.

The results from the Langmuir technique show that the interactions of the two anthracycline drugs are more pronounced with DMPS than DMPC monolayers, and that the drugs – in particular DOx – are disruptive to the lipid packing and may undergo a conformational change with increasing surface pressure. Even so, this technique provides no direct quantification of the extents of interactions as a function of the surface pressure, the location of the drug in the monolayer nor its adopted morphology as a result of the drug-lipid interactions, and as such additional techniques were applied.

### 3.2. Interfacial composition (NR)

The low-Qz implementation of NR was applied to resolve the dynamic interfacial composition of anthracycline drugs interacting with pure and mixed lipid monolayers in situ during compression-expansion cycles. The approach is sensitive to the amounts of two components present in a thin interfacial layer at the air-water interface, and while it has been applied to good effect to polymer/surfactant films during cycling of the surface area [57,58], this is the first time it has been used to study drug-lipid interactions.

#### Table 1

| Subphase | $A_0$ / Å² | $A_{10 \text{ nm}}$ / Å² | $A_{50 \text{ nm}}$ / Å² | $C_{max}$ / mN/m |
|----------|----------|----------------|----------------|-------------|
| DMPC     |          |                |                |             |
| H2O      | 67.6 ± 0.5 | 70.0 ± 0.5     | 52.7 ± 0.5     | 110 ± 8     |
| 10⁻⁵ mol/L DOx | 80.3 ± 1.2 | 82.2 ± 0.5     | 59.6 ± 0.5     | 85 ± 8      |
| 10⁻⁵ mol/L IDA | 86.7 ± 0.1 | 86.7 ± 0.5     | 61.1 ± 0.5     | 75 ± 9      |
| DMPS     |          |                |                |             |
| H2O      | 42.8 ± 1.0 | 46.5 ± 1.4     | 40.2 ± 0.7     | 400 ± 7     |
| 10⁻⁵ mol/L DOx | 51.2 ± 0.7 | 139.9 ± 0.1    | 78.6 ± 0.3     | 132 ± 5     |
| 10⁻⁵ mol/L IDA | 166.3 ± 1.6 | 160.9 ± 1.3   | 120.3 ± 0.1    | 76 ± 5      |
interactions using such a dynamic platform. Here we consider the results of the pure lipid monolayers, and the objectives were three-fold: to quantify directly the relative extents of interactions of DOx with pure DMPC and DMPS monolayers, to resolve the dependence of these extents of interactions on the surface pressure, and to compare the extents of interactions of DOx with IDA with pure DMPS monolayers.

The interfacial composition was resolved during two successive compression-expansion cycles on a Langmuir trough of pure DMPC and DMPS monolayers prepared on 10⁻⁵ mol/L DOx solution, and in the latter case – where the interaction is shown to be much stronger – on 10⁻⁵ mol/L IDA solution as well (Fig. 3). For each of the three systems, plots of the surface pressure/area (panels A/D/G), surface excess/area (panels B/E/H) and surface excess/surface pressure (panels C/F/I) are shown. The surface excess plots are shown for the second cycle only, in order to exclude time-dependent effects of lipid loss at the start of the experiment, as explained in the Experimental Section; time-resolved data for both cycles can be found in Fig. S1 and selected values of the surface excesses are reported for reference in Table S2.

The surface pressure isotherms exhibit mild hysteresis between compression and expansion, which is related to time-dependent relaxation processes during reorganization of the interfacial material [69,70]; the monolayers were not expanded to zero surface pressure, however, so complete reorganization could not take place. The hysteresis appears least pronounced for the interactions of DOx with the DMPS monolayer (Fig. 3 D), coincident with the quasi-plateau in the data that suggests a conformational change in the interfacial material.

Even though the interactions of DOx with the DMPC monolayer result in a shift (Fig. 2A) and hysteresis (Fig. 3A) in surface pressure/area isotherms, the direct quantification of the interfacial material with respect to the surface pressure demonstrates that the extent of interactions is very low, as the surface excess of drug is minimal compared to that of DMPC (Fig. 3B). Indeed, the surface excess of drug is close to the detection limit of the measurement at 0.2 ± 0.2 μmol/m² at zero surface pressure through to −0.1 ± 0.2 μmol/m² at a surface pressure of 40 mN/m. In contrast, the interactions of DOx with the DMPS monolayer is much more significant, as the surface excess of drug exceeds that of lipid over the whole range of surface pressures measured (Fig. 3E). As lower limits, taking into account the experimental uncertainties, the relative extents of interactions of DOx with the DMPS versus DMPC monolayers is greater by a factor of 5 at a surface pressure of 10 mN/m and by a factor of 12 at a surface pressure of 30 mN/m. It has been previously shown that electrostatic attractions between positively charged anthracyclines and negatively charged lipids are the most important factor governing the interactions [3,24,71,72]. The lack of such interactions between zwitterionic DMPC headgroups and positively charged DOx explain the small surface excess determined directly here for the first time using NR.

The dependence of the extent of interactions on the surface pressure of DOx with the DMPC monolayer cannot be resolved outside the error limits of the measurement, unfortunately. There is a
clear trend of decreasing surface excess of drug with increasing surface pressure, consistent with the exclusion of drug from the lipid monolayer [25] (Fig. 3C), but the total apparent loss matches that of the experimental uncertainties. On the other hand, the extent of interactions of DOx with the DMPS monolayer clearly increases with increasing surface pressure, as the surface excesses of the two components increase commensurately (Fig. 3F). There is an upturn in the data around 20–30 mN/m, which is noteworthy given that data of a simple monolayer would instead tend to a plateau at limiting surface coverage. Coincident with the quasi-plateau in the surface pressure/area isotherms, these data support the inference above that a conformational change in the drug-lipid interactions occurs with increasing surface pressure, and suggests the possible preferential accumulation of DOx in a sub-surface layer at higher surface pressures.

As the surface area is compressed by a factor of 2 (from 280 to 140 cm²; Fig. 3E/H), the surface excess of DMPS increases by just 75% (1.2 to 1.8 μmol/m²) as the surface area is compressed by a factor of 2 (from 280 to 140 cm²; Fig. 3E), which directly reveals the expulsion of lipid from the interface. Such a loss of lipid to the subphase may seem counterintuitive given its poor solubility, but we attribute the fact to the strong association of the two components due to their opposite charges, resulting in the formation of drug-lipid aggregates [73,74]. Also, it should be noted that this loss of lipid is consistent with the observation that the isotherm of the mixed system crosses that of pure DMPS > 40 mN/m resulting in lower surface pressure for the mixture at the same nominal area per lipid molecule (Fig. 2B); there is no clear explanation why the drug-lipid interactions would reduce the surface pressure at a constant lipid surface excess, hence it follows that lipid has indeed been lost from the monolayer, consistent with the NR data. This effect has two further consequences: first the values of the reciprocal of the compression modulus stated above should be taken as lower limits, and second any calculation of the excess free energy of the system upon compression would not be physically valid [24,69,70]. Nevertheless, lack of hysteresis in the surface pressure/area and surface excess/area isotherms shows that re-adsorption of material to the interface is faster than the minute time scale of the measurements.

In a comparison of the interactions of DOx and IDA with DMPS monolayers, the surface excesses of drug and lipid at low surface pressures are almost the same for the two systems (<10% difference; Fig. 3F/I). However, in the case of IDA, the upturn in the surface excess is absent, which hints at the existence of a different mechanism of interaction as the surface pressure increases. In this case, the surface excess of DMPS increases by just 65% (1.2 to 2.0 μmol/m²) as the surface area is compressed by a factor of 2 (Fig. 3H), which suggests the expulsion of a greater quantity of drug-lipid aggregates from the interface at the highest maximum surface pressure reached for this system. The hysteresis of the surface pressure/area isotherm for IDA (Fig. 3G) is mirrored by that of the surface excess (Fig. 3H), which can be attributed to a slower re-adsorption of material upon initial expansion of the monolayer. Nevertheless, there is lack of hysteresis in the surface excess/surface pressure plots (Fig. 3I), which suggests that the interfacial layers formed during compression and expansion adopt similar morphologies. For both drugs, the general reproducibility of data features over two cycles, i.e. no clear reduction in the surface excesses of either component during the two expansion phases, indicates that the expulsion and re-adsorption processes are fully reversible (Fig. S1).

Our novel application of the low-Q objective function implementation of NR to the dynamic interactions of anthracycline drugs has allowed us to reach some clear conclusions. First, DOx interacts to a much stronger extent with DMPS than DMPC monolayers; lower limits of the relative amounts of bound drug are a factor of 5 greater at a surface pressure of 10 mN/m and a factor of 12 greater at a surface pressure of 30 mN/m. Second, while there is an indication that DOx may be excluded from the DMPC monolayer during compression, its interaction with the DMPS monolayer is stronger with increasing surface pressure, which highlights the role of electrostatic interactions. Also, although 12.5% of the lipid is lost in drug-lipid aggregates expelled from the interface during compression, the particles must re-adsorb quickly upon expansion. Third, while the extents of interactions of DOx and IDA are similar at low surface pressure, the drugs appear to interact with the lipid monolayers by different mechanisms. The surface excess increases more sharply with surface pressure in the case of DOx, indicating the possible accumulation of drug underneath the phospholipid monolayer, and expelled drug-lipid aggregates accounting for 17.5% of the lipid from the monolayer re-adsorb more slowly in the case of IDA. In spite of this new quantitative information, insight into the structures and morphologies was still required, so additional techniques and methods were applied.

3.3. Interfacial structure (NR)

The structural implementation of NR was then performed in order to resolve the location normal to the interface on the nm length scale of the anthracycline drugs in the monolayers. The objective was to gain structural insight into the similarities and differences in the interaction mechanisms with respect to the surface pressure and type of drug. Data were recorded over the full Q₀ range in 4 isotopic contrasts for pure DMPC and DMPS monolayers on subphases of pure water and 10⁻⁵ mol/L solutions of DOx and IDA at surface pressures of 10 and 30 mN/m. Data of the pure lipid monolayers in the absence of drug were fitted to a model with an upper layer of acyl chains and a lower layer of hydrated head-groups in our recent publication on modelling NR data of phospholipid monolayers [61]. These data provided useful references that allowed the model parameters to be refined following the incorporation of drug into models of the mixed systems. Data of the drug-lipid monolayer systems were subsequently fitted, in which process structural models with the drug present in different locations were compared with reference to the global χ² values to assess their validity. Fig. 4 and Table 2 reports the optimized fitting results and refined model parameters while Figs. S2–S8 show a comparison of different models attempted and state the corresponding global χ² values.

3.3.1. DMPC monolayers

Again, only the interactions of DOx with DMPC monolayers were investigated, as the extent of interactions of the drugs with zwitterionic lipid monolayers has been shown to be minimal (Fig. 3A), and only a small shift of the Langmuir isotherm resulted (Fig. 2A). Unfortunately, it was not possible to distinguish the location of the drug at the interface through the comparison of different structural models, because the data recorded in multiple isotopic contrasts were so similar to those recorded in the absence of drug as a result of the small amount of drug present in the monolayer (Fig. S2). Nevertheless, data from the contrast involving hydrogenous DMPC (weakly scattering phospholipid) in ACMW (null reflecting subphase), which is most sensitive to the presence of drug at the interface, allowed us to refine our quantification of the drug surface excess to 0.30 ± 0.07 μmol/m² at 10 mN/m and 0.09 ± 0.07 μmol/m² at 30 mN/m. These results confirm the exclusion of drug from the DMPC monolayer with increasing surface pressure discussed above, which was observed previously for the interaction of anthracyclines with zwitterionic lipid monolayers [25], and is akin to the process experienced by the small anaesthetic propofol [17] although contrary to the behavior of a short, designed peptide [16].
Fig. 4. Experimental neutron reflectivity data and optimized model fits for DMPS monolayers at 10 mN/m on subphases containing A) $10^{-5}\ \text{mol/L DOx}$ and B) $10^{-5}\ \text{mol/L IDA}$ and DMPS monolayers at 30 mN/m on subphase containing C) $10^{-5}\ \text{mol/L DOx}$ and D) $10^{-5}\ \text{mol/L IDA}$: (purple) $d_{54}$-DMPS/ACMW, (blue) DMPS/ACMW, (green) $d_{54}$-DMPS/D$_2$O, (red) DMPS/D$_2$O; insets: corresponding scattering length density profiles normal to the interface. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Parameters obtained from the optimized model fits to NR data of DMPC and DMPS monolayers in the presence of anthracyclines at 10 mN/m and 30 mN/m.

| Subphase        | Layer 1: thickness (Å)/compaction (%)/drug (%) | Layer 2: thickness (Å)/hydration (%)/drug (%) | Layer 3: thickness (Å)/hydration (%)/drug (%) | Layer 4: thickness (Å)/hydration (%)/drug (%) | $\chi^2$ | $I_{\text{lipid}}$ (μmol/m$^2$) | $I_{\text{drug}}$ (μmol/m$^2$) |
|-----------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|--------|-------------------------------|-------------------------------|
| **DMPC $\pi = 10$ mN/m** |                                               |                                               |                                               |                                               |        |                               |                               |
| $10^{-5}\ \text{mol/L DOx}$ | 9.8/0/0                                        | 8.0/36/10                                     | N/A                                           | N/A                                           | 10     | 2.08                          | 0.30                          |
| **DMPC $\pi = 30$ mN/m** |                                               |                                               |                                               |                                               |        |                               |                               |
| $10^{-5}\ \text{mol/L DOx}$ | 11.6/50/0                                      | 8.0/28/3                                      | N/A                                           | N/A                                           | 10     | 2.67                          | 0.09                          |
| **DMPS $\pi = 10$ mN/m** |                                               |                                               |                                               |                                               |        |                               |                               |
| $10^{-5}\ \text{mol/L DOx}$ | 5.1/56/0                                       | 5.5/68                                        | 2.5/70/30                                     | N/A                                           | 21     | 1.18                          | 1.68                          |
| $10^{-5}\ \text{mol/L IDA}$ | 5.6/56/0                                       | 5.5/65                                        | 2.5/65/35                                     | N/A                                           | 15     | 1.30                          | 1.65                          |
| **DMPS $\pi = 30$ mN/m** |                                               |                                               |                                               |                                               |        |                               |                               |
| $10^{-5}\ \text{mol/L DOx}$ | 10.4/100/0                                     | 7.5/49/0                                      | 36/82/18                                      | 30/93/7                                       | 397    | 2.60                          | 3.22                          |
| $10^{-5}\ \text{mol/L IDA}$ | 7.5/100/12                                     | 5.5/56                                        | 2.5/38/62                                     | N/A                                           | 33     | 1.65                          | 2.05                          |

The uncertainty in the layer 1 thicknesses is ±0.5 Å, which translates to the lipid surface excesses as ±0.10 μmol/m$^2$. The uncertainty in the layer 2 thicknesses, which was iterated in the global fit due to limitations of the software in applying the surface excess constraint between the number of acyl chains and headgroups to respect the molecular stoichiometry (which itself determined the hydration of layer 2), is estimated as ±0.5 Å. The extent of acyl chain compaction was initially constrained from fits to the pure lipid monolayers [61], and adjustment to other values did not improve the quality of the fits. The maximum uncertainty in the percentage of drug in layers 2 and 3 is 3%, which translates to the drug surface excesses as ±0.07 μmol/m$^2$; in the case of DOx + DMPS at 30 mN/m, however, the thicknesses and hydrations of layers 3 and 4 are highly correlated, resulting in uncertainties of ±5 Å and ±5%, respectively.
3.3.2. DMPS monolayers at 10 mN/m

The interactions of DOx and IDA with DMPS monolayers at a surface pressure of 10 mN/m were next investigated. For each system, two structural models were attempted: (1) drug in the acyl chains layer and (2) drug both in the headgroup layer and an additional thin, hydrated layer underneath the lipid necessary because the drug is larger than the lipid headgroups [62].

The best fits to the experimental data for both systems were obtained for model 2 where the drug is located in the regions of the headgroups and underneath the phospholipid (Fig. 4A/B), which resulted in $\chi^2$ values lower by 35% for DOx and 45% for IDA compared with model 1 where the drug is located in the region of the acyl chains (Figs. S3 and S4). This result demonstrated strong drug-lipid association but without penetration into the acyl chains region of the monolayer. The best fits were achieved by keeping the extent of acyl chain compaction as 56%, in line with our previously published data for pure DMPS monolayers at the same surface pressure [61], but with a headgroup layer that has a thickness of 5.5 ± 0.5 Å and zero hydration in comparison with a thickness of 7.5 ± 0.5 Å and 31% hydration for pure DMPS [61]. This reduction in thickness is attributed to strong electrostatic interactions between the drug and the oppositely charged phosphoserine headgroups [24,71]. Lastly, it may be noted that in accordance with the compositional investigation above, the surface excesses of phospholipid and drug are very similar for the two systems (<10% difference), which together with the very similar structural data for the two anthracyclines demonstrate that they interact equivalently with DMPS monolayers at 10 mN/m. Therefore, in spite of the different lipophilicity of the two drugs [49], the interactions are clearly driven by electrostatics at this low surface pressure where the phospholipid molecules are loosely packed, and significant differences in the behavior of the two systems are not apparent.

3.3.3. DMPS monolayers at 30 mN/m

In the next part of the study, the interactions of DOx and IDA with DMPS monolayers were investigated at the higher surface pressure of 30 mN/m. This surface pressure was chosen because it corresponds to the surface pressure occurring in real cell membranes [75]. Given the significant differences observed above in the surface pressure/area isotherms and the dynamic extents of interactions for the two systems at this surface pressure, different structural models were applied according to the nature of the recorded data.

In the case of DOx, the experimental data are visibly distinct to those of any other system with the falloff of the data to high $Q_v$ values much less pronounced for the d-lipid/D$_2$O contrast and more pronounced for the h-lipid/D$_2$O contrast (Fig. 4C). Three models were tested: (1) drug in the acyl chains layer and (2) drug both in the headgroup layer and an additional thin, hydrated layer underneath the lipid, both as above, and (3) drug forming extended layers underneath the headgroups (Figs. S5 and S6). Even though the final $\chi^2$ value remained high due to the imperfect agreement of the model with the data at low-to-mid $Q_v$ values, model 3 clearly results in more satisfactory fits to all 4 isotopic contrasts than either of models 1 or 2 over the whole $Q_v$ range. The best fits were achieved with an extent of acyl chain compaction of 100%, similar to the parameters used for a pure DPPC monolayer at a similar surface pressure of 35 mN/m [61], but in this case the headgroup layer thickness remained at 7.5 ± 0.5 Å as for pure DMPS, and its hydration remained significant at 49%. This reduction in the influence of electrostatic interactions, which given the lack of penetration of DOx into the DMPS acyl chains or headgroups, and the relatively large amount of drug at the interface at 30 mN/m, may be taken as an indication of the segregation of the drug into lateral domains as opposed to its adopting homogeneous binding across the monolayer. Such an explanation would be qualitatively consistent with the conformational change with increasing surface pressure implied from the surface pressure/area isotherm (Fig. 2A) as well as the relatively high $\chi^2$ value of the optimal fit to a homogeneous layer model. A heterogeneous layer model was also attempted in which the reflectivities of a lipid monolayer with limited drug-lipid interaction as well as regions with bound drug aggregates are summed with respect to the area coverage. Such a model is necessary when the domain size exceeds the coherence length of 10–100 μm [76]. While improvements in the model fit were achieved in some isotopic contrasts, there was no improvement to the global fit, which hints at the strong complexity of modeling such systems given that the coherence length varies by an order of magnitude over the $Q_v$-range measured. Even so, the presence of extended drug layers bound underneath the lipid headgroups is unambiguous from the data, and we make no further comment on their structural parameters, e.g. thickness and coverage. This qualitative physical picture is supported and further elaborated through the application of BAM below.

In the case of IDA, the experimental data are visibly more similar to those of IDA at 10 mN/m than DOx at 30 mN/m (Fig. 4D). As there is no indication in the data of the presence of an extended drug layer for this system, the first two models from above were tested (Fig. S7). The best fit was obtained for model 2 where drug is located in the regions of the headgroups and underneath the lipid with $\chi^2$ being 35% lower than model 1. Even so, the model fits remained not as good as those at lower surface pressure, so a further model was tested with drug present in both the acyl chains layer and the region of the headgroups and underneath the lipid, which resulted in a further reduction in the $\chi^2$ value of 45% (Fig. S8). The best fits were achieved with an extent of acyl chain compaction of 100%, in agreement with both the results for pure DMPS and its interactions with DOx, but with a headgroup layer thickness of 5.5 Å and its solvent fully excluded, in keeping with the strong electrostatic interactions observed at low surface pressure for both drugs.

The structural NR results described above reveal that the mechanisms of interaction of DOx and IDA with DMPS monolayers are similar at 10 mN/m and significantly different at 30 mN/m. A related observation has been made previously based on circular dichroism studies where the different interactions of anthracyclines with large unilamellar vesicles were attributed to the greater lipophilicity of IDA [49]. Indeed, it has been suggested previously that, in comparison with DOx, IDA may penetrate the acyl chain region of lipid monolayers more effectively through hydrophobic interactions in addition to electrostatic attractions between positively charged amine groups of the drug and negatively charged phosphoserine headgroups [24]. Clear structural evidence in support of this mechanism is provided by our new results. On the contrary, the lower lipophilicity of DOx leads to the situation where drug does not penetrate acyl chains of the monolayer, and the interaction is dominated by electrostatics at low surface pressure. Even so, it is difficult to explain all of our results with the physical picture of a laterally homogenous interface: the quasi-plateau in the surface pressure/area isotherm (Fig. 2A) as well as the upturn in the surface excess/pressure isotherm (Fig. 3F) imply a conformational change of the drug-lipid interactions with increasing surface pressure, and reduced compaction of the headgroups combined with the presence of extended hydrated layers of drug (Fig. 4C and Table 2) imply reduced electrostatic interactions and possible segregation of drug into domains. The application of an imaging technique at the air/water interface could help to elucidate such a physical picture.
3.4. Interfacial morphology (BAM)

BAM was then used to examine changes in the interfacial morphology on the μm length scale following the interactions of the anthracycline drugs with DMPS monolayers at 10 mN/m and 30 mN/m; measurements on DMPC monolayers were skipped due to the small extent of drug-lipid interactions (Fig. 5). The technique allows one to collect information on the macroscopic characteristics of the monolayer, especially concerning the formation of lateral domains at the air–water interface [32]. The objective of these measurements was to evaluate if there are clear differences in the morphological features of the monolayers involving DOx and IDA, particularly at a physiologically-relevant surface pressure of 30 mN/m where the results from NR indicate the existence of different interaction mechanisms.

The images of DMPS monolayers on pure water at the selected values of the surface pressure exhibit lighter regions that originate from condensed phase domains [37]. Flower-like domains are typically observed for DMPS at the plateau region [71,77]. However, at a surface pressure of 10 mN/m, which corresponds to the region above plateau on the π–A isotherm, there are still dark voids between domains that have lost their characteristic shape and pack closer to each other, which is consistent with the moderate extent of acyl chain compaction of 56% used in the structural NR analysis. At a surface pressure of 30 mN/m, the domains are clearly more closely packed, forming a well-ordered, almost uniform layer. The bright spots observed in the images at the higher surface pressure may be attributed to the formation of regions of solid monolayer, as indicated by the values of the Cs⁻¹ for this surface pressure [63], and which is a physical picture that is broadly consistent with

the extent of acyl chain compaction of 100% used in the structural NR analysis.

The images are completely different when the anthracycline drugs are present in the subphase. At 10 mN/m, the values of the reciprocal of the compression modulus indicate that DMPS forms LE films on both DOx and IDA solutions (Fig. 2B inset). The BAM images show quite a uniform, thin layer, typical for lipid monolayers in the LE phase. Nevertheless, there are some dark regions between the continuous phase, indicating domains where the molecules are more loosely packed.

Compression of the monolayer to 30 mN/m does not induce significant changes in the images obtained for DMPS monolayers formed on subphase containing IDA. The resulting monolayer is more uniform and thinner than in the absence of drug, consistent with the shift to larger areas per lipid molecule at fixed surface pressure in the isotherm data (Fig. 2B). A clearly different situation is observed for DOx. Compression of the monolayer to 30 mN/m leads to the presence of some brighter spots corresponding to thicker aggregates within the monolayer, which is not homogeneous. This observation is in good agreement with the isotherm data showing a quasi-plateau region at 30 mN/m, which suggests a conformational change with increasing surface pressure (Fig. 2B), and the coincidence of the maximum surface excess of drug (Fig. 3E/H) with reduced electrostatic headgroup interactions (Fig. 4C). It can be inferred that the aggregation of DOx molecules leads to the formation of domains of extended layers of the drug underneath the lipid monolayer mediated through hydrogen bonding. Such dimerization or even polymerization of drug molecules is in line with previous literature reports for DOx [78–80] but has not been reported for IDA, which is consistent with the new results presented here.

Fig. 5. BAM images recorded at selected surface pressures for DMPS monolayers formed either on pure water (left), 10⁻⁵ mol/L DOx (middle) and 10⁻⁵ mol/L IDA (right). Images are 800 μm × 430 μm.
3.5. DMPC:DMPS monolayers

Lastly, measurements of the dynamic interfacial composition were performed on mixed DMPC:DMPS (7:3 molar ratio) monolayers on a 10^{-3} mol/L DOx subphase using the low-Q implementation of NR (Fig. S1). Such a lipid composition represents a slightly less crude model biomembrane than the pure lipid monolayers studied above, as the proportion of charged phospholipid is closer to the ~20–30% found in real cells [46,47]. It can be seen that the extent of interactions of the drug with the 30%-charged mixed DMPC:DMPS monolayers at a surface pressure of 30 mN/m is 35% of that with the pure DMPS monolayer, i.e. the interaction is roughly commensurate with the proportion of negatively charged lipid in the monolayer (Table S2). This result demonstrates that even though drug-lipid interactions were studied using different pure lipid monolayers above in order to maximize sensitivity of the techniques to the different molecular interactions and resulting interfacial morphologies, the insight gained can be related to biomembrane models of closer physiological composition and relevance.

4. Conclusions

In this work, we have shown that the interactions of the two anthracycline drugs with zwitterionic and negatively charged lipid monolayers depend strongly on the type of lipid, the type of anthracycline drug and the surface pressure. It has been possible to resolve direct quantitative information about the extent of interaction and solve general interaction mechanisms of relevance to physiological surface pressure thanks to the application of a suite of complementary surface-sensitive experimental techniques. We chose to focus the study mainly on pure lipid monolayers in order to maximize the sensitivity of the applied techniques to the different drug-lipid interactions as well as the interfacial structures and morphologies formed, but even so it was not possible to resolve the location of DOx in the DMPC monolayers due to the small extent of drug-lipid interaction. Data were presented on mixed lipid monolayers, similar to those previously used by other groups in related studies [43,81], which indicates that the interpretations made in this work may be related also to mixed lipid systems.

The interactions of both DOx and IDA with pure DMPC and DMPS monolayers are very different, which is attributed primarily to the different charge of the lipid. In the case of zwitterionic DMPC, the interactions of both drugs are minimal, even though there are small shifts in the surface pressure/area isotherms. This result stays in agreement with previous studies on the interactions of different anthracyclines with lipid monolayers [24]. The converse is true for DMPS, as electrostatics between the positively charged amine group of both DOx and IDA and the negatively charged phosphoserine headgroups dominate the interactions at low surface pressure, resulting in a greater extent of interaction for DOx by at least a factor of 5. Even though the interaction diminishes with increasing surface pressure as the drug is excluded from the DMPC monolayer, the opposite is true for the DMPS monolayer, where the extent of interaction at a physiologically-relevant surface pressure of 30 mN/m is greater by at least a factor of 12. This result is broadly consistent but more quantitative than previous observations [24,71]. It was the recently developed fast compositional implementation of NR that enabled these direct quantitative results to be determined during dynamic compression-expansion cycles on Langmuir trough of a drug-lipid monolayer system for the first time [29,56]. Use of the technique also allowed us to prove that anthracyclines do interact through electrostatic interactions between the negative charge of the phosphatidylserine and the positive charge of the amino sugar and, contrary to previous reports [82], the strong interactions of anthracyclines with lipid monolayers do depend on the presence of anionic lipids.

Although perhaps somewhat counterintuitive given the low solubility of the components, it was also shown directly using the compositional implementation of NR that for all systems studied lipid is expelled from the monolayers during surface area compression, which we have inferred to be attributed to the formation of drug-lipid aggregates mediated by association of the oppositely charged components. The surface pressure/area isotherms support indirectly this interpretation but it is the novel application of NR that has provided the definitive evidence. The result serves as caution that even though the fluidizing effect of the drug on the DMPS monolayers is clear, the calculations of the reciprocal of the compression modulus should be taken as lower limits, which is pertinent given the apparent inconsistency between the relative low value of the reciprocal of the compression modulus and the high value of the extent of acyl chain compaction needed in the structural NR data analysis for the samples at 30 mN/m. The matter also deterred us from performing calculations of the excess free energy of the systems upon compression [24,69,70].

The interactions of DOx and IDA with DMPS monolayers are fundamentally different at a physiologically-relevant surface pressure of 30 mN/m, at which we note that the molecular organization and elastic compressibility modulus for monolayers and bilayers are comparable [75,83,84]. In the case of DOx, the drug clusters into domains of aggregates (observed by BAM) and the electrostatic interactions diminish as the drug is excluded from the lipid monolayer, as evidenced by reduced compaction and increased hydration of the phosphoserine headgroups (resolved using NR). We relate this surface-induced aggregation to the ability of DOx to self-aggregate, mediated by hydrogen bonding, in solution [78–80]. Indeed, such a possibility of drug dimerization or polymerization has been observed previously for DOx but not for IDA [78]. Additionally, the formation of a hydrogen-bonding network of DOx may in turn hinder the penetration of the drug into DMPS monolayer at physiological surface pressure. In the case of IDA, on the other hand, the drug penetrates the acyl chains region of the monolayer, which we relate to its greater lipophilicity [49], allowing its interactions with rigidly-packed acyl chains. This full physical picture of strong relevance to the passive transport of drugs in cells was resolved thanks not only to the traditional, structural implementation of NR [12,29], but also imaging of the interfacial morphology using BAM [32]. A schematic representation of the different equilibrium structures created as a result of the interactions of DOx and IDA with DMPS monolayers at surface pressures of 10 mN/m and 30 mN/m is shown (Fig. 6).

Our results may also be considered in view of MDR, which leads to the transport of cytotoxic drugs out of cells, usually by means of the P-glycoprotein, and is associated with elevated levels of phosphatidylserines in the lipid bilayer [28]. This is a well-known mechanism, which is also a major issue accounting for treatment discontinuation [27]. It has been shown that the lipid composition of human breast cancer MCF-7 cells changes when the MDR condition is developed. DOx-resistant MCF-7 cells exhibit elevated levels of negatively charged lipids with the highest content of phosphatidylserines [47]. Therefore, the results on DMPS monolayers in the present work may also be interpreted in the context of the resistance of cancer cell membranes to anthracyclines. Additionally, the changes in the lipid composition when MDR is developed show that the fluidity of the membranes is decreased, which also has relevance to our new results: at physiologically-relevant surface pressures, in a more organized and less fluid biomembranes, DOx does not penetrate the DMPS layer, instead forming an extended layer under the polar headgroups, yet IDA is present both around the polar headgroups and has penetrated into the acyl chains. These data are in good agreement with literature studies.
showing that the increasing lipophilicity of the drug may lead to the circumvention of MDR [85]. Therefore, it can be concluded from our results on the molecular level that the increase in phosphatidylserines in DOx-resistant cancer cell membranes has dual action: prevention of DOx penetration through the biomembrane through the passive diffusion mechanism and activation of drug removal mechanisms.

CRediT authorship contribution statement

Dorota Matyszewska: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Validation, Writing - original draft, Writing - review & editing. Ewa Nazaruk: Data curation, Investigation, Methodology. Richard A. Campbell: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank the Institut Laue-Langevin for allocations of neutron beam time on FIGARO (https://doi.org/10.5291/ILL-DATA.8-02-797 and https://doi.org/10.5291/ILL-DATA.8-02-805), and Andrea Tummino and Philipp Gutfreund for assistance with the latter experiment. This work was financially supported by Polish National Science Centre (Project No. 2016/23/D/ST4/03200). Part of the study was carried out at the Biological and Chemical Research Centre, University of Warsaw, established within the project co-financed by European Union from the European Regional Development Fund under the Operational Program Innovative Economy, 2007–2013. The open access fee was covered by FILL2030, a European Union project within the European Commission’s Horizon 2020 Research and Innovation programme under grant agreement N°731096.

References

[1] M.K. DeGorter, C.Q. Xia, J.J. Yang, R.B. Kim, Drug transporters in drug efficacy and toxicity, Annu. Rev. Pharmacol. Toxicol. 52 (2012) 249–273, https://doi.org/10.1146/annurev-pharmtox-010611-134529.
[2] G. Zhang, Y. Ma, D. Xi, Z. Rao, X. Sun, X. Wu, Effect of high uric acid on the disposition of metformin: in vivo and in vitro studies, Biopharm. Drug Dispos. 40 (2019) 3–11, https://doi.org/10.1002/bdd.2164.
[3] D. Matyszewska, K. Brzezińska, J. Juhaniewicz, R. Bilewicz, pH dependence of daunorubicin interactions with model DMPC Cholesterol membranes, Colloids Surf. B Biointerfaces 134 (2015) 295–303, https://doi.org/10.1016/j.jcolsci.2015.07.001.
[4] K. Olechowska, M. Mach, K. Hac-Wydra, P. Wydra, The influence of 2-hydroxyoleic acid – an anticancer drug – on model membranes of different fluidity modulated by the cholesterol content, J. Mol. Liq. 283 (2019) 756–762, https://doi.org/10.1016/j.jolq.2019.03.143.
[5] L.A. Clifton, R.A. Campbell, F. Sebastiani, J. Campos-Terán, J.F. Gonzalez-Martinez, S. Björklund, J. Sotres, M. Cárdenas, Design and use of model membranes to study biomolecular interactions using complementary surface-sensitive techniques, Adv. Colloid Interface Sci. 277 (2020), https://doi.org/10.1016/j.cis.2020.102118 102118.
[6] P. Böhm, A. Kourisouhas, J.F. Moulin, J.O. Rädler, E. Sackmann, B. Nickel, Probing the interface structure of adhering cells by contrast variation neutron reflectometry, Langmuir 35 (2019) 513–521, https://doi.org/10.1021/acs.langmuir.8b02228.
[7] M. Ashrafuzzaman, C.Y. Tseng, J.A. Tuszynski, Charge-based interactions of antimicrobial peptides and general drugs with lipid bilayers, J. Mol. Graph. Model. 95 (2020), https://doi.org/10.1016/j.jmgm.2019.107502 107502.
[8] H. Brockman, Lipid monolayers: why use half a membrane to characterize protein-membrane interactions?, Curr. Opin. Struct. Biol. 9 (1999) 438–443, https://doi.org/10.1016/S0959-440X(99)80061-X.
[9] R. Mendelsohn, C.R. Flach, Infrared reflection-absorption spectroscopy of lipids, peptides, and proteins in aqueous monolayers, Curr. Top. Membr. 52 (2002) 57–88, https://doi.org/10.1016/S0959-440X(99)80061-X.
[10] N. Nilsson, P.M. Hansson-Mille, A. Swerin, P.M. Claesson, J. Schoelkopf, P.A.C. Martino, S. Björklund, J. Sotres, M. Cárdenas, Design and use of model membranes to study biomolecular interactions using complementary surface-sensitive techniques, Adv. Colloid Interface Sci. 277 (2020), https://doi.org/10.1016/j.cis.2020.102118 102118.
[11] D. Nieciecka, A. Królikowska, A. Joniec, P. Krysinski, Partitioning of doxorubicin into Langmuir and Langmuir-Blodgett biomimetic mixed monolayers: electrochemical and spectroscopic studies, J. Electroanal. Chem. 710 (2013) 59–69, https://doi.org/10.1016/j.jelechem.2013.03.004.
[12] K. Kim, S.Q. Choi, J.A. Zasadzinski, T.M. Squires, Nonlinear chiral rheology of phospholipid monolayers, Soft Matter. 14 (2018) 2476–2483, https://doi.org/10.1039/c8sm00184g.
[13] T. Narayan, H. Wacklin, O. Konovalov, R. Lund, Recent applications of synchrotron radiation and neutrons in the study of soft matter, Crystalllogr. Rev. 23 (2017) 160–226, https://doi.org/10.1134/S0889311X16127722.
[14] B. Gzyl-Malcher, J. Handzlik, E. Klekowska, Temperature dependence of the interaction of prazosin with lipid Langmuir monolayers, Colloids Surf. B Biointerfaces 112 (2013) 171–176, https://doi.org/10.1016/j.jcis.2013.07.030.
[15] M. Elderdii, A.F. Sikorski, Langmuir-monolayer methodologies for characterizing protein-lipid interactions, Chem. Phys. Lipid. 212 (2018) 61–72, https://doi.org/10.1016/j.chemphyslip.2018.01.008.
[16] W.M. Pazin, G.C.M. Ruiz, O.N. de Oliveira, C.J.L. Constantino, Interaction of Arzepilin C with model membranes: effects of pH and ionic strength, Biochim. Biophys. Acta - Biomembr. 1861 (2018) 410–417, https://doi.org/10.1016/j.bbamem.2018.11.008.
[17] D. Ciumac, R.A. Campbell, H. Xu, L.A. Clifton, A.V. Hughes, J.R.P. Webster, J.R. Lu, Implications of lipid monolayer charge characteristics on their selective interactions with a short antimicrobial peptide, Colloids Surf. B Biointerfaces 150 (2017) 308–316, https://doi.org/10.1016/j.jcis.2016.10.043.
[18] F. Niga, P.M. Hansson-Mille, A. Swerin, P.M. Claesson, J. Schoelkopf, P.A.C. Martino, S. Björklund, J. Sotres, M. Cárdenas, Design and use of model membranes to study biomolecular interactions using complementary surface-sensitive techniques, Adv. Colloid Interface Sci. 277 (2020), https://doi.org/10.1016/j.cis.2020.102118 102118.
