Biochimica et Biophysica Acta 1858 (2016) 422–433

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Langmuir monolayers and Differential Scanning Calorimetry for the study of the interactions between camptothecin drugs and biomembrane models

Ana Casadó [a], M. Chiara Giuffrida [a,c], M. Lluïsa Sagristá [a], Francesco Castelli [c], Montserrat Pujol [b], M. Asunción Alsina [b], Margarita Mora [a,*]

[a] Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain
[b] Department of Physical Chemistry, Faculty of Pharmacy, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain
[c] Department of Drug Sciences, University of Catania, Viale A. Doria 6, 95125 Catania, Italy

ARTICLE INFO

Article history:
Received 8 September 2015
Received in revised form 20 November 2015
Accepted 3 December 2015
Available online 4 December 2015

Keywords:
Camptothecins
Biomembrane models
Langmuir monolayers
Bilayer systems
Liposomes
Membrane interactions

ABSTRACT

CPT-11 and SN-38 are camptothecins with strong antitumor activity. Nevertheless, their severe side effects and the chemical instability of their lactone ring have questioned the usual forms for its administration and have focused the current research on the development of new suitable pharmaceutical formulations. This work presents a biophysical study of the interfacial interactions of CPT-11 and SN-38 with membrane mimetic models by using monolayer techniques and Differential Scanning Calorimetry. The aim is to get new insights for the understanding of the bilayer mechanics after drug incorporation and to optimize the design of drug delivery systems based on the formation of stable bilayer structures. Moreover, from our knowledge, the molecular interactions between camptothecins and phospholipids have not been investigated in detail, despite their importance in the context of drug action. The results show that neither CPT-11 nor SN-38 disturbs the structure of the complex liposome bilayers, despite their different solubility, that CPT-11, positively charged in its piperidine group, interacts electrostatically with DOPS, making stable the incorporation of a high percentage of CPT-11 into liposomes and that SN-38 establishes weak repulsive interactions with lipid molecules that modify the compressibility of the bilayer without affecting significantly neither the lipid collapse pressure nor the miscibility pattern of drug–lipid mixed monolayers. The suitability of a binary and a ternary lipid mixture for encapsulating SN-38 and CPT-11, respectively, has been demonstrated.

1. Introduction

Irinotecan (camptothecin CPT-11; 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin) and SN-38; (irinotecan metabolite, ethyl-10-hydroxy-camptothecin) are antineoplastic agents belonging to the family of topoisomerase I inhibitors that arrest the synthesis of DNA and possess strong antitumor activity (Fig. 1) [1,2].

The sole catalytic mechanism for camptothecin action consists in the formation and stabilization of a reversible enzyme–drug–DNA ternary complex which prevents the ligation step of the nicking/sealing cycle performed by the topoisomerase enzyme. CPT-11 is converted to its metabolite SN-38, with a reported, at least, 100-fold biggest activity, by a human carboxylesterase (hCE), primarily in the liver [3], but also in tumors [4]. CPT-11 is a first-line drug approved for the treatment of a variety of human tumors, including colorectal, lung and gynecological cancers [5]. Both CPT-11 and SN-38 are currently in clinical trial in its liposomal form [6]. However, their severe side effects, such as myelosuppression and gastrointestinal disorders [7,8], impose some restrictions for camptothecin therapies and additional considerations to develop suitable pharmaceutical formulations for clinical purposes. Other drawbacks for their clinical applications are the chemical instability of the lactone ring, which opens to the inactive carboxylate form at physiological pH [9,10] and, in the case of SN-38, the great insolubility in almost all the solvents that could be used to formulate this drug. All of these considerations make evident the importance of protecting the drug from the external environment to maximize its pharmacological potential and the need of using solubilizers or membrane stabilizers [11]. Therefore, current investigations are focused on the development of new forms for camptothecin administration.

Abbreviations: CPT-11, irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin; SN-38, irinotecan metabolite, ethyl-10-hydroxy-camptothecin; DSC, Differential Scanning Calorimetry; P, partition coefficient; DSPC, L-α-Distearoyl-phospathidylcholine; DOPS, L-α-Dioleoyl-phospathidylserine; EPC, Egg Phosphathidylcholine; CHOL, Cholesterol; MLVs, Multilamellar liposomes.

* Corresponding author at: Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, Av. Diagonal 643, Prevosti Building, 1st floor, 08028 Barcelona, Spain.

E-mail addresses: ancasado@clinic.ub.es (A. Casadó), mcgiuffrida@gmail.com (M.C. Giuffrida), mlsagrista@ub.edu (M.L. Sagristá), fcastelli@unict.it (F. Castelli), mopujol@ub.edu (M. Pujol), aalsina@ub.edu (M.A. Alsina), margarita.mora@ub.edu (M. Mora).

http://dx.doi.org/10.1016/j.bbamem.2015.12.007
0005-2736/

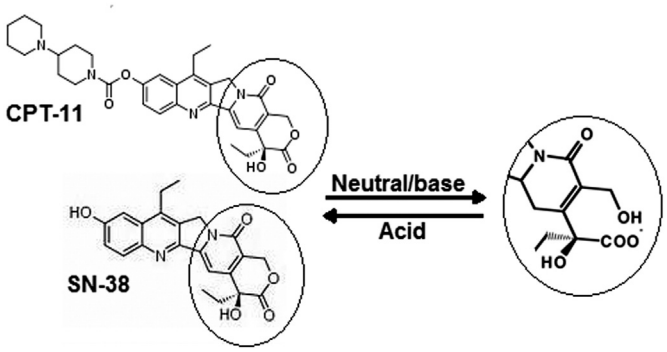

Fig. 1. Molecular structure of camptothecins. Equilibrium between their lactone and carboxylate forms.

The consideration of the pharmaceutical form of anticancer drugs and the procurement of stable formulations can overcome some of the main limitations for their use in clinical applications. Docetaxel, for example, is available in a formulation that contains a high concentration of Tween 80 [12] and Paclitaxel is often provided in Cremophor EL. Unfortunately, the use of both products has been associated with severe side effects related with hypersensitivity reactions [13], nephrotoxicity and neurotoxicity. In order to solve the problems derived from the use of such solubilizers or adjuvants, alternative dosage forms have been developed to improve its clinical administration. Among the potential drug delivery carriers, liposomes or lipid vesicles have endowed with interesting and useful characteristics that make them a pivotal biocompatible and biodegradable drug delivery and formulation platform [14].

Liposomes possess many interesting properties, such as the ability to entrap both hydrophilic and hydrophobic molecules without loss or alteration of their activity, which make them very suitable to create triggered release systems and to provide passive or active targeted strategies [15,16]. They may carry different surface charges, have different sizes and provide long systemic circulation times, depending on their lipid composition. Liposomes can act as sustained depots which release their cargo in a controlled form and in a specific target, giving a preferential accumulation in solid tumors [17]. Moreover, liposomal formulations can reduce the toxicity associated with free anticancer drugs in healthy tissues [18,19], which is severe in the case of CPT-11 and SN-38, and improve drug stability by protecting the compound from chemical degradation or transformation. Several lipid-based and liposomal nanomedicines have been approved in the last 20 years [20–22] and many others are undergoing clinical evaluation [23].

In the case of camptothecins, their encapsulation in liposomes would have the additional advantages of providing a suitable environment to maintain soluble the drug, either in the aqueous phase or in the lipid bilayer, and to afford protection for the lactone ring of the molecule, which is essential for its pharmacological activity, besides being an important structural requirement both for the passive diffusion of these drugs into cancer cells and for their successful interaction with the topoisomerase I enzyme [24,25]. Moreover, CTP-11 liposomalization increases its antitumor activity with an important reduction in the adverse reactions reported for this drug, being the use of carriers completely essential in the case of SN-38, because of its extreme insolubility, to make it a useful drug [26].

Liposomes can be engineered from a wide variety of lipid species, from natural or synthetic origin and can be endowed with special characteristics by adding to their formulation specific components. You can make liposomes sensitive to specific stimuli, stable as pharmaceutical products and in the biological media after administration and that can be vectorized to specific and targeted locations. To develop optimal drug formulations and efficient drug delivery systems it is essential to control the physicochemical parameters of the vehicle and, for this purpose, it is very useful to study how molecular interactions between the constituents of the carrier and the drugs can affect or modify its structure [27,28]. Moreover, the study of drug–lipid interactions can also be used to predict the pharmacokinetic properties of drugs, which are dependent on their chemical stability and, consequently, their bioavailability and efficacy.

When characterizing the interactions of drugs with membrane lipids, it is essential to consider the use of different techniques, each one with advantages or limitations regarding to their applications [29,30]. Among these, it could be outlined Differential Scanning Calorimetry (DSC) [31,32] and Langmuir monolayers [33,34].

DSC is a nonperturbative technique largely employed in pharmaceutical thermal analysis, because its ability to provide information about either the physical or energetic properties of substances. Moreover DSC is one of the more used methods to measure the enthalpy associated with physical processes [35]. As a thermoanalytical method, DSC has definite applications in nanosciences with important features for the development of nanostructured lipid carriers for drug delivery [36,37].

The Langmuir techniques, which use lipid monolayers at the air–water interface as the model for studying the two-dimensional arrangement, are very useful in the area of liposome formulation as they provide information on lipid packaging configuration and so, on liposome stability [34,38]. In addition, the knowledge of the partition coefficients (P) that can be determined in an n-octanol/aqueous medium [39] or by a reverse phase HPLC column [40], gives a good approach to predict the relative tendency of drugs to incorporate into biological membranes.

This work explores the physicochemical interactions of CPT-11 and SN-38 with pure and mixed lipid monolayers and bilayers and informs about the potential usefulness of the liposomal carriers designed for these drugs [41]. The long term stability is an essential parameter of the final formulation because it will control the sustained release of these camptothecins into the cell and because of the protection afforded to their cargos versus its biological degradation. The results will provide comprehensive insights about the possible effects of the molecular interactions of these drugs, on the liposomal formulation, either at the level of the hydrophobic domain of the lipid bilayer in which they can be inserted, or at the level of the polar region when encapsulated in their aqueous space, always in contact with the inner monolayer of the bilayer. It could also be emphasized that, from our knowledge, the molecular interactions between camptothecins and phospholipids have not been investigated in detail.

2. Experimental

2.1. Materials

L-α-Distearoyl-phospathidylcholine (DSPC), L-α-Dioleoyl-phospathidylserine (DOPS), Egg Phosphathidylcholine (EPC) and Cholesterol (CHOL) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). CPT-11, purchased from Afine Chemicals Limited (Hangzhou, China), was pure with a minimal grade of 99%. SN-38 was from Tocris Bioscience (Bristol, United Kingdom). All the organic solvents (Panreac, Montcada i Reixac, Barcelona, Spain) have been distilled before use. Milli-Q water (Millipore Bedford, Massachusetts system, resistivity of 18 MΩ cm) was used. All other chemicals and solvents were of analytical grade.

2.2. Calorimetric studies

Differential scanning calorimetric (DSC) analysis was used to evaluate the thermodynamic aspects of the camptothecin/lipid interactions and was performed by using a Mettler DSC-30 device (Mettler-Toledo, Inc., Columbus, OH, USA) or a MicroCal VP-DSC (GE Healthcare LifeSciences, Uppsala, Sweden). The calorimetric systems were calibrated, in transition temperature and enthalpy changes, by

using indium and zinc or lysozyme (mol wt. 14.3 kDa) for the Mettler or MicroCal devices, respectively. Thermodynamic data were analyzed with Mettler-Toledo STARe or MicroCal-enabled Origin softwares.

2.2.1. Preparation of liposomes

Multilamellar liposomes (MLVs) were used for calorimetric studies and prepared as described previously [41]. DSPC, DOPS, CHOL and EPC were used, in ternary or binary combinations, to prepare lipid films. MLVs containing CPT-11 were prepared as follows: fifteen milligrams of the ternary lipid mixture (DSPC/DOPS/CHOL, 65:35:30) were dissolved in chloroform and mixed with the appropriate amounts of CPT-11 to produce exact molar fractions of the drug. Chloroform was evaporated under a nitrogen stream to get a thin lipid film, which was maintained overnight in a vacuum desiccator to remove all the traces of the solvent and the resulting film was hydrated with 1000 μL of 10 mM lactate buffer (pH 4.4). Finally, the MLV suspension was frozen (liquid N_2) and thawed (water bath above the phase transition temperature, T_m) five times. In the case of SN-38, MLVs were prepared by means of a different protocol: lipid (EPC/DOPS, 9:1, 50 mg)/drug mixtures, giving specific molar fractions of the drug, were hydrated with 1000 μL of 10 mM lactate buffer (pH 4.4), after weighting the appropriate amount of each component, with an Ultra-Turrax IKA T-25 (Staufen, Germany), at 20,000 rpm for 10 min.

Samples with transition temperatures below the freezing point of water (those containing EPC) had 50% ethyleneglycol included in the aqueous phase to prevent the freezing of the bulk solvent phase. It has been taken into account that ethyleneglycol can modify both Tm and ΔH [42,43].

2.2.2. DSC experiments

DSC-30 Mettler experiments for EPC/DOPS MLVs with SN-38 were carried out as follows. Aliquots of 120 μL of the different aqueous suspensions of MLVs were transferred to a 160 μL aluminum calorimetric pan, which was hermetically sealed. The samples were weighted and immediately submitted to DSC analysis, as follows: 1) a heating scan between −50 °C and 20 °C at 2 °C/min; 2) a cooling scan between 20 and −50 °C at 4 °C/min. This low heating scan rate was chosen in order to provide the higher peak resolution. Sharp peaks of a first order gel-to-liquid crystalline phase transition (L_α) in buffered aqueous solution require low scan rates, allowing also the resolution of the possible closely DSC peaks [44,45]. The reference pan contained 120 μL of a 1:1 ethyleneglycol/10 mM lactate buffer (pH 4.4) solution. The temperature of the maximum of the transition endotherm (T_m) and the enthalpy (ΔH_{cal}), calculated from the area under the peak, were determined with a Mettler TC15 TA controller. The cooperativity of the transition was evaluated, approximately, from the widths at half-peak heights (as °C) of the main transition endotherms ($\Delta T_{1/2}$). To carry out the experiments with the MicroCal device, 0.5181 mL of the DSPC/DOPS/CHOL MLV suspension, with and without CPT-11 at different molar fractions, was placed in the calorimetric cell and analyzed as follows: 1) a heating scan in the temperature range of 10–60 °C, at 0.5 °C/min; 2) a cooling scan from 60 to 10° at 1 °C/min. The reference cell contained 10 mM lactate buffer (pH 4.4). As a common procedure both the buffer and the samples were degassed for 10 min before being introduced into the MicroCal cell to avoid any signal artifacts due to air bubbles. In all the experiments, only the heating scans were analyzed. Each experiment was carried out in triplicate to check the result reproducibility. After the DSC analysis, aliquots of all samples were extracted from the calorimetric pan and used to determine the exact amount of lipids by the Stewart's method [46].

2.3. Partition coefficients in n-octanol/buffer solutions

The partition coefficients between n-octanol and buffer solutions (Po/w), at different pH values, were determined by a slight modification of the shake-flask method, earlier reported by Ross et al. [47] and described in the OECD guideline for the testing of chemicals [48]. Stock solutions in DMSO of CPT-11 and SN-38 10 mM were prepared and, prior to use, were diluted in n-octanol to a final concentration of 0.5 mM (1:20). The experiments were carried out as follows: 100 μL of the 0.5 mM camptothecin solutions was diluted with 4.9 mL of n-octanol and mixed with 5 mL of the appropriate buffer solution. The two phases were maintained under constant shaking for 1 h at 25 °C. The n-octanol phase was removed with a Pasteur pipette and both phases were analyzed spectrophotometrically for drug content. The partition coefficient (Po/w) was calculated as the ratio between the molar concentration in n-octanol (Co) and that in the aqueous phase (Cw). Calibration curves in n-octanol and in different buffer solutions were previously performed for both camptothecins at the experimental conditions previously determined (Table S1, Supplementary material).

2.4. Monolayer studies

The Langmuir technique measures the surface pressure as a function of the mean molecular area that occupies a molecule in a monolayer extended on an aqueous surface [49,50]. Experimental measurements were recorded with a Nima Langmuir balance equipped with a Wilhelmy platinum plate (Nima Technology, Coventry) and a Teflon trough that was rinsed with ethanol and distilled water before use. All experiments were performed at room temperature. The monolayer stability was verified by monitoring the change in surface pressure while the area was held constant.

2.4.1. Monolayer compression isotherms

The *π–A* isotherms were recorded with a Nima (U.K.) Langmuir Teflon trough of 595 cm^2 surface area and 297.5 cm^3 volume. Separate stock solutions of individual lipids or lipid mixtures (DSPC/DOPS/CHOL 65:35:30 or EPC/DOPS 9:1) were prepared in chloroform at 1 mg/mL. Required volumes of each of them were mixed with CPT-11 (1 mg/mL) or SN-38 (0.5 mg/mL) solutions to form the lipid–drug spreading solutions containing different drug molar fractions. Monolayers were formed by applying small drops of the spreading solutions on the 10 mM Lactate Buffer subphase (pH 4.4) with a microsyringe (Hamilton Co., Reno, NV, USA). After 15 min, monolayers of the desired composition were continuously compressed with an area reduction rate of 10 $cm^2\ min^{-1}$. The films were compressed to their collapse pressure. Each run was repeated three times and the reproducibility was ± 1 $Å^2\ molecule^{-1}$.

In order to study the effect of the adsorption of CPT-11 on the lipid isotherms and to establish a possible selective interaction with someone of the lipid components, another set of experiments was performed with monolayers made with the ternary mixture of lipids, DSPC/DOPS/CHOL 65:35:30 or with each one of the individual lipid constituents. CPT-11 was injected in the lactate buffer subphase at different molar concentrations, ranging from 5 to 30 μM. The procedure was the same described above.

2.4.2. Surface activity of CPT-11

The surface activity of CPT-11 was studied in order to determine the equilibrium spreading pressure. Using a cylindrical PTFE trough (surface area 19.6 cm^2, volume 27.2 cm^3), that was rinsed with ethanol and distilled water before use, increasing volumes of a 1 mg/mL (1.48 mM) CPT-11 solution in Milli-Q water (18 MΩ cm) were injected below the 10 mM lactate buffer subphase (pH 4.4) through a lateral hole and the adsorption of the drug in the air/water interface was monitored by following the increase in surface pressure as a function of time under continuous stirring of the subphase. All experiments were performed at room temperature.

2.4.3. Insertion of CPT-11 into monolayers

The kinetics of insertion of CPT-11 into monolayers of DSPC/DOPS/CHOL 65:35:30 were measured using the same trough as for the surface

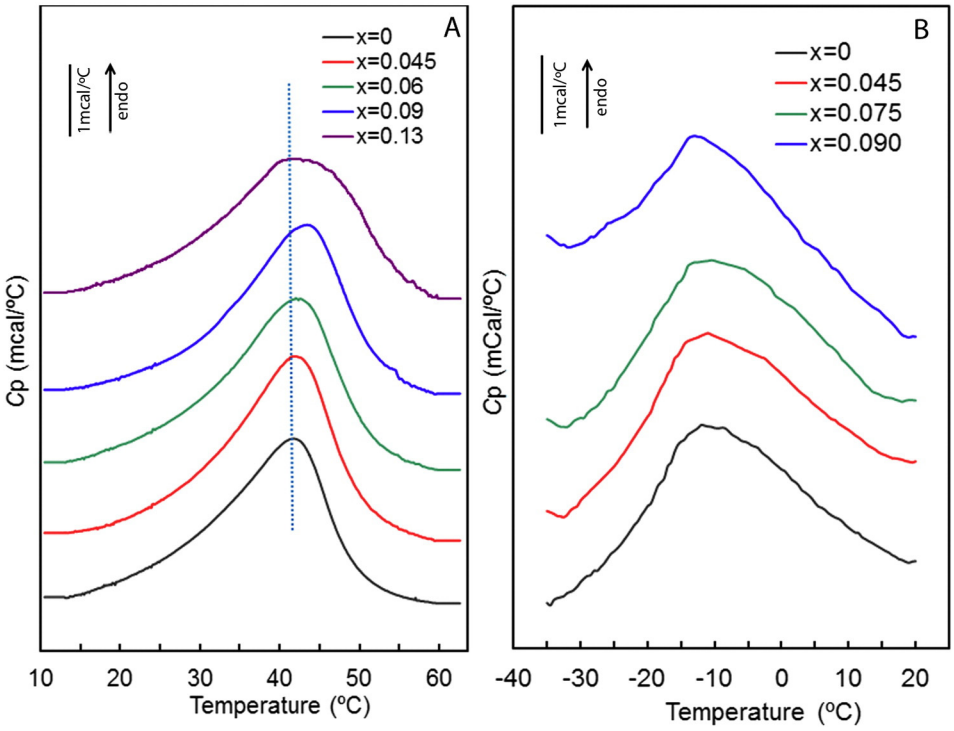

Fig. 2. Endothermic calorimetric curves in heating mode of DSPC/DOPS/CHOL-MLVs (A) and EPC/DOPS-MLVs (B) prepared in the absence and in the presence of increasing molar fractions of CPT-11(A) and SN-38 (B). *x* are the molar fractions of either CPT-11 (A) or SN-38 (B). Enthalpy values were calculated from the area under the peak by using Mettler STAR^e evaluation software. Dotted line in A shows the T_m shift.

activity, which was cleaned in the same way with ethanol and distilled water before use. For these experiments, a lipid stock solution was prepared and added drop wise on the subphase until the desired lipid pressure was achieved. After 10–20 min the equilibrium of the lipid monolayer was reached. Then, a 100 μL of the 1 mg/mL (1.48 mM) CPT-11 solution was injected into the subphase through the side hole of the trough. The subphase was magnetic stirred during the measurements and surface pressure changes were monitored as function of time until it remained constant.

2.4.4. Brewster angle microscopy

Brewster angle microscopy images were obtained in a KSV NIMA MicroBAM instrument (U.K.) mounted on a NIMA Langmuir balance trough. The instrument was equipped with a 30 mW laser emitting p-polarized light a 660 nm, which incises the air/water interface at 53.1° (Brewster angle). The shutter speed used was 1/50 s. All the images were taken at room temperature and under the same acquisition conditions for comparative purposes.

3. Results and discussion

3.1. Calorimetric analysis

Differential Scanning Calorimetry (DSC) was used as a first approach to determine the effect of the incorporation of CPT-11 and SN-38 on the physical state of the liposomal bilayers. Representative DSC thermograms obtained for DSPC/DOPS/CHOL/CPT-11 and EPC/DOPS/SN-38 multilamellar liposomes, acquired with the Mettler DSC-30 device, equipped with a subzero nitrogen-liquid system or with the MicroCal device, are shown in Fig. 2A and B, respectively. These lipid mixtures

Table 1
Thermodynamic parameters of mixed lipid bilayers containing CPT-11 or SN-38.

Sample	ΔHcal[2] (kJ/mol)	Tm[3] (°C)	Δ $T_{1/2}$[4] (°C)
DSPC/DOPS/Chol (x = 0)	11.24 ± 0.55	41.98 ± 1.98	13.28 ± 0.72
DSPC/DOPS/Chol/CPT-11 (x[1] = 0.045)	12.00 ± 0.58	42.19 ± 2.07	12.59 ± 0.61
DSPC/DOPS/Chol/CPT-11 (x = 0.060)	12.36 ± 0.61	42.58 ± 2.12	13.34 ± 0.69
DSPC/DOPS/Chol/CPT-11 (x = 0.090)	12.08 ± 0.57	43.81 ± 2.16	13.27 ± 0,59
DSPC/DOPS/Chol/CPT-11 (x = 0.130)	11.61 ± 0.56	43.76 ± 2.21	15.88 ± 0.81
EPC/DOPS (x = 0)	24.13 ± 1.18	−11.89 ± 0.62	24.16 ± 1.31
EPC/DOPS/SN-38 (x = 0.045)	24.51 ± 1.19	−10.91 ± 0.52	25.55 ± 1.17
EPC/DOPS/SN-38 (x = 0.075)	22.29 ± 1.11	−10.40 ± 0.55	26.38 ± 1.35
EPC/DOPS/SN-38 (x = 0.090)	20.99 ± 1.03	−11.84 ± 0.59	24.44 ± 1.22

Values reported are the mean ± SD of three independent experiments.
[1] x is the molar fraction of CPT-11 or SN-38.
[2] Calorimetric enthalpy calculated from the area under the peak.
[3] Temperature calculated from the maximum of the calorimetric peak.
[4] $\Delta T_{1/2}$ is the width of the calorimetric peak at the half height.

are those chosen, after an accurate selection in our laboratory, for achieving drug formulations [41]. The aim is to study how the molecular interactions between camptothecins and lipids can affect the stability of the liposomal formulations.

The endothermic transition profiles, obtained for the lipid dispersions, in the absence and in the presence of the CPT-11 and SN-38, at the indicated mole fractions of the drugs, showed, in all cases, a broad single endotherm, indicating that the incorporation of both camptothecins into MLVs does not significantly modify the organization of their respective bilayers. However, it could be noted that CPT-11 causes a slight shifting of the transition peak toward a higher temperature (as indicated by considering the dotted line in the Fig. 2A), with respect to the pure lipid mixture and a small broadening of the phase transition: these changes were clear at the highest molar fractions of the drug.

The case of SN-38 was rather different. It appears that SN-38 leaves the transition temperature of EPC/DOPS MLVs almost unchanged, being maintained the asymmetric shape of the peak corresponding to the phase transition observed in the absence of the drug. It should also be remarked that the broadness of the calorimetric peaks is that expected from the complex lipid and fatty acid composition of the bilayers analyzed. The thermodynamic parameters inferred from these thermograms are given in Table 1.

In addition to the aforementioned changes, it could stand out that SN-38 induces a gradual slight reduction of EPC/DOPS ΔH values. Despite the reported information about the changes induced, in both Tm and ΔH_{cal} values, by the presence of ethyleneglycol in the calorimetric pans [42,51], the calorimetric data for the SN-38 containing samples may be compared correctly since all of them include the same percentage of cryoprotectant. From the results of the calorimetric study, it could be underlined that neither the incorporation of CPT-11 nor that of SN-38 disturbs the structure of the complex composited liposome bilayers, despite the special and different solubility properties of these camptothecins.

3.2. Octanol–buffer partition coefficients

The hydrophilic–hydrophobic balance of any drug is generally measured by checking its distribution in a biphasic organic/aqueous system and can be quantitatively expressed as a partition coefficient. CPT-11 and SN-38 Po/w values were determined with an n-octanol/water system, at different pH buffered media (Table 2).

The results show the differences that exist between both camptothecins. At the conditions corresponding to the preparation of the pharmaceutical formulations of both CPT-11 [41] and SN-38 (acidic pH), CPT-11 is mainly in the aqueous media, although moves to the organic phase in neutral an alkaline environments, whereas SN-38 is already in the organic phase in a percentage of almost 93%. The structural characteristics of CPT-11 and SN-38 (Fig. 1) would account for the thermodynamic tendency to partition into the aqueous or organic phases of these drugs: the additional site for protonation of CPT-11 (piperidine group, pKa = 11.20) could influence its solubility in aqueous media [52] and its lactone form possesses more positive charge than the carboxylate form, fact that could be related with the different distribution of this molecule between n-octanol and water phases in function of pH.

Table 2
The octanol/aqueous solution experimental partition coefficients for CPT-11 and SN-38.

	$[Drug]_{oct}$ (μM)	$\%Drug_{oct}$	Po/w	logPo/w
CPT-11				
pH 4.4	1.88	17.15	0.207	−0.684
pH 7.4	10.13	92.43	12.20	1.087
pH 9.0	10.35	94.43	16.97	1.230
SN-38				
pH 4.4	9.23	92.86	13.00	1.11
pH 7.4	9.46	95.25	19.71	1.29
pH 9.0	9.57	96.30	25.86	1.41

The results are the mean values of three individual experiments. The variation coefficients (CV) range from 5.2 to 7.5%.

3.3. Surface studies of CPT-11 and SN-38 camptothecins

Langmuir monolayers of lipids or drugs and of mixed lipids and drugs of different molar compositions have been studied in terms of surface activity and compression isotherms. DSPC/DOPS/CHOL and EPC/DOPS were the lipid mixtures used to form the Langmuir monolayers, as indicated above.

3.3.1. Surface activity

Surface activity of CPT-11 was determined by injecting increasing volumes of a concentrated solution of the drug (1.48 mM) into the lactate buffer subphase of DSPC/DOPS/CHOL (65:35:30) monolayers and recording the surface pressures achieved, for each CPT-11 concentration, as a function of the time until saturation. The surface excess concentration (Γ), expressed in $mol \cdot cm^{-2}$, was calculated by applying the Gibbs equation (Eq. (1), Supplementary material) to the curves.

The value of the surface excess concentration, $\Gamma = 2.08 \times 10^{-10}$ $mol \cdot cm^{-2}$, allows us to calculate the area occupied per CPT-11 molecule at the saturated interface, A. The value obtained, 79.8 $Å^2$ $molecule^{-1}$ (Eq. (2), Supplementary material), correlates well with that expected from the structure and molecular weight (586.68 g/mol) of CPT-11, on the basis of some referenced data for other drugs such as docetaxel (MW = 807.88 g/mol) [12].

The attempts to analyze the surface activity of SN-38 failed because of the inability of this drug to form stable monolayers at the interface.

3.3.2. π–A compression isotherms

Representative compression isotherms of DPPC/DOPS/CHOL/CPT-11 and EPC/DOPS/SN-38, at several drug molar ratios, between zero (only lipids) and one (only drug) are illustrated in Fig. S1A and B (Supplementary material), respectively.

As can be seen, DPPC/DOPS/CHOL and EPC/DOPS form stable Langmuir monolayers at the air–water interface, CPT-11 forms a monolayer with low values of π (~12 mN/m) (Fig. S1A, Supplementary material, red line) and SN-38 does not forms monolayer at the conditions of the assay (Fig. S1B, Supplementary material, dark blue line), similarly to that observed for some resveratrol compounds [53] or for different coumarins [54]. In our case, however, the result should be explained on the basis of the high insolubility of SN-38 that prevents its spreading on the aqueous phase. Both DPPC/DOPS/CHOL and EPC/DOPS lipid mixtures form stable monolayers with CPT-11 and SN-38, respectively, at all the ratios studied (Fig. S1A and B, Supplementary material), being the *π–A* isotherms of the binary monolayers located between those of the pure components. Moreover, increases of the mole percentages of either CPT-11 or SN-38 caused the shift of the isotherms toward smaller areas per molecule.

The values for the collapse pressures of DPPC/DOPS/CHOL and EPC/DOPS monolayers were of about 46.4 and 45.6 mN/m, respectively, whereas the highest surface pressure that pure CPT-11 monolayer can reach on compression was <12 mN/m, probably because of the rigid carbon rings in their structure [55]. On the other hand, mixed DPPC/DOPS/CHOL/CPT-11 monolayers (Fig. S1A, Supplementary material) have collapse pressures similar to that of the lipid mixture for $x_{CPT\text{-}11} \leq 0.5$, slightly decreases when CPT-11 molar fraction was 0.6 (44.9 mN/m) and undergo a dramatic change when the mixed monolayer contained a 0.75 molar fraction of CPT-11; that is, the isotherm stopped at a surface pressure of 22.5 mN/m. Zhao et al. [27], by studying the behavior of DPPC/Paclitaxel mixed monolayers, observed similar results and suggested that they could be interpreted in terms of a squeezing out of paclitaxel from the lipid monolayer during compression. This explanation could also be applied to our results.

When mixed EPC/DOPS/SN-38 isotherms were analyzed (Fig. S1B, Supplementary material), similar considerations can be made. Besides the shifting of the area per molecule toward smaller values, probably because of the smaller area occupied by the SN-38 molecule in relation with that of the molecules of EPC and DOPS, SN-38 causes a slight decrease of the collapse pressure of the lipid mixture from 45.6 mN/m to the value corresponding to the 0.6 molar fraction of SN-38 (43.1 mN/m) and undergo a higher decrease when the molar fraction of the drug was 0.75 (36.4 mN/m). A similar behavior was observed by Jurak and Miñones [56] when studying the binary systems α-tocoferol/POPC and α-tocoferol/DOPC. The curves in Fig. S1 (A and B, Supplementary material), also give the mean molecular area values at the collapse for both lipid monolayers containing CPT-11 or SN-38, respectively.

The existence of a single collapse point in each of the individual drawn isotherms for the lipids/drug mixtures studied could be indicative of the miscibility of camptothecins and the corresponding lipid mixtures in the lipid/drug binary systems. However, if the components of the mixture were miscible, the collapse pressures of the binary mixtures would be, in some extent, dependent on the drug content of the lipid monolayers. Because of this, to discern between miscibility and immiscibility of lipids and drugs and to explore the existence of some kind of interactions between the components of the mixed monolayers in function of the film composition, the applicability of the additivity relationship for the binary systems was further examined and the results were analyzed together.

3.3.3. Isothermal compressibility of mixed monolayers

The isothermal data were also analyzed in terms of isothermal compressibility (C_s), giving additional information about the elasticity and compressibility of the bilayer (Eq. (3), Supplementary material) [57,58]. Its reciprocal, C_s^{-1}, the elastic modulus of area compressibility, is related to the packing degree and can be used to characterize the phase state of the monolayer [59], i.e. a larger C_s^{-1} value indicates a less compressible membrane and a higher degree of viscosity. For liquid expanded films it ranges from 12.5 to 50 mN m^{-1}, while for the liquid condensed phase it ranges from 100 to 250 mN m^{-1} [60].

Both C_s and C_s^{-1} can be calculated directly for DSPC/DOPS/CHOL/CPT-11 and EPC/DOPS/SN-38 mixed monolayers from the slope of their corresponding π–A isotherms. Our results show that the DOPC/DOPS/CHOL monolayer (Fig. 3A) has a value of the compressibility modulus around 83 mN/m at 30 mN/m, which is intermediate between those corresponding to the liquid expanded state and the liquid condensed phase.

The incorporation of CPT-11 increased significantly C_s^{-1}, changing the monolayer to the liquid condensed phase, with Cs^{-1} values higher than 100 mN/m for CPT-11 mole fractions from 0.045 to 0.13. Higher drug concentrations (mole fractions 0.5–0.75) returned the monolayer to the liquid expanded phase, with C_s^{-1} values significantly lower than those corresponding to the pure lipid monolayer. The highest C_s^{-1} values occur at surface pressures ranging between 25 and 40 mN/m, interval at which the compressibility curves display two peaks, the second of which fits with a well-defined shoulder in the lipid compressibility curve.

The plot of C_s^{-1} vs surface pressure for the EPC/DOPS monolayer is given in Fig. 3B. The EPC/DOPS monolayer is in the liquid condensed state (C_s^{-1} value slightly higher than 110 mN/m), with a maximum at a monolayer surface pressure of 30 mN/m. The incorporation of SN-38 into phospholipid monolayers causes a decrease of the C_s^{-1} at all the drug concentrations assayed, and changes gradually the monolayer state toward the liquid expanded phase, making it more compressible. Moreover, the surface pressure at which C_s^{-1} reaches the maximum value decreases down to 20 mN/m as the SN-38 mole ratio increases.

When analyzing together the effect of both camptothecins, the results obtained could be interpreted in terms of changes in the packing of the acyl chains of lipids induced by either CPT-11 or SN-38. The different location of CPT-11 and SN-38 within the liposome structure would account for the different effect of these drugs on the monolayer state.

3.4. Interaction of camptothecins with model lipid membranes

3.4.1. Miscibility analysis of mixed drug–lipid monolayers

The nature of the molecular interactions and also the miscibility of the components of the mixed monolayers, drug and lipids, can be examined by studying the deviations of the area per molecule in the mixed films with respect to the ideality in the context of the additivity rule, which relates the measured molecular area of the mixed drug–lipid film to the areas and mole fractions of the individual film components.

The plots of the average area per molecule of mixed drug–lipid films (DSPC/DOPS/CHOL/CPT-11 and EPC/DOPS/SN-38) versus the molar fraction of the drug, either CPT-11 or SN-38, at different surface pressures, can give information about the nature of the molecular interactions and, also, about the miscibility of the components in the film. A lineal dependence would indicate either ideal mixing of non-interacting molecules or complete immiscibility of two components [61], whereas, according to Costin and Barnes [62], a mixed monolayer would show non-ideal behavior, caused by significant molecular interactions, when its properties do not depend linearly on the monolayer composition. Thus, many authors have examined the area per molecule for a mixed monolayer as a function of its composition, at various surface pressures [63]. The mean molecular area of a monolayer with two components, in the case of an ideal mixture, can be calculated according to Eq. (4) of Supplementary material [64].

The experimental results are given in Fig. 4. The straight broken lines represent the ideal mixing behavior and the solid lines correspond to

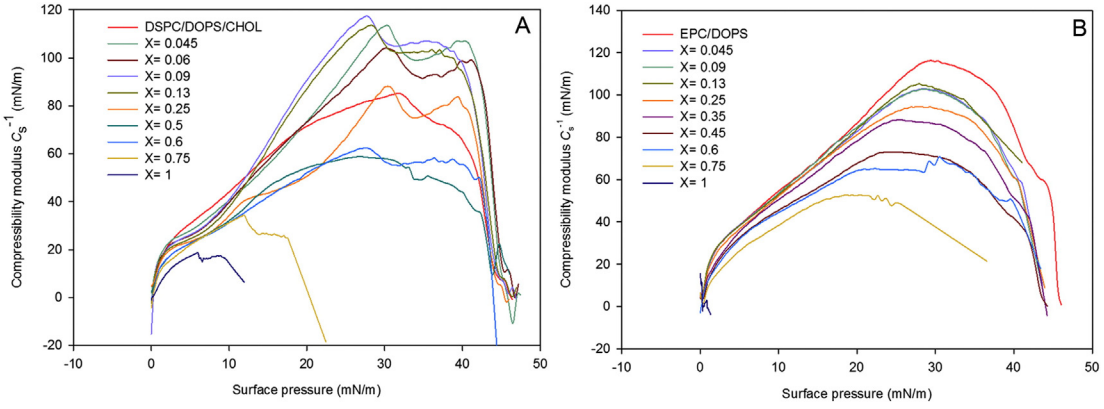

Fig. 3. The compression modulus (C_s^{-1}) of pure DSPC/DOPS/CHOL and mixed DSPC/DOPS/CHOL/CPT-11 (A) and pure EPC/DOPS and mixed EPC/DOPS/SN-38 (B) monolayers as a function of the surface pressure (π). x are the molar fractions of either CPT-11 (A) or SN-38 (B).

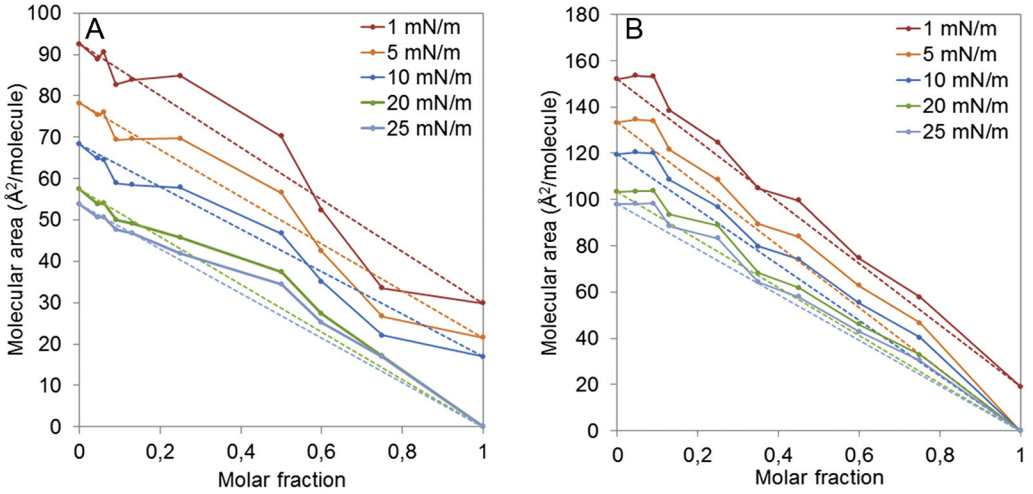

Fig. 4. Mean molecular areas for pure lipid and mixed lipid/drug monolayers as a function of camptothecin molar fractions at 25 °C and the indicated surface pressures. (A) Pure DSPC/DOPS/CHOL and mixed DSPC/DOPS/CHOL/CPT-11. (B) Pure EPC/DOPS and mixed EPC/DOPS/SN-38. The broken lines represent the ideal behavior and the solid lines correspond to the experimental values.

the experimental values and any deviation, positive or negative, from the ideal line would indicate some degree of molecular interactions between the drugs and lipids. In particular, positive deviations suggest that repulsive interactions occur with a perturbation of the regular packing of lipid molecules.

This would be the case of the effect of SN-38 on the monolayer of EPC/DOPS: the repulsive interactions between the drug and lipids would explain the decrease in the order degree of the acyl chains of lipids in a monolayer which becomes more compressible (compressibility analysis). On the contrary, negative deviations are indicative of the existence of increased attractive interactions between the two components in the mixed monolayers [65].

In the case of DSPC/DOPS/CHOL and CPT-11 mixtures, the data in Fig. 4A show the existence of positive and negative deviations from the ideality as a function of the drug molar fraction. It can be observed that the maximum positive deviation occurs at a mole fraction of CPT-11 of 0.5, that the maximum negative deviation is at 0.75, that the increases in the surface pressure up to 20 mN/m leads to lower deviations from the ideal line and that there are only positive deviations when surface pressures were ≥20 mN/m. These facts, together with the consideration of the collapse surface pressures, which do not change for mixtures with CPT-11 < 0.6, could be indicative of the immiscibility between the drug (CPT-11) and the lipid film components (DSPC/DOPS/CHOL) below this mole fraction, whereas for CTP-11 molar fractions higher than 0.6, the film formed would be stable. Moreover, in this range, the collapse pressures exhibit an intermediate value between those corresponding to the pure components individually. As indicated before, when surface pressure is higher than 20 mN/m, the system shifts to a more ideal behavior, being the positive deviations significantly decreased. This result could suggest that the high compression squeezes the CPT-11 molecules out of the lipid monolayer, with its consequent dissolution in the subphase, in agreement with the observations arising from the π–A compression isotherm experiments, and the loss of interaction with lipid molecules. If we also consider the values of the compressibility modulus (Fig. 3A) it would appear that the drug reaches its collapse pressure before and that the compression of lipids continues until their collapse. Thus, for the highest mole fraction of CPT-11 ($x_{CPT\text{-}11} = 0.75$, Fig. 3A) it can be observed a second inflection point at, approximately, 20 mN/m. The compressibility modulus values allow us to understand the miscibility behavior at low CPT-11 mole fractions ($x_{CPT\text{-}11} < 0.13$). In the range $0.045 < x_{CPT\text{-}11} < 0.13$ the system is ideal, with a very small negative deviation at 0.09–0.13 mole fractions, being Cs^{-1} values higher than those of the DSPC/DOPS/CHOL monolayer in this range. Probably CPT-11 and DSPC/DOPS/CHOL mixtures have a mixed miscibility pattern. They both mix well for $x_{CPT\text{-}11} \leq 0.13$ and $x_{CPT\text{-}11} \geq 0.6$, whereas become immiscible at any other mole fractions.

When EPC/DOPS and SN-38 mixtures are analyzed, it can be seen a greater alignment of the dashed lines and the solid lines and that the observed slight deviations are positive (Fig. 4B). This result suggests a mixed behavior close to the additivity rule predictions, unlike that observed for the CPT-11-containing system, indicating either ideal mixing or complete immiscibility of the mixture components. To distinguish between them it would be necessary to consider the isotherms at the collapse pressure. When analyzing together the collapse pressures and the compressibility moduli it can be observed that, despite its high hydrophobicity, the presence of SN-38 in the EPS/DOPS monolayer does not modify neither the shape of the isotherm nor its collapse pressure and, only at the highest mole fraction (x = 0.75), a slight change is produced. The fact that the collapse pressure of the lipid mixture does not change when adding SN-38 might be explained by considering that the molecules of SN-38, because of its apolar character, interact with the hydrophobic chains of lipids, the polar groups of which are in turn interacting with the subphase, and to the fact that it cannot be obtained a monolayer of SN-38. The high hydrophobicity of this drug prevents the spreading of the small drops within the air/water interface, avoiding the formation of the monolayer.

3.4.2. Stability of mixed monolayers

The interaction, either repulsive or attractive, between the two components of a mixed monolayer involves the generation of energy. This energy, known as excess Gibbs energy, G^E, represents the energy associated to the mixing process of the two pure components in the bidimensional phase and can be determined by means of Eq. (5) of Supplementary material [66,67]. The selected pressures (π) were 1, 2, 5 and 10 mN m^{-1}, which are values below the collapse of CPT-11.

The relevance of this study relies on the possibility of performing an analysis of the structural integrity of a system, which can incorporate certain drugs, through the quantitative assessment of the thermodynamic parameter G^E. If the system consists of lipids, and the drug is incorporated into a lipid monolayer, the assembly may be miscible and form a uniform mixed film or, conversely, be immiscible. This last situation would lead to the formation of a structure in which the drug and the lipids would be mixed heterogeneously, probably due to a selective interaction between the drug and some of the lipid constituents of the lipid monolayer [56]. These two situations will translate into different variations of G^E values: in the first case the excess Gibbs energy

should be negative, whereas, when immiscibility occurs, this parameter would have positive values [68].

The data in Fig. 5A show a complex behavior for DSPC/DOPS/CHOL/CPT-11 mixed monolayers, unlike a simple miscibility or immiscibility [69], although the energy values are in agreement with the partial miscibility deducted before from the analysis of Fig. 4A.

The minimal values observed below 0.13 and at 0.75 mole fractions of CPT-11 would account for the great thermodynamic stability of this drug and lipid combination. Additionally, the comparison of the absolute values of the excess Gibbs energy with RT (≈ 2500 J/mol), being R the ideal gas constant and T the experimental temperature, allows analyzing the relevance of such interactions, being significant for G^E values greater than 2500 J/mol [68,70]. A similar evaluation for SN-38-EPC/DOPS mixed monolayers (Fig. 5B), yielded absolute excess energy values very much lower than 2500 J/mol as it was expected according with the miscibility results.

3.5. Interaction studies with CPT-11 in the subphase

The dissolution of a drug in a subphase on which monolayers of lipids have been spread can help to determine the influence of these solutes on the compression isotherms of the lipids and give additional information about the drug–lipid affinity. In our study only the interaction between CPT-11 and lipids has been assessed by means this kind of experiment. The great insolubility of SN-38 has made impossible to introduce this drug in the subphase.

3.5.1. Penetration kinetics of CPT-11 at constant area

The ability of CPT-11 to penetrate into the DSPC/DOPS/CHOL monolayer at the air–water interface was investigated using a fixed 5.44 μM drug concentration in the lactate buffered subphase. The CPT-11 subphase concentration controls the penetration and can be previously determined from the adsorption isotherm profile for the drug. This value is a constant (K) that represents the drug concentration that achieves 1/2 π_{max}. In our case, the determination of the drug concentration to reach the equilibrium pressure and so, to determine the value of K, was not conclusive, probably because of the low surface activity of CPT-11 that hinders to monitor correctly the surface pressure changes with time. Because of this, we have chosen a CPT-11 concentration allowing the correct measurement of the changes in surface pressure in the penetration experiments.

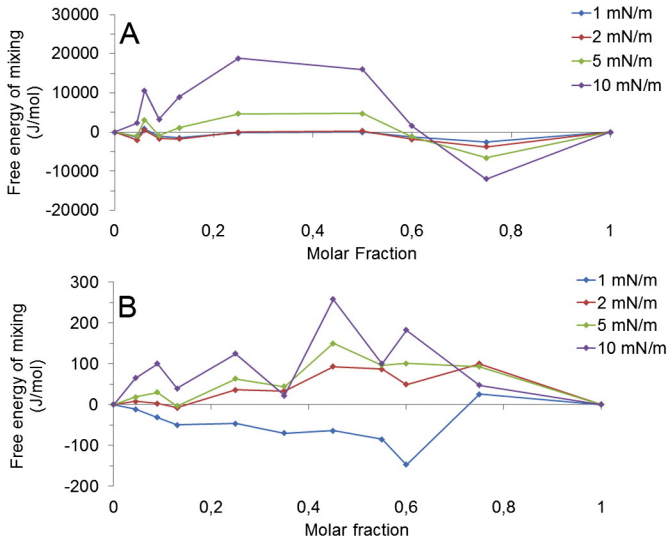

Fig. 5. The excess Gibbs energy (G^E) of DSPC/DOPS/CHOL/CPT-11 (A) and EPC/DOPS/SN-38 (B) mixed monolayers as a function of the monolayer composition, at different surface pressures.

The interaction between CPT-11 and mixed DSCP/DOPS/CHOL monolayers was recorded in terms of the increase of surface pressure for 30 min. It could be observed that CPT-11 insertion promoted an immediate increase in the surface pressure of the system, being remarkable the fact that the surface pressures attained the equilibrium values in no more than 10 min. A similar behavior has been reported for docetaxel insertion in DPPC monolayers [12]. The penetration curves obtained at various initial pressures are shown in Fig. 6.

As shown, these curves tend to an asymptote indicating the end of the penetration process. The graphical plot of the pressure increments vs the initial pressure, at a given drug concentration in the subphase (Fig. 6, upper image), is a straight line which intersection with the horizontal axis gives the critical pressure, above which there will be no penetration of CPT-11 into the lipid monolayer. This value was of ≈23–24 mN/m.

3.5.2. Compression isotherms with CPT-11 in the subphase

Compression isotherms of all the individual lipid components and, also, of the lipid mixture, were done in the absence and in the presence of CPT-11 in the subphase. This set of experiments was carried out in an attempt to go deeply into the potential selectivity of the interactions between the drug and each one of the constituents of the liposome bilayer. The results are plotted in Figs. 7 and S2 (Supplementary material).

When the phospholipid was DSPC, the isotherms recorded in the presence of CPT-11 were identical to that of the lipid alone at surface pressures higher than 25 mN/m, irrespective of the drug concentration, whereas at the lowest values of π (<20 mN/m), if any, a very tiny condensing effect could be observed (Fig. S2A, Supplementary material). In this case, it could be considered that CPT-11 does not modify the

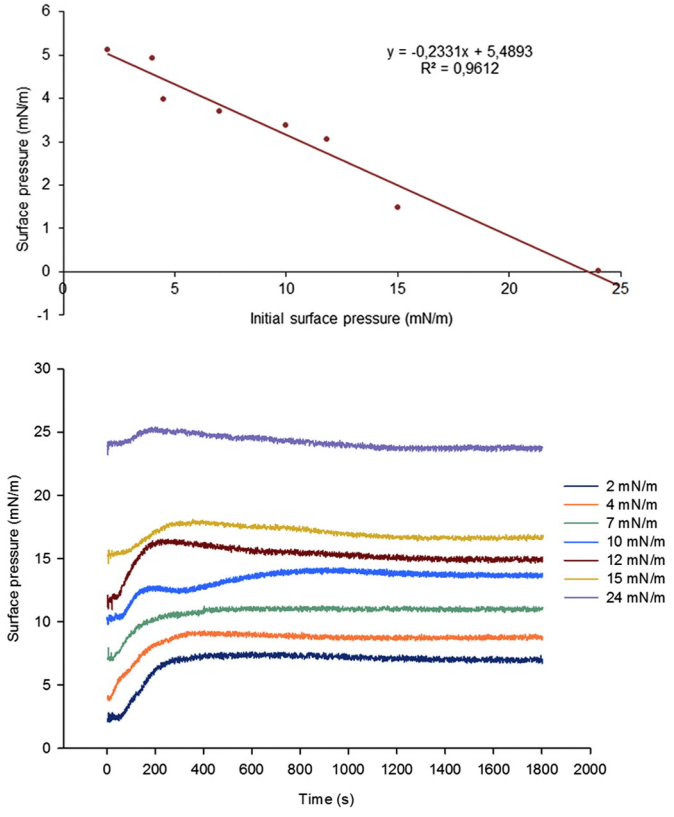

Fig. 6. Pressure increases promoted by CPT-11 when injected under DSPC/DOPS/CHOL monolayers spread at different initial pressures (lower image). The upper image is the linear plot of the increment in surface pressure after CPT-11 injection in the subphase as a function of the initial DSPC/DOPS/CHOL surface pressure and gives the critical surface pressure for CPT-11 penetration into the DSPC/DOPS/CHOL monolayer at the air–water interface.

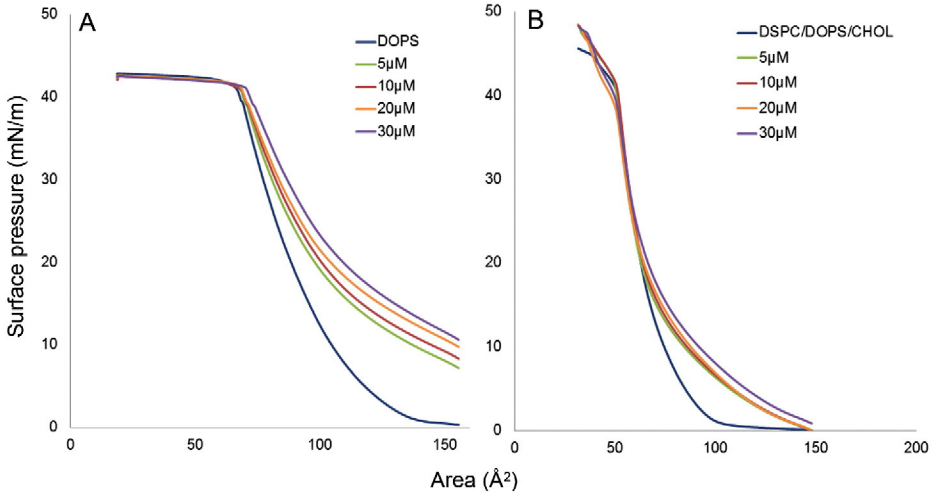

Fig. 7. Compression isotherms of (A) DOPS and (B) DSPC/DOPS/CHOL spread on a lactate buffered subphase alone or with increasing concentrations of CPT-11.

order of the lipid molecules nor establishes any specific interaction with DSPC. This fact was also observed in the case of CHOL (Fig. S2B, Supplementary material). Nevertheless, when the anionic phospholipid DOPS was considered (Fig. 7A), a completely different result was observed. DOPS isotherm was modified when CPT-11 was incorporated in the monolayer subphase, at all the concentrations assayed, and the changes were in the sense of producing an expanding effect within all the range of the surface pressures recorded, although more significant at low surface pressures: the result indicates the incorporation of the drug into the phospholipid monolayer. The interaction CPT-11-DOPS was confirmed by analyzing the effect of the incorporation of the drug in the subphase of a DSPC/DOPS/CHOL monolayer. The expanding effect of the drug was also manifest (Fig. 7B), but only at surface pressures lower than 20 mN/m, being the extent of the effect smaller than the measured in DOPS monolayers in accordance with the fact that the DSPC/DOPS/CHOL mixture only contains a 35% DOPS molar ratio. These results show that CPT-11 interacts specifically with the DOPS constituent of the lipid mixture.

The positive charge of CPT-11 at pH 4.4 and the negative charge of DOPS would account for the observed changes in the compression isotherms, on the basis of an electrostatic interaction. Taking into account the asymmetric distribution of the different lipids between the two leaflets of the bilayers [71] and the fact that the anionic PS is preferentially located in the inner monolayer [72], this result would explain the high percentage of CPT-11 encapsulated into liposomes. The drug encapsulated in their aqueous space, in contact with the inner monolayer, where PS is, will remain electrostatically anchored solving, somehow, the problems associated with the formulation and procurement of carriers for drug soluble molecules.

3.6. BAM images of the mixed CPT-11/lipid films

To get additional information about the influence of CPT-11 on the morphology of the investigated ternary mixed lipid monolayers, a series of BAM images, recorded at different stages of film compression, were analyzed [12,73]. Fig. 8 shows the images obtained at different surface pressures.

In the case of the DSPC/DOPS/CHOL film, the compression of the monolayer induces, according to its π–A isotherm, the formation of a LE phase which reflects in an uniform texture of images until 30 mN/m, when the monolayer becomes less compressible and a bright condensed phase that coexists at 40 mN/m near its collapse pressure. BAM images for CTP-11 demonstrate the ability of the drug to aggregate at the air/water interface at low pressures ($\pi = 3, 7$ mN/m).

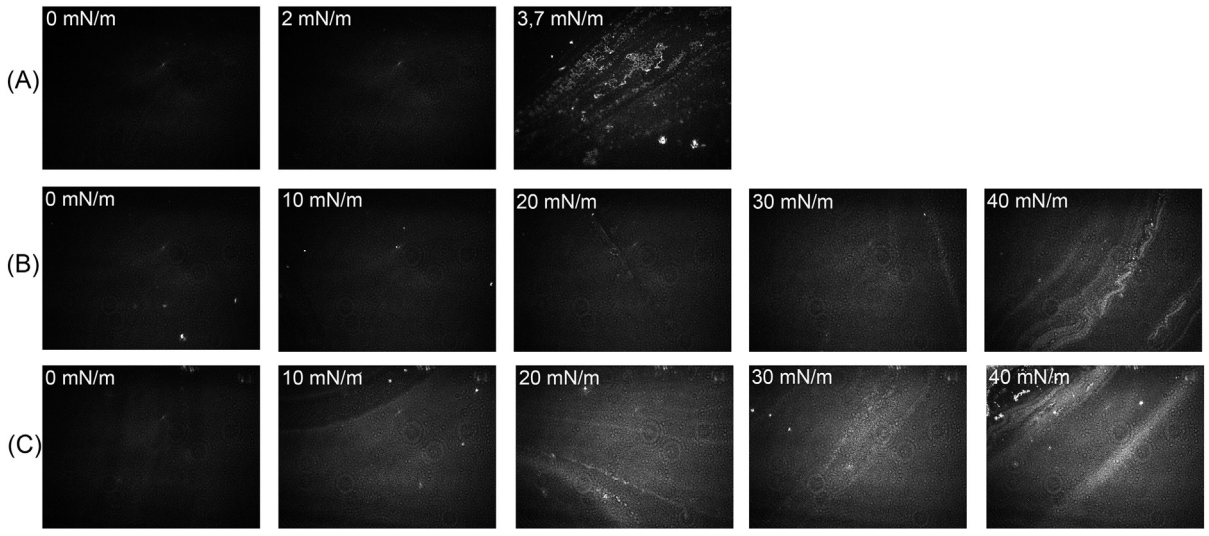

Fig. 8. BAM images of CPT-11 (A), DSPC/DOPS/CHOL (B) and mixed DSPC/DOPS/CHOL/CPT-11 (0.13 drug mole fraction) (C) Langmuir monolayers at the indicated surface pressures and composition. All the images were acquired at the same magnification for comparative purposes.

In the case of the mixtures of DSPC/DOPS/CHOL and CPT-11 (0.13 CPT-11 mole fraction), it can be observed the separation of two phases and the visualization of small nucleation points at a surface pressure of 10 mN/m (Fig. 8C) probably because of the drug, in agreement with the fact that the highest surface pressure that pure CPT-11 monolayer can reach on compression is <12 mN/m. Moreover, bright condensed phases when increasing the compression within the range of 20–40 mN/m (Fig. 8C) were observed, accordingly to the collapse region of the lipid/drug mixture. These phases appear much more marked than for DSPC/DOPS/CHOL monolayers, fact which could indicate that the molecules forming the film are packed more densely when the mole fraction of CPT-11 is 0.13. This result is the expected from the values of the excess area per molecule given in Fig. 4A. Bearing in mind that the properties of the lipid monolayers correlate with the properties of lipid bilayers at higher surface pressures (25 mN m^{-1}–35 mN m^{-1}) [74] it is worth stressing that, for this composition and at these pressures, the little condensation of the area per molecule and the squeezing out of CPT-11 from the lipid monolayer during compression correlate with the incorporation of CPT-11 into liposomes through the above mentioned electrostatic interactions between the drug and the anionic component of the bilayer.

4. Conclusions

The overall results have shown the ability of CPT-11 and SN-38 to interact with and/or to insert among phospholipid molecules in membrane mimetic models: CPT-11 is able to bind effectively to lipid bilayers through electrostatic interactions, whereas SN-38, because of its high lipophilicity, is inserted within the hydrophobic core of the bilayer.

The calorimetric study highlights that neither the incorporation of CPT-11 nor that of SN-38 disturbs the structure of the complex composited liposome bilayers, despite their different solubility properties and their different location within the two possible environments of the liposome structure.

CPT-11 shows surface activity and penetrates the DSPC/DOPS/CHOL monolayer whereas SN-38 does not. The high partition coefficient of SN-38, according with its great hydrophobicity, would explain that SN-38 neither adsorb at the air–water interface, nor form compressible monolayers. Similar results have been reported for some resveratrol and coumarin compounds [53,54].

CPT-11 and SN-38 cause a large contraction effect of the DSPC/DOPS/CHOL and EPC/DOPS Langmuir monolayers, respectively, as can be deduced from the values of the mean molecular area. This result could be explained by a high affinity of both camptothecins for monolayer lipids and would account for increased monolayer stability. On the other hand, the miscibility studies have shown that CPT-11 and DSPC/DOPS/CHOL mixtures mix well in a relatively narrow range of concentrations and that the EPC/DOPS/SN-38 combination exhibit a mixed behavior close to the additivity rule predictions.

The shape of the compression isotherms with CPT-11 in the subphase shows that the interaction between DSPC/DOPS/CHOL and CPT-11, with positive charge at pH 4.4, takes place mainly through the negative charge of DOPS and the miscibility pattern indicates that these interactions are possible at $x_{CPT\text{-}11} \leq 0.13$ and $x_{CPT\text{-}11} \geq 0.6$. This range, thermodynamically favored because their high excess Gibbs free energy values, includes the mole fractions used for the CPT-11 liposomal formulation ($x_{CPT\text{-}11} < 0.13$). Moreover, the existence of such electrostatic interactions would account for the high percentage of CPT-11 encapsulated into liposomes [41] in spite of its water-soluble character and its low n-octanol-water partition coefficient at pH 4.4. There are references in the literature in which CPT-11 is also efficiently encapsulated in liposomes, although neither the method nor the liposome lipid composition is the same [75].

Instead, SN-38 establishes repulsive interactions with the lipid molecules that, although weak, modify the compressibility of the bilayer without affecting significantly neither the collapse pressure of the lipid mixture nor the miscibility pattern of drug–lipid mixed monolayers with an ideal behavior. The decrease of the compressibility modulus and the consequent formation of a more fluid monolayer in the presence of SN-38 suggest the interaction between the drug and the binary EPC/DOPS system; the fact that SN-38 does not induce significant changes in the melting temperature of the EPC/DOPS bilayers, could suggest its localization in the outer hydrophobic zone of the bilayer, as it does paclitaxel when incorporated into DPPC bilayers [27].

Our results provide interesting remarks about the suitability of liposomes as devices for the delivery of camptothecins on the basis of stability criteria, highlight the usefulness of the ternary lipid composition for the delivery of CPT-11, reported previously [41] and supports the choice of the binary EPC/DOPS phospholipid mixture to get a stable formulation for SN-38. The preparation, characterization and evaluation of the efficacy in vitro of a liposomal formulation for SN-38 have already been carried out in our laboratory and the results will be published soon. The characterization of the interactions that occur between drugs and lipids is important when designing liposomal nanocarriers for the delivery of soluble molecules [76].

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgement

This study was funded by the Ministerio de Economía y Competitividad (CTQ2013-48767-C3-3-R) Spain and by the Department d'Universitats, Recerca i Societat de l'Informació (DURSI), Govern de la Generalitat de Cataluña (2009SGR-367). A. Casadó thanks to the University of Barcelona for a predoctoral fellowship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2015.12.007.

References

[1] C.J. Gerrits, M.J. De Jonge, J.H. Schellens, G. Stoter, J. Verweij, Topoisomerase I inhibitors: the relevance of prolonged exposure for present clinical development, Br. J. Cancer 76 (1997) 952–962.
[2] Y. Pommier, DNA topoisomerase I inhibitors: chemistry, biology and interfacial inhibition, Chem Rev. 109 (2009) 2894–2902, http://dx.doi.org/10.1021/cr900097c.
[3] M.H. Wu, B. Yan, R. Humerickhouse, M.E. Dolan, Irinotecan activation by human carboxylesterases in colorectal adenocarcinoma cells, Clin. Cancer Res. 8 (2002) 2696–2700.
[4] P. Tobin, S. Clarke, J.P. Seale, S. Lee, M. Solomon, S. Aulds, M. Crawford, J. Gallagher, T. Eyers, L. Rivory, The in vitro metabolism of irinotecan (CPT-11) by carboxylesterase and β-glucuronidase in human colorectal tumours, Br. J. Clin. Pharmacol. 62 (2006) 122–129, http://dx.doi.org/10.1111/j.1365-2125.2005.02477.x.
[5] S.T. Liew, L.Y. Yang, Design, synthesis and development of novel camptothecin drugs, Curr. Pharm. Des. 14 (2008) 1078–1097, http://dx.doi.org/10.2174/138161208784246180.
[6] V.P. Torchilin, Multifunctional, stimuli-sensitive nanoparticulate systems for drug delivery, Nat. Rev. Drug Discov. 13 (2014) 813–827, http://dx.doi.org/10.1038/nrd4333.
[7] R. García-Carbonero, J.G. Supko, Current perspectives on the clinical experience pharmacology, and continued development of the camptothecins, Clin. Cancer Res. 8 (2002) 641–661.
[8] A. Alimonti, A. Gelibter, I. Pavese, F. Satta, F. Cognetti, G. Feretti, D. Rasio, A. Vecchione, M. Di Palma, New approaches to prevent intestinal toxicity of irinotecan-based regimens, Cancer Treat. Rev. 30 (2004) 555–562, http://dx.doi.org/10.1016/j.ctrv.2004.05.002.
[9] S. Kruszewski, M. Cyrankiewicz, B. Ziomkowska, Biophysical properties of 10-hydroxy-camptothecin determined by fluorescence anisotropy measurements, Pol. J. Environ. Stud. 15 (2006) 69–71.

[10] A. Lansiaux, S. Léonce, L. Kraus-Berthier, C. Bal-Mahieu, R. Mazinghien, S. Didier, M.-H. David-Cordonnier, P. Hautefaye, G. Lavielle, C. Bailly, J.A. Hickman, A. Pierre, Novel stable camptothecin derivatives replacing the E-ring lactone by a ketone function are potent inhibitors of topoisomerase I and promising antitumor drugs, Mol. Pharmacol. 72 (2007) 311–319, http://dx.doi.org/10.1124/mol.107.034637.
[11] M.H. Ali, D.J. Kirby, A.R. Mohammed, Y. Perrie, Solubilisation of drugs within liposomal bilayers: alternatives to colesterol as a membrane stabilising agent, J. Pharm. Pharmacol. 62 (2010) 1646–1655, http://dx.doi.org/10.1111/j.2042-7158.2010.01090.x.
[12] A. Fernández-Botello, F. Comelles, M.A. Alsina, P. Cea, F. Reig, A monolayer study on interactions of docetaxel with model lipid membranes, J. Phys. Chem. 112 (2008) 13834–13841, http://dx.doi.org/10.1021/jp806423k.
[13] A.A. Argyriou, M. Koltzenburg, P. Polychronopoulos, S. Papapetropoulos, H.P. Kalofonos, Peripheral nerve damage associated with administration of taxones in patients with cancer, Critical Rev. Oncol./Hematol. 66 (2008) 218–228, http://dx.doi.org/10.1016/j.critrevonc.2008.01.008.
[14] J.C. Kraft, J.P. Freeling, Z. Wang, R.J.Y. Ho, Emerging research and clinical development trends of liposome and lipid nanoparticle drug delivery systems, J. Pharm. Sci. 103 (2014) 29–52, http://dx.doi.org/10.1002/jps.23773.
[15] S. Mura, J. Nicolas, P. Couvreur, Stimuli-responsive nanocarriers for drug delivery, Nat. Mater. 12 (2013) 991–1003, http://dx.doi.org/10.1038/nmat3776.
[16] K. Sen, M. Mandal, Second generation of liposomal cancer therapeutics: transition from laboratory to clinic, Int. J. Pharm. 448 (2013) 28–43, http://dx.doi.org/10.1016/j.ijpharm.2013.03.006.
[17] M. García-Díaz, M. Kawakubo, P. Mroz, M.L. Sagristá, M. Mora, S. Nonell, M.R. Hamblin, Cellular and vascular effects of the photodynamic agent temocene are modulated by the delivery vehicle, J. Control. Release 162 (2012) 355–363, http://dx.doi.org/10.1016/j.jconrel.2012.07.025.
[18] G. Gregoriadis, Overview of liposomes, J. Antimicrob. Chemother. Suppl. B (1991) 39–48, http://dx.doi.org/10.1093/jac/28.suppl_B.39.
[19] R. Langer, Drug delivery and targeting, Nature 392 (1998) 5–10.
[20] V.P. Torchilin, Recent advances with liposomes as pharmaceutical carriers, Nat. Rev. Drug Discov. 4 (2005) 145–160, http://dx.doi.org/10.1038/nrd1632.
[21] M. Slingerland, H.-J. Guchelaar, H. Gelderblom, Liposomal drug formulations in cancer therapy: 15 years along the road, Drug Discov. Today 17 (2012) 160–166, http://dx.doi.org/10.1016/j.drudis.2011.09.015.
[22] T.M. Allen, P.R. Cullis, Liposomal drug delivery systems: from concept to clinical applications, Adv. Drug Deliv. Rev. 65 (2013) 36–48, http://dx.doi.org/10.1016/j.addr.2012.09.037.
[23] Y. Fan, Q. Zhang, Development of liposomal formulations: from concept to clinical investigations, Asian J. Pharm. Sci. 8 (2013) 81–87, http://dx.doi.org/10.1016/j.ajps.2013.07.010.
[24] Y.H. Hsiang, R. Hertzberg, S. Hecht, L.F. Liu, Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I, J. Biol. Chem. 260 (1985) 14873–14878.
[25] Z. Mi, T.G. Burke, Differential interactions of camptothecin lactone and carboxylate forms with human blood components, Biochemistry 33 (1994) 10325–10336, http://dx.doi.org/10.1021/bi00200a013.
[26] Y. Sadzuka, H. Takabe, T. Sonobe, Liposomalization of SN-38 as active metabolite of CPT-11, J. Control. Release 108 (2005) 453–459, http://dx.doi.org/10.1016/j.jconrel.2005.08.023.
[27] L. Zhao, S.-S. Feng, M.L. Go, Investigation of molecular interactions between Paclitaxel and DPPC by Langmuir Film Balance and Differential Scanning Calorimetry, J. Pharm. Sci. 93 (2004) 86–98, http://dx.doi.org/10.1002/jps.10523.
[28] C. Peetla, A. Stine, V. Labhasetwar, Biophysical interactions with model lipid membranes: applications in drug discovery and drug delivery, Mol. Pharm. 6 (2009) 1264–1276, http://dx.doi.org/10.1021/mp9000662.
[29] A.M. Seddon, D. Casey, R.V. Law, A. Gee, R.H. Templer, O. Ces, Drug interactions with lipid membranes, Chem. Soc. Rev. 38 (2009) 2509–2519, http://dx.doi.org/10.1039/b813853m.
[30] C. Nunes, G. Brezesinski, D. Lopes, J.L.F.C. Lima, S. Reis, M. Lúcio, Lipid–drug interaction: biophysical effects of tolmetin on membrane mimetic systems of different dimensionality, J. Phys. Chem. B 115 (2011) 12615–12623, http://dx.doi.org/10.1021/jp206013z.
[31] M.G. Sarpietro, A. Di Sotto, M.L. Accolla, F. Castelli, Interaction of β-caryophyllene and β-caryophyllene oxide with phospholipid bilayers: differential scanning calorimetry study, Termochimica Acta. 600 (2015) 28–34, http://dx.doi.org/10.1016/l.tca.2014.11.029.
[32] S.-D. Clas, C.R. Dalton, B.C. Hancock, Differential scanning calorimetry: applications in drug development, Pharm. Sci. Technol. Today 2 (1999) 311–320.
[33] M.G. Sarpietro, M.L. Accolla, N. Santoro, F.M. Mansfeld, R. Pignatello, I. Toth, F. Castelli, Calorimetry and Langmuir–Blodgett studies on the interaction of a lipophilic prodrug of LHRH with biomembrane models, J. Colloid Interface Sci. 421 (2014) 122–131, http://dx.doi.org/10.1016/j.jcis.2014.01.040.
[34] B. Moghaddam, M.H. Ali, J. Wilkhu, D.J. Kirby, A.R. Mohammed, The application of monolayer studies in the understanding of liposomal formulations, Int. J. Pharm. 417 (2011) 235–244, http://dx.doi.org/10.1016/j.ijpharm.2011.01.020.
[35] P. Gill, T.T. Moghadam, B. Ranjbar, Differential scanning calorimetry techniques: applications in biology and nanoscience, J. Biomol. Tech. 21 (2010) 167–193.
[36] F. Castelli, M.G. Sarpietro, M. Ceruti, F. Rocco, L. Cattel, Characterization of lipophilic gemcitabine prodrug–liposomal membrane interactions by differential scanning calorimetry, Mol. Pharm. 3 (2006) 737–744, http://dx.doi.org/10.1021/mp060059y.
[37] F. Castelli, M.G. Sarpietro, F. Rocco, M. Ceruti, L. Cattel, Interaction of lipophilic gemcitabine prodrugs with biomembrane models studied by Langmuir–Blodgett technique, J. Colloid Interface Sci. 313 (2007) 363–368, http://dx.doi.org/10.1016/j.jcis.2007.04.018.
[38] M.C. Giuffrida, F. Dosio, F. Castelli, M.G. Sarpietro, Lipophilic prodrug of paclitaxel: interaction with a dimyristoylphosphatidylcholine monolayer, Int. J. Pharm. 475 (2014) 624–631, http://dx.doi.org/10.1016/j.ijpharm.2014.09.022.
[39] J.C. Dearden, G.M. Bresnen, The measurement of partition-coefficients, Quant. Struct.-Act. Relat. 7 (1988) 133–144, http://dx.doi.org/10.1002/qsar.19880070304.
[40] T. Braumann, Determination of hydrophobic parameters by reversed-phase liquid chromatography: theory, experimental techniques, and application in studies on quantitative structure-activity relationships, J. Chromatogr. 373 (1986) 191–225, http://dx.doi.org/10.1016/S0021-9673(00)80213-7.
[41] A. Casadó, M.L. Sagristá, M. Mora, Formulation and in vitro characterization of thermosensitive liposomes for the delivery of irinotecan, J. Pharm. Sci. 103 (2014) 3127–3138, http://dx.doi.org/10.1002/jps.24097.
[42] R.N.A.H. Lewis, B.D. Sykes, R.N. McElhaney, Thermotropic phase behavior of model membranes composed of phosphatidylcholines containing cis-monounsaturated acyl chain homologues of oleic acid: differential scanning calorimetric and 31P NMR spectroscopic studies, Biochemistry 27 (1988) 880–887, http://dx.doi.org/10.1021/bi00403a007.
[43] D. Bach, E. Wachtel, N. Borochov, G. Senisterra, R.M. Epand, Phase behaviour of heteroacid phosphatidylserines and cholesterol, Chem. Phys. Lipids 63 (1992) 105–113, http://dx.doi.org/10.1016/0009-3084(92)90027-M.
[44] M.H. Chiu, E.J. Prenner, Differential scanning calorimetry: an invaluable tool for a detailed thermodynamic characterization of macromolecules and their interactions, J Pharm. Bioallied Sci. 3 (2011) 39, http://dx.doi.org/10.4103/0975-7406.76463.
[45] W. Stillwell, An Introduction to Biological Membranes: From Lipid Bilayers to Lipid Rafts, Academic Press, San Diego (CA), 2013 117–129, http://dx.doi.org/10.1016/B978-0-444-52153-8.00008-8.
[46] J.C.M. Stewart, Colorimetric determination of phospholipids with ammonium ferrothiocyanate, Anal. Biochem. 104 (1980) 10–14, http://dx.doi.org/10.1016/0003-2697(80)90269-9.
[47] D.L. Ross, S.K. Elkinton, C.M. Riley, Physicochemical properties of the fluoroquinolone antimicrobials. III. 1-octanol/water partition coefficients and their relationships to structure, Int. J. Pharm. 88 (1992) 379–389, http://dx.doi.org/10.1016/0378-5173(92)90336-Z.
[48] OECD, Test No. 107: Partition Coefficient (n-octanol/water): Shake Flask Method, OECD Guidelines for the Testing of Chemicals, Section 1, OECD Publishing, Paris, 1995, http://dx.doi.org/10.1787/9789264069626-en.
[49] P. Dynarowicz-Łatka, A. Dhanabalan, O.N.J. Oliveira, Modern physicochemical research on Langmuir monolayers, Adv. Colloid Interf. Sci. 91 (2001) 221–293, http://dx.doi.org/10.1016/S0001-8686(99)00034-2.
[50] M.J. Sánchez-Martín, I. Haro, M.A. Alsina, M.A. Busquets, M. Pujol, A Langmuir monolayer study of the interaction of E1(145–162) hepatitis G virus peptide with phospholipid membranes, J. Phys. Chem. B. 114 (2010) 448–456, http://dx.doi.org/10.1021/jp906900k.
[51] J.C. Domingo, M. Mora, M.A. De Madariaga, The influence of N-acyl chain length on the phase behavior of natural and synthetic N-acylethanolamine phospholipids, Chem. Phys. Lipids 75 (1995) 15–25, http://dx.doi.org/10.1016/0009-3084(94)02397-N.
[52] Y. Tang, P.R. Czuczman, S.T. Chung, A.L. Lewis, Preservation of the active lactone form of irinotecan using drug eluting beads for the treatment of colorectal cancer metastases, J. Control. Release 127 (2007) 70–78, http://dx.doi.org/10.1016/j.jconrel.2007.12.012.
[53] M.G. Sarpietro, C. Spatafora, M.L. Acolla, O. Cascio, C. Tringali, F. Castelli, Effect of resveratrol-related stilbenoids on biomembrane models, J. Nat. Prod. 76 (2013) 1424–1431, http://dx.doi.org/10.1021/np400188m.
[54] M.G. Sarpietro, M.C. Giuffrida, S. Ottimo, D. Micieli, F. Castelli, Evaluation of the interaction of coumarins with biomembrane models studied by differential scanning calorimetry and Langmuir–Blodgett techniques, J. Nat. Prod. 74 (2011) 295-–790, http://dx.doi.org/10.1021/np100850u.
[55] S.-S. Feng, K. Gong, J. Chew, Molecular interactions between a lipid and an antineoplastic drug Paclitaxel (Taxol) within the lipid monolayer at the air/water interface, Langmuir 18 (2002) 4061–4070, http://dx.doi.org/10.1021/la011545p.
[56] M. Jurak, J. Miñones Conde, Characterization of the binary mixed monolayers of α-tocopherol with phospholipids at the air–water interface, Biochim. Biophys. Acta 1828 (2013) 2410–2418, http://dx.doi.org/10.1016/j.bbamem.2013.07.005.
[57] J.B. Rosenholm, P. Ihalainen, J. Peltonen, Thermodynamic characterization of Langmuir monolayers of thiolipids: a conceptual analysis, Colloids Surf. A 228 (2003) 119–130, http://dx.doi.org/10.1016/S0927-7757(03)00301-7.
[58] B. Térová, R. Heczko, J.P. Slotte, On the importance of the phosphocholine methyl groups for sphingomyelin/cholesterol interactions in membranes: a study with ceramide phosphoethanolamine, Biophys. J. 88 (2005) 2661–2669, http://dx.doi.org/10.1529/biophysj.104.058149.
[59] G.L. Gaines, Insoluble Monolayers at Liquid/gas Interfaces, Wiley-Interscience, New York, 1966.
[60] R. Maget-Dana, D. Lelièvre, Comparative interaction of α-Helical and β-Sheet amphiphilic isopeptides with phospholipid monolayers, Biopolymers 59 (2001) 1–10, http://dx.doi.org/10.1002/1097-0282(200107)59.
[61] D.K. Chattoraj, K.S. Birdi, Adsorption and the Gibbs Surface Excess, Plenum Press, New York, 1984 219–223.
[62] I.S. Costin, G.T. Barnes, 2-Component monolayers. 2. Surface pressure-area relations for octadecanol-docosyl sulfate system, J. Colloid Interface Sci. 51 (1975) 106–121, http://dx.doi.org/10.1016/0021-9797(75)90088-0.
[63] M. Pugelli, G. Gabrielli, Behavior of vinyl-polymers in mixed monolayers, J. Colloid Interface Sci. 6 (1977) 420–427, http://dx.doi.org/10.1016/0021-9797(77)90459-3.
[64] A.W. Adamson, Physical Chemistry of Surfaces, John Wiley and Sons, New York, 1990.

[65] M.J.G. Ruiz, M.A.C. Vílchez, A study of the miscibility of bile components in mixed monolayers at the air–liquid interface. 1. Cholesterol, lecithin and lithocholic acid, Colloid Polym. Sci. 269 (1991) 77–84, http://dx.doi.org/10.1007/BF00654662.
[66] R.E. Pagano, N.L. Gershfeld, Physical chemistry of lipid films at the air–water interface. II. Binary lipid mixtures. The principles governing miscibility of lipids in surfaces, J. Phys. Chem. 76 (1972) 1238–1243, http://dx.doi.org/10.1021/j100653a002.
[67] A. Villares, S. Martín, I. Giner, J. Díaz, D.P. Lydon, P.J. Low, P. Cea, The use of scanning polarization force microscopy to study the miscibility of a molecular wire candidate and an insulating fatty acid in mixed LB films, Soft Matter 4 (2008) 1508–1514, http://dx.doi.org/10.1039/b716798a.
[68] A. Eftaiha, M.F. Paige, The influence of salinity on surfactant miscibility in mixed dipalmitoylphosphatidylcholone–perfluorooctadecanoic acid monolayer films, J. Colloid Interface Sci. 353 (2011) 210–219, http://dx.doi.org/10.1016/j.jcis2010.09.045.
[69] F. Bordi, C. Cametti, F. De Luca, T. Gili, D. Gaudino, S. Sennato, Charged lipid monolayers at the air–solution interface: coupling to polyelectrolytes, Colloids Surf. B: Biointerfaces 29 (2003) 149–157, http://dx.doi.org/10.1016/S0927-7765(02)00185-6.
[70] S. Pérez-López, M. Nieto-Suárez, C. Mestres, M.A. Alsina, I. Haro, N. Vila-Romeu, Behaviour of a peptide sequence from the GB virus/hepatitis virus E2 protein in Langmuir monolayers: its interaction with phospholipid model membranes, Biophys. Chem. 141 (2009) 153–162, http://dx.doi.org/10.1016/j.bpc.209.01.007.
[71] A.J. Verkleij, R.F.A. Zwaal, B. Roelofsen, P. Comfurius, D. Kastelijn, L.L.M. van Deenen, The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy, Biochim. Biophys. Acta 323 (1973) 178–193, http://dx.doi.org/10.1016/0005-2736(73)90143-0.
[72] A. Yamaji-Hasegawa, M. Tsujimoto, Asymmetric distribution of phospholipids in biomembranes, Biol. Pharm. Bull. 29 (2006) 1547–1553, http://dx.doi.org/10.1248/bpb.29.1547.
[73] A. Vila-Romeu, M. Nieto-Suárez, P. Dynarowicz-Latka, Miscibility of Gramicidin - ethyl nonadecanoate in Langmuir monolayers in the presence of salts dissolved in the subphase, J. Phys. Chem. B 109 (2005) 14965–14970, http://dx.doi.org/10.1021/jp014092.
[74] D. Marsh, Lateral pressure in membranes, Biochim. Biophys. Acta Rev. Biomembr. 1286 (1996) 183–223, http://dx.doi.org/10.1016/S0304-4157(96)00009-3.
[75] Liposomal irinotecan: formulation development and therapeutic assessment in murine xenograft models of colorectal cancer, Clin. Cancer Res. 10 (2004) 6638–6649, http://dx.doi.org/10.1158/1078-0432.CCR-04-0221.
[76] J. Oliveira Eloy, M. Claro de Souza, R. Petrilli, J.P. Abriata Barcelos, R.J. Lee, J. Maldonado Marchetti, Liposomes as carriers of hydrophilic small molecule drugs: strategies to enhance encapsulation and delivery, Colloids Surf. B: Biointerfaces 123 (2014) 345–363, http://dx.doi.org/10.1016/j.colsurfb.2014.09.029.