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Biophysical study of the non-steroidal anti-inflammatory drugs (NSAID) ibuprofen, naproxen and diclofenac with phosphatidylserine bilayer membranes

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) represent an effective pain treatment option and therefore one of the most sold therapeutic agents worldwide. The study of the molecular interactions responsible for their physiological activity, but also for their side effects, is therefore important. This report presents data on the interaction of the most consumed NSAIDs (ibuprofen, naproxen and diclofenac) with one main phospholipid in eukaryotic cells, dimyristoylphosphatidylserine (DMPS). The applied techniques are Fourier-transform infrared spectroscopy (FTIR), with which in transmission the gel to liquid crystalline phase transition of the acyl chains in the absence and presence of the NSAID are monitored, supplemented by differential scanning calorimetry (DSC) data on the phase transition. FTIR in reflection (ATR, attenuated total reflectance) is applied to record the dependence of the interactions of the NSAID with particular functional groups observed in the DMPS spectrum such as the ester carbonyl and phosphate vibrational bands. With Förster resonance energy transfer (FRET) a possible intercalation of the NSAID into the DMPS liposomes and with isothermal titration calorimetry (ITC) the thermodynamics of the interaction are monitored. The data show that the NSAID react in a particular way with this lipid, but in some parameters the three NSAID clearly differ, with which now a clear picture of the interaction processes is possible.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are a wide heterogeneous group of compounds that have been extensively used for decades as very effective pain-killers [1]. They are medicated for numerous diseases, including arthritis, osteoarthritis and musculoskeletal disorders [2]. NSAIDs are among the most frequently used therapeutic agents not only by prescription [3], but many of them are available over-the counter allowing for self-medication. Since the acid forms of the drugs are poorly soluble in water they are sold as sodium salts to improve their bioavailability. NSAIDs mechanism of action is based on the inhibition of cyclooxygenase (COX) pathway, blocking the production of prostaglandins (PGs) [4]. There are three COX isoforms, but only two are related with the biological activity of NSAIDs. COX-1 is a constitutive enzyme that synthesizes PGs that protects the stomach lining and intestine; COX-2 is induced by cytokines, mitogens, and endotoxins [5], and it is related with the production of PGs associated with inflammation. The therapeutic effects of NSAIDs are due to the inhibition of COX-2 enzyme; the most serious side effects of the NSAIDs such as gastrointestinal injure, kidney and liver damage [6,7] are related to the COX-1 inhibition [8].

Several studies have shown that regular use of NSAIDs reduces the risk of colorectal, breast, gastric and esophageal cancer [9–13], suggesting that anti-inflammatory drugs prevent the malignant cell formation and therefore tumor progression [13–15]. The proposed mechanism behind this chemopreventive property is mainly associated with COX-2 overexpression in tumors; therefore, as COX-2 inhibitors these molecules indirectly control tumor genesis. It has been suggested that NSAIDs should be tested in combination with chemotherapy and radiotherapy to potentiate the anti-tumor effect. Although most of the biological activities of NSAIDs are related with COX-dependent mechanisms, several studies

Abbreviations: ATR, attenuated total reflection; COX, cyclooxygenase; DMPS, dimyristoylphosphatidylserine; DSC, differential scanning calorimetry; FRET, Förster resonance energy transfer spectroscopy; FTIR, Fourier-transform infrared spectroscopy; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; NSAID, non-steroidal anti-inflammatory drugs; PG, prostaglandin.

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have demonstrated that NSAIDs interact with membrane phospholipids in a COX-independent path that could be involved in their biological activity [16–25].

Characterization of drug-phospholipid interactions is fundamental to understand the molecular mechanism behind the physiological activity. Biological membranes are highly complex systems; artificial model membranes are simpler systems in terms of parameters that can be controlled like size, concentration and composition. The most studied models are liposomes, which are spherical lipid bilayer microstructures built of phospholipids that structurally look like biologic cell membranes. Previous studies have reported interactions of NSAIDs with other lipid components of eukaryotic cell membranes [16,17,23,26]; however, as there are no previous reports about NSAIDs interactions with phosphatidylserines (PS), the aim of this study was to investigate the interaction of three of the most widely prescribed NSAIDs, ibuprofen, naproxen and diclofenac (Fig. 1) with dimyristoylphosphatidylserine (DMPS) liposomes. Phosphatidylserine is an anionic phospholipid essentially located in the inner leaflet of eukaryotic cell membranes [27,28]. Alterations of the PS lipid composition, and therefore asymmetry, have been detected under several pathological conditions like apoptosis [29], necrosis [30], cell aging [31], intercellular fusion of myoblasts [32], cell degranulation [33] and malignant cell formation [34]. Furthermore, since the action of the COX molecules is a membrane step initiated from the cytoplasmic side of the cells, a direct interaction of the COX and the inhibition by the NSAID with PS should be physiologically relevant. In the present study, Fourier-transform infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC), Förster resonance energy transfer spectroscopy (FRET) and isothermal titration calorimetry (ITC) were used to characterize the structural effects induced by the three NSAIDs on DMPS liposomes.

2. Material and methods

2.1. Sample preparations

Synthetic DMPS (Lot 14OPS-76, MW 701.8) from Avanti Polar Lipids (Alabaster, AL, USA), sodium ibuprofen (Lot 085K0716, MW 228.3), sodium naproxen (Lot 1241487, MW 252.2) and sodium diclofenac (Lot0751896, MW 318.1) from Sigma (St. Louis, MO, USA) were used without further purification. The lipid samples were prepared as aqueous dispersions in HEPES buffer (20 mM, pH 7.4) at different concentration depending on the sensitivity of the technique: 20–40 mM for FTIR-experiments, 0.4 mM for DSC, 0.1 mM for ITC and 0.1 mM for FRET. In all cases, the lipid was suspended directly in buffer, sonicated, and temperature-cycled several times between 5 °C and 70 °C and then stored overnight at 4 °C before measurements. The drugs were dissolved in buffer and added to the phospholipid liposomes at given molar ratios; the measurements were carried out with freshly prepared mixtures.

2.2. Fourier-transform infrared spectroscopy/attenuated total reflectance (FTIR/ATR)

Infrared spectroscopic measurements were performed on an IFS-55 spectrometer (Bruker, Karlsruhe, Germany) with a MCT (Mercury-Cadmium-Tellurid) detector. For phase transition measurements in transmission, the lipid samples were placed in a CaF_2 cuvette with a 12.5 μm Teflon spacer. Temperature-scans were performed automatically between a low initial temperature (−20 °C to +10 °C) and 70 °C with a heating rate of 0.6 °C min^{-1}. For measurement of hydrated lipid samples in reflection using an attenuated total reflection (ATR) device, they were spread on a germanium plate, and free water was evaporated. For all measurements, 50 interferograms were accumulated, apodized, Fourier-transformed, and converted to absorbance spectra. For the evaluation of the gel to liquid crystalline phase transition, the peak position of the symmetric stretching vibration of the methylene band ν_s (CH_2) around 2850 cm^{-1} was used as it is a sensitive marker of lipid order [35,36]. Furthermore, vibrational bands from the interface region (ester carbonyl stretch around 1725 to 1740 cm^{-1}), and the head group (carboxylate antisymmetric stretching band at 1640 to 1620 cm^{-1} and NH_3^+ antisymmetric bending at 1630 to 1620 cm^{-1}) were analyzed. The instrumental wavenumber resolution was better than 0.02 cm^{-1}, and the wavenumber reproducibility in repeated scans was better than 0.1 cm^{-1}.

2.3. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) measurements were performed in a VP-DSC calorimeter (MicroCal, Inc., Northampton, MA, USA) at a heating and cooling rate of 1 °C·min^{-1}. The DSC samples were prepared by dispersing a known amount of DMPS in 10 mM PBS buffer at pH 7.4, and hydrated in the liquid crystalline phase by vortexing. Prior to the measurements, the samples were stored for a defined time at 4 °C. The measurements were performed in the temperature interval from 5 °C to 65 °C. Temperature ranges are shown in Results only when phase transitions were observed. Five consecutive heating and cooling scans checked the reproducibility of the DSC experiments of each sample [37]. The accuracy of the DSC experiments was ±0.1 °C for the main phase transition temperatures, and ±1 kJ mol^{-1} for the main phase transition enthalpy. DSC data were analyzed using the Origin software, and the phase transition enthalpy was obtained by integrating the area under the heat capacity curve [38].

2.4. Isothermal titration calorimetry (ITC)

Microcalorimetric experiments of drug binding to DMPS were performed on a MCS isothermal titration calorimeter (Microcal Inc., Northampton, MA, USA) at various temperatures. Phospholipid samples in a

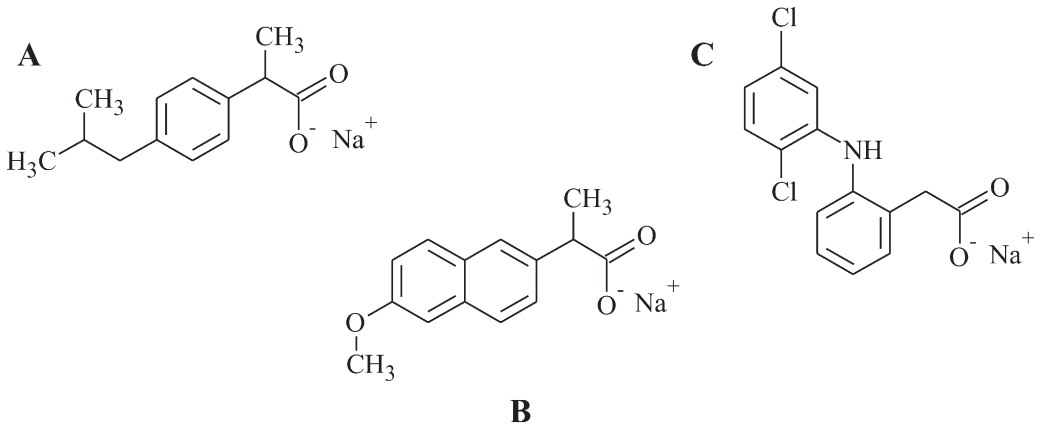

Fig. 1. Chemical structures of A) ibuprofen, B) naproxen, and C) diclofenac sodium salts.

range concentration from 0.05 to 0.15 mM, prepared as described above were filled into the microcalorimetric cell (volume 1.3 ml); the NSAIDs, in the concentration range of 0.5 to 5 mM (pH 7), were filled into the syringe compartment (volume 100 μl), each after thorough degassing of the suspensions. After thermal equilibration, aliquots of 3 μl of the NSAID solution were added every 5 min into the lipid-containing cell, which was constantly stirred, and the heat of interaction after each injection measured by the ITC instrument was plotted versus time. The total heat signal from each experiment was determined as the area under the individual peaks and plotted versus the [NSAID]:[lipid] molar ratios. Since the instrument works in temperature equilibrium at a constant "current feedback" corresponding to a power of approximately 74 μW, the occurrence of an exothermic reaction leads to a lowering of this current and of an endothermic reaction to an increase. All titration measurements, performed at constant temperatures, were repeated at least two times. As control, the drugs were titrated into pure buffer and the dilution enthalpy was subtracted.

2.5. Förster resonance energy transfer (FRET) spectroscopy

Intercalation of NSAIDs into DMPS liposomes (0.01 mM) was determined in 20 mM HEPES, pH 7.4 at three different temperatures (below, at and above the main phase transition temperature) by FRET spectroscopy applied as a probe dilution assay [39,40]. The drugs were added to the lipid aggregates, which were labelled with the donor dye NBD-phosphatidylethanolamine (NBD-PE) and the acceptor dye rhodamine-PE in a 100:1 M ratio (lipid:fluorophore). Intercalation was monitored as the increase of the ratio of the donor fluorescence intensity I_D at 531 nm to that of the acceptor intensity I_A at 593 nm (FRET signal, I_D/I_A) in dependence on time.

3. Results

3.1. Fourier-transform infrared spectroscopy/attenuated total reflectance (FTIR/ATR)

FTIR spectroscopy is a versatile and highly informative technique extensively used for studying interactions of bilayer membranes at the water-lipid interface [41–44]. It provides detailed information about the lipid functional groups in hydrated conditions, and the changes induced by exogenous molecules on the dynamics of the membrane bilayer. Each vibration of the phospholipid-molecule group(s) has a characteristic frequency, absorption maximum and width. The interaction with an exogenous molecule induces changes in the spectroscopic parameters because the frequency depends on the nature and interactions of the atoms involved. FTIR spectra of DMPS liposomes were analyzed in the following regions: phosphate asymmetric stretching vibration PO_2^- (1220–1260 cm^{-1}), carbonyl ester stretching mode (1685–1780 cm^{-1}), and the headgroup (carboxylate antisymmetric stretching band at 1640 to 1620 cm^{-1} and NH_3^+ antisymmetric bending at 1620 to 1600 cm^{-1}). They were selected because of potential binding sites (e.g., by electrostatic interaction) and/or affected by hydration, and/or conformational changes. The symmetric CH_2 stretching vibration (2850–2855 cm^{-1}) was monitored as a parameter associated with the fluidity of DMPS acyl chains. A complete spectrum of pure DMPS and its characteristic vibrational bands is shown in Fig. 2. The asymmetric stretching vibration of the phosphate moiety (ν_{as} PO_2^-) has been extensively studied [45–47] as hydration sensor in the headgroup region of the lipids. The $\nu_{as}PO_2^-$ band shifted from 1250 cm^{-1} for low hydrated to 1220 to 1225 cm^{-1} for highly hydrated PS bilayers, caused by the water molecules hydrogen bonded to the charged phosphate group [48]. For pure DMPS the ν_{as} PO_2^- band was present at 1221 cm^{-1} in accordance to previous result by Bach et al. [30] and Lewis et al. [49]. The region of the spectra of the mixtures of the three NSAIDs with DMPS are shown in Fig. 3, the analysis of the results after the curve fitting revealed that ibuprofen induces a shift in the $\nu_{as}PO_2^-$ band of the phospholipid to

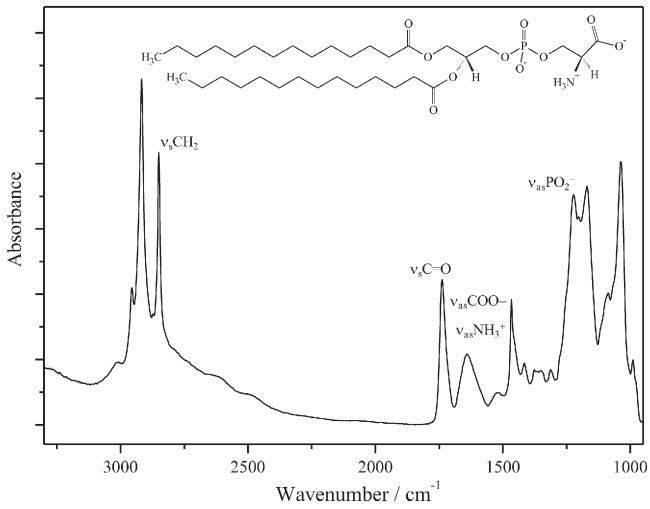

Fig. 2. FT-IR ATR spectrum of 10 mM hydrated film of DMPS in HEPES buffer at pH 7.4, 20 °C.

1211 cm^{-1}, while the interaction of naproxen and diclofenac did not affect the PO_2^- vibration.

The carbonyl stretching vibration of phospholipids is an additional sensitive sensor for the hydration of the lipid interface region, polarity, conformation changes and nature of the hydrogen bonding interactions in the lipid–water interface [45,50]. The C═O vibration for DMPS lead to the splitting of the group vibration into two different bands at 1720–1725 cm^{-1} and 1740–1743 cm^{-1} attributable to subpopulations of hydrogen-bonded and free ester carbonyl groups, respectively [51,52]. Our results showed a smaller band component at 1720 cm^{-1} and a larger one at 1738 cm^{-1}. The carbonyl region is shown in Fig. 4; the analysis of results showed a reduction in the intensity of the hydrogen-bonded component by the presence of ibuprofen. The presence of diclofenac and naproxen does not alter the spectroscopic parameters of the carbonyl band. The negatively charged carboxylate in the serine moiety is an additional site for drug binding and sensitive to hydration/dehydration effects. The same is true for the $-NH_3^+$ symmetric bending band of the serine moiety identified at 1608 cm^{-1} in accordance to previous results by Miller et al. [54]. The antisymmetric stretching vibration of the $-COO^-$ group changes from 1640 cm^{-1} in

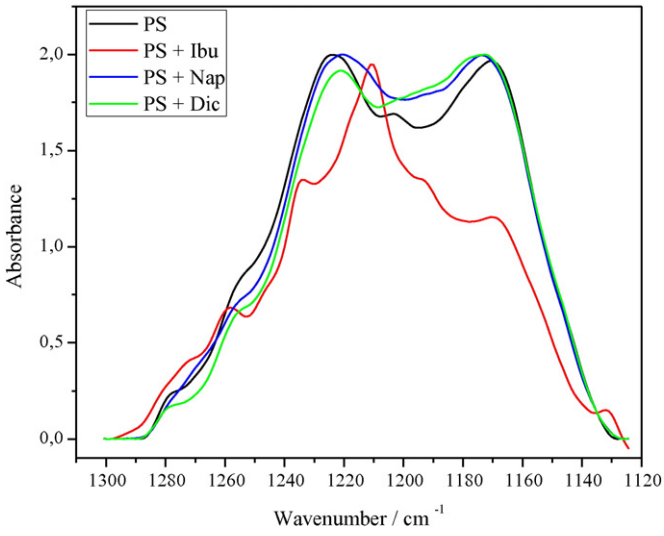

Fig. 3. Infrared spectra of the PO_2^- asymmetric vibration region of – DMPS, — DMPS:ibuprofen, — DMPS:naproxen, — DMPS:diclofenac. The experiments were performed at 1:0.5 lipid:drug molar ratios and at room temperature (18–20 °C).

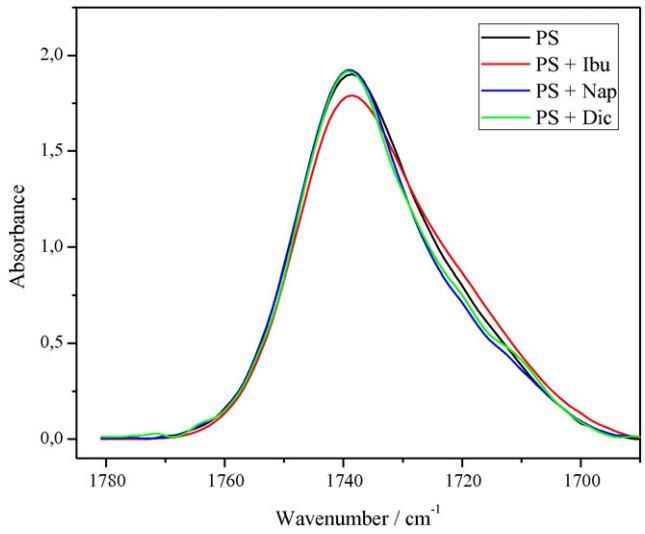

Fig. 4. Infrared spectra of the C=O stretching region of – DMPS, — DMPS:ibuprofen, — DMPS:naproxen, — DMPS:diclofenac. The experiments were performed at 1:0.5 lipid:drug molar ratios and at room temperature (18–20 °C).

the dry state to 1620–1625 in hydrated conditions. Our results confirmed the presence of the band at 1624 cm^{-1} for the PS:Nap sample, which indicated a hydrated environment surrounding the group as reported by Dluhy et al. [53] (Fig. 5). For PS alone and the other two samples this band is only weakly expressed as shoulder of the broad water bending vibration at 1645 cm^{-1}. The curve fitting results showed that the presence of ibuprofen and diclofenac does not affect the COO^- vibration mode. The analysis of the results via curve fitting demonstrated that the interaction of ibuprofen and naproxen with DMPS induced a shift in the δ_{as} $-NH_3^+$ band to slightly above and below 1600 cm^{-1}, respectively. There was no shift in the band for the DMPS:diclofenac mixtures, which gives important evidence that the drug is not interacting with the $-NH_3^+$ group.

The use of FT-IR to study the conformation of the lipid hydrocarbon acyl chains is one of the most common applications of this technique, in particular to follow the gel to liquid-crystalline phase transition due to the temperature dependence of the wavenumber of the CH_2 symmetric stretching mode. The peak positions of the methylene stretching modes

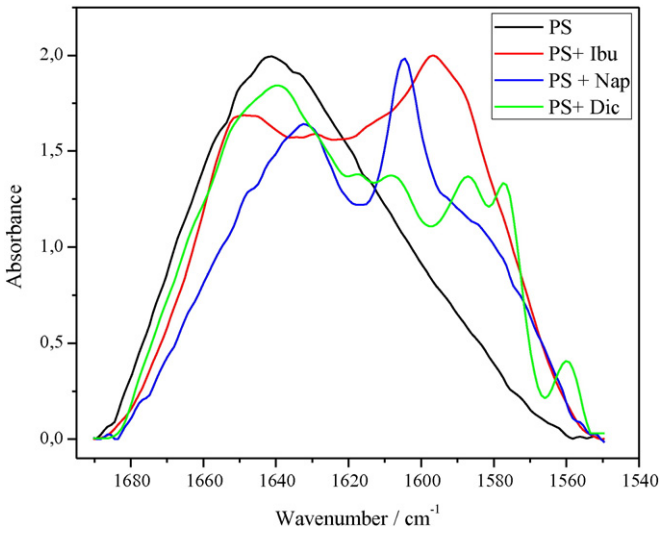

Fig. 5. Infrared spectra of the COO^- asymmetric stretching and NH_3^+ stretching band of DMPS, — DMPS:Ibuprofen, — DMPS:naproxen, — DMPS:Diclofenac. The experiments were performed at 1:0.5 lipid:drug molar ratios and at room temperature (18–20 °C).

($\nu_s CH_2$) in each phase of the lipids have different values: in the gel phase $\nu_s(CH_2)$ lies at 2850 cm^{-1} and in the liquid crystalline phase around 2852 cm^{-1} to 2853 cm^{-1}. At the main phase transition temperature (T_m) the lipid undergoes a conversion from the gel phase to the liquid crystalline phase because of the melting process of the hydrocarbon chain. The T_m has a characteristic value for each phospholipid depending of the length of the acyl chains [55] and the structure of the head groups [56]. The interaction or insertion of molecules into the bilayer increases the space between the fatty acids causing an increase in their mobility of the hydrocarbon chains and their frequency. Therefore, the frequency of this band as a function of the temperature is a recognized parameter to study changes in the lipid order and packing arrangement [35,36]. Fig. 6a-c shows the temperature dependence of the wavenumber values of the peak positions for DMPS acyl chains in the presence of the three NSAIDs under study. The interaction of NSAIDs with DMPS induced a shift in the T_m of the lipid (up to ca. 4 °C for ibuprofen, ca. 5 °C for naproxen and ca. 7 °C for diclofenac). For the DMPS:ibuprofen system, first a minor decrease in T_m is observed, which is in accordance with the calorimetric results. At a DMPS to ibuprofen molar excess ratio of 1:2, a more pronounced T_m decrease of ca. 4 °C is detected. A slight fluidization of the acyl chains is evident for naproxen and diclofenac from the increase in the wavenumbers of the $\nu_s CH_2$ band at fixed temperature. The fluidization effect is more pronounced for diclofenac and it might give evidence of the destabilizing effect of the drug on the lipid systems.

3.2. Differential scanning calorimetry (DSC)

Calorimetric analyses are performed in order to determine the thermotropic phase behavior (T_m) and the energetic of the lipid phase transitions, i.d., it was used to detect interactions between the NSAIDs and the phospholipid. The analysis was performed for pure DMPS and for mixtures of DMPS and the drugs at different molar ratios. The influence of the NSAIDs on the thermotropic transitions of DMPS liposomes is presented in Fig. 7a-d. In the temperature range of 20–50 °C, hydrated DMPS liposomes dispersed in PBS buffer at pH 7.4 underwent a sharp main transition at 36 °C with a corresponding phase transition enthalpy $\Delta H_m = 32.2$ kJ mol^{-1} [49]. This transition corresponds to the conversion of the gel phase (L_β) to the lamellar liquid-crystal (L_α) phase. Fig. 7a shows the heating thermograms obtained with different DMPS:ibuprofen mixtures. Increasing concentrations of ibuprofen produced a broadening of the main phase transition peak without a significant change of the maximum of the heat capacity curve (T_m). However, ΔH decreased up to 28.7 kJ mol^{-1} from the lowest molar ratio (1:0.1), when ibuprofen was added above 1:2 M ratio, the main transition temperature was shifted only 0.5 °C to lower values, and ΔH decreased up to up to 19 kJ mol^{-1} (Table 1). The experiments with naproxen did not produce strong shifts in the phase transition temperature (Fig. 7b), or in the enthalpy change associated with the L_β/L_α phase transition of the lipid. However, the thermogram of DMPS:naproxen at 1:0.1 M ratio exhibited a shoulder above the main transition temperature (at 37.5 °C), indicative of a possible phase separation. With increasing concentrations of naproxen a broadening effect of the main phase transition peak took place. Similar effects were observed for DMPS:diclofenac mixtures; at the lowest molar ratio (1:0.1) the presence of a very small phase transition ($\Delta H < 1$ kJ mol^{-1}) above the T_m was detected (Fig. 7c). At a 1:0.5 M ratio a small phase transition around 44 °C was observed (Fig. 7d), which was also present at higher molar ratios. These results suggested that the three NSAIDs were able to influence the cooperativity and therefore the thermotropic behavior of the lipid.

3.3. FRET (Förster resonance energy transfer) spectroscopy

FRET spectroscopy was applied in order to determine a possible intercalation of the NSAIDs into DMPS liposomes by using liposomes fluorescently labelled by NBD-PE (donor) and RhoPE (acceptor) dyes. With

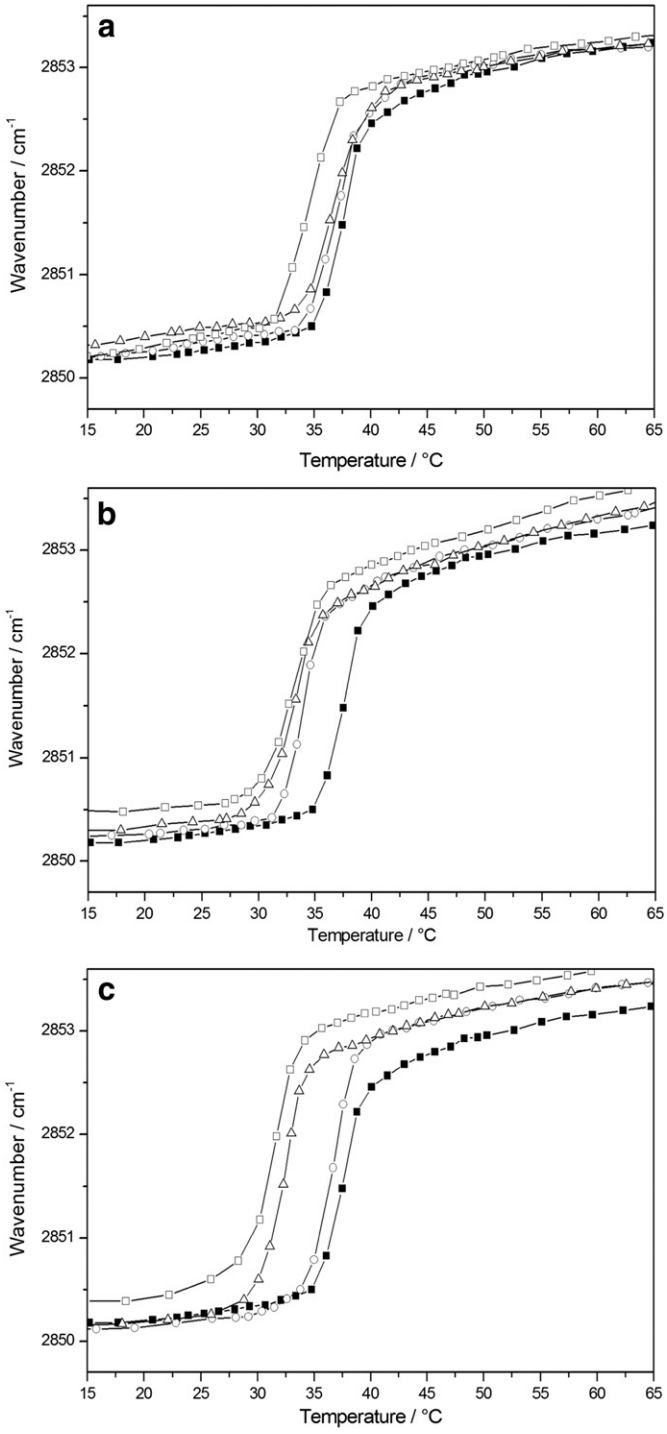

Fig. 6. Peak positions of the symmetric stretching vibration band of methylene groups in dependence of the temperature. DMPS in the presence of (a) ibuprofen, (b) naproxen, and (c) diclofenac at ■ 1:0, ○ 1: 0.5, ▲ 1:1 and □ 1:2 M ratios.

this method a direct intercalation of external agents into the hydrocarbon chain region of membranes can be monitored, whereas a pure adsorption to the membrane surface gives no signal. The ratio of the donor to acceptor signal (FRET signal) is a direct measure of an intercalation into the liposome because of the strong dependence of this signal (Förster energy transfer) on the distance between the two fluorescence markers. The experiments were done at three different temperatures: below, at and above the DMPS phase transition (at 25 °C, 37 °C, and 42 °C, respectively). In Fig. 8 five titrations at equimolar steps are presented for the three drugs at 37 °C corresponding to 1:1 up to 1:5 DMPS:NSAID molar ratios. The results show a slight change of the FRET signal ($I_{donor}/I_{acceptor}$ values) for diclofenac, which is indicative of a very weak intercalation into the DMPS acyl chains. The results obtained at 25 °C and 42 °C also showed a similarly weak intercalation of the NSAIDs into the lipid hydrocarbon chain moiety of DMPS (data not shown).

3.4. Isothermal titration calorimetry (ITC)

ITC is a valuable technique for measuring and characterizing the thermodynamic of binding interactions. It is based on the detection of heat production by monitoring a reaction between a macromolecule and a ligand. When two substances associate and interact, heat is either generated or absorbed. With ITC it is possible to measure in one single experiment binding constants (K_B), reaction stoichiometry (n), enthalpy (ΔH), Gibbs free energy (ΔG), and entropy (ΔS) thereby providing a complete thermodynamic profile of the molecular interaction [57]. Experiments were done below and above the DMPS main transition (T_m) with the three drugs. Results of the titration of ibuprofen with DMPS liposomes are shown in Fig. 9. As can be seen, the results are very noisy over the entire concentration range, characteristic for unspecific effects, except from dilution enthalpy during titration. Also an increase of the DMPS as well as the ibuprofen concentration by a factor of 3 each did not change the picture. Therefore, the lack of an enthalpic reaction is indicative that the interaction process is governed by entropy.

4. Discussion

The widespread use of NSAIDs as painkillers has positioned them among the most consumed non-prescription drugs in the market [3,58]. The study of NSAIDs-membrane interaction is fundamental to understand the biological activity of these drugs. Several studies have demonstrated their potential to establish molecular interactions with the lipid components of cell membranes by a COX-independent mechanism. Previous investigations in our laboratory demonstrated that NSAIDs are able to alter the thermotropic behavior and lipid organization of phosphatidylcholines and phosphatidylethanolamines liposomes [16,17,19,20]. In the present paper, the interactions of ibuprofen, naproxen and diclofenac with the important acidic phospholipid phosphatidylserine (in the form of DMPS liposomes) were investigated by applying a combination of different spectroscopic and calorimetric techniques. Although under physiological conditions in natural immune cells PS is located on the inner side of the cytoplasmic membrane, our approach seems to be relevant due to the fact that membrane steps are necessary for efficient action of the NSAID.

According to the FTIR–ATR results the incubation of ibuprofen with DMPS liposomes induced significant changes in spectroscopic parameters of polar groups of DMPS (Figs. 3–5). The polar groups of the head group region are known sensors to detect hydration changes; the water molecules in the polar region affect the electrostatic properties of the phospholipids and therefore the thermodynamic behavior of PS liposomes. The results showed that the interaction of ibuprofen with DMPS altered the characteristic phosphate, amino and carbonyl vibration modes of the lipid (Figs. 3–4). The analysis of the naproxen:DMPS mixtures showed significant perturbations in the ν_{as} COO^- and δ_{as} NH_3^+ lipid vibrations. Changes in the wavenumbers suggest alterations in head group organization of the lipid. The $-COO^-$ and $-NH_3^+$ groups are intermolecularly associated with the neighboring phosphatidylserines stabilizing the headgroup-headgroup interaction. Local changes in the membrane packing induces the formation of defects or alterations in the bilayer structure, from the structural view these alterations are associated with changes in the spectroscopic and thermodynamic properties of membranes. The FT-IR results suggested that ibuprofen and naproxen molecules are located preferentially in the polar region rather than in DMPS hydrophobic core. The analysis of the head group marker bands, also suggest that the location of ibuprofen and naproxen in the head group region is different, with a deeper penetration for ibuprofen into

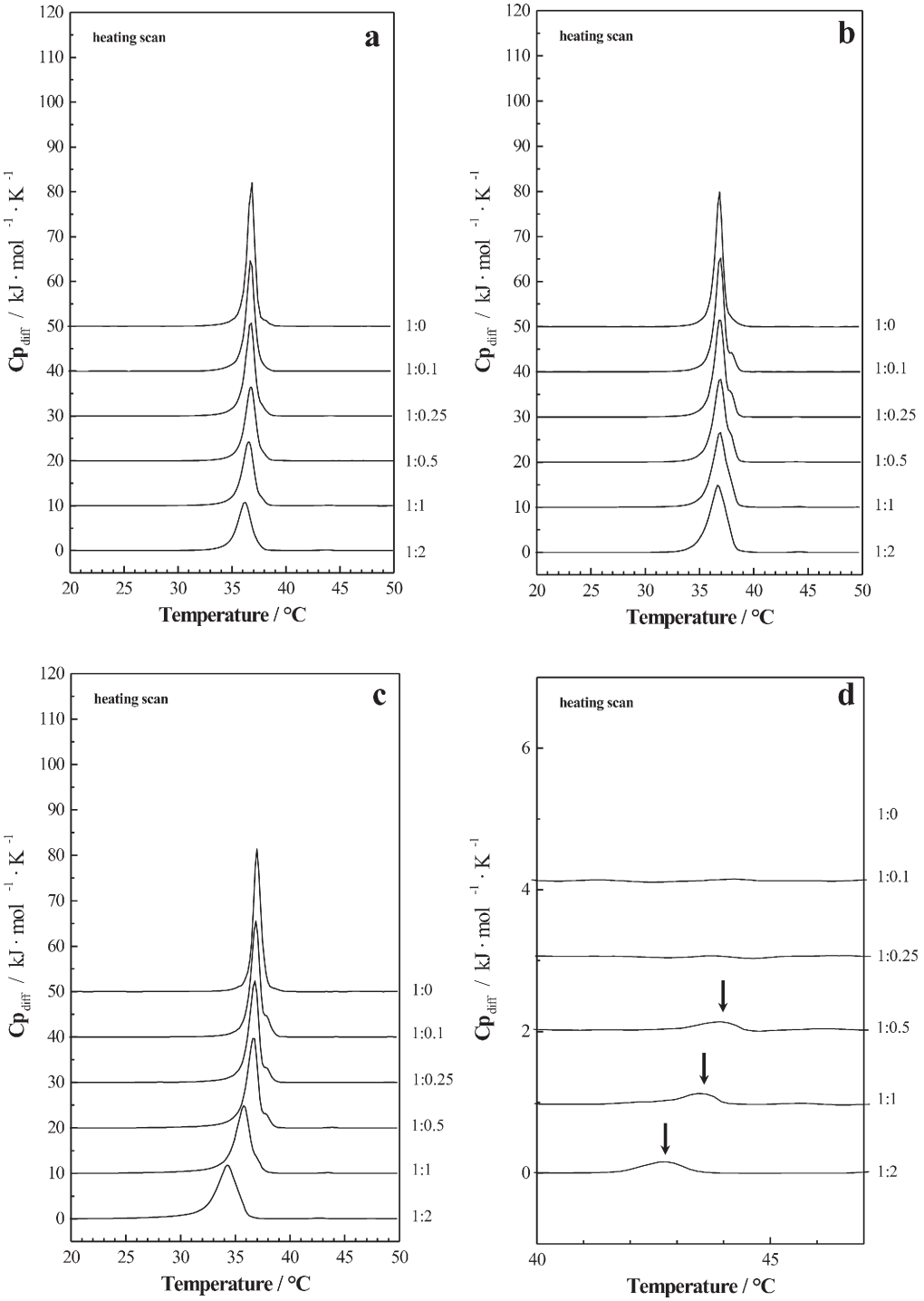

Fig. 7. Representative DSC thermograms obtained for multilamellar DMPS liposomes in the presence of (a) ibuprofen, (b) naproxen, and (c–d) diclofenac. Numbers at the right side represent lipid:NSAID molar Ratios.

Table 1
Enthalpy change (ΔH/kJ mol^{-1}) obtained by DSC for DMPS:NSAIDs mixtures at different molar ratios.

Lipid:drug molar ratio	ΔH/kJ mol^{-1}		
	DMPS:Ibuprofen	DMPS:Naproxen	DMPS:Diclofenac
1:0	32	32	32
1:0.1	28.7	32.1	32
1:0.25	27.4	32	31.3
1:0.5	23.9	30.6	31.6
1:1	22.7	30.7	31.1
1:2	19	30.6	31.5

the polar lipid interface, compared to naproxen (see impact on the carbonyl and phosphate vibrations). Maybe this is a reason that induces a decrease of the phase enthalpy of the system. The results of the diclofenac:DMPS mixtures, in contrast, show only a slight shift in the observed wavenumbers (Figs. 3–5).

Lipids are characterized by a sharp phase transition temperature that depends on the length of the alkyl chains [55] and the type of polar head groups [56]. Below the T_m, the acyl chains are tightly packed forming the gel phase, whereas above the T_m the liquid crystalline phase is characterized by a low order of the acyl chains together with an increase in the headgroup hydration. The phase transitions at T_m takes

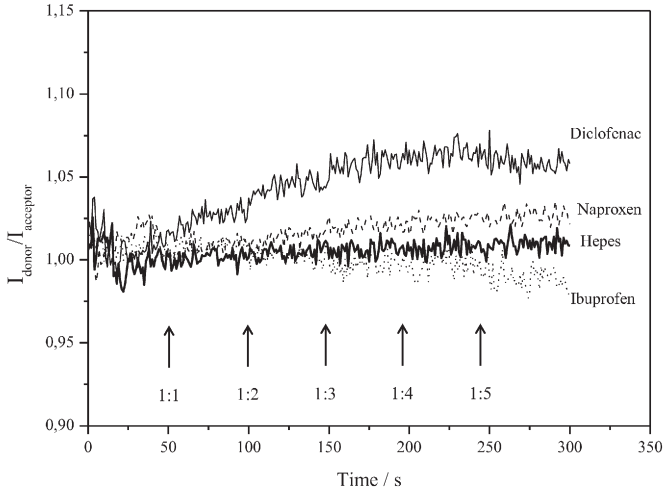

Fig. 8. Fluorescence resonance energy transfer spectroscopy (FRET) of 0.01 mM DMPS liposomes at 37 °C with NSAIDs: ibuprofen (dotted line), naproxen (dashed line) and diclofenac (straight line). Every 50 s, 0.5 μl of a stock solution of 0.1 mM NSAID was added to 1 ml of liposomes, and the FRET signal I_D/I_A was monitored in dependence on time.

place when the entropic contributions balance the enthalpic ones in the transition process. The phase transition measurements followed by FT-IR spectroscopy are based in the detection of changes in the T_m due to the interaction of exogenous molecules with the lipids; if the exogenous molecules are capable of inducing a perturbation in the bilayer, the thermodynamic parameters related to the phase transition are changed and the consequence is a shift in T_m. A shift of the wavenumber of the CH_2 symmetric vibration is closely related to the degree of order of the acyl chains. The results of the phase transition measurements also indicated interaction of the three NSAIDs with DMPS liposomes (Figs. 6A-C). In fact, results showed that the three drugs induced a shift of the T_m to lower temperatures, inducing a fluidization of the DMPS membrane. It is interesting to note that the shift in T_m of DMPS observed for ibuprofen (Fig. 6a) apparently is not observed in the DSC experiment (Fig. 7a), for which, however, a significant reduction in the phase transition enthalpy

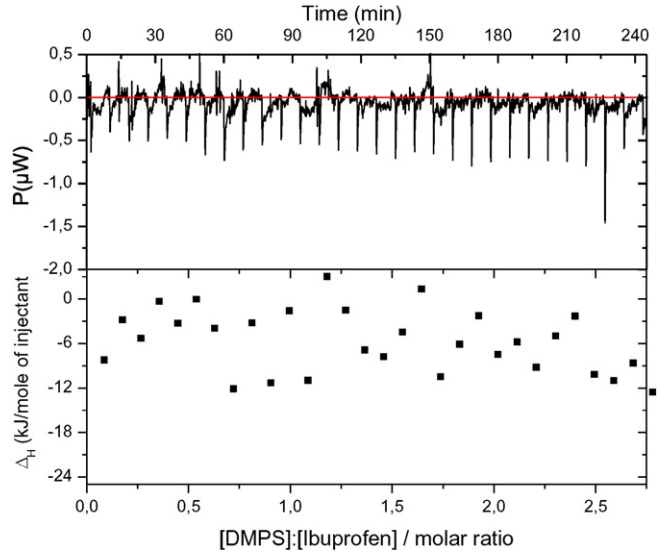

Fig. 9. Isothermal calorimetric titrations at 37 °C of a solution of 2 mM ibuprofen into 0.05 mM DMPS liposomes. For this, the ibuprofen solution was titrated to the liposomes in 30 injections with each 3 μl, and the resulting thermal reaction was recorded by the instrument indicating exothermic reactions when peaks are directed upwards and endothermic reactions with peaks directed downwards.

takes place (Table 1). The begin of the phase transition in the IR experiment corresponds to an increasing number of *gauche* conformers, starting in the head group distal region and ending at the proximal methylene groups. As illustrated in Figs. 6A and 7A, the strong reduction of the enthalpy change with increasing ibuprofen concentrations is not reflected by a corresponding decrease of the change of the wavenumber values between the two phases, showing that both techniques give complimentary information.

The overall effect on the phase transition is stronger in the order diclofenac > naproxen > ibuprofen, a result that indicates the fluidization of the bilayer. The partition coefficient (log P) is used as a potential predictor of the interaction with the hydrophobic core of the lipid bilayer. The reported log *P* values of ibuprofen, naproxen and diclofenac are 3.97, 3.18 and 4.51, respectively [59]. Based on the coefficient for diclofenac, it would be expected that the drug is partially inserted in the hydrophobic core affecting the structural molecular packing.

According to the ATR-FTIR results the ibuprofen and naproxen locate near the head group region inducing changes in the water organization and therefore modifying the hydration shell of the lipid. This effect can be explained as the negatively charged drugs compete with water molecules for hydrogen bonds with DMPS affecting the head group–head group interactions. These entropic changes have an effect on the characteristic T_m of the lipids, inducing the fluidization of the system [60]. The results described above are in accordance with our previous studies with liposomes built of phosphatidylcholines and phosphatidylethanolamines, which demonstrated that NSAIDs were mainly located in the headgroup region of these lipids [16,17]. The interaction of NSAIDs with PC liposomes resulted in the shift of the T_m as well of a fluidization of the lipid system. Naproxen and ibuprofen induced a shift of 5 °C and 7 °C, respectively, and diclofenac induced the strongest perturbation shifting the T_m down by ca. 15 °C at a 1:2 M ratio. The fluidization of the acyl chains was also evident when the $\nu_s CH_2$ vibration was analyzed at a fixed temperature [16]. Similar results were obtained with DMPE liposomes, in which the fluidization effect was detected from 1:0.1 M ratio for the three drugs, without a considerable shift in the T_m. The results for higher molar ratios revealed a shift up to 12 °C for diclofenac and the evidence of phase segregation due to strong destabilization of DMPE bilayers [17].

Calorimetric techniques have significantly contributed to the understanding of physicochemical properties of lipids, and particularly to the characterization of thermotropic phase transitions. The thermograms obtained for the three NSAIDs were in accordance with the FT-IR phase transition measurements, where the T_m values were also shifted to lower temperatures inclusive a broadening of the phase transition. The three NSAIDs also induced changes in the sharpness of the phase transition peaks, a result that might imply a destabilization of the phospholipid intermolecular cooperativity. This property is associated with the number of acyl chains that are able to convert from the gel phase to the liquid crystalline phase, and reflect the degree of aggregation of the lipid.

The analysis of the ibuprofen:DMPS results showed that increasing concentrations of the drug induced a decrease until 40% of the overall melting enthalpy, which is associated with a decrease in the cooperativity unit (Fig. 7). However, even at the highest lipid-drug molar ratio (1:2) there is no significant change in the main transition temperature, which is directly correlated with the phase transition measurements where ibuprofen induced a minor fluidization effect in comparison with the other drugs. Naproxen and diclofenac thermograms showed that both NSAIDs are able to induce changes in the thermotropic properties of the DMPS liposomes by lowering the phase transition temperature and broadening of the endothermic peaks (Fig. 7b,c). Additionally, at the lowest molar ratio (1:0.1) the presence of a small shoulder in the thermograms suggests the possibility of a phase separation process. Thus, one phase should be mainly composed of lipid molecules that underwent the melting phase transition at 36 °C, and another phase probably enriched with NSAID:lipid complexes that

underwent the transitions at a higher temperature. However, from DSC data no further information can be derived with regard to the nature of the formed phases. Regarding to the enthalpy change associated to the lipid $L_{\beta'}/L_{\alpha}$ phase transition, the overall ΔH value was negligibly affected by the incubation with naproxen or diclofenac, rather a decrease was observed when ibuprofen was used. This suggests that naproxen and diclofenac are preferentially located at the water/lipid interphase, with diclofenac penetration deepest into the interface. This is related to the positive FRET effect indicating a drug intercalation in the lipid bilayer. This slightly different penetration effect of diclofenac compared to the other two NSAID drugs (Fig. 8), might be the reason for a more pronounced destabilization of the gel phase of DMPS.

Despite the three NSAIDs share the common feature of being negatively charged in solution, the physicochemical and structural properties of the compounds are quite different. Ibuprofen and naproxen have an elongated conformational structure, whereas the two aromatic rings of diclofenac are always twisted against each other [3]. These structural properties and the amphipathic nature of the NSAIDs have implications in the drug-membrane interactions. According to the DSC results, ibuprofen induced the highest change in the ΔH (Fig. 7a), which indicates a deep interaction of the drug as a substitutional impurity in the liposomes. This effect could be associated with the small size and conformational structure of the drug. The location of a small charged molecule like ibuprofen at the lipid–water interface can easily influence the ordered water layer at the polar interface. The effect of ibuprofen was directly related to the increasing drug concentration, indicating that more molecules of ibuprofen contribute to the head group-head group interactions of the DMPS liposomes. As lipid bilayers have an equilibrium between the polar and the hydrophobic regions, changes of the hydration shell of the polar head groups have an important effect in the packing properties of the hydrophobic region. Our previous studies with PC and PE lipids suggested that alterations of the polar headgroup hydration conditions were directly related to thermodynamic and physical properties of the bilayer, such as cooperativity [16,17]. These statement were taken from the results obtained with ITC, where no enthalpic reaction of the drug: lipid complex was detected (Fig. 9). The comparison of the present results with those found for phosphatidylcholine as main phospholipid of eukaryotic cells shows that similar as found here a decrease of Tm and a reduction of the peak height of the melting endotherm is observed. The data for the NSAID:DMPC interaction, however, exhibit a phase transition broadening, but left the overall phase transition enthalpy constant [16]. Additionally, in contrast to the findings presented here phase separation was observed, by the formation of a NSAID-rich and a NSAID-poor phase. Despite the strong influence of the drugs on the DMPC acyl chain moiety, FRET data did not reveal any evidence for drug incorporation into the lipid matrix, and ITC measurements performed did not exhibit any heat production due to the interaction to drug binding. This implies that the interaction process of DMPC as well as of DMPS with the NSAIDs is governed by only entropic reactions.

Naproxen, with a planar structure but a larger size in comparison to ibuprofen, had a different behavior according to the DSC transition enthalpy, an superficial interaction with the PS liposomes takes place. The phase transition measurements showed a fluidization effect on the liposomes, supporting the broadening effect detected in the DSC measurements. FRET results showed that only diclofenac was slightly intercalating into DMPS liposomes at 37 °C (Fig. 8), which is in accordance with the phase transition results where the drug induced the highest effect on the main transition temperature. This insertion may be favored because the temperature of the experiments is almost at the phase transition of the lipid. During the main transition there is an increase in the head group hydration, van der Waals attraction between the chains is reduced and therefore the acyl chains are exposed to the polar-apolar interface. If the drugs were intercalating into the liposomes, the effect would be detected below and above the main transition temperatures. In case of a dominant electrostatic interaction between a molecule and its ligand, the enthalpy contribution is higher than the entropy one. It thus can be concluded that the interactions between DMPS and the NSAIDs are mediated only by entropic processes.

5. Concluding remarks

Our experimental results demonstrated that the NSAIDs ibuprofen, naproxen and diclofenac destabilized DMPS bilayers affecting their thermodynamic properties. There are several parameters that play an important role in the drug-membrane interaction. Hydration plays an important role in the stability of the bilayer. The alterations of the hydration shell and lipid packing might have consequences for cell membranes, like changes in the semipermeable properties, rate and efficiency of the cell growth, and modulate the activity of a variety of membrane-associated enzymes.

Conflict of interest

The authors declare that they have no conflict of interest.

Transparency document

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

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