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The membrane-activity of Ibuprofen, Diclofenac, and Naproxen: A physico-chemical study with lecithin phospholipids

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) represent non-specific inhibitors of the cycloxygenase pathway of inflammation, and therefore an understanding of the interaction process of the drugs with membrane phospholipids is of high relevance. We have studied the interaction of the NSAIDs with phospholipid membranes made from dimyristoylphosphatidylcholine (DMPC) by applying Fourier-transform infrared spectroscopy (FTIR), Förster resonance energy transfer spectroscopy (FRET), differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC). FTIR data obtained via attenuated total reflectance (ATR) show that the interaction between DMPC and NSAIDs is limited to a strong interaction of the drugs with the phosphate region of the lipid head group. The FTIR transmission data furthermore are indicative of a strong effect of the drugs on the hydrocarbon chains inducing a reduction of the chain–chain interactions, i.e., a fluidization effect. Parallel to this, from the DSC data beside the decrease of T_m a reduction of the peak height of the melting endotherm connected with its broadening is observed, but leaving the overall phase transition enthalpy constant. Additionally, phase separation is observed, inducing the formation of a NSAID-rich and a NSAID-poor phase. This is especially pronounced for Diclofenac. Despite the strong influence of the drugs on the acyl chain moiety, FRET data do not reveal any evidence for drug incorporation into the lipid matrix, and ITC measurements performed do not exhibit any heat production due to drug binding. This implies that the interaction process is governed by only entropic reactions at the lipid/water interface.

1. Introduction

The nonsteroidal anti-inflammatory drugs (NSAIDs) are a wide non-chemically related group of compounds that are also called "aspirin-like drugs" for sharing similar therapeutic actions. They have been used since the introduction of acetylsalicylic acid (Aspirin) in 1899 [1]. NSAIDs are a large family of compounds classified into several subgroups, based on their chemical structure, like salicylates (Aspirin, Diflunisal), phenylacetic acids (Diclofenac), indoles (Indomethacin), oxicams (Piroxicam, Meloxicam), pyrazoles (Phenylbutazone) and sulphonanilides (Nimesulide) [2]. Despite their structural diversity, NSAIDs share most of the therapeutic actions as well as side-effects. Within the most widely prescribed and recognized drugs, Ibuprofen, Naproxen and Diclofenac are well-established NSAIDs, exhibiting anti-inflammatory, analgesic, antithrombotic and antipyretic properties [3]. Since 1974, Diclofenac is widely used in the long-term treatment of degenerative diseases. Some of its associated side-effects are hepatoxicity and liver injury [4]. Naproxen is used for the treatment of mild to moderate pain and fever. The most common side effects from Naproxen are headaches, dizziness, abdominal pain, nausea, and shortness of breath. Naproxen is associated with an increase in the risk to suffer heart attack and malignant effect on mucosal hydrophobicity [5]. One severe challenge encountered with the use of NSAIDs is their little gastrointestinal tolerability [6–9].

In most countries, Ibuprofen is freely available which allows self-medication. It has a wide range of side-effects including cardiovascular [10], renal and hepatic damage associated with long-time treatments [11]. They are also extensively medicated for rheumatoid arthritis, osteoarthritis, and musculoskeletal disorders. Recently, some of the NSAIDs have emerged as part of a new cancer chemotherapeutic and chemopreventive therapy [12,13].

The common mechanism of action of all NSAIDs is the inhibition of the cyclooxygenase (COX) enzyme. The COX pathway converts arachidonic acid in prostaglandins and thromboxans, which contribute

Abbreviations: NSAIDs, Nonsteroidal anti-inflammatory drugs; DMPC, dimyristoylphosphatidylcholine; FTIR, Fourier-transform infrared spectroscopy; ATR, attenuated total reflection; ITC, isothermal titration calorimetry; FRET, Förster resonance energy transfer spectroscopy; DSC, differential scanning calorimetry; T_m, temperature of main phase transition; COX, cyclooxygenase; Pβ, ripple phase; Lα, liquid crystalline phase

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to several physiological functions [14,15]. It is well known that there are at least two COX isoenzymes. The isoforms are called cyclooxygenase 1 and 2, and possess distinct enzymatic activities [16]. COX-1 is expressed constitutively in most tissues types and it is involved in the synthesis of prostaglandins which protect the stomach lining, intestine and kidney from damage. COX-2 is only expressed in a limited number of cell types and contributes among other activities to the inflammation processes in cells like fibroblasts, macrophages and monocytes [17]. Because of the high degree of structural similarity, most of the NSAIDs are non-selective inhibitors of COX-2 [18]. Most serious and sometimes fatal side-effects are gastrointestinal damage including ulceration and haemorrhage, renal toxicity, inhibition of the platelet aggregation that can lead to coagulation disorders which are side effects of non-selective (non-selective) COX-1 inhibition [19]. Additionally, studies have shown alternative COX-independent mechanisms of action inducing local ulcerations in the gastrointestinal tract [20]. These findings suggest the possibility of a direct membrane activity.

Artificial membranes are commonly used as a model for natural membranes to study drug–membrane interactions. The major constituents of the membrane are phospholipids. They are responsible for several features of the bilayer like stability and semi-permeable properties. Major lipidic components of eucaryotic cell membranes are phosphatidylcholines. The interactions of the NSAIDs with phospholipids can change the physicochemical properties of the membranes, which may be essential to understand the dynamics of the mechanism of action. The aim of this study was to investigate the interactions between dimyristoyl-phosphatidyl choline (DMPC) liposomes and three NSAIDs (Ibuprofen, Naproxen and Diclofenac as sodium salts). The results describe the molecular details of these interactions and the consequences for the membranes properties, by using Fourier-transform infrared spectroscopy (FTIR) in transmission and with attenuated total reflection (ATR), differential scanning calorimetry (DSC), isothermal titration calorimetry (ITC), and Förster resonance energy transfer spectroscopy (FRET).

2. Materials and methods

2.1. Lipid sample preparation

The lipid samples were prepared as aqueous dispersions at varying buffer (20 mM HEPES pH 7.4) content, depending on the sensitivity of the technique: 0.1 mM for the FRET-, 0.4 mM for the DSC-, and 20–40 mM for the FTIR-experiments. In all cases, the lipids were suspended directly in buffer, sonicated, and temperature-cycled several times between 5 and 70 °C and then stored at 4 °C before measurement. The drugs were dissolved in buffer and added to the phospholipid liposomes at the given molar ratios, and the measurements were done with these freshly prepared dispersions.

2.2. Fourier-transform infrared spectroscopy

The infrared spectroscopic measurements were performed on an IFS-55 spectrometer (Bruker, Karlsruhe, Germany). For phase transition measurements, 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, these were spread on a ATR Ge plate, and free water was evaporated. Every 3 °C, 50 interferograms were accumulated, apodized, Fourier-transformed, and converted to absorbance spectra.

For the evaluation of the gel to liquid crystalline phase behaviour, the peak position of the symmetric stretching vibration of the methylene band $\nu_s(CH_2)$ around 2850 cm^{-1} was taken, which is a sensitive marker of lipid order [21,22]. Furthermore, vibrational bands from the interface region (ester carbonyl stretch around 1725 to 1740 cm^{-1}), and the head group (antisymmetric stretch at 1260 to 1220 cm^{-1}, the choline group around 1100 to 1000 cm^{-1}, and the N–H stretching band at 1000 to 950 cm^{-1}) were analyzed. The instrumental wavenumber resolution is better than 0.02 cm^{-1}, the wavenumber reproducibility in repeated scans is better than 0.1 cm^{-1}.

2.3. Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were performed with 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 (5.6 mM) in 10 mM PBS buffer at pH 7.4. The samples were hydrated in the liquid crystalline phase by vortexing. Prior to the measurements the DSC samples were stored for a defined time at 4 °C (see text). The measurements were performed in the temperature interval from 5 °C to 65 °C. In the figures only the temperature range is shown where phase transitions were observed. Five consecutive heating and cooling scans checked the reproducibility of the DSC experiments of each sample [23]. 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. The DSC data were analyzed using the Origin software. The phase transition enthalpy was obtained by integrating the area under the heat capacity curve [24].

2.4. Isothermal titration calorimetry

Microcalorimetric experiments of drug binding to DMPC were performed on a MCS isothermal titration calorimeter (Microcal Inc., Northampton, MA, USA) at various temperatures. The phospholipid samples at a concentration of 0.05 to 0.15 mM – prepared as described above – were filled into the microcalorimetric cell (volume 1.3 ml) and the drugs in the concentration range 0.5 to 5 mM 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 stirred constantly, 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 [drug]:[lipid] molar ratio. 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 2 times. As control, the drugs were also titrated into pure buffer.

2.5. FRET (Förster resonance energy transfer) spectroscopy

Intercalation of the NSAIDs into phospholipid liposomes composed of DMPC was determined in 20 mM Hepes, 150 mM NaCl, pH 7.4 at 37 °C by FRET spectroscopy applied as a probe dilution assay [25,26]. The drugs were added to the lipid aggregates which were labelled with the donor dye NBD-phosphatidylethanolamine (NBD-PE) and acceptor dye Rhodamine-PE. 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) in dependence on time.

3. Results

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

FTIR spectroscopy is most suitable for studying the interactions at the water lipid interface because this technique provides information

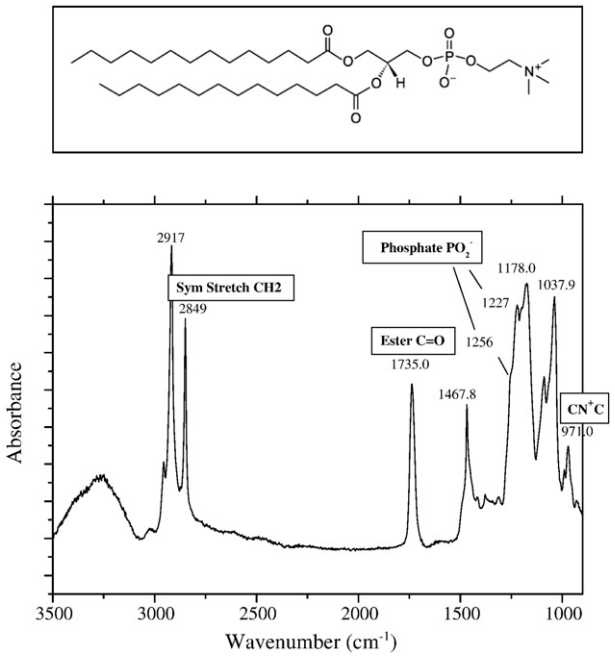

Fig. 1. Infrared ATR spectrum of a 10 mM hydrated film of DMPC in HEPES buffer at pH = 7.4 taken at 20 °C. At the top, the chemical structure of DMPC is plotted.

about the functional groups of the phospholipid under investigation and its dynamic information from all the regions of the molecule in one single experiment. Each band of the spectrum has a characteristic position of the band maximum, denoted by its wavenumber, absorption maximum and width. If an exogenous molecule interacts with the phospholipid bilayer the spectroscopic parameters change, because the vibrational band contours depend on the nature of the atoms involved and their conformation in aqueous environment.

The infrared spectra of DMPC were analyzed in the following regions: phosphate asymmetric stretching vibration PO_2^- (1220–1260 cm^{-1}), ester carbonyl stretching mode (1680–1800 cm^{-1}) and the antisymmetric CN^+C stretching mode ($\nu(CN^+)$) (950–1000 cm^{-1}). These groups were selected in the study because they are potential binding sites (e.g. by electrostatic interaction) or affected by hydration due to interaction or conformational changes. The symmetric CH_2 stretching vibration (2850–2855 cm^{-1}) was analyzed as a parameter associated with the order (or fluidity) of the acyl chains of the phospholipids. A complete spectrum of pure DMPC and its characteristic vibrational bands are shown in Fig. 1.

3.1.1. Phosphate region as a sensor for hydration of phospholipids

The asymmetric stretching vibration of the PO_2^- moiety $\nu_{as}(PO_2^-)$ has been extensively studied [27–30] as sensor for interactions in the headgroup region. The $\nu_{as}PO_2^-$ band is very sensitive to the hydration conditions, in that the wavenumber values decrease substantially with increasing hydration, i.e., they are shifted from 1250 for low hydrated to 1230 cm^{-1} for highly hydrated bilayers, caused by the hydrogen-bonded water molecules to the charged phosphate group [28,29]. For DMPC both band components are present, one lying at 1227 and one at 1256 cm^{-1} (see Fig. 1), in accordance to previous result by Pohle et al. [30]. A shift in the wavenumbers is caused when external molecules modify the number and orientation of hydrogen bonded water molecules around the head group as well as changes in the polarity of the environment.

Fig. 2A–C shows the spectra of three mixtures of DMPC with the NSAIDs in the region of the $\nu_{as}(PO_2^-)$ phosphate vibrations: The figures on top are a mathematical addition of the pure spectra of DMPC and the NSAIDs, the figures on bottom represent the interacting DMPC:NSAID mixtures. Therefore, in the case of non-existing interactions between the compounds both spectra should be identical. As can be seen from the comparison, the analysis of the bands reveals an interaction of the three drugs with this lipid group. For example, both bands of the antisymmetric PO_2^- mode in the pure DMPC spectrum are modified by the presence of Ibuprofen in that the band intensity corresponding to a highly hydrated state is reduced while the band intensity from the band belonging to low hydration is enhanced (Fig. 2A). A similar effect is observed for the DMPC:Naproxen mixture (Fig. 2B), whereas the

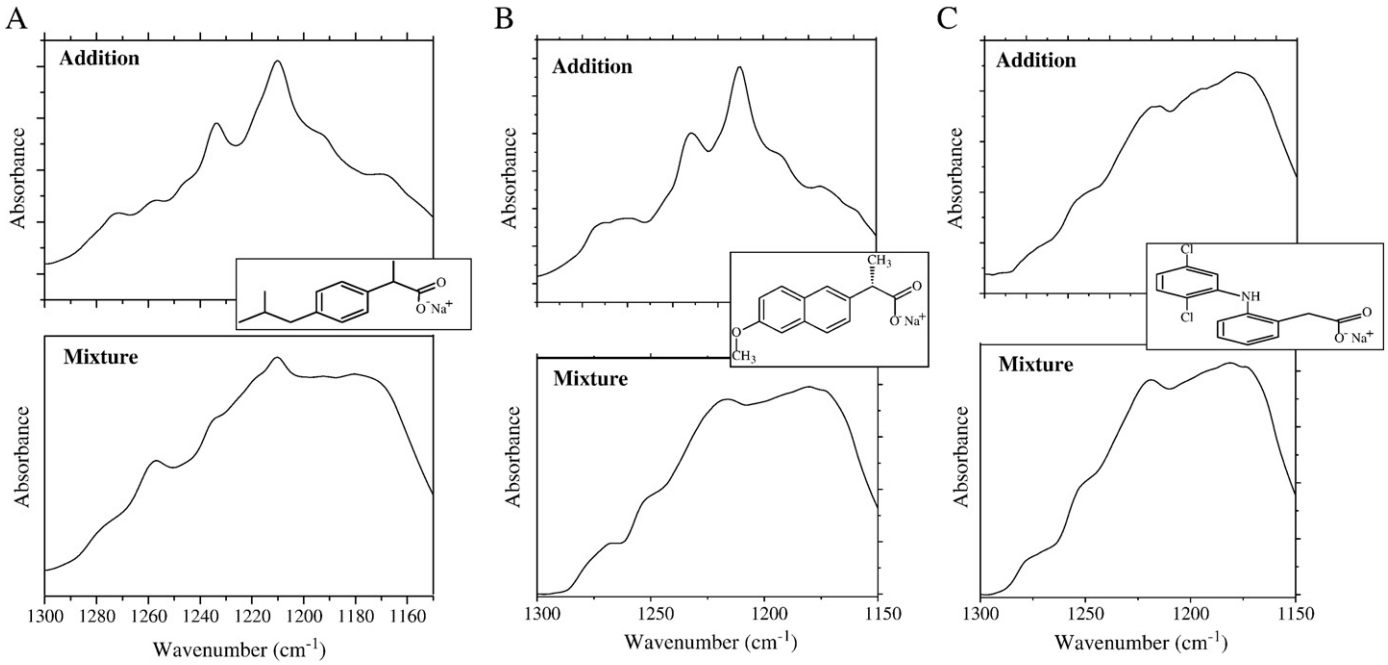

Fig. 2. IR spectra of the asymmetric PO_2^- stretching region for mixtures of DMPC and the three. NSAIDs, Ibuprofen (A) Naproxen (B), and Diclofenac (C). The spectra on top are mathematical additions of the pure spectra of the compounds, the spectra on bottom are the spectra of the interacting mixtures. All experiments were done at a 1:0.5 molar ratio [DMPC]:[drug].

DMPC:Diclofenac mixtures exhibit only slight changes in this spectral region (Fig. 2C).

3.1.2. Carbonyl stretching region

The carbonyl stretching vibration of phospholipids C=O group is a sensitive sensor for the hydration of the lipid interface region, being sensitive to the hydration state, polarity, degree and nature of the hydrogen bonding interactions in the lipid–water interface [27,31]. Different hydration of the carbonyl groups leads to the splitting of the group vibration into two different bands at 1723–1728 cm^{-1} for the hydrogen bonded and 1740–1742 cm^{-1} for the non-hydrogen bonded C=O group [32]. In Table 1 the results are shown after curve fitting of the single band components for pure DMPC and the three mixtures of the lipid with the NSAIDs at a 1:0.5 molar ratio. There is no shift in this band which gives important evidence that the drug is not interacting in the carbonyl region, in contrast to the results presented for the phosphate region. The FTIR transmission data were also analyzed with the same conclusion (data not shown).

3.1.3. Amino stretching region

In literature, the interaction of water with the choline groups has been studied with different phospholipids. A hydrogen bonding formation between water and the choline group is not possible due to the positive charge of the nitrogen. However, the choline group is associated with water molecules by dipole interactions [33], and an interaction with the NSAIDs could cause a shift in the position of this vibration because of the negative charge of the drugs. The results of the analysis via curve fitting in the region of the amino vibrational band (data not shown) do not reveal any kind of interaction between the drug and the amino group. These data are indicative for the absence of a direct interaction between the NSAIDs and this moiety, i.e. the absence of the drug in this region of the phospholipid.

3.1.4. Symmetric stretching vibration of CH_2 groups

The wavenumber of the peak position of the CH_2 band ($\nu_s CH_2$) as a function of the temperature is a recognized parameter sensitive to lipid order and packing [21]. Insertion of molecules into the bilayer increases the space between the fatty acids causing an increase in their mobility and their frequency. The peak positions of the methylene stretching modes ($\nu_s CH_2$) 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 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 [34] and the structure of the head groups [35]. Fig. 3 shows the temperature dependence of the wavenumbers values of the peak positions of the DMPC acyl chains for the pure lipid and for the mixtures, DMPC/NSAIDs, at different molar ratios. The interaction of the NSAIDs results in a shift in T_m as well as a fluidization of the system. The strongest change is observed for Diclofenac, for which T_m is shifted up to ca. 15 °C downwards at a 1:2 molar ratio. At the same molar ratio, Naproxen induces a shift of ca. 5 °C and Ibuprofen of ca. 7 °C. A slight fluidization of the acyl chains is evident from the increase in the wavenumbers of the $\nu_s(CH_2)$ band at fixed temperature, and might give evidence either for a direct interaction of the drug with the phospholipids membrane, or only with the pure head group connected with a change of the lipid packing.

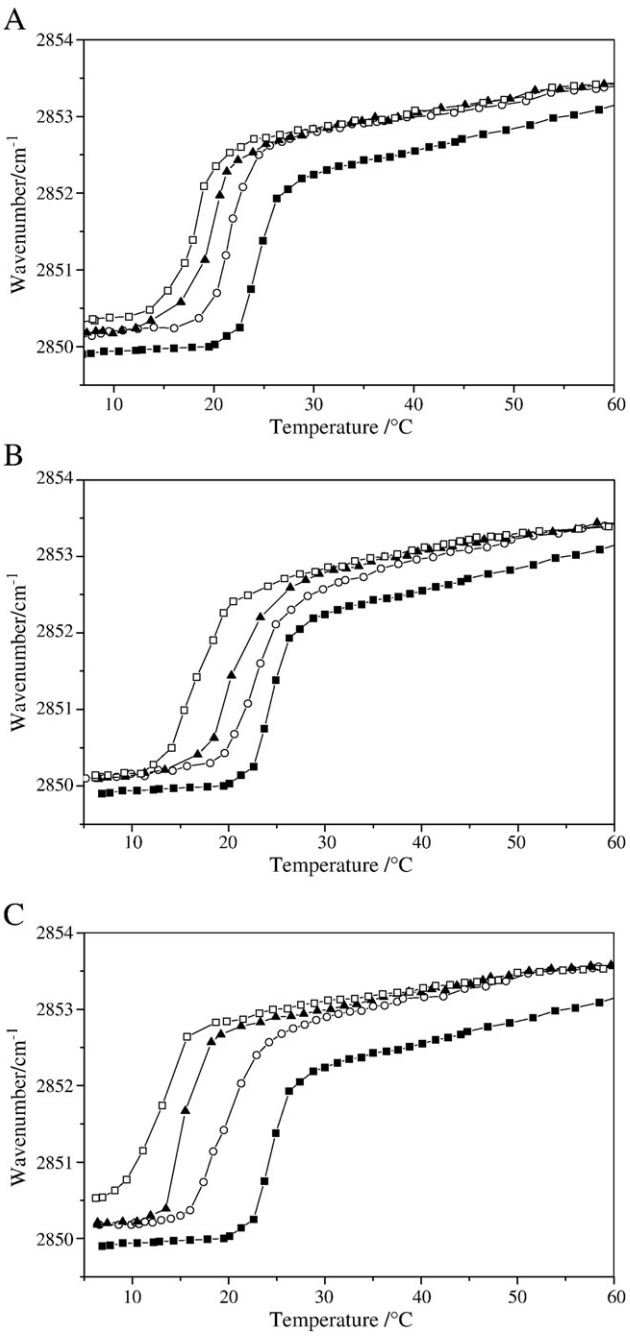

Fig. 3. Peak positions of the symmetric stretching vibration bands of the methylene groups in dependence of temperature. DMPC in the presence of (A) Ibuprofen (B) Naproxen (C) Diclofenac at -■ 1:0, -○ 1:0.5, -▲ 1:1 and -□ 1:2 molar ratios.

Table 1
C=O Stretching band vibration after curve fitting procedure for DMPC and three mixtures [DMPC]:[NSAID] at a molar ratio of 1:0.5

Sample	C=O Peak position/cm^{-1}	
	Hydrogen bonded	Non-hydrogen bonded
DMPC	1726	1739
DMPC + Ibuprofen	1727	1740
DMPC + Naproxen	1725	1739
DMPC + Diclofenac	1724	1739

3.2. Differential scanning calorimetry

The calorimetric analysis was performed in order to determine the heat flow associated with the lipid phase transitions. It can be used to detect interactions between the NSAIDs and the phospholipid. The analysis was performed for pure DMPC and for mixtures of DMPC and the drugs at different molar ratios. The results of the experiments are shown in Fig. 4A–C. The thermogram for pure DMPC shows two peaks,

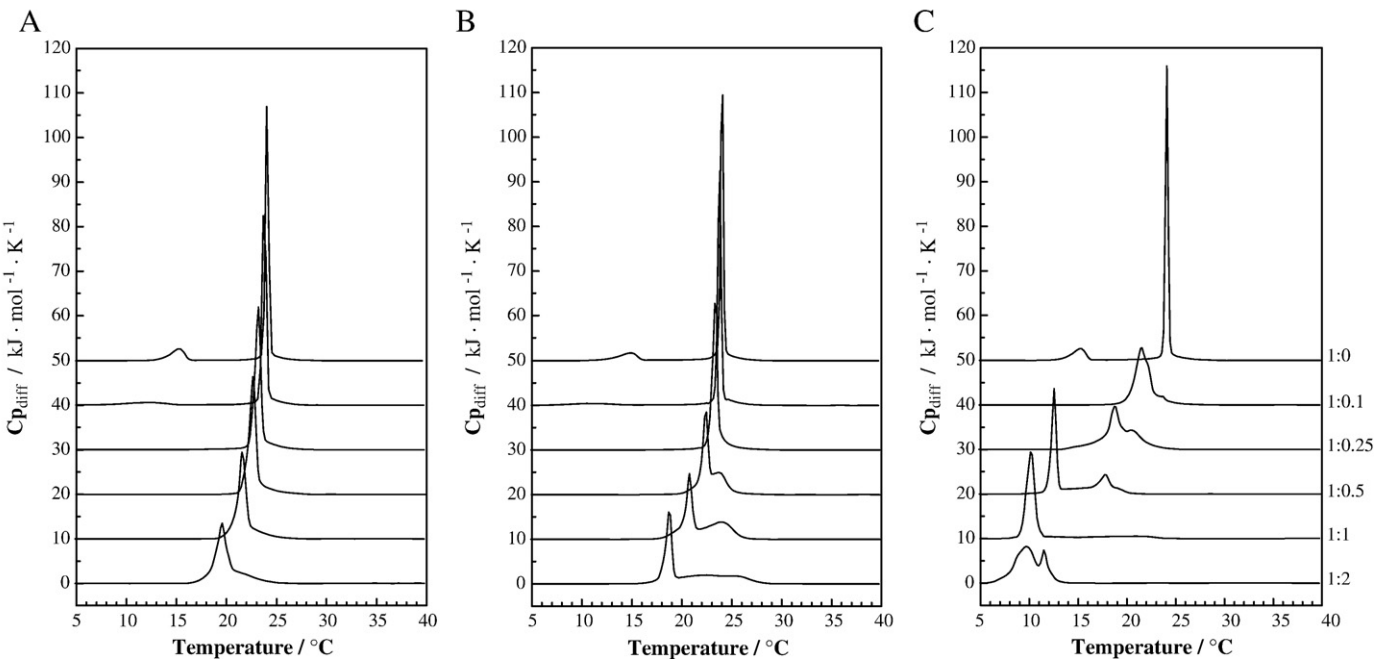

Fig. 4. DSC thermograms of DMPC liposomes + in the presence of (A) Ibuprofen (B) Naproxen and (C) Diclofenac. The numbers on the right side represent the different molar ratios of the mixtures (DMPC:NSAID). DMPC to NSAID molar ratio: 1:0 (top), 1:0.1, 1:0.25, 1:0.5, 1:1, 1:2 (bottom)

a small and a large endothermic one. The small peak at lower temperature (ca. 15 °C) (pretransition) corresponds to the transition from Lβ into the ripple phase (Pβ) and the large peak at higher temperature (ca. 24 °C) corresponds to the main transition [36]. The Pβ-phase is one of the gel phases which occurs due to structural constraints between the packing characteristics of the two acyl chains and the headgroup [37]. For the three NSAIDs at a 1:0.1 molar ratio the gel to liquid crystalline main phase transition is still observed but broadened and shifted to lower temperatures, whereas the metastable ripple phase disappears. This is in accordance to Lygre et al. [38] and Du et al. [39].

The three NSAIDs are able to alter the cooperativity of the phase transition of the phospholipid. Diclofenac has the strongest effect at a 1:2 molar ratio, shifting the T_m to 10 °C. At low molar ratios (1:0.1) a phase separation occurs. This is especially pronounced for Diclofenac. At a molar ratio DMPC:Diclofenac of 1:0.5 two phase transitions are observed with their maxima located at 12 and 18 °C. The phase separation is dependent on the lipid to Diclofenac ratio. The same phenomenon of phase separation is obvious for Naproxen at a 1:0.5 molar ratio (Fig. 4B). At higher molar ratios (1:2) the T_m is shifted below 18–19 °C. Ibuprofen at the same molar ratio shifts the T_m close to 20 °C and has a broadening effect on the endothermic peak. A clear phase separation in the presence of Naproxen is not so pronounced as for the other two NSAIDs systems. The DSC results are in accordance to the infrared measurements where the T_m was also shifted to lower temperature, indicating a strong destabilization of the gel phase. The DSC results confirm the drug–lipid interaction and association due to the shift in the peak position of the T_m. With increasing amount of NSAIDs the phase transition enthalpy of the higher melting phase (phase composed of mainly pure lipid) decreases, whereas the phase transition enthalpy of the lower melting phase (phase containing higher amounts of DMPC-NSAID "complexes") increases (see Fig. 4B, C). In contrast to the evident influence on the phase transition temperature, however, the overall molar enthalpies of the transition remain constant at all molar ratios of the NSAIDs:DMPC mixtures (data not shown). However, it has to be considered, that the overall phase transition enthalpy is the sum of the phase transition of the different phases formed. This is especially evident by analyzing the Diclofenac system. The phase transition enthalpy of the single phase transition, as observed for example for the system DMPC: Diclofenac at a molar ratio of 1:0.5, is strongly reduced compared to the main phase transition enthalpy of the pure lipid. From the DSC data no information can be derived with regards to the nature of the formed phases.

3.3. FRET spectroscopy

Förster resonance energy transfer spectroscopy (FRET) was applied to determine a possible intercalation of the NSAIDs into DMPC liposomes, by using liposomes fluorescently labelled by NBD-PE (donor) and RhoPE (acceptor) dyes. The ratio of the donor to acceptor signal (FRET signal) is a direct measure for an intercalation into the liposome membrane because of the strong dependence of this signal (Förster energy transfer) on the distance between the two fluorescence markers.

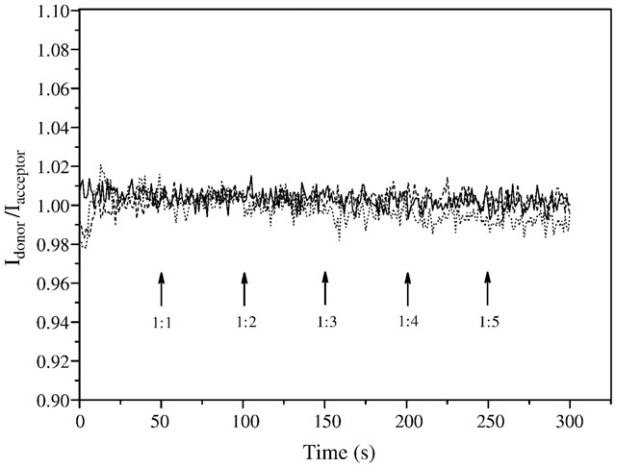

Fig. 5. Förster fluorescence resonance energy transfer spectroscopy (FRET) of 0.01 mM DMPC liposomes at 25 °C with the three NSAIDs: Ibuprofen (dotted line), Naproxen (dashed line) and Diclofenac (straight line). Every 50 s, 0.5 μl of a stock solution of 0.1 mM NSAID was added to 1 ml of liposomes, and the FRET signal I_D/I_A was monitored in dependence on time.

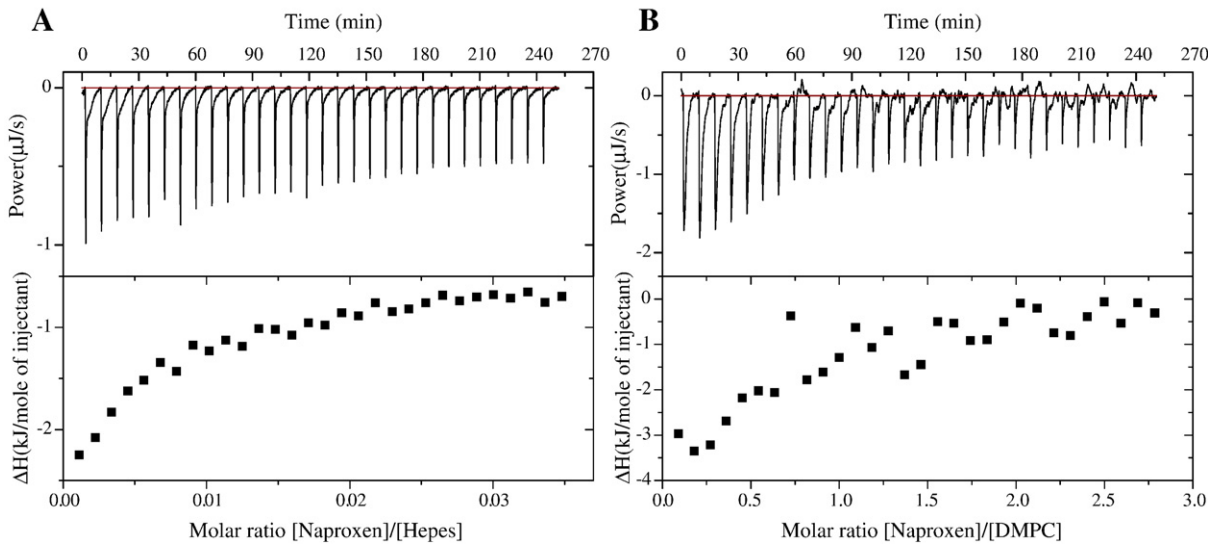

Fig. 6. Isothermal calorimetric titrations at 27 °C of (A) Hepes 20 mM with 30 injections of 3 μl of 10 mM Naproxen and (B) DMPC liposomes 0.05 mM with 30 injections of 3 μl of 2 mM Naproxen.

The experiments were done at three different temperatures; below, during and above the phase transition of DMPC, at 15 °C, 25 °C, and 37 °C, respectively. In Fig. 5 five titrations at equimolar steps are presented for the three drugs at 25 °C corresponding to molar ratios of 1:1 up to 1:5 (DMPC:NSAID). The results do not show any changes of the FRET signal ($I_{donor}/I_{acceptor}$ values), which demonstrate that the drugs are not intercalating into the acyl chains DMPC liposomes. This, however, does not rule out an interaction with the head group. The data are similar at 15 °C and 37 °C, showing no intercalation of the NSAIDs with the lipid moiety independent of the phase state of DMPC (data not shown).

3.4. Isothermal titration calorimetry

ITC is an invaluable technique used for measuring and characterizing the thermodynamic of binding interactions. The technique is based on the detection of heat production by monitoring a reaction between a molecule 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 [40]. Further experiments were done below and above T_m of the phospholipid with the different drugs. The experiments do not show any kind of heat production except from dilution enthalpy. Exemplary experiments are shown in Fig. 6.

4. Discussion

The investigated NSAIDs are known to represent non-specific inhibitors of the cycloxygenase pathway thus suppressing the formation of inflammation mediators such as prostaglandins. The conversion of arachidonic acid as starting point of this inflammation path is a membrane-bound step and should depend on physical–chemical properties of the latter and the changes due to drug binding. In the present paper, the interaction of the NSAID drugs with model membranes from synthetic lecithin (DMPC) was investigated by applying a combination of different spectroscopic and thermodynamic techniques. According to the FTIR–ATR results the drugs are located preferentially in the polar head groups of the phospholipids. There is evidence after the analysis of the vibrational bands in the 1300 to 1150 cm^{-1} spectral range (Fig. 2), that the drugs are located close to the phosphate region. Lichtenberger et al. [41], have suggested that the anionic groups of the NSAIDs can chemically associate with the positively charged choline group of PC. The analysis of the carbonyl and amino vibrations of the choline group of DMPC:NSAID mixtures did not show a significant shift in the observed wavenumbers, discarding the possibility of interactions with these moieties. On the other hand, the spectra changes, or in the case of DMPC:Ibuprofen and DMPC:Naproxen mixtures, the intensity shifts of the PO_2^- asymmetric stretching vibrations in favour of the band corresponding to low hydration (Fig. 2A), are proofs for the location of the drugs in this region of the headgroup.

The observed shifts in the PO_2^- asymmetric vibrations can be understood as changes in the order of the water molecules in the hydration shell of the head group. This can be explained by the presence of a negatively charged group (COO^-) of the drug near the phosphate region of DMPC: These two negative charges may be bridged by positive counterions, responsible for this (weak) interaction. That such interactions – favoured by the action of counterions – may take place, were shown in experiments with negatively charged lipids, which do incorporate into negatively charged, but not into uncharged liposomes [25,42].

The presence of this charge can compete for the formation of hydrogen bonds with the water molecules of the hydration shell, which is an entropy-driven process, also in accordance with the ITC results that exclude enthalpic contributions of the reaction.

The analysis of the phase transition behaviour also reveals an interaction of the NSAIDs with the DMPC molecules. In the gel phase below T_m, the mobility of the hydrocarbon chains is low, whereas the liquid crystalline phase is characterized by disordered lipid chains. During the phase transition, there is an increase in the entropy with chain rotational disorder, together with an increase in the intermolecular entropy and an increase in the head group hydration. The van der Waals attraction between the chains is reduced, and there is an increase in the exposure of the acyl chains to the polar–apolar interface. The phase transitions at T_m take place when the entropic contributions balance the enthalpic ones in the transition process. If an exogenous molecule is capable of inducing a perturbation in the bilayer, the thermodynamics 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 infrared results show that the three drugs shift the T_m to lower temperatures, indicating a strong

fluidization of the system. The shifts in the T_m can be induced by alteration of the hydration of the lipid headgroups and thus headgroup–headgroup interactions, which leads to changes in the packing density in the headgroup region. Previous studies have also suggested that the NSAIDs are able to locate near the headgroup of DPPC molecules with the capability to form hydrogen bonding with the water molecules or the headgroup itself [26]. It is known that the DPPC headgroup is inclined thus allowing an interaction with the phosphates but not with the amine N^+-Me_3 group. The observed weakening of the water binding to the phosphate region modifies the membrane affinity for water and the packing of the bilayers, leading to a change in the composition of the aqueous phase, and the equation $\Delta T_t = \Delta G_t/\Delta S_t$ demonstrates the relation between the shift in T_m and the entropy of the water molecules of the hydration in the bilayer [43].

Another phenomenon important to analyze is the increase of the net surface charges. The three drugs are negatively charged. The presence of these molecules in the polar region is modifying the electrostatic properties of the phospholipids, which could explain the shifts in the T_m of DMPC as a concentration-dependent perturbation. All these interactions influence the lipid bilayer structure and of course the lipid phase behaviour. The three NSAIDs induce a change in the sharpness of the phase transition peak as measured by DSC, caused by broadening of the phase transition temperature range (Fig. 4), indicating that the NSAIDs cause a destabilization of the phospholipid intermolecular cooperativity. This property is related with the number of acyl chains that are able to convert from the gel to liquid crystalline phase and reflect the high degree of aggregation in these systems. The DMPC:Ibuprofen:Naproxen systems undergo a small shift in the T_m and a broadening of the phase transition peak. Furthermore, the latter and the DMPC:Diclofenac system show also a phase separation which results in the presence of a second peak in the DSC thermograms (Fig. 4B, C). The presence of another phase is probable due to the induction of domains by the NSAIDs. Diclofenac induces a higher decrease in T_m in comparison with the other two NSAIDs and also a clear phase separation. As the overall ΔH value does not change, it indicates that the NSAIDs are located at the water/lipid interphase. The location of the drugs in the polar head groups in some way generates a reorganization of water molecules affecting the cooperativity of the phospholipid. In the case of Diclofenac, the reorganization must be higher to explain the strong shift in the T_m value. This destabilization is directly related to the increasing drug concentration, indicating further reorganization of water molecules in the head group region with the increase in NSAID concentration. T_m depends to a certain extent on the structure of the polar head groups; therefore the NSAIDs induce changes in the packing of the polar head group region, by modifying the surface-bond water molecules in the bilayer. The number of water molecules within the phospholipid liposomes is a determinant for the functions of the bilayer in an aqueous environment.

The lack of interaction found in the FRET experiments (Fig. 5) is in accordance to the ITC data showing no enthalpic reaction of the drug: lipid complex (Fig. 6). These findings are also consistent with the DSC results: The constant phase transition enthalpy implies that the NSAIDs do not intercalate into the acyl chain region of the bilayer, but are adsorbed at the lipid–water interface, influencing the ordered water layer at the polar interface. These conclusions are compatible with previous studies of other research groups. Castelli and coworkers found that the acid form of Naproxen, covalently linked to a water-soluble polymer, induces a shift in the T_m of DPPC [44] and DMPC [45] liposomes without variations in the enthalpy. The Gibbs energy of binding is dictated by $K_a = e^{-\Delta^{G/RT}}$, however, ΔG depends on two different parameters, $\Delta G = \Delta H - T\Delta S$, which contribute to the process. In case of a dominant electrostatic interaction between a molecule and its ligand, the enthalpy contribution is higher than the entropy one. As the experiments did not detect any kind of enthalpic contribution, it can be concluded that the interactions between the phospholipid and the NSAIDs are mediated only by entropic processes. Taking into account that the interaction of the three drugs with the phosphate group is similar, it is important to check the structural similarities in the three NSAIDs. The COO^- moiety in the drugs is the common feature. It is well known that the phosphate group is sensitive to hydration alterations in membranes. The hydration plays an important role in the stability of the bilayer. The alterations of the hydration shell have consequences for the membrane, like changes in the semi-permeable properties, rate and efficiency of the cell growth and modulate the activity of a variety of membrane-associated enzymes.

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