NSAIDs Interactions with Membranes: A Biophysical Approach

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ABSTRACT:

This work focuses on the interaction of four representative NSAIDs (nimesulide, indomethacin, meloxicam, and piroxicam) with different membrane models (liposomes, monolayers, and supported lipid bilayers), at different pH values, that mimic the pH conditions of normal (pH 7.4) and inflamed cells (pH 5.0). All models are composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) which is a representative phospholipid of most cellular membranes. Several biophysical techniques were employed: Fluorescence steady-state anisotropy to study the effects of NSAIDs in membrane microviscosity and thus to assess the main phase transition of DPPC, surface pressure–area isotherms to evaluate the adsorption and penetration of NSAIDs into the membrane, IRRAS to acquire structural information of DPPC monolayers upon interaction with the drugs, and AFM to study the changes in surface topography of the lipid bilayers caused by the interaction with NSAIDs. The NSAIDs show pronounced interactions with the lipid membranes at both physiological and inflammatory conditions. Liposomes, monolayers, and supported lipid bilayers experiments allow the conclusion that the pH of the medium is an essential parameter when evaluating drug–membrane interactions, because it conditions the structure of the membrane and the ionization state of NSAIDs, thereby influencing the interactions between these drugs and the lipid membranes. The applied models and techniques provided detailed information about different aspects of the drug–membrane interaction offering valuable information to understand the effect of these drugs on their target membrane-associated enzymes and their side effects at the gastrointestinal level.

INTRODUCTION

Inflammatory processes induce the production of the membrane-associated enzyme cyclooxygenase-2 (COX-2) which is the principal target of nonsteroidal anti-inflammatory drugs (NSAIDs). In order to achieve their main target, NSAIDs have first to pass through the membranes, and hence the drug–membrane interaction constitutes an important step to predict the distribution of these drugs in the body and ultimately to understand their therapeutic effects. Lipophilicity, acid–base properties, and structural chemical features of drugs may play a determinant role among various factors that rule the drug–membrane interactions. In this regard, the acid–base properties of NSAIDs determine the different ionization states according to the biological environments encountered by the drug. Therefore, this work involved studies conducted at physiological conditions (pH 7.4) and at pathological conditions representative of inflamed tissues (pH ≈ 5.0). The acidic pH is also important in the study of NSAIDs–membrane interplay at the stomach level, where a local effect of the drugs may disrupt the protective lipid barrier and cause gastric toxicity.

Hence, the main objective of this research is to gain further insight into the NSAID–membrane interaction at the two most relevant pH values mentioned above. To achieve this aim, four NSAIDs belonging to different chemical groups were chosen: meloxicam, piroxicam, nimesulide, and indomethacin (Figure 1).

Membrane mimetic models are widely used to study the interactions of drugs with membranes, once they avoid the complexity of the biological membranes, providing experimental information.
conditions to contribute to a better understanding of the molecular mechanisms that rule these interactions. In this work, three types of membrane models were used to assess the influence of NSAIDs on membranes: liposomes, monolayers, and supported lipid bilayers (SLB). All models were composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). The advantage of using a single-component system instead of a mixture of natural phospholipids is the unambiguous determination of the most important biophysical parameters. Furthermore, DPPC was chosen because phosphatidylcholines are one of the most abundant phospholipids of natural plasma membranes and line the luminal aspects of the mucus gel layer providing the stomach with a protective layer of surface-active phospholipids. Moreover, DPPC is an endogenous component of the joints and represents approximately 45% of the total synovial fluid lipid component, which constitutes the main target of many inflammatory diseases such as rheumatoid arthritis and scleroderma.

Regarding the type of membrane models used, liposomes and monolayers allow study of the influence of dimensionality and curvature, which is relevant to understanding what happens at the cellular level. Liposomes labeled with an interfacial probe, 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were used to assess the effect of the NSAIDs on the main phase transition temperature (T<sub>m</sub>) of DPPC at pH 7.4 and 5.0 by steady-state fluorescence anisotropy measurements. However, in three-dimensional (3D) spherical membrane mimetic systems such as liposomes, membrane parameters as packing density, mobility of the molecular components, and topology of the surfaces are mutually affected by interactions with drugs, and it is very difficult to extract individual contributions of any of these parameters. In this context, Langmuir monolayers at the air/liquid interface complemented the information gathered with 3D lipid models, and allowed study of the adsorption and penetration of the NSAIDs at the membrane surface. Moreover, these 2D membrane model systems provide many possibilities to control, in an effective way, a wide range of experimental variables, such as phase state of the film-forming molecules, packing density, surface viscosity, and mobility of the molecules.

When coupled with infrared reflection–absorption spectroscopy (IRRAS), isotherms of lipid monolayers provide a description on the molecular level of the modifications that occur due to the interactions with NSAIDs including changes in chain conformation and hydrogen bonding or ionic bonds.

Supported lipid bilayers (SLB) are extensively studied membrane models that provide information on lipid properties and organization. The lateral mobility of lipids in supported bilayers makes them particularly useful models because they retain much of the fluidity associated with actual cellular membranes. Changes in the morphology of the SLB after interaction with the NSAIDs were thus assessed by atomic force microscopy (AFM) that has been shown to be ideally suited for obtaining detailed morphological information on effects of drugs on membranes as, i.e., phase separation processes.

In summary, the main aim of this work is to contribute to a better understanding of drug–membrane interactions by the use of a variety of membrane model systems (liposomes, monolayers, and supported lipid bilayers) and of several sophisticated biophysical techniques (fluorescence steady-state anisotropy to study the effects in membrane microviscosity, surface pressure—area isotherms to evaluate adsorption and penetration of NSAIDs into membranes, IRRAS to acquire structural information regarding the drug—membrane interaction, and AFM to study changes in surface topography during the interaction of NSAIDs with lipid membranes). The gathered results will lead to a better understanding of the NSAIDs mode of action and will shed some light on their local gastric toxicity.

### MATERIALS AND METHODS

**Reagents.** The anti-inflammatory drugs indomethacin, nimesulide, piroxicam, and meloxicam were obtained from Sigma-Aldrich, and DPPC was supplied by Avanti Polar Lipids, Inc. (Alabama, USA). TMA-DPH was purchased from Molecular Probes (Invitrogen Corporation, Carlsbad, California, USA). These reagents were used without further purification. All other chemicals were purchased from Merck (Darmstadt, Germany).

Drug solutions were prepared with either Hepes buffer (pH 7.4) or acetate buffer (pH 5.0) and the ionic strength I = 0.1 M was adjusted with NaCl. The buffers were prepared using double deionized water (conductivity <0.1 μS cm<sup>-1</sup>).

**Phase Transition Temperature Studies.** Phase transition temperature studies were performed using DPPC liposomes labeled with 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene probe (TMA-DPH). DPPC and TMA-DPH were co-dissolved in chloroform/methanol mixture (3:1) to give a lipid/probe molar ratio of 300:1. The mixture was evaporated to dryness with a stream of nitrogen and the lipid film was left under vacuum overnight to remove all traces of the organic solvent. The resultant dried lipid film was hydrated with the desired buffer (either Hepes or Acetate) and vortexed to yield unilamellar liposomes (MLVs). Lipid suspensions were equilibrated at (45.0 ± 0.1) °C for 30 min, and extruded 10 times through polycarbonate filters with a pore diameter of 100 nm to form unilamellar vesicles (LUVs). Buffered NSAIDs solutions were added to liposomes to reach final drug and liposome concentrations of 40 μM and 500 μM, respectively.

The transition temperature was assessed by fluorescence anisotropy measurements in a Perkin—Elmer LS 50B steady-state fluorescence spectrometer equipped with a constant-temperature
cell holder. All data were recorded in 1 cm cuvettes with excitation and emission slits between 2.8 and 3.0 nm. The excitation and emission wavelengths were set to 361 and 427 nm, respectively. The anisotropy was recorded at several temperatures between 28.0 and 52.0 °C, with an accuracy of 0.1 °C.

**Isotherm Measurements.** Surface pressure—area isotherms (\(\pi/A\)) were measured in a PTFE Langmuir trough (Riegler & Kirstein, Potsdam, Germany), equipped with barriers for changing the surface area, and a Wilhelmy microbalance with filter paper plate (accuracy superior to 0.1 mN m\(^{-1}\)) for measuring the surface tension of the monolayer covered surface.

Monolayers of DPPC were obtained after spreading phospholipid/chloroform solutions (1 mM) onto the buffer subphase (Hepes or Acetate buffer) or onto the drug subphase (50 \(\mu\)M) prepared in the same buffers. After an equilibrium time of 10 min, the monolayers were compressed at a rate of 5 Å\(^2\)/molecule/min. Before each compression, the trough was cleaned thoroughly with chloroform and double deionized water. Cleanliness was confirmed by compressing a water subphase and keeping zero surface pressure reading. All measurements were performed at 20 °C.

**Infrared Reflection Absorption Spectroscopy (IRRAS).** Spectra were acquired with an IFS 66 FT-IR spectrometer from Bruker (Ettlingen, Germany) equipped with an external reflectance unit (XA - 511, Bruker) containing a Langmuir trough setup (R&K) and a liquid nitrogen-cooled MCT detector. The IR beam is directed through the external port of the spectrometer and is subsequently reflected by three mirrors in a rigid mount perpendicular to the plane of incidence, had an angle of incidence of 40° with respect to the surface normal. This angle of incidence is obtained by a computer-driven rotation of the mount holding the mirrors. The reflected light was collected at the same angle as the angle of incidence. Measurements were made by switching between two interconnected troughs (to ensure the same surface height on both sides) at regular intervals using a trough shuttle system controlled by the acquisition computer. One trough contained the monolayer system under investigation (sample), whereas the other (reference) is filled with the pure subphase. The spectra from the reference trough were subtracted from the sample spectra in order to eliminate the water vapor signal. To reduce relative humidity fluctuations, the setup was placed in a hermetically sealed container. Spectra were recorded with a spectral resolution of 8 cm\(^{-1}\) and accumulated using 200 (for s-polarized light) scans.

**Atomic Force Microscopy (AFM).** The AFM studies were performed on supported lipid bilayers (SLB). The SLB were prepared by the fusion of LUVs on solid supports of freshly cleaved mica surfaces and heated for 45 min at 60 °C, in order to form the SLB. After slowly cooling the system down to room temperature, the samples were carefully rinsed with buffer (Acetate or Hepes) in order to remove the excess of LUVs. AFM images were taken at room temperature using a Nano-scope III AFM in tapping mode (Veeco/Digital Instruments, Santa Barbara, CA). Cantilevers with 0.01 N m\(^{-1}\) spring constants and oxide-sharpened tips in the liquid cell (multimode liquid cell using O-ring) were used. At least three regions of the surface were examined to verify if similar morphology existed throughout the sample.

After recording the lipid bilayer in buffer and choosing an image of a region of interest, that is, a region which is representative of the entire SLB, the buffer was exchanged for the NSAID solution (1 mM). Several images were recorded for at least 2 h. The images were analyzed using the Nanoscope V software from the AFM supplier.

**RESULTS AND DISCUSSION**

**Phase Transition Temperature Studies.** The effect of NSAIDs on the main phase transition temperature of the lipid membrane \(T_m\) was assessed by steady-state fluorescence anisotropy studies determining the degree and extent of rotational diffusion of a membrane-bound fluorophore (probe) during the lifetime of the excited state. Small changes in the microstructure of the lipid bilayer due to NSAIDs—lipid interactions produce changes in the rotational movement of the probe and, as such, promote changes in anisotropy. The fluorophore used in this study was TMA-DPH which contains a DPH phenyl ring located within the hydrophobic acyl chains of the membrane phospholipids and a cationic group that anchors the probe in the polar headgroup region of the phospholipids. Therefore, TMA-DPH reports the interfacial region of lipid membranes.

The effects of drug—membrane interactions on the microstructure of the lipid bilayer were measured comparing fluorescence anisotropy data in the presence and absence of NSAIDs. Figure 2 presents, as an example, the results obtained with DPPC liposomes labeled with TMA-DPH in the presence and absence of indomethacin at pH 5.0 (Figure 2 A) and pH 7.4 (Figure 2 B).

The data was fitted by the following equation:

\[
\frac{1}{r_s} = \frac{1}{r_{s1}} + \frac{p_1 T + r_{s2} - r_{s1} + p_2 T - p_1 T}{1 + 10^{(1/T - 1/T_m)}} \tag{1}
\]

where \(T\) is the absolute temperature, \(p_1\) and \(p_2\) are the slopes of the linear fits to the data before and after the phase transition region, respectively, and \(r_{s1}\) and \(r_{s2}\) are the corresponding \(y\) intercepts. From this equation, it is also possible to determine the cooperativity \((B)\) and the main phase transition temperature \(T_m\) of the lipid bilayers from the gel phase (\(T_{g2}\)) to the liquid-crystalline phase \(T_{l2}\). For all the studied systems, the values of cooperativity \((B)\) and main phase transition temperature \(T_m\) are presented in Table 1.

The \(T_m\) values obtained for DPPC liposomes at pH 7.4 (Table 1) are consistent with previously reported studies. Comparing \(T_m\) of DPPC at both pH values shows that \(T_m\) at pH 5.0 is approximately 2 °C lower compared to the \(T_m\) value at pH 7.4, and the cooperativity is enhanced at pH 5.0. The liquid-crystalline phase is stabilized at pH 5.0 due to higher solvation of the lipid head groups, which is also responsible for the increase in the cooperativity at pH 5.0.

Regarding the effects of NSAIDs, it is important to mention that the qualitative and quantitative differences in the lipid phase transition characteristics arise from a localization of these drugs in different regions of the bilayer. Therefore, although the molecular details of the drug—membrane interaction and the changes of the physical state of the membrane cannot be readily...
discerned from these experiments, it is still possible to draw conclusions about the location of the drugs in the membrane, and to establish correlations between the ionized forms of the NSAIDs studied and their proposed membrane locations.

The membrane is characterized by a fluidity gradient both above and below the transition temperature, i.e., the part of the hydrocarbon chains near the center of the bilayer is more "fluid" or disordered than the part of the chains near the headgroup region.28,30 Accordingly, along the acyl chains there is a constant segmental order parameter between C1 and C9. However, at the chain end (C10 until C16) there is considerable disorder.28,35 Therefore, the size of the cooperative unit that undergoes the transition is largely regulated by the interaction in the C1 until C9 regions of the acyl chains, where the membrane is more ordered.31 Changes in the cooperativity and in the transition temperature induced by a drug can be taken as indication for the location of the drug, i.e., the drug is located in the chain region close to the head groups (C1—C9).36,37 On the other hand, as the bilayer core is relatively disordered, a drug located in this region (C12—C16) would have little or no effect on the transition profile. Thus, if the drug penetrates deeper into the membrane, one would expect a change in the packing (lowering of the transition temperature) but only a slight (if any) change in the size of the cooperative unit, i.e., no or very subtle alterations in the cooperativity.36,37

Following these arguments, it is possible to predict the membrane location of NSAIDs according to their effects on the transition parameters. Therefore, at pH 7.4, all the NSAIDs reduced the Tm values and changed the cooperativity (Table 1) meaning that the drugs must be located in the higher ordered region close to the head groups (C1—C9). This location is in agreement with the fact that the NSAIDs are negatively charged at this pH which may be the reason for their distribution closer to the polar head groups. Furthermore, these results are consistent with previous X-ray diffraction studies where indomethacin and nimesulide reduced the Tm of liposomes of DPPC without changing the phase transition enthalpy (ΔH), which is consistent with the location proposed.5

All the NSAIDs reduced the cooperativity of the transition at pH 7.4, except indomethacin, that caused a cooperativity increase (Table 1), consistent with a location of this NSAID in the headgroup region of the bilayer.25,38 Increased values of cooperativity were observed in the literature for interactions of ions with membranes.38 Hence, the charged ions are able to screen the charges on the phosphate and on the quaternary ammonium group of the DPPC polar head, reducing the electrostatic interactions between DPPC dipoles. In this way, the polarity of the interfacial zone of the bilayer is reduced, with a consequent increase of the attractive van der Waals forces between the phospholipid molecules, giving rise to a more cooperative phase transition.38 Similarly, the observed increase in cooperativity induced by indomethacin can be interpreted as an indication of tighter intermolecular binding, probably between the negatively charged indomethacin and the positively charged choline. This observation is consistent with previous X-ray diffraction studies performed using the same lipid and the same pH conditions that provided evidence for an interaction between indomethacin and the PC head groups.5,39

In contrast to pH 7.4, NSAIDs at pH 5 have smaller effects on the Tm. In the case of nimesulide and meloxicam, the cooperativity is also kept constant within the error (Table 1), pointing to a deeper location of these drugs in the membrane core (at the level of C12—C16).36,37 Indeed, if the drugs are located close to the bilayer core, where the bilayer is in a relatively disordered state, this explains why the drugs have little or no effect on the transition profile (either transition temperature or cooperativity). At pH 5.0, indomethacin and piroxicam are still located in the chain region closer to the head groups (C2—C8), although they penetrate deeper than at pH 7.4, as demonstrated by the subtle variations of the transition profile (Table 1).
Isotherm Measurements. Monomolecular lipid films show ordered phases similar to three-dimensional smectic liquid crystals. Surface pressure/area ($\pi/A$) isotherms give information about the phases and the type of phase transitions (first or second order) of a lipid monolayer, which in the case of phospholipids can be gaseous (G), liquid expanded (LE), condensed (LC), and solid phases (S), depending on the temperature, pressure, and pH. The condensed phases can be specified based on grazing incidence X-ray diffraction (GIXD) measurements. Figure 3A,B shows, respectively, the $\pi/A$ isotherms of a DPPC monolayer at pH 5.0 and 7.4 (black lines). The $\pi/A$ isotherm of DPPC at pH 7.4 is in good agreement with literature data showing the same values for the transition pressure and the limiting area per lipid molecule.\(^5\) The $\pi/A$ isotherm of DPPC at pH 5.0 is very similar. This could be expected since a zwitterionic monolayer is much less sensitive to changes of pH or salt concentration.\(^5\)

The NSAIDs studied (nimesulide, indomethacin, meloxicam, and piroxicam) are amphiphilic molecules that can adsorb and penetrate into the lipid monolayer. The penetration and/or adsorption of NSAIDs to the lipid monolayers was evaluated comparing $\pi/A$ isotherms of DPPC monolayers on a buffer subphase (pH 5.0 and 7.4) in the absence and presence of drugs (Figure 3A,B).

From $\pi/A$ isotherms obtained, it was possible to calculate the minimum area per lipid molecule ($A_{\text{min}}$), the elastic modulus ($C_s^{-1}$), and the lipid monolayer collapse pressure ($\pi_{\text{collapse}}$). The effect of NSAIDs was also quantified in terms of these parameters (Table 2).

The elastic modulus ($C_s^{-1}$) of the DPPC monolayers was calculated from the $\pi/A$ isotherms by the following equation:

$$C_s^{-1} = -A(d\pi/dA)$$  \hspace{1cm} (2)

where $A$ is the area per lipid molecule, and $\pi$ is the surface pressure.

$C_s^{-1}$ describes the relationship between the surface pressure increase and the area strain (area decrease),\(^4\) so a higher value of $C_s^{-1}$ indicates a less compressible lipid monolayer.\(^4\) Accordingly, in the absence of drugs, the $C_s^{-1}$ and $A_{\text{min}}$ values obtained for DPPC on both pH subphases indicate that the DPPC monolayer exhibits very similar properties (Table 2). Therefore, we assume that the packing density in the LC phase is not influenced by the buffer used.

All the NSAIDs studied (indomethacin, nimesulide, piroxicam, and meloxicam) are weak acids with $pK_a$ values between 4.50 and 6.50 (see Figure 1).\(^4\) Therefore, these drugs have a higher ionization degree at pH 7.4, and are negatively charged. Hence, the interaction between these NSAIDs and DPPC monolayers is highly dependent on the pH of the study. DPPC is a zwitterionic phospholipid with a negatively charged phosphate group ($\text{PO}_2^-$) and a positively charged trimethylammonium group ($[(\text{CH}_3)_3\text{N}]^+$).\(^4\) The headgroup is oriented almost parallel to the surface and strongly hydrated. This causes a large area requirement of the headgroup and forces the chains to be tilted in the condensed phase to optimize their van der Waals interactions. The drug interactions with lipid monolayers are

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**Table 2. Characteristic Parameters (Minimum Area Per Lipid Molecule, Elastic Modulus and Collapse Pressure) of the Langmuir DPPC Monolayers on the Aqueous Subphases Containing 50 $\mu$M of Indomethacin, Nimesulide, Piroxicam, and Meloxicam at pH 5.0 and 7.4**

<table>
<thead>
<tr>
<th>Subphase</th>
<th>pH</th>
<th>$A_{\text{min}}$ ($\AA^2$/molecule)</th>
<th>$C_s^{-1}$ (mN/m)</th>
<th>$\pi_{\text{collapse}}$ (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>5.0</td>
<td>53.8 ± 0.5</td>
<td>260 ± 10</td>
<td>54 ± 1</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>55.0 ± 0.5</td>
<td>220 ± 10</td>
<td>52 ± 1</td>
</tr>
<tr>
<td>Inclomethacin (50 $\mu$M)</td>
<td>5.0</td>
<td>52.1 ± 0.5</td>
<td>172 ± 10</td>
<td>34 ± 1</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>68.3 ± 0.5</td>
<td>169 ± 10</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>Nimesulide (50 $\mu$M)</td>
<td>5.0</td>
<td>48.4 ± 0.5</td>
<td>193 ± 10</td>
<td>51 ± 1</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>66.5 ± 0.5</td>
<td>220 ± 10</td>
<td>44 ± 1</td>
</tr>
<tr>
<td>Piroxicam (50 $\mu$M)</td>
<td>5.0</td>
<td>53.0 ± 0.5</td>
<td>153 ± 10</td>
<td>37 ± 1</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>55.4 ± 0.5</td>
<td>109 ± 10</td>
<td>52 ± 1</td>
</tr>
<tr>
<td>Meloxicam (50 $\mu$M)</td>
<td>5.0</td>
<td>57.7 ± 0.5</td>
<td>174 ± 10</td>
<td>40 ± 1</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>52.1 ± 0.5</td>
<td>228 ± 10</td>
<td>58 ± 1</td>
</tr>
</tbody>
</table>

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Figure 3. Surface pressure/area ($\pi/A$) isotherms of DPPC at pH 5.0 (A) and pH 7.4 (B) in buffer (black), indomethacin (green), nimesulide (magenta), piroxicam (cyan), and meloxicam (gray).
strongly dependent on the molecular packing density of the lipids. In the liquid expanded phase (LE), phospholipid acyl chains have considerable degree of rotational freedom, and the lower lipid density facilitates interactions and insertion of the drugs in comparison to the lipid condensed phase.

The NSAIDs clearly change the π/A isotherm profile of the pure DPPC monolayers at both pH 5.0 (Figure 3A) and pH 7.4 (Figure 3B). In most cases, the NSAIDs studied shift the isotherms to larger molecular areas (expansion), and lead to a decrease in the cooperativity of the phase transition (the coexistence region of the first-order phase transition is smeared over a larger pressure range). The differences between isotherms measured at different pH values will be discussed for each drug separately.

At pH 5.0, meloxicam and indomethacin shift the DPPC π/A isotherm to larger molecular areas in the disordered LE state (see Figure 3A). The increase in the molecular area indicates that the NSAIDs penetrate into the DPPC monolayer. In agreement with the experiments in liposomes, meloxicam could penetrate into the hydrophobic region of the DPPC layer, but will be clearly squeezed out upon compression. Piroxicam and nimesulide do not show any shift of the molecular area in the LE state. Obviously, these drugs do not penetrate deeply into the DPPC layer, but they are located close to the headgroup region. In the condensed phase, the interactions of the DPPC monolayer with indomethacin, piroxicam, and nimesulide lead to clearly smaller molecular areas. The reason could be that an interaction between these NSAIDs and the lipid headgroups occurs in the condensed phase with a consequent changed orientation and/or hydration of the bulky PC head groups. This would reduce the area requirement of the head groups, reduce the mismatch between the headgroup and chain areas, and allow the chains to be less tilted. GIXD will in future show if this assumption is justified. Contrarily, meloxicam does not influence the headgroup region in the condensed state, as discussed for the other NSAIDs.

At pH 7.4, the drugs are negatively charged. The negatively charged groups of the drugs are therefore able to interact electrostatically with the positively charged choline, which might possibly change the orientation of the headgroup and influence the packing density. However, the charge effect is clearly not the only factor conditioning the interaction of the drugs with the lipid monolayers: the charge at the nitrogen is screened by the 3 bulky methyl groups. In addition, the effects of NSAIDs on the elastic modulus (C_{11}^{-1}) and the collapse pressure (\pi_{\text{collapse}}) indicate that the drugs interact with the DPPC monolayer at both pH values, i.e., the interaction between NSAIDs and membranes occurs even when the drugs are less charged. In most cases, the condensed lipid films spread on the subphases containing NSAIDs are more compressible than the films spread on the buffer. Similarly, the stability of the monolayers decreases in the presence of drugs, as indicated by the decreasing values of \pi_{\text{collapse}}. In agreement with these results, literature reports that π/A isotherms of DPPC monolayers on subphases containing piroxicam, indomethacin, and nimesulide at pH 7.4 are shifted to larger molecular areas, which is an indication of the penetration of drugs into the lipid monolayer leading to a fluidization of the phospholipid system. Furthermore, other studies with different NSAIDs and different lipid monolayers consistently revealed an increase in the mean molecular area of the lipid layer, confirming the capacity of NSAIDs to interact with lipid membranes.

Meloxicam at pH 7.4 causes only a slight decrease in the area per DPPC molecule, but similarly to what has been observed at pH 5.0, this NSAID is clearly squeezed out upon compression of the monolayer (Figure 3B). However, it is important to note that the squeezing-out pressure of meloxicam is reduced on the pH 7.4 subphase compared to the pH 5 subphase, and at lateral pressures above 30 mN/m, which is discussed as the lateral pressure of bilayers, the molecular area of the lipid monolayer with meloxicam is the same as observed for DPPC on the bare buffer surface. This shows that meloxicam has the smallest effect on the DPPC isotherm at this pH and that this drug does not influence the headgroup region in the condensed state, similarly to what has been discussed at pH 5.0. Compared with meloxicam, piroxicam exhibits higher effects on DPPC monolayers at pH 7.4. At low pressure, a shift to larger molecular areas can be seen. The transition range is shifted to higher pressures and shows a reduced cooperativity. Above 30 mN/m, the drug is squeezed out from the condensed DPPC phase.

Nimesulide and indomethacin lead clearly to the expansion of the DPPC film at pH 5.0. The transition region cannot be identified from the isotherms of these NSAIDs; therefore, it remains unclear whether the DPPC layer is in a condensed state at high pressure.

Figure 4A,D shows pressure–time isotherms demonstrating the different ability of the drugs to penetrate into the LE phase of the DPPC monolayer. In all cases, the starting pressure of the DPPC monolayer was zero. At both pH values, indomethacin is the drug with the largest equilibrium pressure after long adsorption time. The isotherms measured after reaching the equilibrium adsorption pressure (Figure 4B,E) are slightly shifted compared to the ones measured directly after spreading the monolayer (Figure 3A,B), but the trend discussed above is not changed. This shows that the adsorption process needs a certain time for reaching the equilibrium. These isotherms will be compared with the IRRAS results, since IRRAS measurements have always started 1 h after spreading the DPPC monolayer and the IRRAS experiment along an isotherm takes several hours. To estimate the transition pressure from the isotherms is extremely difficult since the first-order transition and the squeezing-out of the drug overlap. From the analysis of Figure 4C and F, where the ΔA values (the difference in area per molecule with respect to the pure DPPC) are plotted versus π at the two pH values studied, it can be concluded that the NSAIDs show different effects at the two pH conditions studied. Upon interaction with the NSAIDs, the LE phase is more expanded (the largest shift is observed for indomethacin), and the LC phase is not influenced (due to the fact of drugs being squeezed out of the monolayer upon compression), is shifted to higher molecular areas (in the case of nimesulide and indomethacin at pH 7.4), or is shifted to smaller molecular areas (due to changes in the area requirement of DPPC due to interactions of the head groups with the drugs).

This latter effect is in agreement with the study of the interaction between dimyristoylphosphatidylcholine (DMPC) monolayers and oximes, where meloxicam induced negative ΔA values at comparable concentrations. This shift to lower molecular areas was discussed as a solubilization effect of the lipids into the subphase. Indeed, similar behavior has been reported in the literature, where changes in the molecular area of the lipids have been attributed to a solubilization effect of the lipid molecules.

At pH 7.4 and at the pressure of the biomembranes (π ≈ 30 mN/m), indomethacin and nimesulide are still incorporated.
into the lipid monolayer, while at pH 5.0, all the NSAIDs are squeezed out from the monolayer.

**Infrared Reflection/Absorption Spectroscopy Measurements.** Langmuir isotherms provide important information, but do not allow a complete molecular description of the interaction of the drugs with the lipid monolayers. Therefore, to complement the information already gathered, infrared reflection-absorption spectroscopy (IRRAS) measurements were performed to acquire structural insights into the influence of the pH on the biophysical properties of the lipid membrane and detailed information about the effect of the NSAIDs studied on chain conformation, hydrogen bonding, and ionic interactions.

The reflectance-absorbance (RA) intensity is presented as $-\log(R/R_0)$, where $R$ and $R_0$ are the reflectivity values of the monolayer-covered and pure buffer solution surfaces, respectively. Accordingly, IRRAS data are plotted as RA intensity versus wavenumber for different compression states of the monolayer.

Figure 5 shows the IRRAS spectra of a DPPC monolayer at pH 5.0 in buffer (black), indomethacin (green), nimesulide (magenta), piroxicam (cyan), and meloxicam (gray) during intermittent compression.

Figure 6 shows the surface pressure versus frequency of the methylene asymmetric stretching vibration for a DPPC monolayer at pH 5.0 in the LE phase ($\pi = 5$ mN/m, black line) and in the LC phase ($\pi = 30$ mN/m, gray line) where the wavenumbers of the most characteristic vibrations of DPPC in a monolayer are indicated.

In the previously presented Langmuir isotherm measurements, the phase transition between LE and LC could not be clearly identified. In contrast to the isotherm measurements, IRRAS measurements offer the possibility to plot the wavenumbers ($\nu_{as(CH_2)}$) of DPPC on buffer and on the different drug subphases, thus providing information about the influence of drugs in the LE-LC phase transition. Figure 6 shows as an
example of the wavenumbers (νs(CH2)) of DPPC on the different subphases (pH 5) plotted versus the lateral pressure. Values around 2925 cm\(^{-1}\) indicate disorder in the chain region (presence of gauche conformers), and values below 2920 cm\(^{-1}\) are typical for all-trans conformation. On the pure pH 5 subphase, the transition occurs at about 4.5 mN/m in agreement with the Langmuir isotherm experiments. The phase transition pressure in presence of the NSAIDs is shifted to higher values (Figure 6) and shows that the transition pressure increases in the following order: DPPC on pH 5 buffer (~4.5 mN/m) < on the meloxicam solution (~8.8 mN/m) < on the piroxicam solution (~9.5 mN/m) < on the nimesulide solution (~11.5 mN/m) < on the indomethacin solution (~16.5 mN/m). This information cannot be obtained by measuring only the isotherms. In many systems studying the interaction between insoluble phospholipids and soluble drugs, the transition pressure cannot be connected with pronounced features in the isotherm because the transition from LE to LC and the squeezing out of the drug are superimposed processes. These results show the importance of using more than one method for such studies. The wavenumbers in the LC phase are for all the systems very similar (with slightly higher values in the case of meloxicam) typical for the expected all-trans conformation of the hydrocarbon chains in a rotator phase.

Besides analyzing the (νs(CH2)), it is also important to study the effects on the ν(C=O) and on the νas(PO\(_2\)\(^-\)) bands. The ν(C=O) band of DPPC monolayers on a pH 5 subphase exhibits a pronounced asymmetry at low lateral pressures (data not shown). It consists of two overlapping bands, one at 1738 cm\(^{-1}\) and another at 1727 cm\(^{-1}\). The high-frequency component of this band is assigned to the non-hydrogen-bonded (free) carbonyl group and the lower-frequency component to the hydrogen-bonded carbonyl group. An increase of the intensity of the free carbonyl band can be seen upon compression. This can be explained with a tighter packing of DPPC in the LC phase and hence with a reduction of the hydration of the carbonyl group.\(^{58-60}\)

This observation indicates that a higher number of carbonyl groups are hydrogen-bonded at pH 5\(^{19}\) and confirms the existence of a high-ordered monolayer at acidic conditions.\(^61\) Moreover, it is observed that at pH 5.0 and for both pressures assayed (γ = 10 and 30 mN/m) the νas(PO\(_2\)\(^-\)) bands appear at lower wavenumbers than at pH 7.4 (Table 3) indicating hydrogen-bonded water molecules to the phospholipid head groups,\(^62\) which is also in very good agreement with the hydration behavior observed for the isotherm experiments.

Generally, the IRRAS data obtained show that, despite the pH conditions, the NSAIDs penetrate more easily into the less ordered phases of the monolayer, which is in agreement with the isotherm measurements (Figure 4C,F) and with previous reported studies.\(^53\)

The subsequent analysis of Table 3 will focus on the effects of NSAIDs on the different regions of the phospholipids through the analysis of the phosphate asymmetric stretching vibration and the carbonyl stretching mode. This analysis will also be performed taking into consideration the pH of study and the lipid phase. Previously, it was proposed that, because of being negatively charged at pH 7.4, the NSAIDs studied would interact preferentially with the headgroup region of the monolayers. With the exception of nimesulide, this preferential interaction can indeed be confirmed by the shift of the νas(PO\(_2\)\(^-\)) to much smaller values compared with pure DPPC (see Table 3, at pH 7.4 and at both pressures), indicating that NSAIDs changed the number and orientation of hydrogen-bonded water molecules around the phospholipid head groups and modified the polarity of the environment.\(^{19,63,64}\) Other studies are consistent with these observations, and suggested that NSAIDs are able to be located near the DPPC head groups, being capable of forming hydrogen bonds with the water molecules or with the headgroup itself.\(^65\) The same observations were made when studying the interaction of NSAIDs with other lipids, e.g., it was reported that NSAIDs interact directly with the phosphate groups of DMPC or DMPE leading to a change of water binding.\(^{66}\) and other studies showed that adding the NSAID diclofenac to lecithin—water systems revealed changes in the hydration properties of the membrane.\(^65\) Nimesulide presents a different behavior at pH 7.4, because unlike the other NSAIDs, this drug does not cause any shift in the νas(PO\(_2\)\(^-\)) (Table 3 at pH 7.4 and both pressures) which gives important evidence that nimesulide is not interacting in the phosphate region. This may be a result of the different ionization state of nimesulide. Unlike the other NSAIDs studied, nimesulide does not possess acidic groups, like indomethacin that contains a carboxylic group, or oxicams (meloxicam and piroxicam) that contain an enolic group. Therefore, the pKₐ of nimesulide is higher than the pKₐ of the other NSAIDs assayed (Figure 1), and although the anionic form of nimesulide is dominant (80%) at pH 7.4, a neutral counterpart is also apparent (20%). Therefore, it seems clear that a less ionized molecule is less capable of interacting with the polar region of the membrane. Despite not being able to interact with the head groups of the membrane, nimesulide exhibits interaction with the acyl groups of the monolayer which is evident

<table>
<thead>
<tr>
<th>Subphase</th>
<th>pH 5.0</th>
<th>Indomethacin</th>
<th>Nimesulide</th>
<th>Piroxicam</th>
<th>Meloxicam</th>
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<td>v(_s)(CH(_2)) (cm(^{-1}))</td>
<td>2851</td>
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<td>2922</td>
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</tr>
<tr>
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<td>1227</td>
<td>1228</td>
<td>1227</td>
<td>1227</td>
</tr>
<tr>
<td>ν(C=O) (cm(^{-1}))</td>
<td>1738</td>
<td>1743</td>
<td>1729</td>
<td>1730</td>
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</tr>
</tbody>
</table>

Table 3. Vibrational Wavenumbers of DPPC Monolayers on the Aqueous Subphases Containing Indomethacin, Nimesulide, Piroxicam, and Meloxicam at pH 5.0 and 7.4 for γ = 10 and 30 mN/m
from the increase in the wavenumbers of the $\nu_{\text{C=O}}(\text{CH}_2)$ and $\nu_{\text{C=O}}(\text{CH}_3)$ bands (Table 3 at pH 7.4 and $\pi = 10 \text{ mN/m}$).

Being weak acids, the NSAIDs studied are less ionized at pH 5, and thus more prone to penetrate deeper within the phospholipids possibly establishing hydrophobic interactions at the bilayer core. This fact observed in the anisotropy studies (where the analysis of Table 1 pointed to a deeper location of NSAIDs within the membrane core) is confirmed by the IRRAS measurements which indicate that, at pH 5.0, the NSAIDs are able to penetrate within the acyl chains of phospholipids, thus shifting the phase transition pressure (Figure 6). The deeper membrane penetration of NSAIDs at pH 5.0 is hence indicative that these drugs induce changes in lipids conformers from trans to gauche and consequently have a lipid disordering effect.\textsuperscript{19} Despite being less charged at pH 5.0, indomethacin still interacts with the headgroup region of the monolayer. Interestingly, the $\nu(C=O)$ band of DPPC is composed of two components as on the pure pH 5 buffer; however, the intensity of the components changes in a different way (data not shown). With the exception of nimesulide, the high-frequency band increases upon compression as observed for DPPC on the pure buffer. In the case of nimesulide, the intensity ratio does not change upon compression. The C=O group is sensitive to the hydration state, polarity, and the degree and nature of hydrogen bonding interactions;\textsuperscript{64} therefore, this shift to higher wavenumbers reflects the dehydration of the carbonyl moieties,\textsuperscript{33} upon interaction with drug molecules.

Besides considering the great influence of the pH on the interactions between drugs and membrane, it should be mentioned that the lipid phase has also a role defining the interactions between the drugs and different phospholipid groups. In this context, in the condensed phase ($\pi = 30 \text{ mN/m}$), the most affected groups are the polar head groups and the chains are in all-trans conformation. Moreover, in the condensed phase ($\pi = 30 \text{ mN/m}$) the interactions at the carbonyl level of the lipid monolayer are more significant at pH 5.0 than at pH 7.4. Contrastingly, the interactions of NSAIDs (except nimesulide) at the phosphate level are more evident at pH 7.4 than at pH 5.0. These results confirm that, despite interacting less with the monolayer in the condensed phase, NSAIDs, which are squeezed out upon compression, still interact with the headgroup region of the monolayer, and at the acidic pH, they penetrate even up to the carbonyl groups of the phospholipid polar heads, while at pH 7.4, NSAIDs interact preferentially with the outer phosphate groups.

**Atomic Force Microscopy.** Atomic force microscopy (AFM) is a direct imaging tool for visualizing lipid bilayers on a substrate\textsuperscript{66} providing images of biosurfaces at high resolution and in buffer solutions. With this technique, it is possible to explore the nanoscale properties of membranes\textsuperscript{22} and follow the evolution of the interaction of supported lipid bilayer (SLB) model systems with drugs.\textsuperscript{67}

The process of formation of SLB from liposomes involves adsorption of the vesicles on the surface, rupture, and flattening to form a planar phospholipid bilayer.\textsuperscript{22,68} After preparation, SLBs were kept hydrated (by addition of either pH 5.0 or pH 7.4 buffer) at room temperature. At these conditions, the bilayer of DPPC is in gel phase ($l_{\text{FF}}$) mimicking the protective gastric layer made of phosphatidylcholine.\textsuperscript{9} The SLB produced will be used to study the so-called “topical” effect(s) of the NSAIDs. This term is used for the nonprostaglandin mediated effects of NSAIDs which occur when high concentrations of the drugs are in contact with the mucosa, following ingestion or via biliary excretion. Indeed, in the circulation, NSAIDs are extensively protein-bound, and the effective free plasma concentration of the drugs is within the micromolar range, whereas the concentration required for COX inhibition is even smaller (within the submicromolar range). Therefore, to assess the acute gastrointestinal toxicity of NSAIDs, high concentrations of drugs should be tested to simulate the topical concentration by NSAID accumulation.\textsuperscript{70–72}

The accumulation of NSAIDs is not only relevant to their topical gastric toxicity, but it also occurs at the target tissues of these drugs. In inflamed tissues some NSAIDs were reported to accumulate, leading, for example, to concentrations in the synovial fluid of inflamed joints about three to eight times higher than those in plasma and also in control joints.\textsuperscript{73} Whether the apparent safety of the NSAIDs will be kept up at the higher doses needed for rheumatoid arthritis, and also at the higher concentrations reached by accumulation, is still a matter of investigation, and in this regard the interaction between NSAIDs and SLB was tested at high drug concentrations. Hence, after scanning SLB of DPPC in buffer for one hour to ensure stability of the membrane and to choose a region which is representative of the entire SLB, the buffer in the cell was exchanged for a 1 mM solution of drug in the same buffer by injection into the cell using an O-ring device.

The injection of NSAIDs on the SLB of DPPC altered dramatically their nanoscale organization. The effects consisted of a time-dependent erosion of the gel phase domains that might be due to the interaction between NSAIDs and DPPC molecules. Presumably, disruption of the lipid–lipid interactions due to the intercalation of the drug is responsible for the progressive disappearance of the domains. Figure 7 shows a typical example

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**Figure 7.** AFM image of DPPC supported lipid bilayer at pH 7.4 in the presence of meloxicam with different incubation times.
of the time-dependent interactions of meloxicam with DPPC SLBs at pH 7.4.

From Figure 7, one can conclude that the interaction of meloxicam with the SLB results in a decrease of the bilayer surface coverage. Several holes appear in the bilayer, and their size and number increase with time of interaction between the SLB and the drug. Figure 8 illustrates the variation of the radius of the holes formed in the bilayer in a time course upon interaction with meloxicam. Simultaneously, the thickness of the supported lipid bilayer is reduced upon interaction of meloxicam and in a time course process.

These effects were observed for all NSAIDs used and at the two pH conditions (data not shown), except for nimesulide. Figure 9 shows a time course interaction between DPPC and nimesulide at pH 7.4.

Nimesulide seems to have no effect on the surface topology of the lipid bilayer even for long time exposures. This is in agreement with previous X-ray diffraction studies revealing that nimesulide has the smallest effect on the membrane structure in the gel phase.\textsuperscript{5,36,74} Therefore, considering that nimesulide presents minimal effects in the lipid gel phase, it is predictable that this drug has the least damaging gastrointestinal profile. Furthermore, the fusogenic-like effects observed for indomethacin, meloxicam, and piroxicam are in agreement with reported fusogenic effects of oxycams.\textsuperscript{75}

**CONCLUSIONS**

Both the techniques and the membrane models employed in this study provide complementary information that leads to a better understanding of the complex mechanism of interaction of the NSAIDs with the membranes. This is a fundamental topic, once the physical—chemical properties of the membrane and the changes due to NSAID—membrane interactions may limit or enhance their effect as anti-inflammatory therapeutic drugs.

Liposomes, monolayers, and SLB lead to the same conclusion that the pH medium is an essential parameter, since it conditions the structure of the membrane and the ionization state of NSAIDs influencing the interactions established between these drugs and the lipid membranes.

The different dimensionality and curvature of the lipid models used is also a fundamental aspect to obtain complementary information and understand the behavior of drugs when interacting with these membrane model systems. Finally, the lipid phase constitutes the third essential parameter that should be considered when evaluating the interactions between drugs and membranes. Therefore, the studies performed covered the interactions of NSAIDs with lipid membranes in both gel (L\textsubscript{gel}) and liquid-crystalline (L\textsubscript{w}) phases. Although the fluid phase is the most biologically relevant, ordered domains are also present in biomembranes and these domains share common properties with the lipid gel phase, a fact that is most times overlooked. Additionally, the interaction between NSAIDs and lipid membranes in the gel phase is particularly interesting given that the gastric protective mucous layer is also a lipidic membrane in the gel phase.

Overall, this work provides important biophysical studies regarding the effect of several NSAIDs on the lipid membrane, since this can be correlated with significant therapeutic effects and can offer valuable information for drug design. Indeed, within the cell membrane, NSAIDs affect many processes and are responsible for the inhibition of membrane-associated enzymes like cyclooxygenase and phospholipase. Additionally, the degree of water solubility and ionization, as well as liposolubility, of the NSAIDs is believed to play a role in both their absorption and GI side effects. Therefore, the rational development of safer NSAIDs will depend upon the determination of the various effects of these drugs on membranes and vice versa, as this is a precondition to understanding how these drugs act. In this context and from the obtained results, it can be concluded that the NSAIDs are able to interact with the membranes at both pH values. However, while at pH 7.4 the ionized forms of NSAIDs prevailed and the drug location at the membrane level is shallower, at pH 5 (the pH of inflamed tissues) the neutral forms of NSAIDs have stronger interactions with the membrane, which correlate well with their efficacy as inflammation inhibitors.

In the SLB models, all drugs (except nimesulide) increase visibly the fluidity of the lipid gel domains, which may be related to the reduction in $T_{in}$ observed in the lipid models of similar dimensionality (liposomes). These results in SLB models are also in agreement with the increase of the mean molecular area observed by the compression isotherms of the lipid monolayers. Nimesulide is an exception and does not affect the SLB structure, which can be correlated to its well described smaller local effect interacting with the phospholipid protective layer of the gastric mucosa. This biophysical interpretation of the topical effects of high concentrations of nimesulide gives a good argument for the least gastric toxic effects presented by this drug. By the same reasoning, the biophysical interpretation of the erosion of the gel phase domains in SLB caused by high concentration of the other NSAIDs constitutes a proof-of-concept for their documented gastric toxicity.
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