PAPER

Effects of non-steroidal anti-inflammatory drugs on the structure of lipid bilayers: therapeutical aspects

Cláudia Nunes,[a] Gerald Brezesinski,[b] José L. F. C. Lima,[a] Salette Reis[a] and Marlene Lúcio[*a]

Inflammatory processes are phenomena that initiate at the cellular surface. Therefore, the study of interactions of non-steroidal anti-inflammatory drugs (NSAIDs) with membranes or model membranes is a fundamental step for the enlightenment of their pharmacological activity. Despite their similar chemical structures, NSAIDs have different biological impact, concerning their therapeutic effects. The mechanisms leading to these differences may be related to their different effects on the structural parameters of lipid bilayers. In this study, the molecular interaction between five NSAIDs (piroxicam, meloxicam, tolmetin, indomethacin and nimesulide), widely used in the clinical practice, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) as a membrane model was investigated by small-angle X-ray scattering (SAXS) and wide-angle X-ray scattering (WAXS). The present study provides evidence that NSAIDs produce distinct biophysical effects depending on the initial organization of the membrane. All the NSAIDs studied reduce or abolish the pre-transition temperature ($L_{\beta'} \rightarrow P_{\beta'}$) indicating a surface-disordering effect of these drugs. The main lipid phase transition ($P_{\beta'} \rightarrow L_{\alpha}$) is also affected by the NSAIDs studied, with the exception of nimesulide. Meloxicam, tolmetin and indomethacin have the major effects on the L_{α} phase structure of the DPPC bilayers, which can also be related to their enhanced therapeutic effect as anti-inflammatory drugs. The structural disorder effects found for the NSAIDs studied in the $L_{\beta'}$ phase of the bilayers follow the order: meloxicam > indomethacin > tolmetin > piroxicam and can be also correlated with the anti-oxidant effect of these NSAIDs.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most prescribed group of drugs for the control of pain, especially when it is associated with inflammatory conditions. They also represent the main therapy for chronic pathologies like arthritis or Crohn's disease being prescribed in a daily basis. Despite presenting diverse chemical structures, NSAIDs act similarly by blocking the prostaglandin synthesis through the inhibition of cyclooxygenase enzyme (COX).[1,2]

Notwithstanding the similar chemical structures of NSAIDs, these drugs have different biological impact, concerning their therapeutic effects. The mechanisms leading to these differences may be related with their interaction with the biological membranes. Indeed, NSAIDs can be distributed between the membrane and the aqueous phase. This distribution determines the concentration of drug in each phase and thereby controls the extent of NSAIDs penetration into the membrane and/or interactions with phospholipids or other membrane components, such as COX enzymes, which are embedded in the lipid bilayers.[3] For this reason, the present study is focused on the interaction of NSAIDs with a model membrane which encloses the ability of these drugs to bind to phospholipids and their effect on the dynamic properties of the lipid bilayers. Lipid bilayer systems are valuable biomimetic model systems. Since biological membranes are of highly complex composition, these models can be used as a comparative system since they are well characterized and provide experimental simplicity.

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was the chosen lipid for this study, since phosphatidylcholines are one of the most abundant phospholipids of natural plasma membranes.[4] DPPC spontaneously forms multilamellar structures with a water layer between the bilayers, defined by a balance of forces that include van der Waals attraction, repulsive, hydration and steric forces.[5] The multilayers formed exhibit a rich polymorphism. Crystalline, gel, and liquid-crystalline phases are formed depending on the environmental conditions, such as the level of hydration, pH, ionic strength, temperature, and pressure.[5,6]

Structural information of DPPC bilayers was accessed by two techniques widely used in biophysical studies: small angle X-ray

[a]Departamento de Química, REQUIMTE, Serviço de Química-Física, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal; Fax: +351-222078961; Tel: +351-222078966
[b]Max Planck Institute of Colloids and Interfaces, Science Park Golm, D-14476 Potsdam, Germany

scattering (SAXS) and wide angle X-ray scattering (WAXS). These techniques are one of the most powerful tools available for characterizing the structure and packing parameters of the model membranes as well as for studying structural changes.[7] Therefore, SAXS and WAXS studies were performed aiming to investigate at a molecular level the structural modifications induced by NSAIDs (piroxicam, meloxicam, tolmetin, indomethacin and nimesulide) in biomembrane models.

In chronic pathologies such as rheumatoid arthritis, higher doses of NSAIDs are needed and drugs tend to accumulate in inflamed tissues, leading to much larger concentrations in the lipid membranes than in the aqueous phase.[8,9] In this regard, the range of concentrations under study were enlarged to put in evidence some concentration-dependent features that may happen in a daily basis administration of NSAIDs. The chemical structures of the studied NSAIDs are represented in Fig. 1.

This work, contributing to identify NSAIDs-induced alterations on membrane lipid physical properties putatively correlated with their pharmacological activities, may provide significant insights for predicting or modeling the impact of other related compounds, potentially used for therapeutic purposes, on the basis of their behavior as membrane perturbing agents. X-Ray studies of the interaction of DPPC membranes with indomethacin and nimesulide have already been performed by our group.[10] However, there are no studies in the literature covering such a wide range of chemically different NSAIDs and concentrations, and therefore this study is innovative since the conclusions provided can lead to a deeper understanding of the interactions between NSAIDs and membrane bilayers.

Results and discussion

It is well known that zwitterionic DPPC molecules spontaneously form lyotropic lamellar phases in excess water, whose structure and long-range organization are temperature dependent. As temperature is increased from room temperature, DPPC presents three different phases: $L_{\beta'}$, $P_{\beta'}$ and L_{α}. $L_{\beta'}$ and $P_{\beta'}$ are

Fig. 1 Chemical structures of the studied NSAIDs: piroxicam (A), meloxicam (B), nimesulide (C), indomethacin (D) and tolmetin (E).

solid like gel phases with tilted acyl chains predominantly in all-trans conformation, and L_{α} is the lamellar fluid phase with disordered acyl chains due to conformational changes (trans–gauche isomerization). The degree of perturbation of the different DPPC phases by NSAIDs was evaluated by the X-ray diffraction patterns at small and wide angles yielding information respectively on the long range bilayer organization and the hydrocarbon chain packing, in a drug concentration dependent manner.

Typical SAXS and WAXS patterns obtained for $L_{\beta'}$, $P_{\beta'}$ and L_{α} phases of fully hydrated DPPC at physiological pH are presented in Fig. 2 and are in good agreement with the literature.[5]

The pre-transition ($L_{\beta'} \rightarrow P_{\beta'}$) and the main lipid phase transition ($P_{\beta'} \rightarrow L_{\alpha}$) were determined to be at (34.5 ± 0.5) °C and (41.5 ± 0.5) °C, respectively, which agree well with previously published data (Table 1).[11–13] From the SAXS patterns of DPPC (Fig. 2) the lamellar spacing was determined in each lipid phase. In the $L_{\beta'}$ gel phase, the bilayer thickness including a water layer between the bilayers was determined to be (6.38 ± 0.05) nm (see also Table 2) similar to values that have been reported in the literature.[14] On heating, the spacing increases to (7.30 ± 0.05) nm in the ripple gel phase $P_{\beta'}$, and decreases again to (6.58 ± 0.05) nm (Table 2) in the liquid-crystalline phase (L_{α}). Deconvolution of the WAXS patterns gives the lattice constants of the pseudo-hexagonal lattice of the chain packing, i.e., (4.09 ± 0.05) Å and (4.20 ± 0.05) Å, which are also in good agreement with the literature.[15] Moreover, the SAXS diffraction peaks exhibit a small FWHM (full-width at half maximum) showing high correlation length (ξ) between the bilayers. Such correlation is profoundly reduced by the addition of all the NSAIDs studied, manifesting the disturbance effect of these drugs on the membrane structure.

Results from SAXS and WAXS experiments of DPPC in Hepes buffer (pH 7.4) and in buffered solutions with 20 mol% of the NSAIDs at 20 °C are shown in Fig. 3.

As it can be seen in Fig. 3, for a molar fraction of 80 mol% of DPPC, both SAXS and WAXS patterns of piroxicam and nimesulide are similar to those of pure DPPC, indicating that the two drugs do not modify the initial DPPC lamellar organization at this concentration. In fact, in the case of nimesulide, increasing drug concentrations only lead to an insignificant increase of the long repeat distance d in the $L_{\beta'}$ phase from 6.38 nm for pure DPPC to 6.43 nm for a nimesulide molar fraction of 60 mol% (Table 2). The observed differences are within the experimental error. The correlation length decreases only slightly. The chain packing is also not significantly influenced by the addition of 20 mol% of nimesulide although the WAXS peaks positions are slightly deviated, which might be explained by a reduction in the chain tilt. The same behavior is observed for higher concentrations of the drug.

Looking at the SAXS region in the L_{α} phase, the addition of nimesulide led to a slight raise of the d value to a maximum of 6.80 nm for the highest concentration of drug added (Table 2). Such an increase of the long spacing could be the result of a change in the hydration behavior of DPPC due to interaction with nimesulide in the disordered liquid-crystalline phase. Moreover, from the analysis of the temperature scans (not shown) it was possible to conclude that the main transition temperature was not affected by the presence of nimesulide, and
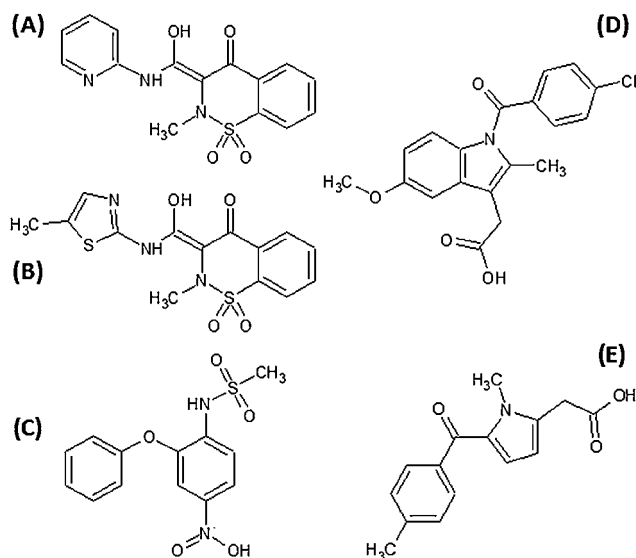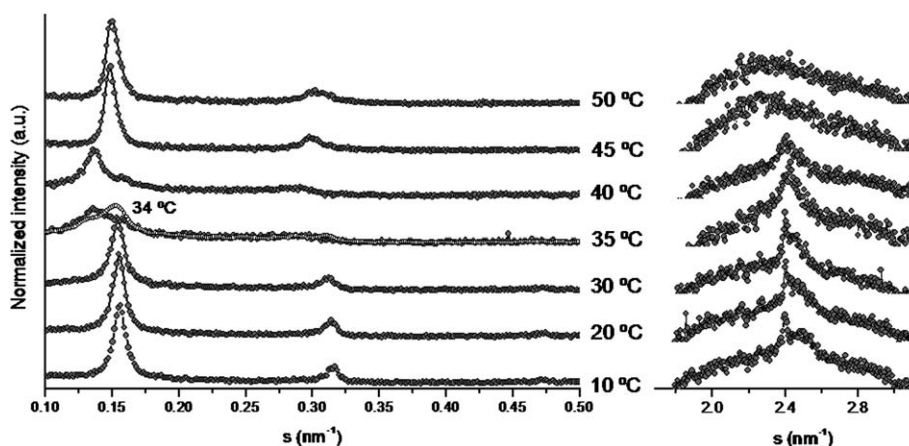
Fig. 2 Temperature dependent small and wide angle X-ray diffraction patterns (SAXS and WAXS) of DPPC bilayers at physiological pH (7.4).

Table 1 Pre-transition (T_p) and main transition temperatures (T_m) of DPPC and subsequent mixtures with 20 mol% of piroxicam, meloxicam, tolmetin, indomethacin and nimesulide

	$T_p/°C$ ($L_{\beta'} \rightarrow P_{\beta'}$)	$T_m/°C$ ($P_{\beta'} \rightarrow L_\alpha$)
DPPC	34.5 ± 0.5	41.5 ± 0.5
DPPC + piroxicam	28.5 ± 0.5	40.5 ± 0.5
DPPC + meloxicam	—	39.0 ± 0.5
DPPC + tolmetin	24.5 ± 0.5	38.5 ± 0.5
DPPC + indomethacin	—	39.5 ± 0.5
DPPC + nimesulide	32.5 ± 0.5	41.5 ± 0.5

only the pre-transition ($L_{\beta'}$–$P_{\beta'}$) temperature was markedly reduced to (32.5 ± 0.5) °C (Table 1).

In the $L_{\beta'}$ phase, piroxicam behaves very similarly to nimesulide. This means that piroxicam also does not perturb much the membrane in this phase state, and only a small increase of the

d value is observed, most probably due to an interaction of the drug molecules with the phospholipid polar headgroups. Such interactions could reduce the effective area requirement of the headgroups by changing the hydration behavior and/or the headgroup orientation leading to a reduction of the tilt angle of the chains (increased d value). This is corroborated by the fact that the pre-transition of DPPC in the presence of piroxicam decreases to (28. 5 ± 0.5) °C and the main transition decreases to (40.5 ± 0.5) °C. Other authors who observed the decrease of the pre-transition and main transition temperatures as well as the disturbance of the $L_{\beta'}$ phase have also attributed such behavior to the interaction of the molecules with the polar headgroups of the phospholipids.[16]

The WAXS patterns of piroxicam show a slight perturbation of the chain packing with a shift of the Bragg peaks to smaller d values.

Table 2 Long and short distance (d) and correlation length (ξ) determined from SAXS and WAXS diffraction patterns, respectively, at 20 °C and 50 °C[a], at physiological pH (7.4). The data are presented as a function of the mol% of each drug

pH 7.4	χ_{DRUG}/mol%	20 °C ($L_{\beta'}$)					50 °C (L_α)	
		Long distances		Short distances			Long distances	
		d (Å)	ξ (Å)	d_1 (Å)	d_2 (Å)	ξ (Å)	d (Å)	ξ (Å)
DPPC	0	63.8 ± 0.5	648 ± 10	4.20	4.09	232 ± 10	65.8 ± 0.5	621 ± 10
DPPC + piroxicam	20	64.6 ± 0.5	467 ± 10	4.17	4.05	125 ± 10	67.9 ± 0.5	667 ± 10
	40	64.1 ± 0.5	485 ± 10	4.17	4.04	11 ± 10	68.0 ± 0.5	554 ± 10
	60	64.4 ± 0.5	480 ± 10	4.14	3.98	31 ± 10	68.1 ± 0.5	614 ± 10
DPPC + meloxicam	5	79.8 ± 0.5	99 ± 10	4.11	4.00	42 ± 10	92.5 ± 0.5	80 ± 10
	10	84.3 ± 0.5	62 ± 10	4.08	–	46 ± 10	93.6 ± 0.5	79 ± 10
	20	108.0 ± 0.5	52 ± 10	4.07	–	92 ± 10	94.7 ± 0.5	91 ± 10
DPPC + tolmetin	5	64.7 ± 0.5	640 ± 10	4.17	4.05	209 ± 10	69.1 ± 0.5	610 ± 10
	10	64.5 ± 0.5	541 ± 10	4.16	4.01	68 ± 10	69.7 ± 0.5	499 ± 10
	20	72.9 ± 0.5	242 ± 10	4.11	–	46 ± 10	98.0 ± 0.5	78 ± 10
DPPC + indomethacin	20	79.5 ± 0.5	272 ± 10	4.09	3.95	49 ± 10	69.2 ± 0.5[b]	36 ± 10
	40	80.8 ± 0.5	274 ± 10	4.09	3.94	43 ± 10	67.0 ± 0.5[b]	61 ± 10
	60	78.2 ± 0.5	519 ± 10	4.12	3.94	34 ± 10	62.6 ± 0.5[b]	385 ± 10
DPPC + nimesulide	20	63.9 ± 0.5	569 ± 10	4.18	4.02	86 ± 10	66.7 ± 0.5	521 ± 10
	40	63.9 ± 0.5	524 ± 10	4.17	4.02	93 ± 10	67.1 ± 0.5	863 ± 10
	60	64.3 ± 0.5	307 ± 10	4.14	4.02	100 ± 10	68.0 ± 0.5	660 ± 10

[a] Above the main phase transition only the long spacing is presented corresponding to the SAXS patterns, since the WAXS patterns give a very broad halo in this phase. [b] In this case an additional nonlamellar phase was found with the following long spacing: 81.8 (20 mol%), 82.3 (40 mol%) and 75 (60 mol%). The correlation length was in this case drastically reduced and was not calculated given the very broad peaks obtained.
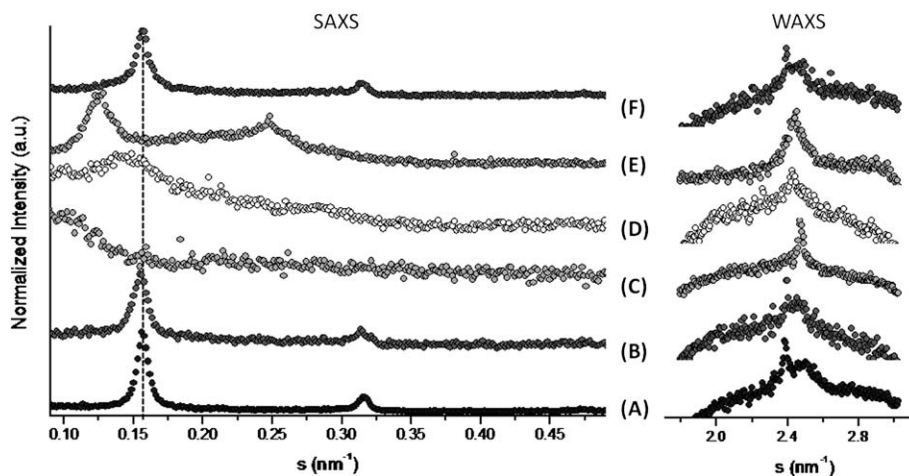
Fig. 3 Small and wide-angle X-ray diffraction patterns (SAXS and WAXS) at 20 °C for DPPC (A) and subsequent mixtures with 20 mol% of piroxicam (B), meloxicam (C), tolmetin (D), indomethacin (E) and nimesulide (F).

Regarding the L_α phase, a subtle shoulder at smaller s values can be seen (Fig. 4), which appears only at 20 mol% of piroxicam and disappears for higher molar fractions (data not shown), evidencing a lipid phase separation. The coexistence of a non-influenced lipid phase and an influenced lipid phase indicates that at the lower concentration of the drug there is not enough piroxicam to reach a homogeneous distribution of the drug within the lipid membrane; however, for higher concentrations of piroxicam, a homogeneous phase is observed.

Although meloxicam belongs to the same chemical group as piroxicam, its behavior is quite different. In the $L_{\beta'}$ phase, 5 mol% of meloxicam is sufficient to increase the d value from 6.38 to 7.98 nm, without any phase separation and the bilayer correlation is drastically reduced (Fig. 5). Higher concentrations of the drug enhance the increasing d value effect, leading to extremely large d values of 10.8 nm for a molar fraction of 20%. This huge increase of the d value implies that the interaction of meloxicam with DPPC induces possibly a change of the polar headgroups orientation in a perpendicular position to the bilayer

Fig. 4 Small angle X-ray diffraction patterns (SAXS) at 50 °C for DPPC (A) and subsequent mixtures with 20 mol% of piroxicam (B), meloxicam (C), tolmetin (D), indomethacin (E) and nimesulide (F).

plane. This change reduces the area requirement of the headgroups and allows the chains to be upright oriented. Nevertheless, this phenomenon does not explain by itself such an increase in the d values. Indeed, assuming that the chains are in an *all-trans* conformation, the change from the tilted (30°) into the non-tilted state would increase the d-value by approximately 0.6 nm. Together with the changed headgroup orientation an increase of the bilayer thickness of ~1.5 nm seems to be reasonable. Thus the greater increase of d-values indicates that the hydration behavior must be also affected by the drug, leading to a much thicker water layer between the lipid bilayers.

The chain packing is also significantly influenced by meloxicam. Only one Bragg peak at 4.05 nm is observed, indicating a change from the orthorhombic unit cell of tilted chains to a hexagonal packing of nontilted chains which correlates well with the already discussed change in the headgroup orientation to a more upright position leading to the decrease of the chain tilt. Furthermore, it is possible to assume that for increasing drug concentration (10 mol% and 20 mol% of meloxicam), the progressive transformation of the two WAXS peaks (characteristic of the $L_{\beta'}$ phase with tilted chains) into a single WAXS peak, together with the increase of the lamellar periodicity observed, suggests that meloxicam is able to change the gel phase into a $P_{\beta'}$ phase at room temperature (20 °C). Examples reported in the literature of similar effects on lipid bilayers found for other drugs corroborate the described effects of meloxicam on the lipid organization, namely the ability to change the $L_{\beta'}$ gel phase into a $P_{\beta'}$ phase.[17,18]

The effect of meloxicam on the biophysical properties of the lipid membrane is not restricted to the pre-transition. Indeed, from the analysis of the temperature scans it was possible to conclude that the main transition temperature is also reduced by the presence of meloxicam to (39.0 ± 0.5) °C confirming a fluidizing effect of this NSAID.

Concerning the L_α phase, the addition of meloxicam leads to larger d values as observed in the gel phase. However, in contrast to the gel phase, the d values in the liquid-crystalline L_α phase are much less influenced by the concentration of the drug used (*e.g.* for 5 mol% and 20 mol% the d values obtained were 9.25 nm and
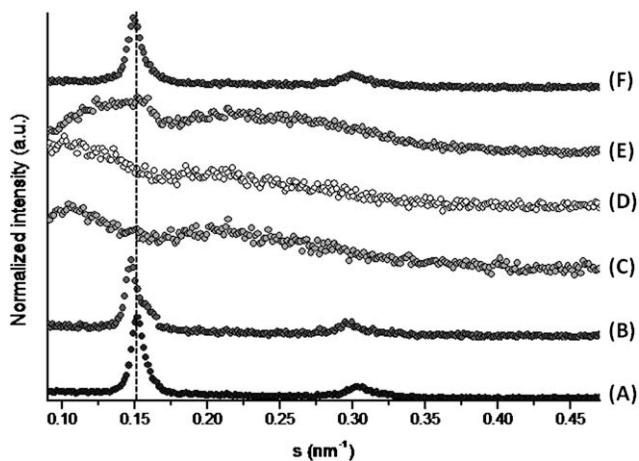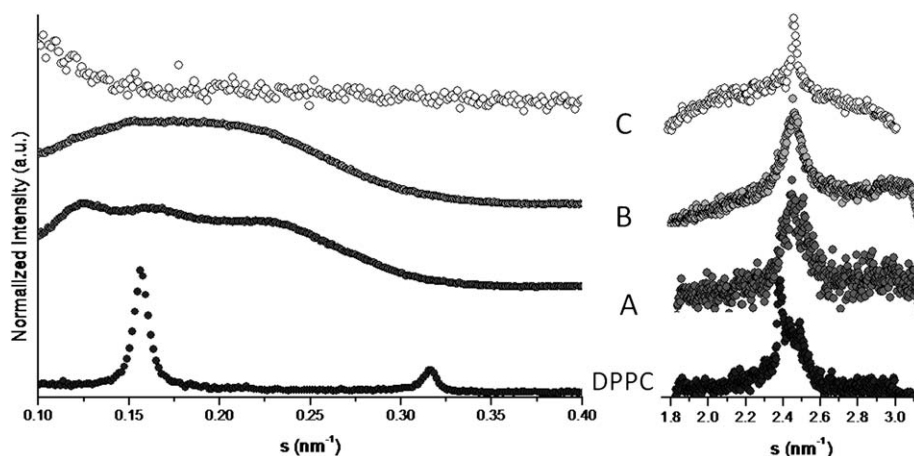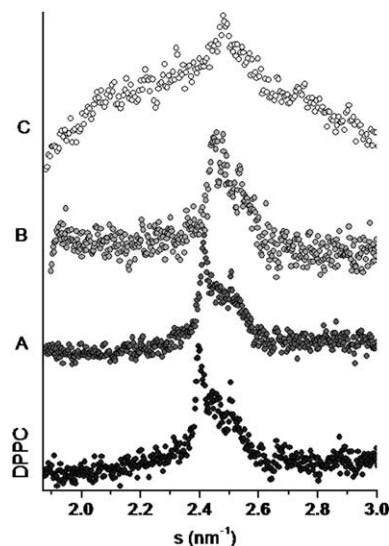
Fig. 5 Small and wide-angle X-ray diffraction patterns (SAXS and WAXS) at 20 °C for DPPC and subsequent mixtures with meloxicam at 5 mol% (A), 10 mol% (B) and 20 mol% (C).

9.47 nm, respectively—Table 2). This result indicates that in the L_α phase, a concentration of meloxicam as small as 5 mol% is enough to cause a significant change of the lipid bilayer spacing. The increase of the d value compared with pure DPPC shows that meloxicam increases the thickness of the water layer between the bilayers. The hydration of the headgroups in the liquid-crystal-line phase is obviously not further influenced by increasing drug concentrations. Comparing the changes of the d values in the gel and liquid-crystalline phases allows us to assume that the increase in the water layer thickness is maximal 3 nm in both phases. However, this thickness increase can be reached by much smaller drug concentrations in the L_α phase compared to the $L_{\beta'}$ gel phase.

Regarding the SAXS patterns of tolmetin in the $L_{\beta'}$ phase of DPPC, the position of the Bragg peak and the correlation length of the bilayers do not change much with the addition of 5 mol% of the drug compared to pure DPPC. As the concentration of the drug increases, the d values increase up to 7.29 nm which can be explained by the interaction of tolmetin with the polar region of the phospholipids, leading to a changed headgroup orientation and/or hydration allowing the chains to be less tilted as already observed for meloxicam and piroxicam. As previously discussed, a decrease of the chain tilt explains an increase in the d value of 0.6 nm. The remaining 0.3 nm can be attributed to a changed headgroup orientation and most probably to a changed hydra-tion. Increasing concentrations of tolmetin lead to an increase of the water layer thickness between the lipid bilayers.

Moreover, the analysis of the temperature scans showed that both the pre-transition and main phase transition temperatures were reduced by the presence of tolmetin to (24.5 ± 0.5) °C and (38.5 ± 0.5) °C, respectively. In the L_α phase, the behavior is very similar to the one observed in the $L_{\beta'}$ gel phase indicating that the interaction of tolmetin with the lipid bilayers is qualitatively the same in both phases.

The WAXS patterns also change as a function of the concentration of tolmetin (Fig. 6). The peaks become broader and less asymmetric with the increase of the drug concentration. This change must likely be due to a change in the lattice structure from clearly orthorhombic to more hexagonal, i.e., the lattice distortion decreases with increasing drug concentration. This is
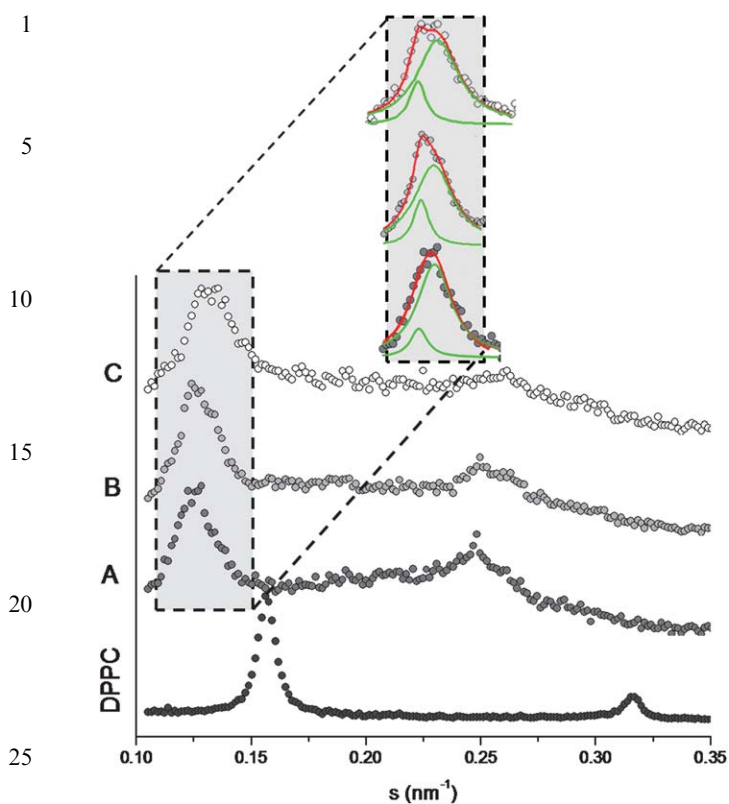

Fig. 6 Wide angle X-ray diffraction patterns (WAXS) at 20 °C for DPPC and subsequent mixtures with tolmetin at 5 mol% (A), 10 mol% (B) and 20 mol% (C).

in accordance with the discussed decreased tilt angle of the chains.

It should be noticed that concerning tolmetin and meloxicam the studied molar fractions were smaller than those used for the other NSAIDs because the effect of both drugs was much more pronounced, and for higher concentrations, the Bragg peaks got very broad and disappeared. Therefore, no structural informa-tion could be obtained at higher drug concentrations.

Regarding the effect of indomethacin in the $L_{\beta'}$ phase, all molar fractions tested led to approximately the same increase of the d value. This increased d value relatively to the one of pure DPPC may again indicate the decrease of the chain tilt angle once indomethacin molecules penetrate into the polar phospholipid headgroup region (Fig. 7). This behavior is in good agreement with previous studies reported in the literature[10] where increasing concentrations of indomethacin have also shown to favor the appearance of a larger lamellar spacing and the coexistence,

Fig. 7 Small angle X-ray diffraction patterns (SAXS) at 20 °C for DPPC and subsequent mixtures with indomethacin at 20 mol% (A), 40 mol% (B) and 60 mol% (C).

within this concentration range, of two distinct lamellar structures (splitting of the Bragg peak in the SAXS region).

The penetration of indomethacin into the polar phospholipid headgroups has a fluidizing effect on the lipid bilayers confirmed by the temperature scans from which one observes that this NSAID reduces the main phase transition temperature to (39.5 ± 0.5) °C.

The WAXS patterns show a shift of the Bragg peaks to larger s values (smaller d values), and the cross-sectional area of the chains is decreased to 19 Å^2 indicating a much higher packing density compared to pure DPPC with a cross-sectional area of 20.2 Å^2. The decreased tilt angle and the obviously enhanced attraction between the hydrophobic chains due to the presence of indomethacin indicate a changed headgroup packing due to interactions with the drug.

Above the phase transition and for the concentrations of 20 and 40 mol% of indomethacin, a diffraction pattern with very broad and overlapping Bragg peaks has been found. The deconvolution of these peaks indicates that two different phases with extremely low correlation lengths might coexist: a lamellar and a hexagonal phase. The d spacing decreases slightly upon increasing concentration of indomethacin from 6.92 nm (20 mol%) to 6.7 nm (40 mol%). The hexagonal phase has a much larger d spacing of approximately 8.2 nm, which does not change significantly upon increasing drug concentration. The main question is now whether this phase is a normal or an inverted hexagonal phase. DPPC alone does not form a hexagonal phase since according to the molecule shape concept the molecule can

be regarded as a cylinder which prefers lamellar phases. The interaction between indomethacin and the headgroups changes obviously the molecule shape drastically so that the lamellar phase is not longer energetically favorable. It seems reasonable to assume that the headgroups require now a much larger space than the tails, so that the hexagonal phase should be a normal one. The still observable lamellar phase with the usual d values must be the part of the sample which is not markedly influenced by the drug.

The addition of 60 mol% of indomethacin leads to a further decrease of the d value of the lamellar phase to 6.26 nm, which is even smaller than that for pure DPPC (6.58 nm). The Bragg peaks of the hexagonal phase became again broader, so that the determination of the peak position is connected with a larger error bar. The d value in the hexagonal phase is also smaller (7.5 nm). This indicates that the presence of high concentrations of indomethacin might be responsible for the formation of a structure where the water layer is clearly reduced or/and where the hydrocarbon chains from opposite monolayers may be partially interdigitated. The same type of interdigitated lipid structures have been described in the literature.[19] It is thus possible that the hydrophilic part of indomethacin interacts with the phospholipid headgroups forming in some cases hydrogen bonding while the hydrophobic part interacts with the hydrophobic lipid acyl chain region. Therefore, the chains partly interdigitate to fill in the high energy voids created by such drug insertions, which simultaneously increases the preferable van der Waals interactions between the acyl chains. The possible smaller water thickness may be due to the presence of indomethacin molecules that block out the water interacting with the polar headgroups. Furthermore, the phenomenon of bilayer interdigitation has been also explained by strong electrostatic interactions.[20] Accordingly, this might be also happening with indomethacin which is negatively charged at the pH of the studies and, as such, can establish strong electrostatic interactions with the positively charged choline from the headgroups, especially in the case of higher concentrations of the drug.

Experimental

Materials

The anti-inflammatory drugs nimesulide, tolmetin, piroxicam, meloxicam and indomethacin were obtained from Sigma-Aldrich, and DPPC was supplied by Avanti Polar-Lipids Inc. All compounds were used without further purification.

All other chemicals were obtained from Merck. Solutions were prepared with water from a Milli-Q plus system with specific conductivity less than 0.1 μS cm^{-1}.

Lipid dispersion preparation

Different amounts of NSAIDs were mixed with DPPC in a chloroform–methanol mixture (3 : 1 v/v) according to the required molar fraction of the drug. Lipid films were formed from these solutions, dried at 50 °C under a stream of N$_2$ and left overnight under reduced pressure to remove all traces of the organic solvents.

The lipid films were hydrated by adding 10 mM HEPES buffer (pH 7.4) and then heated above the lipid phase transition in

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a water bath at 60 °C, mixed by vortexing for about 5 min and centrifuged for 30 s at 2000g. This procedure was repeated three times. Finally, the samples were aged overnight at 4 °C and shaken by vortex at room temperature for 5 min. The dispersions were transferred into glass capillaries, which are transparent to X-rays, of 1.5 mm diameter (Hilgenberg, Malsfeld, Germany). The flame-sealed capillaries were stored at 4 °C until the time of the measurements.

SAXS and WAXS measurements

SAXS and WAXS experiments were performed at the beamline A2 of Doris III at HASYLAB (DESY, Hamburg, Germany) with a monochromatic radiation of wavelength 0.15 nm. The SAXS detector was calibrated with rat-tail tendon and the WAXS detector by polyethylene terephthalate (PET). Heating and cooling scans were performed at a rate of 1 Kmin^{-1} in the range of 10 °C to 70 °C. Data was recorded for 10 s every min. Static exposures were also taken below and above the main transition temperature and compared with the same temperatures of the heating/cooling cycles to check for possible radiation damage. In order to minimize the X-ray exposure to the sample, a shutter mounted before the sample was kept closed when no data were acquired.

Lamellar lattice constants, d, were calculated from the small angle Bragg reflections using $s = n/d$, where s is the lamellar spacing and n the order of the reflection ($n = 1, 2,...$). To obtain a more precise position for s, the diffraction peaks were fitted with Lorentzians and the positions of maximum intensities and half widths of peaks at one half of their intensity were determined. The cross-sectional area A of the aliphatic chain was calculated from the position of WAXS peaks as previously described.[21]

Conclusions

The liquid-crystalline phase also known as fluid phase is the most biologically relevant phase of the lipid membrane. However, the main difference encountered in the actual membrane model from the Fluid-Mosaic model is that a high degree of spatiotemporal order also prevails in the fluid membrane and in membrane domains and this order seems to be essential for the functioning of lipid-embedded and integrated proteins.[22] Lipid alkyl chains in the membrane domains have common properties to those of gel phase since they are extended and ordered, and this justifies studying the effect of drugs in more ordered lipid phases, rather than just exclusively evaluating their effect in the fluid phase. According to this, in the present study the effect of NSAIDs was evaluated in different lipid phases and results provide evidence that NSAIDs promote distinct biophysical effects depending on the initial organization of the membrane. The biophysical effects of NSAIDs can be further correlated with the different modes of action of this therapeutic group of drugs from which results their antioxidant and anti-inflammatory properties.

On the basis of this consideration, it is important to start commenting about the relevance of the effects of drugs in the lipid gel phase regarding their antioxidant properties. It has been found that at the cellular level, free radicals oxidize the polyunsaturated fatty acids of phospholipids, thereby increasing the saturated fatty acid content of a membrane and consequently decreasing membrane fluidity in a process called membrane peroxidation.[23,24] The peroxidation process has an effect of destroying the spatial arrangement of the lipid bilayer and impairing the crucial membrane functions of transport and permeability, also hindering homeostasis. The studies of membrane peroxidation and the effect of antioxidants on this process have been conducted in liposomes and since that peroxidized membranes are rigid and stiff, they are better modeled by lipid systems in the ordered gel phase.[25,26] The dependence of peroxidation on the lipid phase has been further addressed by previous studies concluding that the peroxidation of liposomal lipids is faster when the oxidizable lipids reside in the ordered gel phase bilayers than in the less tightly packed liquid-crystalline bilayers.[27] Hence the effects of drugs and other bioactive compounds in the more ordered L$_{\beta'}$ phase of the membrane are particularly important to establish correlations with their antioxidant effect. Indeed it has been proved that dietary antioxidants such α-tocopherol induce a disordering effect in the gel phase of phospholipid membranes which can be related with its antioxidant action since membranes become less tightly packed and thus less prone to suffer peroxidation.[27–31] Additionally, several authors have demonstrated that free radical scavenger antioxidants could interact more efficiently with lipid radicals in a disordered lipid bilayer.[32–34] Thus, the fluidifying effect of tocopherols and phenolic compounds could favor the known antioxidant capability and scavenging characteristics of these compounds.[33,35]

In this context, the fluidizing effect on the membrane gel phase observed for piroxicam, tolmetin, indomethacin and meloxicam could favor their known scavenging characteristics and this is in agreement with their reported antioxidant capability.[8,25,26] In fact, the anti-oxidant effect of NSAIDs has been ordered in previous published studies as meloxicam > indomethacin > tolmetin > piroxicam, which is exactly the same order that is obtained herein in terms of structural lipid disorder promoted by these drugs in the membrane gel phase. Furthermore, nimesulide which did not perturb the membrane in the gel phase has been described to have the least anti-oxidant effect.[36]

On the other hand, the effect of NSAIDs on the L$_{\alpha}$ phase can be associated with their anti-inflammatory action given that these drugs must pass through the cell membrane, which is in the fluid state, and either enter or pass through the interior of the endoplasmic membrane to reach the COX enzyme. According to this, the NSAIDs studied revealed perturbing effects of the membrane liquid-crystalline phase, as observed by SAXS studies and even nimesulide, which has shown almost no interaction in the gel phase, was able to interact with the L$_{\alpha}$ phase of the membrane, as required to exert its anti-inflammatory effects *in vivo*. The observed effects of the NSAIDs are in agreement with other biophysical techniques that have reported the fluidizing effect of these drugs by means of fluorescence measurements of steady-state anisotropy, infrared spectroscopy and differential scanning calorimetry measurements.[37–41]

Though all studied NSAIDs have shown an interaction with the membrane in the L$_{\alpha}$ phase, the different degrees of membrane disturbance are related with their chemical structure, lipophilicity and their state of protonation.

Meloxicam is considered as one of the most potent NSAIDs, and its efficacy has been related with the fact that its structure contains a sulfonamide group that forms hydrogen bonds with the amino acids present in the side pocket of the COX-2 enzyme.[42] Nonetheless its higher anti-inflammatory efficiency can also be related with an enhanced membrane penetration[43] as it has been verified in this work. This can be a very plausible explanation once piroxicam is chemically similar to meloxicam and exhibits also a sulfonamide group, and yet it presents less anti-inflammatory efficiency. The higher degree of penetration into the membrane can be related to the fact of meloxicam being more lipophilic[37] than piroxicam. Also the fact of meloxicam being negatively charged at the physiological pH justifies the enhancement of the water layer by a solvation effect.[3]

Also tolmetin revealed a strong interaction with the head-groups of the phospholipid bilayer in the fluid phase. This can explain its great therapeutic efficacy, and the preferable use of this drug in post-chirurgic stages as a potent analgesic, a thera-peutic class of drugs that is also known for perturbing the membrane biophysics.

In addition to the fluidizing effects in lamellar phases, indo-methacin has shown here to be responsible for forming non-lamellar phases that add versatility to lipid structures, and might have important biological effects. Indeed, indomethacin is known for inducing lipid peroxidation followed by mitochon-drial dysfunction and membrane fluidity enhancement.[44] According to the literature[45] the effect of mitochondrial dysfunction is related to the ability of drugs to induce non-lamellar hexagonal phases that coexist with lamellar phases in the lipid bilayer, similar to what has been observed in this work. Furthermore, indomethacin at high concentrations favors the interdigitation of the lipid layers possibly due to strong electro-static interactions between indomethacin (which is negatively charged at the pH of the studies) and the positively charged choline from the phospholipid headgroups. This is in agreement with previously reported zeta potential measurements where the charge effects of indomethacin in membrane surface potential where high, especially at high concentrations of drug.[46] Inter-digitation of lipid molecules can in principle couple together the two opposing leaflets of a bilayer, and thus may have profound biological effects. As the difference in the bilayer hydrophobic thickness between gel and liquid crystalline phase can be extreme, interdigitation is potentially a factor in membrane microdomain formation and organization that in the particular case of NSAIDs may interfere with biological parameters such as binding of COX to its different lipid domains. Consequently, the effect of indomethacin causing membrane interdigitation can also be correlated with its potent anti-inflammatory effect.

Overall this work provides important biophysical studies regarding the effect of several NSAIDs on the structural poly-morphism of the lipid membrane and explores the potential biological consequences of such interaction since this could be correlated with significant therapeutic effects and offer valuable information for drug design.

Acknowledgements

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