Effect of Increase in Orientational Order of Lipid Chains and Head Group Spacing on Non Steroidal Anti-Inflammatory Drug Induced Membrane Fusion

ARTICLE *in* LANGMUIR · DECEMBER 2010

Impact Factor: 4.46 · DOI: 10.1021/la103802d · Source: PubMed

3 AUTHORS, INCLUDING:

Sutapa Mondal Roy
Sardar Vallabhbhai National Institute of Tec…
11 PUBLICATIONS **90** CITATIONS
SEE PROFILE

Munna Sarkar
Saha Institute of Nuclear Physics
45 PUBLICATIONS **1,170** CITATIONS
SEE PROFILE

Langmuir
Article
pubs.acs.org/Langmuir

Effect of Increase in Orientational Order of Lipid Chains and Head Group Spacing on Non Steroidal Anti-Inflammatory Drug Induced Membrane Fusion

Sutapa Mondal Roy,[†] Amol S. Bansode,[‡,§] and Munna Sarkar*,[†]

[†]*Chemical Sciences Division, Saha Institute of Nuclear Physics, 1/AF, Bidhannagar, Kolkata − 700064, India, and* [‡]*National Institute of Pharmaceutical Education and Research (Kolkata), Indian Institute of Chemical Biology, Jadavpur, Kolkata − 700032, India.* [§] *Project student, involved in the initial part of the work*

Received September 22, 2010. Revised Manuscript Received November 11, 2010

Membrane fusion is a key event in many biological processes. The fusion process, both in vivo and in vitro, is induced by different agents which include mainly proteins and peptides. For protein- and peptide-mediated membrane fusion, conformational reorganization serves as a driving force. Small drug molecules do not share this advantage; hence, drug induced membrane fusion occurring in absence of any other fusogenic agent and at physiologically relevant concentration of the drugs is a very rare event. To date, only three drugs, namely, meloxicam (Mx), piroxicam (Px), and tenoxicam (Tx), belonging to the oxicam group of non steroidal anti-inflammatory drugs (NSAIDs), have been shown by us to induce fusion at very low drug to lipid ratio without the aid of any other fusogenic agent. In our continued effort to understand the interplay of different physical and chemical parameters of both the participating drugs and the membrane on the mechanism of this drug induced membrane fusion, we present here the effect of increase in orientational order of the lipid chains and increase in head group spacing. This is achieved by studying the effect of low concentration cholesterol (< 10 mol %) at temperatures above the chain-melting transition. Low concentration cholesterol (< 10 mol %), above the gel to fluid transition temperature, is mainly known to increase orientational order of the lipid chains and increase head group spacing. To isolate the effect of these parameters, small unilameller vesicles (SUVs) formed by dimyristoylphosphatidylcholine (DMPC) with an average diameter of 50−60 nm were used as simple model membranes. Fluorescence assays were used to probe the time dependence of lipid mixing, content mixing, and leakage and also used to determine the partitioning of the drugs in the membrane bilayer. Differential scanning calorimetry (DSC) was used to study the effect of drugs in the presence of cholesterol on the chain-melting temperature which reflects the fluidization effect of the hydrophobic tail region of the bilayer. Our results show contradictory effect of low concentration cholesterol on the fusion induced by the three drugs, which has been explained by parsing the effect of orientational order and increase in head group spacing on the fusion process.

1. Introduction

Vital cellular processes, such as neurotransmission,[1] fertilization,[2] trafficking,[3] viral infection,[4] and so forth, all require membrane fusion. Membrane fusion is controlled both in vivo and in vitro by different fusogenic agents. The nature of the fusogenic agents plays a crucial role in determining the exact mechanism by which membrane fusion proceeds toward completion. Even though proteins and peptides constitute the largest group of fusogenic agents in vivo, other agents such as polymers, for example, poly(ethylene glycol),[5] and small cations[6] such as Ca^{2+} are also known to induce membrane fusion in vitro. All membrane fusion is characterized by three major steps which involve membrane contact, followed by lipid rearrangements to form the stalk intermediate, and finally pore formation leading to content mixing of the two cells or cell organelles involved.[7] Each of these steps is controlled by a specific energy barrier which can be easily overcome by fusogenic proteins/peptides through the conformational reorganization of their own structures that provides the driving force.[7,8] Small drug molecules do not share this advantage; hence, membrane fusion induced solely by a drug molecule without the aid of other fusogenic agents and at physiologically relevant concentration of low drug to lipid ratio is indeed rare in the literature.[9] Our group is the first group to show that three painkillers belonging to the oxicam group of non steroidal anti-inflammatory drugs (NSAIDs), namely, meloxicam, piroxicam, and tenoxicam, can induce membrane fusion at physiologically relevant very low drug to lipid ratio of 0.018 without the aid of any other agent. Direct imaging using transmission electron microscopy (TEM) has shown the presence of fused membranes arrested at different stages of the fusion process.[10,11] Not only that, piroxicam has also been shown to induce fusion even in vivo, being able to fuse and rupture the mitochondrial outer membrane leading to the release of cytochrome C in the cytosol and subsequent activation of downstream pro-apoptotic caspase-3.[12]

*To whom correspondence should be addressed. Fax: +91-33-23374637. E-mail: munna.sarkar@saha.ac.in.

(1) Wiedemann, C. *Nat. Rev. Neurosci.* **2009**, *10*, 172.
(2) Stein, K. K.; Primakoff, P.; Myles, D. *J. Cell Sci.* **2004**, *117*, 6269–6274.
(3) Rothman, J. E.; Söllner, T. H. *Science* **1997**, *276*, 1212–1213.
(4) Harrison, S. C. *Nat. Struct. Mol. Biol.* **2008**, *15*, 690–698.
(5) Lentz, B. R. *Eur. Biophys. J.* **2007**, *36*, 315–326.
(6) Jena, B. P. *Protein Pept. Lett.* **2009**, *16*, 712–717.
(7) Chernomordik, L. V.; Kozlov, M. M. *Nat. Struct. Mol. Biol.* **2008**, *15*, 675–683.
(8) Tamm., L. K.; Crane, J.; Kiessling, V. *Curr. Opin. Struct. Biol.* **2003**, *13*, 453–466.
(9) Swift, J. L.; Carnini, A.; Dahms, T. E. S.; Cramb, D. T. *J. Phys. Chem. B* **2004**, *108*, 11133–11138.
(10) Mondal, S.; Sarkar, M. *J. Phys. Chem. B* **2009**, *113*, 16323–16331.
(11) Chakraborty, H.; Mondal, S.; Sarkar, M. *Biophys. Chem.* **2008**, *137*, 28–34.
(12) Chakraborty, H.; Chakraborty, P. K.; Raha, S.; Mandal, P. C.; Sarkar, M. *Biochim. Biophys. Acta, Biomembr.* **2007**, *1768*, 1138–1146.

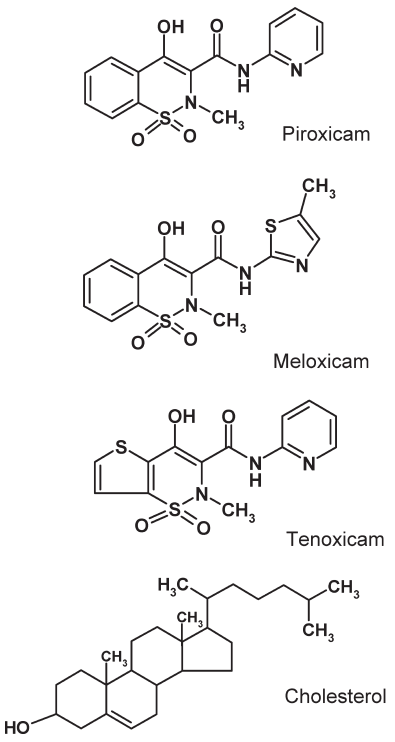

Figure 1. Chemical structures of piroxicam, meloxicam, tenoxicam, and cholesterol.

Protein induced membrane fusion and drug induced membrane fusion where proteins play no role are expected to be different. Hence, understanding how these small drug molecules can induce membrane fusion and lead it to completion is a critical question that needs to be answered. This will allow these painkillers to be better used, to control membrane fusion both in vivo and in vitro, thereby modulating biochemical processes in biotechnology where fusion plays a central role.[13] Membrane fusion is controlled by the interplay of different physical and chemical parameters of both the participating lipids and the drugs.[14,15] The dependence of drug concentration and temperature on the fusion process has already been studied using fluorescence assays and imaging techniques.[10] In our continued effort to unravel the influence of different parameters on drug induced membrane fusion, we present in this study the effect of an increase in orientational order of the lipid chains and increase in head group spacing. This is achieved by studying the effect of low concentration cholesterol (< 10 mol %) at temperatures above the chain-melting transition.

Cholesterol (Figure 1) has three distinct regions: a small polar hydroxyl group, a rigid steroid ring region, and a fatty acyl chain region. Lipid−cholesterol interaction plays an important role in the modulation of the physical properties of the lipid membranes, and the concentration of cholesterol in lipid bilayer dictates the function it performs in the membranes. That is why the structure and the phase behavior of the cholesterol−phospholipid mixtures have been the subject of extensive studies in the past decades.[16] At low concentration ≤10 mol %, above the chain melting temperature, in mixtures of DMPC/cholesterol and DPPC/cholesterol, the liquid-crystalline (L_α) phase is characterized by a large increase in the average orientational order of the lipid chains as seen both by ^{2}H NMR studies[17,18] and ^{13}C NMR studies[19,20] and an increase in head group spacing with increasing cholesterol in this range.[18,21] Low concentration cholesterol is also found in many internal cell organelles, for example, ∼ 8 wt % in the Golgi apparatus, ∼6 wt % in the endoplasmic reticulum, and only ∼3 wt % in the mitochondria.[22,23] It should be mentioned that an increase in orientational order of the lipid chains simply means that the tilting of the lipid tails is inhibited. This results in bilayer thickening as observed by small angle neutron scattering (SANS) studies on the effect of cholesterol on the lipid bilayer.[24]

Biological membranes have complex architectural constituents containing a wide variety of phospholipid molecules, with differences in the structure of their polar head groups, hydrocarbon chain length, the degree of unsaturation in the fatty acyl chain, cholesterol content, and so forth. To achieve a clear understanding of how individual parameters modulate drug induced membrane fusion, it is essential that we study their effect on simple membrane model systems, which will facilitate the parsing of effects of individual parameters. Once this is achieved, elucidating the effect of the drugs on biological membrane fusion would be complete. Therefore, a simple model membrane consisting of small unilamellar vesicles (SUVs) formed by the phospholipid dimyristoylphosphatidylcholine (DMPC) with an average diameter of 50−60 nm was used for this study as well as for our previous studies. Since the advantage of using this model system has already been discussed in our previous studies, it will not be repeated here. The only thing we would like to point out is that the SUVs do not undergo spontaneous fusion under the experimental conditions studied, as was shown by us in carefully designed control experiments.[10,11] Besides, SUVs having 50−60 nm diameter as used in this case do not have enough material to form the inverted hexagonal phase (H_{II}) that promotes fusion.[15,25]

The effect of an increase in the average orientational order of the lipid chains and head group spacing was followed by varying cholesterol concentration in the region of 1−10 mol %. To monitor the time course of the three major events in the fusion process, namely, lipid mixing, content mixing, and leakage, three different fluorescence assays were used. Content mixing and leakage were monitored by the Tb^{3+}/DPA assay, whereas lipid mixing was monitored using the standard N-NBD-PE/N-Rh-PE assay at physiological pH of 7.4. Kinetic parameters were extracted from the time courses of the change in fluorescence intensity of the probes used in the three different assays. The effect of increased head group spacing was monitored by determining the partition coefficient with increasing cholesterol. Differential scanning calorimetry (DSC) was used to monitor the change in chain melting temperature in the presence of the three drugs both in the presence and in the absence of cholesterol. This allows us to have an idea of the fluidization effect of the drugs and how the presence of low concentration cholesterol modulates it.

(13) Chen, E. H.; Olson, E. N. *Science* **2005**, *308*, 369–373.
(14) Kinnunen, P. K. J. *Chem. Phys. Lipids* **1992**, *63*, 251–258.
(15) Cevc, G.; Richardsen, H. *Adv. Drug Delivery Rev.* **1999**, *38*, 207–232.
(16) Karmakar, S.; Raghunathan, V. A. *Phys. Rev. Lett.* **2003**, *91*, 098102–098105.
(17) Vist, M. R.; Davis, J. H. *Biochemistry* **1990**, *29*, 451–464.
(18) Haberkorn, R. A.; Griffin, R. G.; Meadows, M. D. *J. Am. Chem. Soc.* **1977**, *99*, 7353–7355.
(19) Wittebort, R. J.; Blume, A.; Huang, T. H.; Das Gupta, S. K. *Biochemistry* **1982**, *21*, 3487–3502.
(20) Cornell, B. A.; Keniry, M. *Biochim. Biophys. Acta, Biomembr.* **1983**, *732*, 705–710.
(21) Ghosh, R. *Biochemistry* **1988**, *27*, 7750–7758.
(22) Jamieson, G. A.; Robertson, D. M. In *Mammalian cell membrane*, Butterworth: London, 1977, Vol. *2*.
(23) Lemmich, J.; Mortensen, K.; Ipsen, J. H.; Hønger, T.; Bauer, R.; Mouritsen, O. G. *Eur. Biophys. J.* **1997**, *25*, 293–304.
(24) Pencer, J.; Nieh, M.-P.; Harroun, T. A.; Krueger, S.; Adams, C.; Katsaras, J. *Biochim. Biophys. Acta, Biomembr.* **2005**, *1720*, 84–91.
(25) Parente, R. A.; Lentz, B. R. *Biochemistry* **1986**, *25*, 6678–6688.

2. Materials and Methods

Dimyristoylphosphatidylcholine (DMPC), cholesterol (5-Cholesten-3β-ol), DPA (dipicolinic acid), Triton X-100 (ultra pure), terbium (Tb^{3+}) chloride, (3-[*N*-morpholino]propanesulfonic acid) sodium salt (MOPS buffer), piroxicam [(8*E*)-8-[hydroxy-(pyridin-2-ylamino)methylidene]-9-methyl-10,10-dioxo-10λ^6-thia-9-azabicyclo[4.4.0] deca-1,3,5-trien-7-one], and tenoxicam [(3*E*)-3-[hydroxy(pyridin-2-ylamino)methylene]-2-methyl-2,3-dihydro-4*H*-thieno[2,3-*e*] [1,2]thiazin-4-one 1,1-dioxide] were purchased from Sigma-Aldrich. Meloxicam [(8*E*)-8-[hydroxy-[(5-methyl-1,3-thiazol-2-yl)amino] methylidene]-9-methyl-10,10-dioxo-10λ^6-thia-9-azabicyclo[4.4.0]deca-1,3,5-trien-7-one] was from LKT Laboratories. *N*-NBD-PE [*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triammonium salt] and N-Rh-PE [Lissamine rhodamine B-1,2-dihexadecanoyl-*sn*-glycero-3-phosphanolamine, triethylammonuim salt] were from Invitrogen Life Science Corporation. 2-[Tris(hydroxymethyl) methylamine]-1-ethanesulfonic acid (TES buffer) and sodium ethylene diaminetetraaceate (EDTA sodium salt) were purchased from SRL (India). All were used without further purification. Water was quartz distilled thrice before use. Stock solutions of meloxicam, piroxicam, and tenoxicam were prepared in dimethyl sulfoxide (DMSO) (Merck, Germany), and the exact concentration was adjusted by the corresponding buffer. The dilution of the drugs was done in such a way so that each sample contains 0.5% (v/v) DMSO, which had no significant effect over the SUV structure or membrane fusion process.[10]

2.1. Preparation of SUVs. SUVs of DMPC and different mole % of cholesterol (1, 3, 5, and 8 mol %) were prepared using the sonication method.[26] To prepare SUVs of DMPC in the presence of cholesterol, the phospholipid was dissolved in 2:1 (v/v) chloroform/methanol solution, and the solvent was evaporated under a stream of argon. The lipid film thus produced was then dried overnight in vacuum desiccator at −20 °C. The dried films were hydrated and swelled in 10 mM TES buffer and at pH 7.4 along with or without 60 mM NaCl, as required for $TbCl_3$ (8 mM), dipicolinic acid (DPA) (80 mM) containing vesicles, or N-NBD-PE and N-Rh-PE containing vesicles, respectively.[10] Along with the buffer, suitable probes were added in requisite amounts during the process of hydration for respective fusion assays. After hydration, the mixture was vortexed to disperse the lipids. The dispersion was then sonicated for about 10 min, in three equal time intervals, using dr. Heilscher (Germany) probe sonicator (200 W). The samples were then allowed to stand for 40 min at 39 °C to be hydrated completely. The sonicated samples were centrifuged at 10 000 rpm for 15 min to remove titanium particles, introduced as an impurity from the sonicator probe during the process of sonication, and aggregated lipids.[27]

2.2. Estimation of Phosphate. The phospholipid concentration was measured following the published protocol already used in previous experiments.[10,28] The intensity of the blue color, as developed after the treatment with ANSA, and the amount of phosphate were estimated from the absorbance measured at 660 nm using a Shimadzu UNICAM-UV-500 absorption spectrophotometer.

2.3. Lipid Mixing Assay. Förster resonance energy transfer (FRET) assay was used as described in previous works[29,30] for measuring the rate of lipid mixing during the drug induced membrane fusion process. Both of the FRET probes N-NBD-PE (donor) and N-Rh-PE (acceptor) were used at concentration of 0.8 mol % each, in the probe containing vesicles, during the assay. Two sets of vesicles, one set of probe containing vesicles, and another set of probe free vesicles were prepared in 10 mM TES, 100 mM NaCl, and 0.1 mM EDTA buffer at pH 7.4. The probe containing vesicles were mixed with probe free vesicles at a ratio of 1:9 for measurement in the quartz fluorescence cell. Membrane fusion decreased the FRET due to enhanced probe distances which resulted in fluorescence dequenching of N-NBD-PE. This was monitored with time using a Hitachi F-7000 fluorescence spectrophotometer with an excitation at 460 nm and emission at 530 nm. It should be mentioned that all fluorescence measurements for this assay and subsequent assays, including that for determining partition coefficients, were done using the same instrument and at constant temperature of 39 °C. The fluorescence data were taken at a constant concentration of the oxicam NSAIDs at 30 μM, that is, D/L ratio at 0.03 and at constant temperature of 39 °C but varying concentration of cholesterol (1, 3, 5, and 8 mol %). The oxicam drugs have some quenching effect on the fluorescence intensity of the lipid probes. This quenching effect was monitored and taken into consideration in the same way as was done in our previous work.[10] The actual fluorescence intensity (F) for lipid mixing was thus calculated by considering the initial fluorescence of the labeled liposomes as 0% fluorescence, and the 100% fluorescence was determined by lysing the vesicles with Triton X-100 at 1% (v/v) to the final concentration. The 100% fluorescence intensity is used to calibrate the scale for the determination of percentage (%) lipid mixing. At this maximal (or 100%) fluorescence, the signal obtained from N-NBD-PE fluorescence is considered to be at its infinite dilute condition where the energy transfer will be completely eliminated. The ultrapure Triton X-100 used here does not affect the N-NBD-PE fluorescence, and hence, the correction factor of 1.4−1.5 was not used.[31]

The lipid mixing is given by the following equation:

$$\%\ \text{lipid mixing} = \frac{F - F_0}{F_\infty - F_0} \times 100 \qquad (1)$$

where F = fluorescence intensity at time t, F_0 = the residual fluorescence intensity, and F_∞ = maximal (or 100%) fluorescence intensity. Each experiment is repeated at least three to four times, and the error bar in the data points represents the standard deviation of that data point. All the time courses of lipid mixing were fitted to a single exponential rate equation: $f = a(1 - \exp(-kt))$, where the exponential constant k is referred to as "rate constant" and the extent of % lipid mixing at infinite time is given by the pre-exponential factor a.

2.4. Content Mixing Assay. The content mixing assay or the Tb/DPA (terbium chloride/dipicolinic acid) assay was based on those originally proposed and modified by Wilschut et.al.[32,33] Vesicles were prepared in either 80 mM DPA or 8 mM $TbCl_3$ along with the respective buffers as required at pH 7.4, as mentioned in the section 2.1. The untrapped probes, present in the external buffer of the vesicles, were removed using a Sephadex G-50 (Amersham Biosciences) column equilibrated with the assay buffer (10 mM TES, 100 mM NaCl, 1 mM EDTA at pH 7.4). The lipid concentration in all the experiments was kept at 1.0 mM as determined by the phosphate estimation method (data not shown). Membrane fusion was monitored in the presence of various concentrations of cholesterol (1, 3, 5, and 8 mol %) in 1.0 mM DMPC vesicles, induced by constant concentration of oxicam NSAIDs of 30 μM, that is, D/L ratio of 0.03.

The time courses of the content mixing were monitored in terms of an increase in fluorescence intensity at 490 nm, due to the formation of a highly fluorescent Tb/DPA complex, which is

(26) Huang, C. H. *Biochemistry* **1969**, *8*, 344–352.
(27) Chakraborty, H.; Roy, S.; Sarkar, M. *Chem. Phys. Lipids* **2005**, *138*, 20–28.
(28) Fiske, C. H.; Subbarow, Y. *J. Biol. Chem.* **1925**, *66*, 375–400.
(29) Struck, D. K.; Hoekstra, D.; Pagano, R. E. *Biochemistry* **1981**, *20*, 4093–4099.
(30) Hoekstra, D.; Düzgüneş, N. In *Methods in Enzymology*; Düzgüneş, N., Ed.; Academic Press, Inc.: San Diego, 1993; Vol. *220*, pp 15−32.
(31) Düzgüneş, N.; Bagatolli, L. A.; Meers, P.; Oh, Y.-K.; Straubinger, R. M. *Liposomes: A Practical Approach*, 2nd ed.; Torchilin, V., Weissig, V., Eds.; Oxford University Press: Oxford, UK, pp 113−116.
(32) Wilschut, J.; Düzgüneş, N.; Fraley, R.; Papahadjopoulos, D. *Biochemistry* **1980**, *19*, 6011–6021.
(33) Düzgüneş, N.; Wilschut, J. In *Methods in Enzymology*; Düzgüneş, N., Ed.; Academic Press, Inc.: San Diego, 1993; Vol. *220*, pp 3−14.

excited at 275 nm. For the determination of drug induced content mixing, specific amounts of the stock drug solutions were added to a (1:1) mixture of Tb^{3+} and DPA-containing vesicles. The calibration of the fluorescent scale (100% content mixing) was done following the same procedure as in our earlier work.[10,33]

The percent of content mixing was calculated in the following way:

$$\% \text{ content mixing} = \frac{(F - F_0)/F_0}{(F^{\mathrm{l}} - F_0^{\mathrm{l}})/F_0^{\mathrm{l}}} \times 100 \quad (2)$$

where F = fluorescence intensity of Tb/DPA complex in presence of drug at time t, F_0 = fluorescence intensity of Tb/DPA complex in the presence of drug at $t = 0$, F^{l} = Fluorescence intensity of rechromatographed, lysed Tb vesicles in the presence of an adequate amount of DPA, and F_0^{l} = fluorescence intensity of rechromatographed, lysed Tb vesicles. Similar to lipid mixing assay plots, all the time courses of content mixing were fitted to a single exponential rate equation: $f = a(1 - \exp(-kt))$, where the exponential constant k is referred to as "rate constant" and the extent of % content mixing at infinite time is given by the pre-exponential factor a.

2.5. Leakage Assay. The leakage assay was done by using coencapsulated Tb/DPA vesicles, already referred in our previous paper.[10,34] Tb^{3+} (8 mM) and DPA (80 mM) coencapsulated DMPC vesicles with varying concentration of cholesterol (1, 3, 5, and 8 mol %) were prepared in 10 mM TES, pH 7.4 and were chromatographed on a Sephadex G-50 column equilibrated with 10 mM TES, 100 mM NaCl, 1 mM EDTA, pH 7.4 to eliminate unbound probe in the external buffer. The decrease in fluorescence intensity in the presence of drugs (30 μM Mx, Px, and Tx) was measured at 490 nm with the excitation wavelength at 275 nm. The drop in fluorescence intensity due to the leakage of contents, that is, coencapsulated Tb^{3+}/DPA, occurs owing to the quenching of the Tb^{3+} by EDTA present in the external buffer. The 0% leakage and the 100% leakage were characterized by the same method as was done previously using 0.1% (w/v) Triton X-100.[10] The percentage leakage was calculated as

$$\% \text{ leakage} = \frac{\left(F_{\mathrm{CO}}^{\mathrm{d},\,t=0} - F_{\mathrm{CO}}^{\mathrm{d,\,det}}\right) - \left(F_{\mathrm{CO}}^{\mathrm{d},\,t} - F_{\mathrm{CO}}^{\mathrm{d,\,det}}\right)}{F_{\mathrm{CO}}^{\mathrm{d},\,t=0} - F_{\mathrm{CO}}^{\mathrm{d,\,det}}} \times 100 \quad (3)$$

where $F_{\mathrm{CO}}^{\mathrm{d},t\,=\,0}$ = fluorescence intensity of Tb/DPA coencapsulated vesicles in presence of drug at time $t = 0$ (first data in the kinetics measurement), $F_{\mathrm{CO}}^{\mathrm{d},t}$ = fluorescence intensity of Tb/DPA coencapsulated vesicles in presence of drug with time, and $F_{\mathrm{CO}}^{\mathrm{d,det}}$ = fluorescence intensity of Tb/DPA coencapsulated vesicles in presence of drug after treatment of 0.1% (w/v) Triton X-100. All the time courses of the leakage assay thus obtained were also fitted to a single exponential rate equation: $f = a(1 - \exp(-kt))$, where k measured the rate constant of leakage and pre-exponential factor a measured the extent of the leakage of contents.

2.6. Fluorescence Emission and Calculation of Partition Coefficient (K_P). The partition coefficient (K_P) of the three oxicam NSAIDs, namely, Mx, Px, and Tx, between the aqueous phase, that is, the buffer phase at pH 7.4, and the lipid phase, that is, the DMPC vesicles, along with varying concentrations of cholesterol (1, 3, 5, and 8 mol %) was measured using a Hitachi F-4010 fluorescence spectrophotometer, measuring the change in fluorescence intensity of the drugs. All the emission spectra were corrected for instrument response at each wavelength. A 2 mm × 10 mm path length quartz cell was used for all the fluorescence measurements to avoid any blue edge distortion of the emission spectrum due to inner filter effect.

At pH 7.4, all the three oxicam NSAIDs exist in their anionic forms.[35] The absorption maxima of the anionic forms of Mx, Px, and Tx in hydrophobic environment are at 362, 363, and 368 nm respectively and the emission maximum of Mx, Px, and Tx is at 500 nm.[27,36] The partition coefficient values were estimated from the changes in the fluorescence intensity of the respective drugs at λ_{em} = 500 nm, with increasing concentration of DMPC for varying concentration of cholesterol. The respective plots are hyperbolic in nature, which indicate a noncooperative partitioning of the drugs in the lipid vesicles.

From the fluorescence emission spectra, the partition coefficients (K_P) of the three oxicam drugs were calculated using the following equation.[37]

$$I = \frac{I_{\mathrm{W}} + K_{\mathrm{P}} \times \gamma \times I_{\max} \times [L]}{1 + K_{\mathrm{P}} \times \gamma \times [L]} \quad (4)$$

where I is the fluorescence intensity of the drugs, that is, Mx, Px, and Tx, at any concentration of DMPC, I_{W} is the fluorescence intensity of the drugs, that is, Mx, Px, and Tx in the aqueous phase (i.e., buffer phase), $I_{\max}$ is the maximum fluorescence intensity of the oxicam drugs at saturating DMPC concentration, K_P is the partition coefficient of the oxicam drugs between the lipid phase (i.e., DMPC vesicles)and the aqueous phase (i.e., buffer phase), γ is the molar volume of DMPC, and $[L]$ is the concentration of DMPC added during titration. We could use eq 4 for calculation of the partition coefficient because, for all cases, the fluorescence intensity increased in going from the aqueous phase to the lipid phase without any shift in their respective emission maximum. Nonlinear least-squares fitting of the I versus $[L]$ plot was done using eq 4. The value of γ was considered as 0.95.[38] The maximum fluorescence intensity $I_{\max}$ was obtained using the following equation.[39]

$$\frac{1}{\Delta I} = \frac{1}{\Delta I_{\max}} + \frac{1}{K_{\mathrm{app}} \times \Delta I_{\max}} \times \frac{1}{[\mathrm{DMPC}] - [\mathrm{drug}]} \quad (5)$$

where ΔI is the change in fluorescence intensity at any concentration of DMPC, $\Delta I_{\max}$ is the maximum change in fluorescence intensity at the saturating molar concentration of lipid, and K_{app} is the apparent partition coefficient of the drug between the lipid and aqueous phase. Since the concentration of the drugs, that is, [drug], is almost negligible compared to the concentration of the lipid added, that is, $[L]$, during the calculation of partition coefficient values [drug] was neglected. A plot of $1/\Delta I$ versus 1/[DMPC] gave a straight line with a correlation coefficient around 0.99, whose intercept on the y-axis gives the value of $1/\Delta I_{\max}$. Knowing $\Delta I_{\max}$, $I_{\max}$ can be calculated by subtracting the value of the initial fluorescence intensity from $\Delta I_{\max}$. This value of $I_{\max}$ is used in eq 4 to calculate the value of K_P by nonlinear curve fitting analysis of the eq 4.

2.7. Differential Scanning Calorimetry (DSC). The lipid vesicles were prepared for the DSC experiments following a similar method as described before. The dried lipid films were hydrated in 10 mM MOPS buffer at pH 7.4 instead of in 10 mM TES buffer, with other associated components remaining same. Two sets of lipid vesicles were prepared, with one set having 1 mM DMPC and the other set having 1 mM DMPC + 8 mol % cholesterol. A constant concentration of 30 μM of the oxicam NSAIDs was also used. The DSC scans were taken after 2 h to ensure the completion of the fusion event in presence of the drugs. The DSC measurements were done using a Microcal, LLC (Northampton, MA) VP-DSC microcalorimeter. All the samples

(34) Lentz, B. R.; Talbot, W.; Lee, J.; Zheng, L.-X. *Biochemistry* **1997**, *36*, 2076–2083.
(35) Tsai, R. S.; Carrupt, P. A.; Tayar, N. E.; Giroud, Y.; Andrade, P.; Testa, B. *Helv. Chim. Acta* **1993**, *76*, 842–854.
(36) Banerjee, R.; Chakraborty, H.; Sarkar, M. *Spectrochim. Acta, Part A* **2003**, *59*, 1213–1222.
(37) Santos, N. C.; Prieto, M.; Castanho, M. A. R. B. *Biochim. Biophys. Acta* **2003**, *1612*, 123–135.
(38) Barrantes, F. J.; Antollini, S. S.; Blanton, M. P.; Prieto, M. *J. Biol. Chem.* **2000**, *275*, 37333–37339.
(39) Wang, J. L.; Edelman, G. M. *J. Biol. Chem.* **1971**, *246*, 1185–1191.

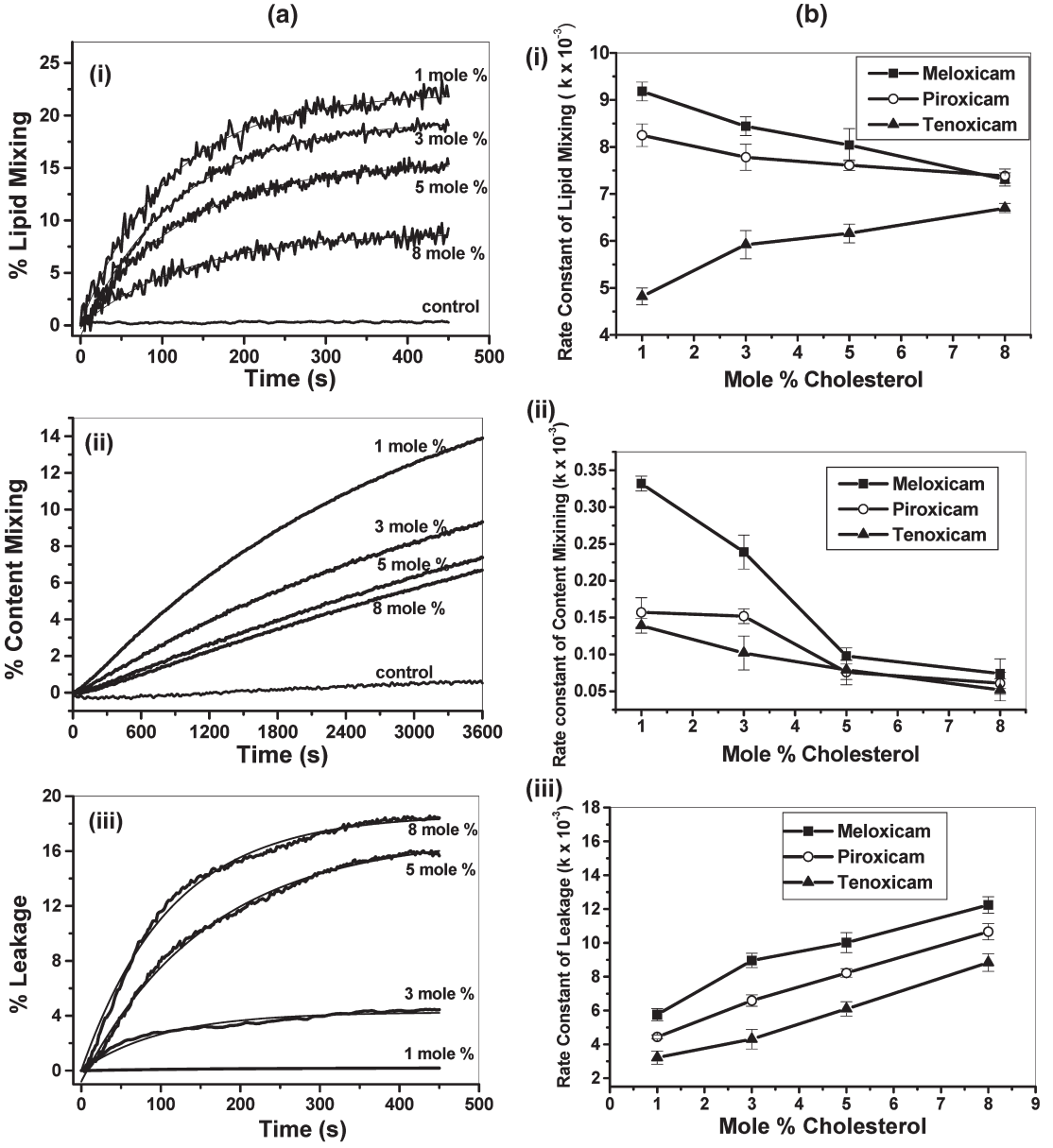

Figure 2. (a) Drug dependent lipid mixing followed by N-NBD-PE/^{N}Rh-PE assay (i), content mixing assay followed by Tb/DPA assay (ii), and leakage followed by Tb/DPA assay (iii) of DMPC vesicles containing cholesterol of different concentrations as mentioned with time for 30 μM Mx. The Time courses were fit to single exponential curves [$f = a\{1 - \exp(-kt)\}$] using Origin 7.0. The control experiments were done for the lipid mixing assay (i) and content mixing assay (ii) without the addition of any drugs. The temperature was kept constant at 39 °C throughout the experiments. (b) Plots of calculated values of rate constants of lipid mixing (i), content mixing (ii), and leakage (iii) of DMPC vesicles with 1, 3, 5, and 8 mol % concentration of cholesterol in presence of 30 μM Mx, Px, and Tx versus the mol % of cholesterol at similar experimental conditions.

and the buffer were degassed by spinning them in the Eppendorf centrifuge 5415D at 13 000 rpm for 30 min and were loaded into the sample and reference cell. All the samples were scanned four to five times from 5 to 40 °C with a scan rate of 20 °C/h. In all cases, the last two scans were identical. The DSC curves were analyzed by using the fitting program Origin 7.0, provided by Origin Lab (Northampton, MA).

3. Results

The three fusion assays, namely, lipid mixing assay, content mixing assay, and leakage assay, were done in presence of four different mole concentrations of cholesterol, that is, 1, 3, 5, and 8 mol % cholesterol, in the presence of 30 μM Mx, Px, and Tx at 39 °C and pH 7.4. The representative time courses of all the three fusion assays in presence of 30 μM Mx are given in Figure 2a. Control experiments in the absence of the drugs were done, and the data were also included in Figure 2a (i) and (ii). It is apparent that in absence of the drugs there is no fusion. All the time courses could be fitted to a single exponential rate equation as has already been stated before. The time courses in presence of 30 μM Px and 30 μM Tx under the same experimental conditions gave similar kind of plots as for Mx, and the plots are given as Supporting Information (Figures S1a and S1b). The details of the study are described under separate headings.

3.1. Effect on Lipid Mixing Assay. The rate constants obtained from the kinetics of the lipid mixing in the presence of the three drugs with varying concentrations of cholesterol are shown in Figure 2b (i). The rate of lipid mixing assay, in presence of four different mole concentrations of cholesterol, was found to show some differential behavior on the fusion process induced by

the three oxicam NSAIDs. From Figure 2b (i), it is clear that, with increasing cholesterol content in the vesicles, the rate constant of the lipid mixing process is found to diminish for Mx and Px. The extent of decrease of rate constants is greater in the case of lipid mixing in the presence of Mx compared to that in the presence of Px. However, in the presence of Tx, the rate constant of lipid mixing is found to increase. This increase in rate constant of lipid mixing in the presence of Tx with an increase in mol % of cholesterol is a quite unique phenomenon that is not found in the other fusion assays, namely, content mixing assay.

The increased lipid chain orientational order in presence of low concentration cholesterol means that the tilting of the lipid tails is inhibited. This is expected to increase the energy barrier to the formation of stalk intermediate, thereby inhibiting the process of lipid mixing during the initial stages of membrane fusion. Further clarification of this point is included in the Discussion section. The inhibition of lipid tail tilting is reflected in the decreased rate of lipid mixing as seen in case of Mx and Px induced fusion. However, why the rates of lipid mixing increase with increasing cholesterol concentration in case of Tx induced fusion needs to be probed further.

3.2. Effect on Content Mixing Assay and Leakage Assay. The rate constants obtained from the kinetics of the content mixing in the presence of the three drugs with varying concentrations of cholesterol are shown in Figure 2b (ii). In contrast to the lipid mixing rate constants, for content mixing, the rate constants steadily decrease with an increase in cholesterol content in the vesicles in the presence of all three drugs. The maximum decrease occurs in the presence of 30 μM Mx and the minimum in the presence of 30 μM Tx, with Px showing intermediate values.

Figure 2b (iii) shows the rate constants of leakage with increasing cholesterol. There is a steady increase in the leakage rates that reflects the effect of increased head group spacing, which is the other bilayer parameter affected by low concentration cholesterol. The increase in rate constants of leakage is maximum in the presence of Mx and minimum in the presence of Tx, with Px having intermediate values. It should be mentioned that this differential behavior depends on the nature and location of the drugs in the bilayer, and was already reflected in the concentration effect of the drugs.[10,40]

It is well-known that content mixing and leakage are two competitive events that occur simultaneously during the fusion process. The dominance of leakage reduces the rates of content mixing, as was seen by us in the case of increasing drug concentration.[10] From the rate constant data, it is evident that a very high rate constant value of leakage is found during the fusion process. This high rate of leakage overwhelms the initial portion of content mixing kinetics. Only after 500 s, the content mixing starts to overpower the leakage, tapering down the contribution of the leakage process.

3.3. Effect on Partition Coefficient (K_P). The partition coefficients of the oxicam NSAIDs, namely, Mx, Px, and Tx, were determined at pH 7.4 in DMPC vesicles containing varying concentration of cholesterol, using the increase in fluorescence intensity of the drugs with an increase in lipid concentration. Representative curves for 1 mol % concentration of cholesterol are shown in Figure 3a. The partition coefficients were determined from nonlinear fit of the data using eq 4. The value of I_{max} in eq 4 was calculated from the linear plot of eq 5, which are given in the insets of Figure 3a. Similar kinds of plots as in Figure 3a were obtained for all other concentrations of cholesterol. These

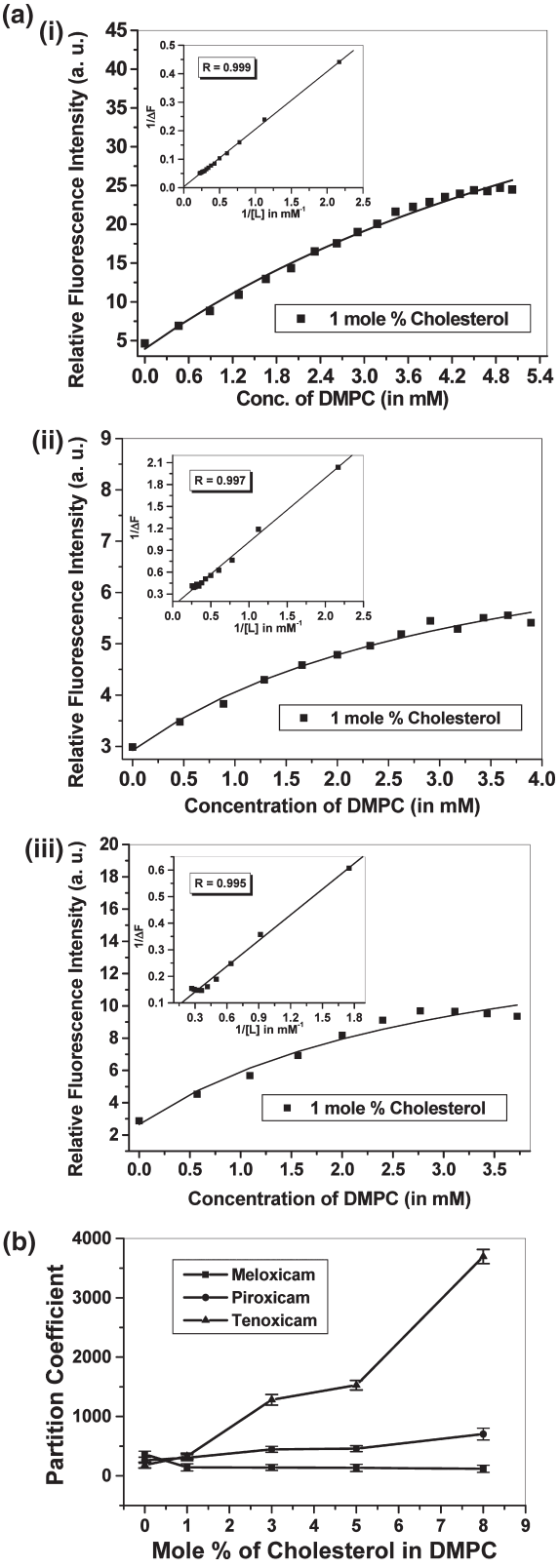

Figure 3. (a) Plots of relative fluorescence intensity of Mx (i), Px (ii), and Tx (iii) versus mole concentration of DMPC added, containing 1 mol % cholesterol. All the drugs used have the concentration of 30 μM, and the temperature and the pH of the experiments were 39 °C and 7.4, respectively. Plots of $1/\Delta I$ versus $1/[L]$ for the respective drugs are given in the insets. (b) Plots of calculated values of partition coefficient (K_P) of the oxicam NSAIDs, namely, Mx, Px, and Tx, in the DMPC vesicles containing varying concentration of cholesterol versus the mol % of cholesterol. The concentration of the drugs remained constant at 30 μM. Other experimental conditions remained the same.

(40) Kyrikou, I.; Hadjikakou, S. K.; Kovala-Demertzi, D.; Viras, K.; Mavromoustakos, T. *Chem. Phys. Lipids* **2004**, *132*, 157–169.

Table 1. Calculated Values of Partition Coefficient (K_P) of the Oxicam NSAIDs, namely, Mx, Px, and Tx, between DMPC Vesicles Containing Varying Concentration of Cholesterol[a]

	partition coefficient		
mol % cholesterol	meloxicam	piroxicam	tenoxicam
0	364.2	262.2	181.2
1	142.6	304.0	329.4
3	139.8	446.3	1281.2
5	133.8	457.4	1527.3
8	118.3	703.0	3695.2

[a] The concentration of drugs remained constant at 30 μM, and the temperature and pH were kept at 39 °C and 7.4, respectively, throughout the experiments. The error limit of the partition coefficient data was within 0.05−0.15% in all cases.

plots are given as Supporting Information (Figure S2a−S2c). Figure 3b shows the change in the partition coefficient of the three drugs with increasing cholesterol. There is a large increase in the partition coefficient of Tx, whereas, compared to Tx, there occurs only a small change in partition coefficients of Px and Mx on increasing cholesterol concentration. The change in the values of partition coefficients is given in Table 1. From the values, it is evident that for Px there is an increase and for Mx there is a decrease in the values of the partition coefficients with increasing cholesterol concentration. It may be small compared to that of Tx, but it is significant. This indicates that, with increasing cholesterol content in the DMPC vesicles, the most hydrophilic drug Tx gets much more incorporated inside the vesicles, whereas less hydrophilic Px shows a smaller increase in the incorporation of the drug inside the vesicles. For the most hydrophobic Mx, the small decrease in partition coefficient values signifies that the addition of cholesterol actually inhibits the incorporation. The reason will be elucidated later. It should be mentioned that the partition coefficient data presented in Figure 3b and the data obtained in our earlier work[27] show some significant discrepancy in the values of partition coefficient of the anionic form of Px and Mx (at pH 7.4) and in DMPC vesicles without added cholesterol. The values obtained in this study are more than that recorded previously. This is because, in our previous work,[27] the working solutions had 6% (v/v) ethanol, which is quite large compared to 0.5% (v/v) DMSO that was used in the present experiments. Ethanol being present in rather high concentration compared to DMSO competed with Px and Mx in partitioning in the vesicles, thereby decreasing the partition coefficient values of Px and Mx in DMPC vesicles compared to that in the present study.

3.4. DSC and Effect on Phase Transition Temperature (T_M). Representative DSC thermograms of DMPC and DMPC + 8 mol % cholesterol containing 30 μM Mx, Px, and Tx are presented in Figure 4a (i) and (ii), respectively. After analysis, the gel to fluid transition temperature, T_M, values for all the samples were obtained and plotted in Figure 4b. It is seen that, in the absence of cholesterol, the shift in values of T_M with the incorporation of the drugs is quite pronounced, with Mx showing the maximum lowering of temperature followed by Px and then Tx. This lowering of T_M reflects the fluidization effect of the drugs in the tail region of DMPC. Interestingly, in the presence of cholesterol, the effect of the drugs on T_M is more or less obliterated. Incorporation of the bulky cholesterol molecule in DMPC vesicles in the absence of the drugs causes an almost −0.70 °C shift in the phase transition temperature, causing maximum fluidization. The presence of cholesterol masks the effect of incorporation of the drugs on the fluidization of the tail region. Table 2 shows the change in enthalpy ΔH along with the corresponding changes in T_M. Even though there is a decrease in ΔH on addition of drugs to the SUVs with and without cholesterol, the trend shown by ΔH does not match the changes in T_M.

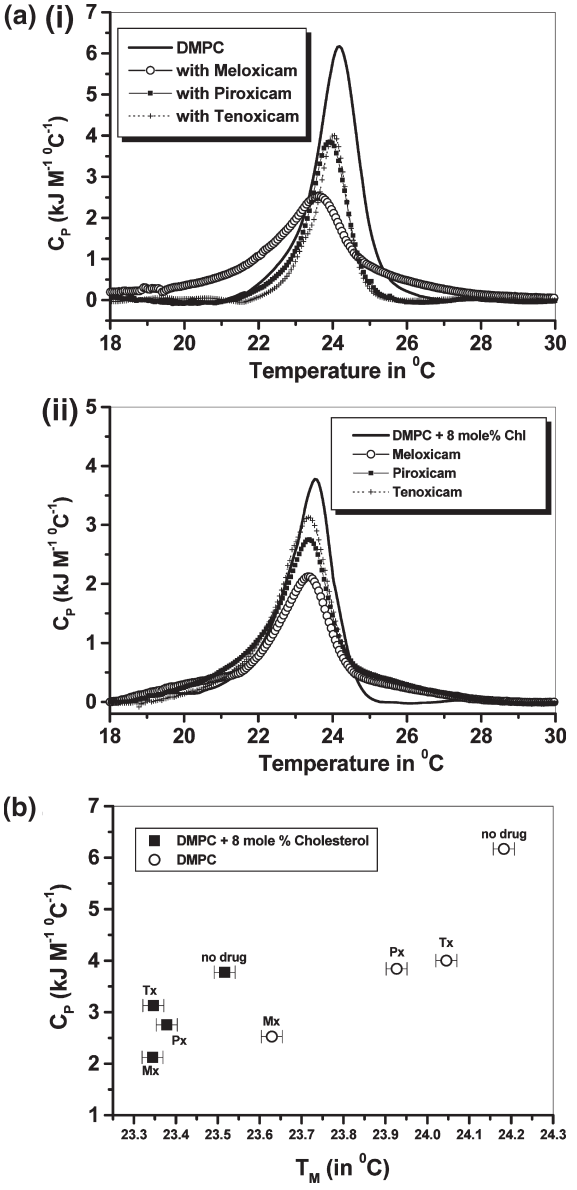

Figure 4. (a) DSC thermograms showing the plot of specific heat (C_P) as a function of temperature for simple DMPC vesicles (i) and DMPC vesicles containing 8 mol % cholesterol (ii) in the presence of 30 μM oxicam NSAIDs, namely, Mx, Px, and Tx, for both the cases and at pH 7.4. The scan rates were 20 °C/h for all the experiments. (b) Plot of specific heat (C_P) versus phase transition temperature (T_M) for DMPC vesicles and DMPC vesicles containing 8 mol % cholesterol in the presence of 30 μM oxicam NSAIDs, namely, Mx, Px, and Tx, at pH 7.4.

4. Discussion

It is already known that cholesterol plays a key role in membrane lateral organization, which in turn is thought to modify the membrane fusion properties. As mentioned before, two membrane parameters are mainly affected when low concentrations of cholesterol (< 10 mol %) are incorporated in the DMPC bilayer at temperatures above the chain-melting transition, that is, there is a large increase in the average orientational order of the tail region and increased head group spacing.

Table 2. Gel to Fluid Phase Transition Temperatures and the Corresponding Change in Enthalpy of DMPC Vesicles As Obtained from the DSC Experiments, without Cholesterol and Containing 8 mol % Cholesterol in the Absence and Presence of 30 μM Oxicam NSAIDs

	T_M (in °C)		enthalpy of phase transition (kJ M^{-1})	
drugs used	DMPC vesicles without cholesterol	DMPC vesicles containing 8 mol % cholesterol	DMPC vesicles without cholesterol	DMPC vesicles containing 8 mol % cholesterol
no drug	24.18	23.52	10.24	9.58
meloxicam	23.63	23.34	7.59	4.79
piroxicam	23.93	23.38	5.31	6.11
tenoxicam	24.04	23.35	4.60	6.13

The increase in average orientational order of the tails manifests in the inhibition of lipid tail tilting. However, the decrease in the gel to fluid transition temperature, T_M, as derived from the maximum value on the DSC curves, highlights the overall fluidization effect of low concentration cholesterol on the bilayer. The enhanced dynamics of the bilayer due to the fluidization effect of cholesterol apparently seems to contradict our results of decrease in the lipid mixing rates in drug treated SUVs. This paradox is solved if one considers the effect of lipid tail tilting in promoting lipid mixing to form the stalk intermediate. We have already shown by TEM imaging that the NSAIDs induced fusion proceeds via the formation of a stalk intermediate.[10,11] Originally, the stalk membrane was assumed to have a semitoroidal shape.[41] This model was plagued by excessive stalk energy due to the presence of "voids" in the hydrophobic interstices which gave stalk energy as high as $\sim 200kT$. The maximum energy barrier that a membrane can overcome within a characteristic time scale of biological fusion is $\sim 40kT$. To overcome this large energy barrier to stalk formation, a modified intermediate stalk structure was predicted based on the necessity of having lipid tail tilt motion.[42,43] This tilt model facilitates the oblique packing of the hydrocarbon chains filling up the hydrophobic "void", thereby reducing the stalk energy and solving the energy crisis of the stalk formation. Therefore, inhibition of this tilting motion due to increased orientational order of the chains in the presence of a low concentration of cholesterol is expected to raise the energy barrier to stalk formation. This is reflected in the decrease in the lipid mixing rates with increasing cholesterol concentration for both Mx and Px. Unexpected behavior is shown by Tx induced fusion, where the lipid mixing rate increases with cholesterol concentration. To explain this, the change in the other lipid parameter, that is, the effect of increased head group spacing, needs to be considered.

Before discussing the effect of increased head group spacing, it should be mentioned that the extent of fusion induced by the drugs in the absence of cholesterol correlates with the hydrophobicity of the drugs.[10] The most hydrophobic Mx shows a maximum extent of fusion, followed by Px and Tx the most hydrophilic showing the minimum extent of fusion. The hydrophobicity also guides the location of the drugs. It is known that Mx, being the most hydrophobic, resides inside the fatty-acyl chain region of the membrane bilayer. Both Px and Tx are located at the interface that is near the head group region, with Tx being more hydrophilic and nearer to the aqueous phase than Px.[40]

Increased head group spacing of the DMPC vesicles with increasing cholesterol concentration will lead to increased water penetration in the bilayer.[44] It is well-known that increasing cholesterol results in hydration up to a penetration depth of C7−C9 of the fatty acyl chain of DMPC[44,45] Water penetration would modulate the environment deeper in the bilayer, making it more hydrophilic, thereby facilitating the partitioning of the most hydrophilic molecules like Tx to a larger extent. The partitioning of Px with intermediate hydrophobicity is increased to a lesser extent, whereas, for hydrophobic Mx, increased water penetration inhibits its partitioning. Increase in drug incorporation increases membrane fusion as seen from our previous studies on the concentration effect of the drugs on the fusion process.[10] Hence, for Tx and to a smaller extent for Px, the effect of the increase in head group spacing on the fusion process will counter the effect of the increase in orientational order of the tails as cholesterol concentration is increased within 10 mol %. This is reflected in the rates of the lipid mixing of Figure 2b (i).

Interestingly, Figure 2b (ii) also shows that, unlike lipid mixing, the rate of content mixing decreases for all the drugs. The rate of leakage, on the other hand, increases for the three NSAIDs. Leakage is known to compete more with content mixing, the last step of the fusion process, than lipid mixing which precedes content mixing.[46] Increased head group spacing in the presence of cholesterol also increases leakage which affects the content mixing step in a similar way for the three drugs, irrespective of their extent of incorporation in the vesicles.

The fluidization effect reflected in the change in gel to fluid transition temperature, T_M, determined from the DSC curves, correlates well with the difference in hydrophobicity of the drugs, which in turn also guides their location in the bilayer. Cholesterol increases head group spacing, resulting in increased water penetration and thereby increasing incorporation/penetration of the hydrophilic Px and Tx. This masks the differential effect of the drugs on the fluidization of the bilayer as reflected by the decrease in T_M values in the presence of both the drugs and cholesterol. The change in enthalpy ΔH, which decreases on addition of the drugs in bilayers with or without cholesterol, does not follow the same trend as T_M. This is not unusual when one considers the fact that the gel to fluid transition temperature is dependent on the free energy ΔG, which is zero at the T_M, giving $T_M = \Delta H/\Delta S$, where ΔS is the change in entropy. It is therefore evident that ΔH will not alone reflect the extent of fluidization of the lipid bilayer since change in entropy plays an important role. Therefore, the values of ΔH and T_M do not follow the same trend.

In our continued effort to elucidate the effect of different physicochemical parameters of both the participating lipids and the drugs on membrane fusion, we have been able to identify and parse the effect of two parameters affected by low concentration of cholesterol, namely, increased orientational order of the tail region and increased head group spacing. However, there exist several other parameters whose effects need to be identified and parsed before we can claim to understand the complete mechanism of NSAIDs induced membrane fusion.

(41) Kozlov, M. M.; Markin, V. S. *Biofizika* **1983**, *28*, 242–247.
(42) Kuzmin, P. I.; Zimmerberg, J.; Chizmadzhev, Y. A.; Cohen, F. S. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 7235–7240.
(43) Kozlovsky, Y.; Kozlov, M. M. *Biophys. J.* **2002**, *82*, 882–895.
(44) Jendrasiak, G. L.; Hasty, J. H. *Biochim. Biophys. Acta* **1974**, *337*, 79–91.
(45) Marsh, D. *Eur. Biophys. J.* **2002**, *31*, 559–562.
(46) Weinreb, G.; Lentz, B. R. *Biophys. J.* **2007**, *92*, 4012–4029.

5. Conclusion

The study on the effect of low concentration cholesterol on membrane fusion induced by oxicam NSAIDs deciphers the effect of two main parameters, namely, increased orientational order of the lipid tails and increased head group spacing. Increased orientational order leads to a decrease in fusion as reflected in the rates of lipid mixing and content mixing for Mx and Px with increasing cholesterol. For the most hydrophilic drug Tx, lipid mixing rates show an unexpected increase. This has been explained in terms of increased head group spacing, that results in deeper water penetration in the bilayer, which facilitates enhanced partitioning of hydrophilic Tx leading to increase in fusion. Content mixing does not reflect this behavior because of the competitive leakage process that overwhelms the later part of the fusion process more than the initial steps of lipid mixing. The decrease in the gel to fluid transition temperature, that reflects the fluidization effect of the drugs on the bilayer, is masked by the presence of cholesterol.

Acknowledgment. We acknowledge Mr. Shekhar Bhattacharyya of the Biophysics Division of Saha Institute of Nuclear Physics for his help in the loading of samples during the DSC experiments. We also thank Prof. Dipak Dasgupta of the Biophysics Division for useful suggestions.

Supporting Information Available: Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.