

## RESEARCH COMMUNICATIONS

## Differential scanning calorimetric studies on the interaction of *N*-acylethanolamines with cholesterol

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Earlier studies have suggested the formation of a 1 : 1 (mol/mol) complex between *N*-myristoylethanolamine (NMEA) and cholesterol in aqueous dispersion. In this study, this interaction has been investigated further by differential scanning calorimetry (DSC) on dry mixtures of NMEA, *N*-palmitoylethanolamine (NPEA) and *N*-stearoylethanolamine (NSEA) with cholesterol. The results obtained indicate that addition of cholesterol to NMEA leads to a new phase transition at 86.5°C, besides the solid–liquid phase transition of NMEA at 95°C. The intensity of the peak corresponding to the new transition increases with cholesterol content up to 50 mol%, but decreases thereafter, whereas the intensity of the peak corresponding to the melting of NMEA decreases with increasing cholesterol content, with concomitant and gradual shift to lower temperatures and vanishes at 50 mol% cholesterol. These results are consistent with the formation of a 1 : 1 molar complex between NMEA and cholesterol proposed earlier and indicate that these two amphiphiles are associated in the solid state as well. DSC studies on hydrated mixtures of NPEA and NSEA with cholesterol yielded results that parallel those obtained with the NMEA/cholesterol system, indicating that these two long-chain NAEs also form 1 : 1 (mol/mol) complexes with cholesterol.

**Keywords:** 1 : 1 complex, hydrogen bonding, lipid membrane, phase transition.

LONG-CHAIN *N*-acylethanolamines (NAEs) and their precursors, *N*-acylphosphatidylethanolamines (NAPEs) accumulate in plants and animals when the parent organism is subjected to stress such as injury in animals or dehydration in plants. Besides, NAEs exhibit a variety of interesting biological properties such as binding to types-I and II cannabinoid receptors, inhibition of gap-junction conductance and reduction of sperm fertilizing capacity as well as anti-inflammatory, antibacterial and antiviral properties, which are also of considerable interest and potential application<sup>1–4</sup>.

In view of the foregoing, it is important to characterize the physical properties of NAEs and NAPEs, and investigate their interaction with other membrane constituents such

as cholesterol, phospholipids and integral membrane proteins in order to develop structure–function relationships to rationalize their properties. In view of this, we have initiated a long-term programme aimed at investigating the structure, physico-chemical properties, and interactions of NAEs and NAPEs with other membrane lipids. In initial studies the thermotropic phase transitions of a homologous series of NAEs were investigated by differential scanning calorimetry (DSC)<sup>5,6</sup>. In other studies reported from our laboratory and elsewhere, the three-dimensional structures of *N*-stearoylethanolamine (NSEA) and *N*-myristoylethanolamine (NMEA) were determined by single-crystal X-ray diffraction studies and the molecular packing and intermolecular interactions in the solid phase were analysed<sup>7–9</sup>. In a recent study from our laboratory, two structural polymorphs of *N*-palmitoylethanolamine (NPEA) were investigated by single-crystal X-ray diffraction and their packing properties and intermolecular interactions were characterized<sup>10</sup>. These studies revealed that in the crystal lattice NAE molecules pack in a bilayer format, analogous to that found in phospholipid membranes<sup>7–10</sup>.

The interaction of NPEA with dipalmitoylphosphatidylcholine (DPPC) in aqueous dispersion, investigated by DSC, <sup>31</sup>P-NMR and small-angle X-ray diffraction, indicated that both components mix well up to 60 mol% of NPEA with phase separation occurring at higher contents of the NAE<sup>11</sup>. Calorimetric, <sup>31</sup>P-NMR and spin-label ESR studies on the phase behaviour of mixtures of NMEA and NPEA with diacyl phosphatidylethanolamines (PEs) containing matched, saturated acyl chains have shown that the two components mix well only up to 35–40 mol% of NAE and that phase separation occurs at higher NAE content<sup>12</sup>. DSC, fast-atom-bombardment mass spectrometric (FAB-MS) and molecular modelling studies on the interaction of NMEA with cholesterol indicated the formation of a 1 : 1 (mol/mol) complex between them<sup>13</sup>. Here, this interaction has been further investigated by DSC studies on solid mixtures of NMEA, NPEA and NSEA with cholesterol as well as on hydrated mixtures of NPEA and NSEA with cholesterol.

NMEA, NPEA and NSEA were synthesized and characterized as described earlier<sup>5</sup>. Cholesterol was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Dry mixtures of NMEA, NPEA and NSEA with cholesterol for DSC measurements were prepared by weighing out appropriate amounts of the two lipids into a dry glass test tube to give the desired ratio and then dissolving the mixture (total weight ~5 mg) in ca 0.5 ml of dichloromethane with mild vortexing to ensure uniform mixing of the dissolved components. The solvent was then removed by blowing dry nitrogen gas slowly on the surface of the sample while the sample tube was maintained in a warm water bath, followed by vacuum desiccation for at least 3 h. The dry mixture was then transferred to a pre-weighted aluminium DSC pan, which was sealed by crimping and weighed again. Samples for experiments with hydrated

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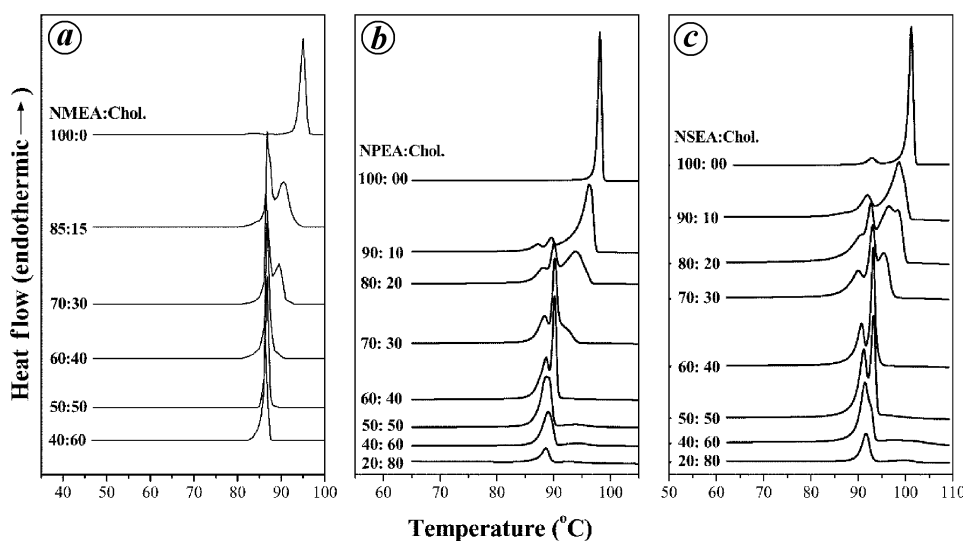
mixtures of NPEA and NSEA and cholesterol were also prepared in a similar manner, except that, after preparation of the dry mixture, the sample (1–3 mg) was transferred to a pre-weighted stainless steel DSC pan, which was weighed again. Then about 30  $\mu$ l of double-distilled water was added and the sample pan was sealed by crimping. Reference pans were prepared with only water.

DSC measurements were performed on a Perkin-Elmer DSC-4 calorimeter equipped with a data station or a Perkin-Elmer Diamond DSC, which is computer controlled. For dry samples, DSC scans were carried out by placing the DSC pan containing the appropriate lipid or lipid mixture in the sample compartment of the calorimeter, with an empty pan in the reference compartment. Then two heating and two cooling scans were performed at a scan rate of 2.5°/min. The scan range was 30–100°C for NMEA/cholesterol, and 40–110°C for NPEA/cholesterol and NSEA/cholesterol mixtures. DSC measurements on hydrated samples were performed essentially as described earlier<sup>13</sup>. Briefly, the DSC pans containing the lipid or lipid mixture were hydrated by incubation at 90°C for 30 min in the calorimeter and then cooled to 35°C. After incubation at this temperature for 20 min, two heating scans and two cooling scans were performed at a scan rate of 2.5°/min. After each scan, the sample was incubated for 10 min at the extreme. Transition enthalpies were evaluated by integrating the area under each peak using the software supplied by the instrument manufacturers.

Heating thermograms of dry samples of NMEA and NMEA/cholesterol mixtures of various compositions are shown in Figure 1 *a*. Consistent with earlier results, dry NMEA shows a reversible, highly cooperative solid–liquid phase transition centred at 95°C, which is in good agreement with the literature value<sup>5</sup> of 93.9°C. Addition of chole-

sterol at low mole fractions results in two distinct changes in the thermograms. First, intensity of the peak corresponding to the chain-melting transition decreases, becomes somewhat broader and also shifts to a slightly lower temperature. In addition, a second peak appears around 86.5°C, indicating a new phase transition, the relative intensity of which increases with increasing NMEA:cholesterol ratio up to 1:1 (mol/mol). The two peaks partially overlap and the overlap increases with increasing cholesterol content, because the shift in the peak corresponding to NMEA alone becomes larger with increasing cholesterol content. For the sample with 40 mol% cholesterol, the thermogram appears to consist of two overlapping peaks – one broad and the other sharp – indicating that the two transitions occur at the same temperature. Cooling scans indicate that both the transitions are reversible (not shown). Cholesterol alone did not show any phase transition between 35 and 100°C (not shown).

In view of the overlapping transitions, it was not possible to obtain reliable quantitative information on the enthalpies of the two transitions as a function of cholesterol content. However, the above observations are qualitatively similar to the results obtained earlier on hydrated mixtures of NMEA and cholesterol<sup>13</sup>, and suggest that NMEA and cholesterol form a 1:1 (mol/mol) complex in the solid state also. These results taken together with the those obtained earlier, indicate that NMEA and cholesterol form a 1:1 (mol/mol) complex in the solid phase also and suggest that other NAEs may also form similar complexes with cholesterol. Such defined stoichiometry of interaction would indicate specific interaction between the two components, which is consistent with the results of computational modelling reported earlier<sup>13</sup>. In such a case, the ability of NAEs to interact with cholesterol may be relevant to the functional roles



**Figure 1.** Heating thermograms of dry mixtures of (a) NMEA, (b) NPEA and (c) NSEA with cholesterol. Composition of the lipid mixture (in mol ratio) is indicated. Scan rate is 2.5°/min. Y-scale is not the same for all the scans shown.

that they play in different organisms. In order to investigate this aspect further, interaction of NPEA and NSEA (which are the major NAEs found in various living systems) with cholesterol was studied by DSC.

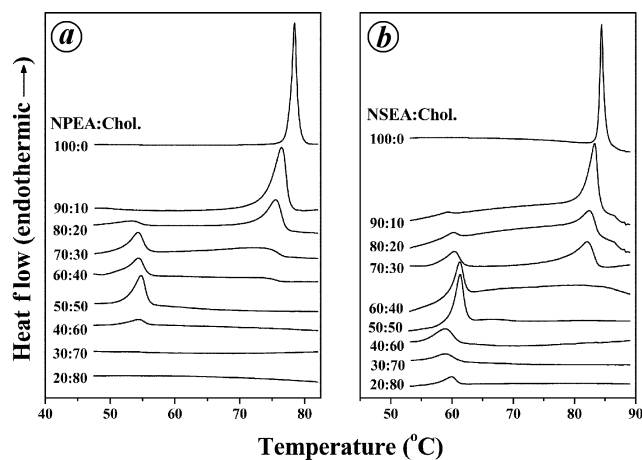
Thermograms corresponding to heating scans of dry samples of NPEA and its mixtures with cholesterol and of NSEA and its mixtures with cholesterol are shown in Figure 1 *b* and *c* respectively. Dry NPEA and NSEA yielded solid–liquid phase transitions at 98.2 and 101.2°C respectively, which are in good agreement with the values of 99.7 and 102.5°C respectively, reported earlier<sup>5</sup>. Dry NSEA also yielded a small peak at 93°C, which has been attributed earlier to a solid–solid phase transition<sup>5</sup>. Addition of cholesterol to NPEA led to considerable broadening of the endotherm corresponding to NPEA melting, accompanied by a shift of the transition to lower temperatures. In addition, two new peaks are seen at 87–88.5°C and 89–90°C (Figure 1 *b*). The intensity of the peak corresponding to NPEA decreases with increasing cholesterol and vanishes completely at NPEA/cholesterol (mol/mol) ratio of 60:40, whereas the intensity of the two new peaks increases at the same time. The two new peaks coalesce at 1:1 (mol/mol) ratio and give a single, slightly broad isotherm. At higher cholesterol contents, a single isotherm is observed. These results are consistent with the formation of a 1:1 (mol/mol) complex when the two components are present in equal proportion as well as at higher cholesterol fractions. At lower fractions of cholesterol, the picture is somewhat unclear and additional studies are required to understand the structures of the phases formed. Qualitatively similar results are observed for mixtures of NSEA and cholesterol, with the exception that the 1:1 (mol/mol) mixture gave two endothermic peaks, which coalesced into a single peak when the NSEA/cholesterol ratio was reduced to 40:60.

Earlier, computational modelling studies have shown that two strong hydrogen bonds between the hydroxy groups of NMEA and cholesterol and between the amide carbonyl of NMEA and the hydroxy group of cholesterol, as well as significant dispersive interactions between the hydrophobic regions of the two molecules stabilize the 1:1 stoichiometric complex<sup>13</sup>. A close matching in the size and hydrophobic/hydrophilic regions of the partner molecules also contribute to the stabilization of the complex. The above observations indicate that increasing the length of the hydrophobic part, as in NPEA and NSEA, may alter the interaction and lead to differences in the thermograms observed above. However, in the hydrated samples the difference in the length of the hydrophobic part between 14 and 18 C-atoms does not seem to significantly affect the interaction between NAE and cholesterol (see below).

Heating thermograms of hydrated samples of NPEA and NPEA/cholesterol mixtures are shown in Figure 2 *a* and thermograms corresponding to hydrated samples of NSEA and NSEA/cholesterol mixtures are shown in Figure 2 *b*. Overall the results obtained with these two sys-

tems are qualitatively rather similar to and parallel to those obtained earlier with hydrated mixtures of NMEA and cholesterol<sup>13</sup>. Therefore, results obtained with NPEA/cholesterol and NSEA/cholesterol mixtures are discussed together here.

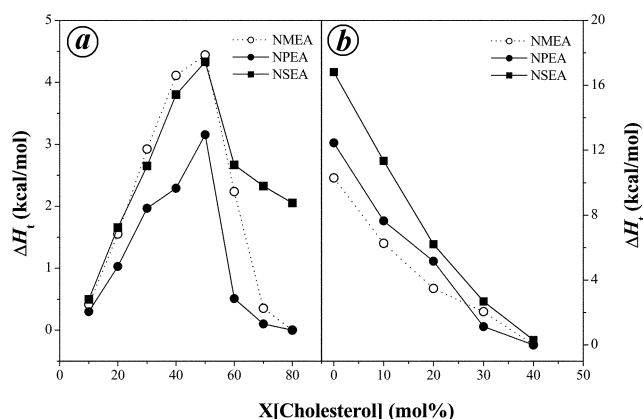
From the thermograms shown in Figure 2 *a* and *b*, it can be seen that hydrated samples of NPEA and NSEA show highly cooperative gel–fluid chain–melting phase transitions centred at 78.4 and 84.4°C respectively. These values are in good agreement with the previously published results<sup>5</sup>. Addition of cholesterol at low mole fractions results in two distinct changes in the thermograms. First, the intensity of the peak corresponding to the chain–melting transition decreases with slight broadening and a shift to lower temperature. In addition, a second peak appears around 53–55°C for the NPEA/cholesterol mixture and 59–61°C for the NSEA/cholesterol mixture, indicating a new phase transition in each case. In both cases, intensity of this new peak increases with increasing cholesterol content up to 1:1 mol ratio of the two components in the mixture, whereas intensity of the peak corresponding to chain melting of the NAE decreases steadily and disappears completely at the same ratio. These observations are similar to the results obtained with the NMEA/cholesterol system and suggest the formation of 1:1 (mol/mol) complexes of both NPEA and NSEA with cholesterol. Hence this new peak will be referred to as the complex peak. Intensity of the complex peak also decreases with increasing cholesterol content and becomes nearly zero at 0.7 mol fraction of the sterol (for the NPEA/cholesterol mixture), whereas for the NSEA/cholesterol mixture even at 0.8 mol fraction of the sterol, the complex peak has some intensity. Cooling scans with both sets of mixtures indicate that both the transitions are reversible (not shown). Cholesterol alone did not show<sup>13</sup> any phase transition between 35 and 90°C.



**Figure 2.** Heating thermograms of aqueous dispersions of (a) NPEA/cholesterol and (b) NSEA/cholesterol mixtures. Composition of the lipid mixture (in mol ratio) is indicated. Scan rate is 2.5°/min. Y-scale is not the same for all the scans shown.

A plot depicting variation of the change in enthalpy ( $\Delta H_t$ ) of the two transitions as a function of cholesterol content in NPEA/cholesterol and NSEA/cholesterol mixtures is given in Figure 3. For the sake of comparison, the corresponding data for the NMEA/cholesterol system, taken from our previous work<sup>13</sup>, are also shown in Figure 3. In each case,  $\Delta H_t$  for the transition corresponding to the NAE/cholesterol complex increases gradually up to 50 mol% cholesterol and then decreases steadily, becoming negligible around 70–80 mol% cholesterol for NMEA and NPEA, whereas for NSEA the decrease is more gradual, remaining even at 80 mol% cholesterol.  $\Delta H_t$  values corresponding to the chain-melting transition of each NAE decrease monotonically with increasing cholesterol content and approach zero at 50 mol% cholesterol. These results are consistent with the formation of a 1 : 1 complex between the different NAEs investigated and cholesterol, proposed earlier for hydrated mixtures of NMEA and cholesterol. These results further suggest coexistence of the 1 : 1 complex and NAE in mixtures containing <50 mol% cholesterol, and coexistence of the complex and cholesterol in mixtures containing >50 mol% cholesterol. The mixture having 50 mol% cholesterol appears to contain the complex exclusively.

Our previous computational modelling studies carried out under water-soaked condition, suggested that several hydrogen bonds between the hydroxy groups of NMEA and cholesterol, besides dispersive interactions stabilize the interaction between them<sup>13</sup>. In view of the strong similarities between NMEA and its higher homologues, NPEA and NSEA in the chemical structure as well as in the results obtained in the DSC studies on the aqueous dispersions of their mixtures with cholesterol, it is likely that similar hydrogen bonding and hydrophobic forces stabilize the complexes formed in hydrated mixtures of NPEA and NSEA with cholesterol as well.



**Figure 3.** Dependence of change in transition enthalpy ( $\Delta H_t$ ) on cholesterol content in hydrated mixtures of cholesterol with NMEA, NPEA and NSEA. **a**, Transition corresponding to the 1 : 1 complex. **b**, Transition corresponding to chain-melting of NAEs. Transition enthalpies in (a) were calculated using the weighted average of the molecular weights of NMEA and cholesterol, whereas those in (b) were calculated based on the molecular weight of NAE alone.

Due to the ubiquitous presence of cholesterol in animal plasma membranes, its interaction with different phospholipids, sphingolipids and glycolipids has been investigated in great detail<sup>14–18</sup>. There is renewed interest in such studies due to the presence of cholesterol in significant amounts in membrane rafts, along with phospholipids and sphingolipids, including glycosphingolipids<sup>15,18–20</sup>. Such studies have suggested that cholesterol forms novel ‘condensed complexes’ with different phospholipids with simple integral stoichiometry<sup>21–26</sup>. Spin-label ESR and Langmuir film balance studies on the interaction of cholesterol with *N*-myristoyl DMPE have shown that cholesterol exerts the classic chain condensing effect on the *N*-acyl chain of NAEs also and indicated the possibility of formation of complexes between NAEs and cholesterol as well<sup>27,28</sup>. It has been suggested that the condensed complexes of phospholipids and cholesterol play a role in the formation of lipid rafts and modulate the chemical activity of cholesterol, which could possibly regulate its biosynthesis<sup>29,30</sup>. The formation of relatively strong stoichiometric complexes between NAEs such as NMEA, NPEA and NSEA with cholesterol demonstrated earlier<sup>13</sup> and in the present study, provide an attractive model system for investigating the importance of lipid–lipid interactions in the formation and dynamics of rafts. Such specific complexes could be important in the putative cytoprotective and stress-combating actions of NAEs.

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## Studies on lower epidermal papillae, the site of storage of basmati rice aroma compounds in *Pandanus amaryllifolius* Roxb.

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***Pandanus amaryllifolius* Roxb. is the only species belonging to the family Pandanaceae that has fragrant leaves. In the higher plants, aroma compounds in leaves are stored in vacuoles and epidermal outgrowths like papillae, glandular hairs and trichomes. The lower epidermis of *P. amaryllifolius* has papillae as protrusions of lower epidermal cells. The papillae run parallel along the leaf length and are absent over the veins and midrib. The number of papillae varied from one to seven per cell. Papillae were also found surrounding the stomata forming a necklace-like structure. Quantitative analysis yielded 3.10 mg of 2-acetyl-1-pyrroline per kg of fresh leaves. Cell size, area and number of papillae were more in the clone of 'Sawantwadi' than in 'Pune'.**

**Keywords:** 2-Acetyl-1-pyrroline, basmati aroma, lower epidermal papillae, *Pandanus amaryllifolius* Roxb.

THE genus *Pandanus*, family Pandanaceae comprises approximately 600 species that are widely distributed in tropical and subtropical regions<sup>1</sup>. Thirty-six species of *Pandanus* have been recorded in India, among which *P. odoratissimus* Linn. and *P. amaryllifolius* Roxb. are being exploited commercially by the flavour industry. In *P. odoratissimus* the flowers are scented, while in *P. amaryllifolius* the leaves are scented<sup>2,3</sup>. *P. amaryllifolius* Roxb. is a native of the Philippines and Thailand<sup>4</sup>. It was introduced<sup>5</sup> into India from Indonesia through the Botanical Garden at Kolkata in 1798. The principal aroma compound, 2-acetyl-1-pyrroline (2AP) is ten times higher in this

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