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Effect of variation in the chain length and number in modulating the interaction of an immunogenic lipopeptide with biomembrane models

Maria Grazia Sarpietro [a], Dorotea Micieli [a], Rosario Pignatello [b], Ming Tao Liang [c], Istvan Toth [c], Francesco Castelli [a,*]

[a] *Dipartimento di Scienze Chimiche, Università degli Studi di Catania, Viale Andrea Doria, 6, 95125 Catania, Italy*
[b] *Dipartimento di Scienze Farmaceutiche, Università degli Studi di Catania, Viale Andrea Doria, 6, 95125 Catania, Italy*
[c] *School of Molecular and Microbial Sciences, The University of Queensland, St. Lucia, Brisbane 4072 Qld, Australia*

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Abstract

A differential scanning calorimetry study was carried out to investigate the effect exerted by immunogenic synthetic lipopeptides obtained by the conjugation of $LCMV_{33–41}$ peptide with lipoamino acids (Laas) bearing different alkyl chain lengths (C_{12} and C_{16}) and number of chains ($2 \times C_{12}$) on the thermotropic behaviour of dimyristoylphosphatidylcholine (DMPC) liposomes. The aim of this work was to study the ability of these compounds to be carried by a liposomal system and released to a biomembrane model.

The examined compounds caused variations of the thermotropic parameters that characterise the liposomal system (transition temperature, T_m and enthalpy variation, ΔH), and interacted with the biomembrane models in different way. The interaction was found to be modulated by the length and number of chains present in the examined compounds. In fact, the compounds with higher number of lipid chain showed a stronger interaction with the biomembrane models with respect to the pure peptide and the compounds with a single lipid chain. These results suggest that the lipoamino acid moiety could favour the peptide to be carried by the liposomal system and released to biomembrane.

Keywords: Lipopeptides; Dimyristoylphosphatidylcoline; Differential scanning calorimetry; Liposomes

1. Introduction

Lipopeptides as vaccines are currently under intensive investigation, because they can generate comprehensive immune responses without the use of conventional adjuvants [1]. The lipopeptide vaccines consist of synthetic peptide epitopes of interest and a covalently linked fatty acid moiety. Lipoamino acids (Laas) have also received considerable attention for developing self-adjuvanting peptide vaccines. Laas are highly versatile and unique in their capacity to be conjugated with peptides through either their amine or carboxylic acid functional groups [1–3]. The chemical structures of Laas are adaptable allowing for the possibility of modification in the length, number and type of lipidic chains (saturated and unsaturated) as well as their stereochemistry. The length and structure of the lipidic chain and the number of Laa residues can ultimately modulate the lipophilicity, stability and solubility of the resulting conjugate.

Peptides usually demonstrate poor stability within biological matrices and are poorly absorbed across biological membranes. The objective of the conjugation of the peptide to the Laas is to change the physico-chemical and the pharmacologic characteristics allowing its better absorption by biomembrane. Particularly the conjugation confers increased lipophilicity to the peptide that, as a result, increases its membrane permeability [4]. Conjugation of peptides to Laas has also been shown to protect the peptide from enzymatic digestion [5,6]. As a result of the increased lipophilicity, the aqueous solubility of the lipopeptide constructs can be dramatically reduced, frequently requiring administration as a suspension. The nature of the Laa conjugates (particularly those with short peptides) also makes them poorly soluble in a range of solvents including H-bonding solvents such as short chain alcohols and dipolar solvents such as dichloromethane. It is really important to determine the best

* Corresponding author. Tel.: +39 095 7385099; fax: +39 095 580138.
E-mail address: fcastelli@dipchi.unict.it (F. Castelli).

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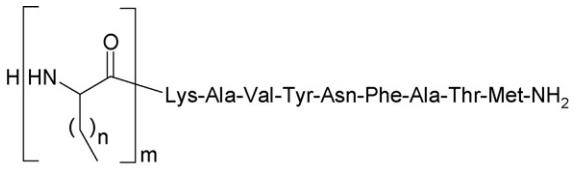

n=9	C_{12}-LCMV
n=13	C_{16}-LCMV
n=9, m=2	$2C_{12}$-LCMV

Fig. 1. Chemical structure of synthetic lipopeptides.

lipophilic/hydrophilic balance of lipopeptides which increases their interaction with biomembrane.

Employing differential scanning calorimetry (DSC) technique and DMPC multilamellar vesicles (MLV) as biomembrane models, we investigated whether the Laa moiety can improve the interaction of lipopeptides with biomembranes compared to the pure peptide. DMPC liposomes (a model biomembrane chosen for the low transition temperature, T_m), made by a single species of phospholipid, when heated, exhibit a gel-to-liquid crystal ($L_\beta \rightarrow L_\alpha$) phase transition [7–10] that can be easily revealed by DSC by measuring the associated thermodynamic parameters (transition temperature, T_m, and enthalpy changes, ΔH). Amphiphilic substances interacting with lipid bilayer should cause a destabilisation of the lipid chain packing resulting in a change of the transition thermodynamic parameters, like a depression of T_m [10–19]. This behaviour could be analysed by the Van't Hoff model of the freezing point depression of solutions, which had been verified for several classes of chemical compounds, such as anaesthetics [11], and applied on theoretical basis by some researchers [11,20,21], taking into account also the deviation from the model due to the complex structure as well as the size of the compounds [22,23].

In this study, the effects exerted by lipopeptides on the thermotropic behaviour of DMPC liposomes were examined. The investigated lipopeptides were obtained by the conjugation of $LCMV_{33–41}$ (LCMV) peptide (KAVANFATM, an epitope expressed by the Lewis lung carcinoma cell line [24]), with Laas bearing different alkyl chain lengths (C_{12} and C_{16}), and the C_{12} construct was also prepared in dimeric form ($2 \times C_{12}$) (Fig. 1).

2. Experimental

2.1. Materials

Lipopeptides were synthesised according to the previously reported method [5]. The purity of the lipopeptides was determined by analytical RP-HPLC using a Vydac C_{18} 22 mm × 4.6 mm column, and the purity of LCMV and the other lipopeptides such as C_{12}-LCMV, C_{16}-LCMV and $2C_{12}$-LCMV was above 95%. 1,2-Dimyristoyl-sn-glycero-3-phoshatidylcholine (DMPC) was supplied by Genzyme Pharmaceuticals (Liestal, Switzerland). Lipids were chromatographically pure as assessed by two-dimensional thin-layer chromatography. Lipid concentration was determined by the phosphorus analysis [25]. 50 mM Tris buffer solution, adjusted to pH 7.4, was employed. All reagents were of commercial grade quality and were used as received (Merck, Sigma–Aldrich); solvents were distilled and dried using standard techniques.

2.2. Liposome preparation

DMPC multilamellar vesicles were prepared in the absence and presence of increasing molar fractions of the lipopeptides as follows: stock solutions of DMPC were prepared using chloroform/methanol (1:1, v:v). Aliquots of DMPC solution were distributed in glass tubes in order to have 0.01032 mmol of DMPC in all the tubes. Solvents were removed under nitrogen flow and the resulting films were freeze dried under vacuum to eliminate solvent residues. An exact amount of each compound, to have a defined molar fraction with respect to the DMPC, was added to the phospholipidic film. 168 μL of a 50 mM Tris buffer (pH 7.4) was added to the films and the samples were heated at 37 °C for 1 min and successively shaken for 1 min. This procedure was repeated for other two times. The samples were then kept at 37 °C for 1 h to allow MLV to homogenise and the compounds to equilibrate between the lipid and aqueous phases. Aliquots (120 μL, 0.00737 mmol) of blank MLV or loaded with different molar fractions of lipopeptides were transferred into a 160 μL aluminium pan, hermetically sealed and submitted to DSC analysis.

2.3. Differential scanning calorimetry

A Mettler Toledo STAR^e system equipped with a DSC-822^e cell and a Mettler TA-STAR^e software were used. The scan rate employed was 2 °C/min (heating scan) or 4 °C/min (cooling scan) in the temperature range 5–37 °C. The sensitivity was automatically chosen as the maximum possible by the calorimetric system and the reference pan was filled with buffer solution. The calorimetric system was calibrated, in transition temperature and enthalpy changes, by using indium, stearic acid and cyclohexane by following the procedure of the DSC-822^e Mettler TA-STAR^e instrument. The samples were cooled and heated four times to cheek the reproducibility of results. After the DSC analysis all samples were extracted from the pan and aliquots were used to determine the amount of phospholipids by the phosphorus assay [25].

3. Results and discussion

DSC techniques were used to study the effect of the length and/or numbers of lipid chains of lipopeptides on their interaction with DMPC MLV biomembrane models. MLV were prepared in the absence and presence of increasing molar fractions of the examined compounds and the related calorimetric curves were compared with that of pure DMPC MLV (Figs. 2–5). Pure DMPC curve is characterised by a pretransition peak at about 15.8 °C, associated to the phospholipid hydrophobic chain tilt, and a main peak at 24.4 °C, associated to phospholipid gel–liquid crystalline main phase transition, related to the disor-

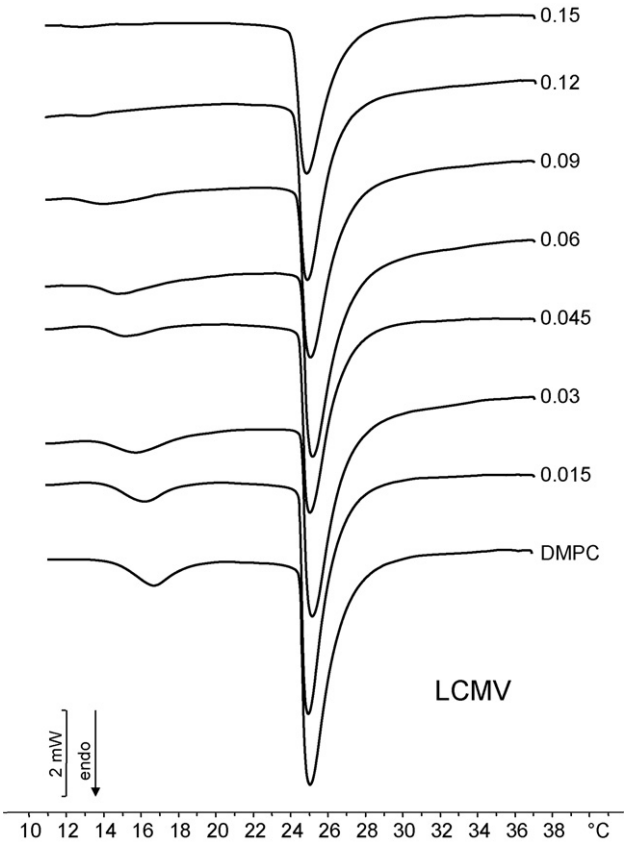

Fig. 2. Calorimetric curves, in heating mode, of DMPC MLV prepared in the presence of increasing molar fractions of LCMV.

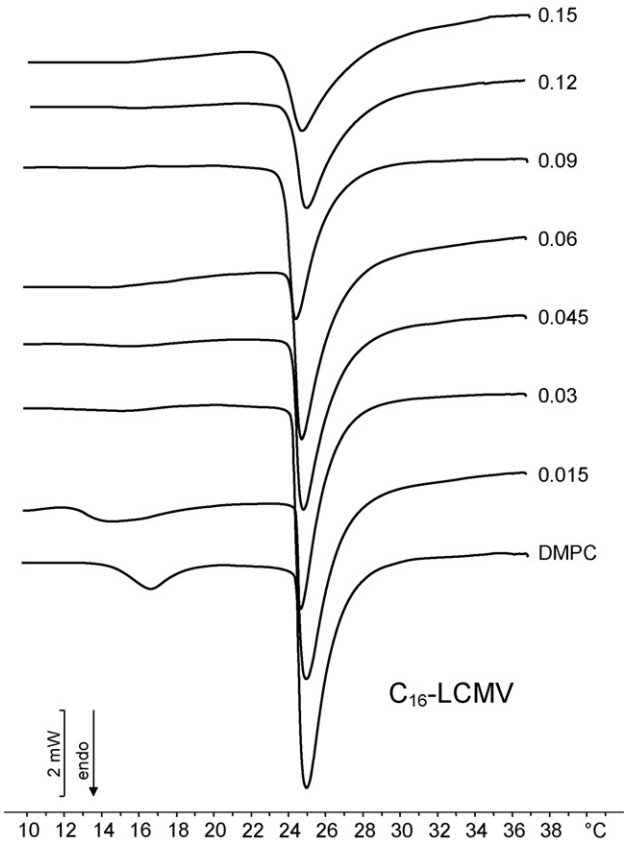

Fig. 4. Calorimetric curves, in heating mode, of DMPC MLV prepared in the presence of increasing molar fractions of C_{16}-LCMV.

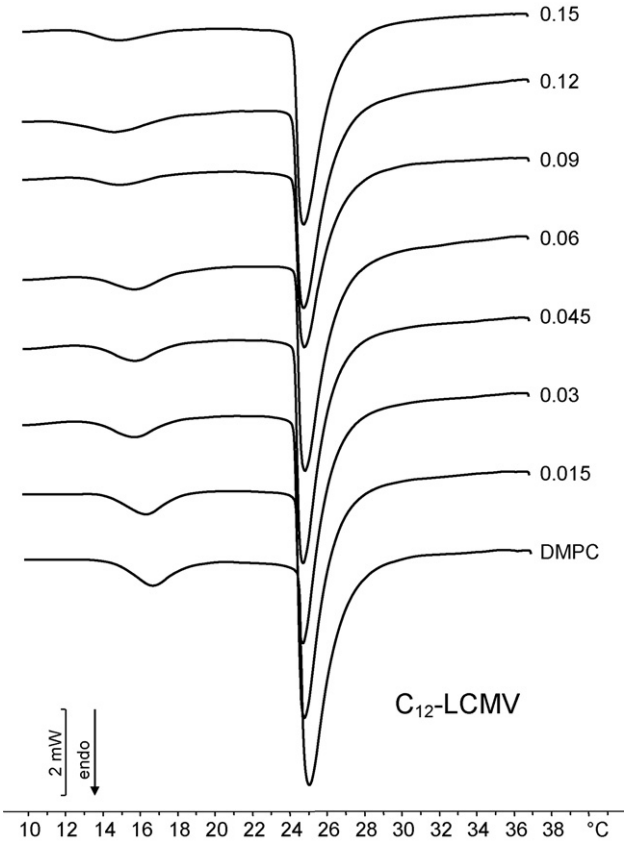

Fig. 3. Calorimetric curves, in heating mode, of DMPC MLV prepared in the presence of increasing molar fractions of C_{12}-LCMV.

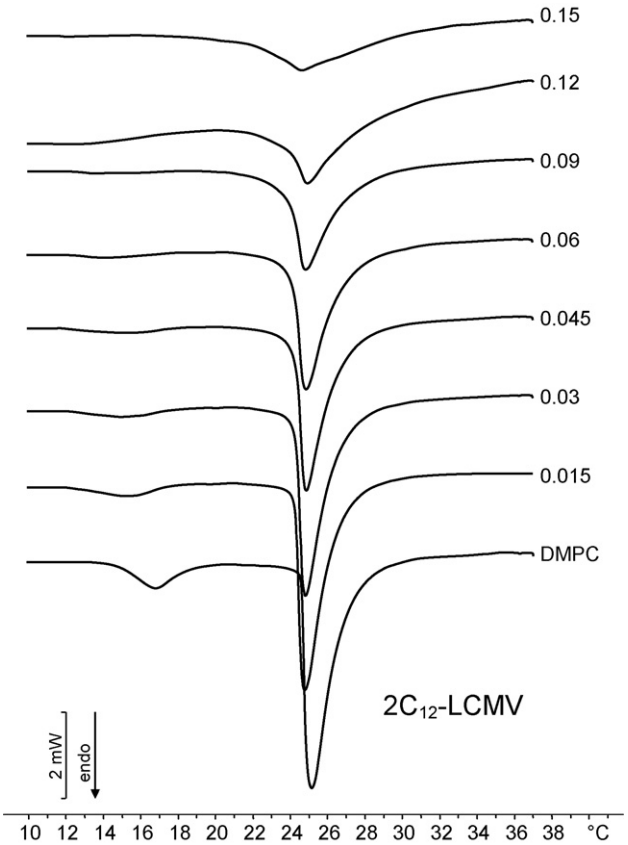

Fig. 5. Calorimetric curves, in heating mode, of DMPC MLV prepared in the presence of increasing molar fractions of $2C_{12}$-LCMV.

der caused by the temperature increase on the lipid multilayer. Changes of calorimetric curves (i.e.: shape and/or transition temperature) of liposomes prepared in the presence of increasing molar fractions of the examined compounds indicated that an interaction between the DMPC and the compounds occurred. Such interaction is explained in terms of a "fluidifying" effect usually due to the introduction of the amphiphilic molecules into the ordered structure of the phospholipid bilayers. Drug molecules act as spacers in this structure causing a destabilisation of the lipid mosaic with a decrease of T_m of the gel-to-liquid crystal phase transition. Fig. 2 shows the calorimetric heating curves of DMPC liposomes in the presence of different molar fractions of the pure peptide (LCMV). LCMV caused the DMPC pretransition peak to gradually disappear and the main peak to shift towards lower temperature whereas its shape remains unchanged for all the tested concentrations. This behaviour indicates a very low interaction between the compound and the DMPC MLV.

Fig. 3 shows the calorimetric heating curves of DMPC liposomes in the presence of different molar fractions of C_{12}-LCMV lipopeptide. The pretransition peak shifted towards a lower temperature and became gradually smaller but did not disappear with increasing the C_{12}-LCMV molar fraction. The mean calorimetric peak was not changing with the changes of the molar fraction, and its shape also remained the same, suggesting that C_{12}-LCMV is not able to strongly interact with the biomembrane model.

Fig. 4 shows the behaviour of DMPC liposomes in the presence of different amount of C_{16}-LCMV lipopeptide. Increasing amount of C_{16}-LCMV suppressed the pretransition peak (at molar fraction >0.015), indicating the location of C_{16}-LCMV in the polar region of the bilayer probably due to the peptide moiety. DMPC transition peak was broadened and shifted to a lower temperature with increasing the molar fraction of C_{16}-LCMV in the MLV. This denotes a decrease of the cooperativity, mainly at high molar fractions, of the phase transition and a disordering in the phospholipid bilayer probably due to the high length of the Laa moiety.

Fig. 5 reports the calorimetric heating curves of DMPC liposomes in the presence of different molar fractions of $2C_{12}$-LCMV. This lipopeptide caused the disappearance of the DMPC pretransition peak for molar fractions higher than 0.03; the main peak shifted towards a lower temperature and broadened in a more evident way due to the presence of the double Laa moiety, which penetrate the lipidic layers in a more destroying way with respect to the single Laa compounds.

The transition temperature (T_m), enthalpy changes (ΔH) and peaks width ($\Delta T_{1/2}$, width at half peak height) variations obtained from Figs. 2–5 are reported in Figs. 6–8 as a function of the compounds molar fraction.

The T_m is expressed as $\Delta T/T_m^0$, where $\Delta T = T_m - T_m^0$, T_m is the transition peak temperature of DMPC MLV prepared in the presence of the examined compound and T_m^0 is the transition peak temperature of pure DMPC MLV; the ΔH is expressed as $\Delta\Delta H/\Delta H^\circ$, where ΔH is the value of enthalpy change of DMPC vesicles prepared in the presence of the individual compounds and ΔH° is the enthalpy variation of pure DMPC MLV.

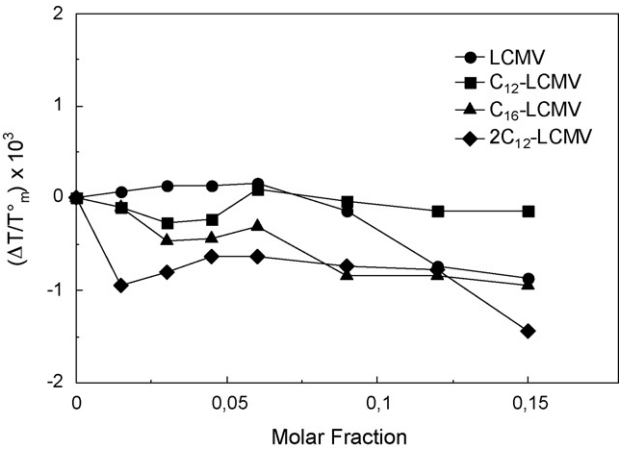

Fig. 6. Transition temperature variations of DMPC MLV prepared in the presence of increasing molar fractions of LCMV, C_{12}-LCMV, C_{16}-LCMV, $2C_{12}$-LCMV, expressed as $\Delta T/T_m^0$ ($\Delta T = T_m - T_m^0$ where T_m^0 is the transition temperature of pure DMPC MLV and T_m is the transition temperature of DMPC MLV prepared in presence of the compounds), as a function of compounds molar fraction in MLV aqueous dispersion.

A small decrease in the transition temperature variation was observed in Fig. 6 for LCMV, C_{12}-LCMV and C_{16}-LCMV by increasing their molar fractions, whereas a significant decrease in the transition temperature change was observed with $2C_{12}$-LCMV.

The enthalpy changes reported in Fig. 7 reveal that only $2C_{12}$-LCMV exhibits a nearly regular and well-defined decrease in the value related to the calorimetric peak whereas the other compounds show a less defined behaviour.

This behaviour is then more significant in the case of more lipophilic conjugates (Figs. 7 and 8). The results highlight a strong deviation from the ideal behaviour predicted by the Van't Hoff equation which predicts an almost linear relationship between the lowering of the solvent melting temperature and concentration of the solute. Deviations are ascribed to the non-

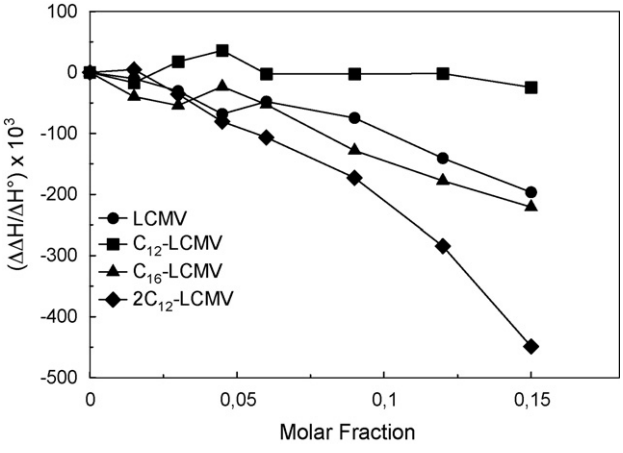

Fig. 7. Enthalpy change variations of DMPC MLV prepared in the presence of increasing molar fraction of LCMV, C_{12}-LCMV, C_{16}-LCMV, $2C_{12}$-LCMV, expressed as $\Delta\Delta H/\Delta H^\circ$ ($\Delta\Delta H = \Delta H - \Delta H^\circ$, where ΔH° is the enthalpy change of pure DMPC MLV and ΔH is the enthalpy change of DMPC MLV prepared in presence of the compounds), as a function of compounds molar fraction in MLV aqueous dispersion.

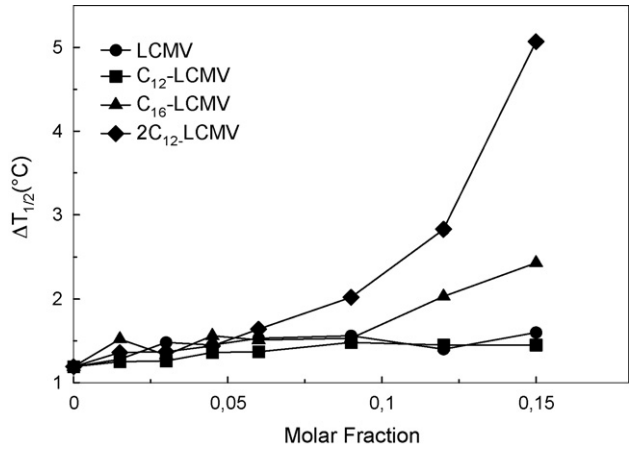

Fig. 8. Peak width variations ($\Delta T_{1/2}$) of DMPC MLV prepared in the presence of increasing molar fraction of LCMV, C_{12}-LCMV, C_{16}-LCMV, $2C_{12}$-LCMV, as a function of compounds molar fraction in MLV aqueous dispersion.

ideal solute–solvent mixing [22,23]. Since we observe either positive and negative deviations in the temperature shift depending on the studied compounds, we may conclude that both the hydrophilic heads and hydrophobic tails contribute in an opposite way to the temperature shift originated by the interaction of the lipoconjugate with the DMPC MLV.

The peak width is a parameter that indicates the cooperativity degree of the transition and the disorder degree in the lipid bilayer [26]. Particularly, when the LCMV and C_{12}-LCMV were used the peak width remained almost unchanged, whereas increasing the chain length (C_{16}-LCMV) and the number of the lipid chains ($2C_{12}$-LCMV) the increase of the peak width value occurred indicating, mainly for high molar fractions, a decrease of cooperativity of the transition and the induction of a disorder in the phospholipid bilayer.

These results suggested that the tested peptide and lipopeptides interact with the bilayers in different way. It is not simple to define the nature of the interaction between peptide and phospholipid as it depends on several parameters such as the length [27] and the amino acids sequences [28] of the peptide.

The pure peptide (LCMV) caused a small decrease of the thermotropic parameters. The disappearance of the pretransition peak at high molar fractions showed that LCMV interacts weakly with the bilayers surface, probably because of its poor stability within biological matrices [4]. On the basis of the classification proposed by Papahadjopoulos et al. [29] and McElhaney [30] that put in relation the effects on the thermotropic parameters and the protein localisation in the phospholipid bilayer, the peptide could localise at the bilayer interface where it interacts primary with the polar head group and glycerol backbone of the phospholipid molecules, although some interaction with the region of the hydrocarbon chains near the bilayer interface also could occur. The conjugation of LCMV with Laas with longer acyl chain (C_{16}-LCMV) or with two short chains ($2C_{12}$-LCMV) strongly perturbs the biomembrane models, mainly at high molar fractions of compounds.

In particular, with the increase of the number of the lipid chains, the interaction is stronger, causing the melting to be less endothermal and cooperative.

The weaker interaction of C_{12}-LCMV may not be due to the limited insertion in the phospholipid bilayer, as the length of the C_{12} chain allowed an interaction but was not long enough for the full penetration of the phospholipids bilayer.

4. Conclusion

In this work we studied the interaction of the peptide LCMV and some of its lipopeptide conjugates with a membrane model made of multilamellar vesicles of DMPC. The aim of this work was to investigate whether the Laa moiety improves the interaction with DMPC with respect to the pure peptide. The peptide, alone or conjugated with a single acyl chain, showed a very low interaction with DMPC bilayer, whereas the conjugation of the peptide with two acyl chains enhanced its ability to interact with the biomembrane model, showing that the interaction strongly depended on the lipid chains number, because they caused a decrease of the cooperativity of the phase transition and the induction of disorder in the phospholipid bilayer. Consequently, compounds with higher number of lipid chain showing a stronger interaction with the biomembrane model, could let the conjugated peptide to be carried more efficiently by a liposomal formulation.

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