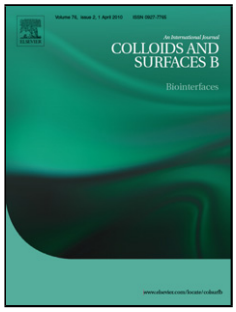

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Interaction of fengycin with stratum corneum mimicking model membranes: a calorimetry study

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Abstract

Based on its outstanding antifungal properties, it is reasonable to believe that fengycin might be efficient to topically treat localized dermatomycoses. Since most of the fungi species involved in the formation of those mycotic skin diseases colonize primarily the stratum corneum (SC), studying the interaction between fengycin and SC-mimicking lipid membranes is a primary step to determine the potential of fengycin to overcome the physical barrier of the skin.

In this respect, multilamellar lipid vesicles (MLVs), with a lipid composition mimicking that of the SC, were prepared and characterized by differential scanning calorimetry (DSC). The critical micelle concentration (CMC) of fengycin was also assessed under skin conditions and found to be 1.2 ± 0.1 μM. The molecular interactions of fengycin with SC-mimicking MLVs were investigated by both DSC and isothermal titration calorimetry (ITC). Results showed that the interactions were considerably affected by changes in lipid phase behaviour. At 40 °C and below, fengycin induced exothermic changes in the lipid structures suggesting that less-ordered lipid domains became more-ordered in presence of fengycin. At 60 °C, clearly endothermic interaction enthalpies were observed, which could arise from the "melting" of remaining solid domains enriched in high melting lipids that without fengycin melt at higher temperatures.

1. Introduction

Fengycin is a bioactive amphiphilic lipopeptide produced by the *Bacillus subtilis* strain S499 in an optimized culture media [1]. It is a decapeptide containing a *β*-hydroxy hydrocarbon chain (Figure 1). It is composed of closely related variants, which differ both in the length of the hydrocarbon chain (13 to 17 carbon atoms) and in the nature of the amino acid in position 6 of the peptide moiety (*D*-Ala for fengycin A and *D*-Val for fengycin B). Fengycin includes three amino acid residues that can be protonated or deprotonated according to pH. At neutral pH, the lipopeptide has two negatively charged (glutamic acid) residues and one positively charged (ornithine). The environmental conditions play an important role in its intermolecular interactions [2] and on the turn conformation adopted by the molecule [3]. This relatively low haemolytic lipopeptide is active against a large number of fungi [4-7], especially against filamentous fungi [8]. The antifungal activity of fengycin most likely arises from its interaction with cytoplasmic membranes where it is supposed to form a complex with ergosterol causing a change in bilayer permeability [8]. Such mechanism of action has been also reported for other antifungal compounds such as amphotericin B [9,10] and mycosubtilin [11]. Its remarkable antifungal properties make fengycin an excellent candidate to topically treat localized dermatomycoses which are common cutaneous fungal infections caused by filamentous fungi [12]. Since these filamentous fungi have a high affinity for keratin, they invade and colonize primarily the stratum corneum (SC), i.e. the keratinized uppermost layer of the skin, as well as the hair follicles and the nails which have extremely high keratin content [12].

Located at the interface between the body and the environment, the SC plays a considerable role in the barrier function of the skin. While this layer constitutes only 10 % of the entire skin, it contributes to over 80 % of the cutaneous barrier function [13]. SC consists of keratin-filled dead cells (corneocytes) embedded in an extracellular matrix of lipid lamellae

[14]. The extracellular lipids constitute the sole continuous regions of the SC, and molecules passing the skin barrier must be transported through this lipid matrix [15-18]. Unlike other biological membranes, SC is almost devoid of phospholipids and its major constituents are ceramides (40–50 wt %), cholesterol (20–33 wt %) and long-chain, predominantly saturated, free fatty acids (7–13 wt %) [19-22].

It is clear that the action of bioactive molecules on the skin requires their interactions with skin lipids. Studying the molecular interaction between fengycin and lipid membranes mimicking both the lipid composition and organization of the SC is a primary step to determine the potential of fengycin to overcome the physical barrier of the skin. Such interactions can be studied using several types of model membrane systems, including lipid monolayers, supported lipid bilayers and lipid vesicles. Although these model systems strongly differ in term of lipid organization, they can be combined to provide a comprehensive and detailed analysis of molecular interactions between lipid constituents and potential drugs.

A previous study performed on skin-mimicking lipid monolayers [23] showed that fengycin was able to modulate ceramide properties such as molecular organization, phase separation and fusogenic activity. This phenomenon was found to be influenced by both the environmental conditions (temperature, pH) and fengycin concentration. Another work [24] revealed that the insertion of fengycin into SC-mimicking models is more favourable when cholesterol is added to a pure ceramide model. The lipopeptide preferentially partitions into cholesterol-enriched phases rather than into phase(s) with lower cholesterol content. The cholesterol-rich phase is less condensed than the cholesterol-poor one, which probably facilitates the molecular interactions of fengycin with cholesterol-containing model membranes.

In the present study, the combination of differential scanning calorimetry (DSC) and high-sensitivity isothermal titration calorimetry (ITC) was used to further investigate the interaction of fengycin with skin lipid vesicles. Multilamellar vesicles were prepared from an equimolar mixture of ceramides, cholesterol and free fatty acids. They are appropriate model membrane systems to mimic both the composition and organization of SC [25] since the lipid components of the SC that are located in the intercellular space between corneocytes are arranged in a lamellar structure [26].

The pH gradient as well as other gradients in the non-equilibrium SC system [27-31] makes it challenging to choose the conditions that best mimic a relevant physiological situation. It was decided to work at pH 7.4 in an excess PBS buffer containing 150 mM NaCl because SC model lipids suspensions were found to be more stable at higher pH. The conditions in terms of water activity and pH should better mimic the conditions found in the lower layers of the human SC rather than the upper layers [32], expect for occluded skin (skin covered by impermeable dressings, tapes, gloves, or transdermal devices) [27,33,34]. Moreover, the colonization and invasion of the SC by filamentous fungi most likely induce changes of the various gradients in the non-equilibrium SC as the germ tubes resulting from the fungal development invade individual corneocytes, grow transversally and through the whole thickness of the SC, and form branching hyphae [12]. As this is also the case in many other superficial skin disorders, it can be assumed that the environmental conditions found in the upper layers of SC infected by filamentous fungi tend to be similar to the ones found in the lower layers of SC.

Under physiological conditions, fengycin bears two negative charges (glutamic acid residues) and one positive charge (ornithine residue) (Figure 1) [2] although the SC-mimicking MLVs are partially negatively charged (at pH 7.4, the fatty acid under

investigation is partially dissociated [35]). However, the high ionic strength in the PBS buffer may partially screen the electrostatic effects associated with the negative charges.

DSC was performed on pure SC-mimicking MLVs to characterize the specific SC model membrane under investigation. ITC measurements were then carried out at four different temperatures, 20, 32, 40 and 60 °C, to investigate the impact of both the lateral packing and phase behaviour of the lipid bilayers on the molecular interactions with fengycin. Ultimately, the effect of fengycin on the phase transitions of our specific SC model membrane was explored by performing DSC experiments on the vesicle suspensions after they have been titrated with fengycin (using the ITC technique).

2. Materials and Methods

2.1. Chemicals

Fengycin A with a β-hydroxy hydrocarbon chain of 16 carbon atoms (Fengycin A C_{16}; molar mass: 1462.8 g/mol) was prepared from the culture media of Bacillus subtilis S499 as described previously [1,36]. Isolation from crude fengycins was performed by preparative reverse phase chromatography. The identification and verification of the purity were made by amino-acid analysis [37], analytical RP-HPLC and MALDI-TOF spectrometry (Ultraflex TOF, Bruckner, Karlsruhe, Germany). The purity of fengycin samples was always higher than 95 %.

N-lignoceroyl-D-sphingosine (C_{24}-Ceramide 2) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Tetracosanoic acid (C_{24}-fatty acid) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemicals were used without further purification.

A phosphate buffered saline (abbreviated PBS buffer) was used in this study. It was composed of $NaH_2PO_4.H_2O/Na_2HPO_4$/NaCl 20/20/150 mM. Its pH was adjusted to 7.4 by adding an adequate amount of NaOH 7.5 M.

2.2. Multilamellar lipid vesicle

The multilamellar lipid vesicles (MLVs) consisted of an equimolar mixture of C_{24}-ceramide 2, cholesterol and tetracosanoic acid (C_{24}-fatty acid). The latter was chosen since together with the C_{22}-fatty acid they are the most abundant free fatty acids in intact SC [21,38,39]. C_{24}-ceramide 2 was incorporated in our SC model because ceramides of type 2 with 24 (C_{24}) and 26 (C_{26}) carbon chains are the most prevalent in epidermal SC [19,20,25,40]. The ceramides of type 1 or 4, which are responsible for the formation of the

long periodicity lamellar phase [41], were not included in our model mixture. Our SC-mimicking model also contained 33 mol% cholesterol, which is similar to the cholesterol content in the human SC extracellular lipids [22].

For the preparation of the MLVs, ceramide, cholesterol and fatty acid were first co-dissolved in chloroform/methanol 2/1 (v/v). Equimolar mixtures of the three lipids were prepared and the solvent was left to evaporate under a stream of nitrogen. Traces of solvents were removed by evacuation under reduced pressure (vacuum) for at least 12 hours. The dried samples were then hydrated in a large excess of buffer above 95 °C by vigorous vortexing. To favour hydration of dried SC lipids, sonication was performed at temperature close to 95°C and for one minute periods using a bath-type sonicator (Starsonic 90). The final concentration of total lipids in the vesicle suspension was 5 mM. Before beginning the DSC or ITC experiments, the hydrated samples were kept at room temperature during 30 minutes.

2.3. Differential Scanning Calorimetry

Differential scanning calorimetry measurements were performed using a high-sensitive differential scanning calorimeter MicroCal VP-DSC (Microcal Inc., Northampton, MA, USA), equipped with two total-fill cells of 0.507 ml, one for the reference and one for the sample to be studied. The reference PBS buffer was degassed using a Nueva II stirrer (Thermolyne) before being transferred into the cell using a Hamilton syringe. The suspension of SC-mimicking multilamellar lipid vesicles (MLVs) was transferred into the sample cell without extra degassing thirty minutes after preparation. After 15 minutes at 15 °C, the temperature of both the reference and sample cells was increased from 15 to 99 °C, with a heating scan rate of 0.5 °C/min. A reference thermogram was recorded with both cells filled with the reference solution. Identical DSC conditions were applied when performing DSC experiments on the suspensions of SC-mimicking MLVs that were titrated with fengycin

using the ITC technique. At least three independent samples were measured and showed good reproducibility in both the transition maxima and the size of the peaks (see Figure 3 for an example). Data were analysed using Origin Scientific plotting software, version 5 after subtraction of the reference thermogram.

2.4. High-Sensitivity Isothermal Titration Calorimetry

High-sensitivity isothermal titration calorimetry was used to investigate the thermodynamic changes associated with the interaction of fengycin with SC-mimicking MLVs. In addition, ITC experiments have been performed to determine the critical micelle concentration (CMC) of fengycin.

A Microcal VP-ITC instrument (Microcal Inc., Northampton, MA, USA) was used. Millipore water was used as reference solution as it gave the same results as the PBS buffer. All measurements were carried out at least in triplicate. Data analysis was done using Origin® software (version 7.0) from Microcal (Microcal Inc., Northampton, MA, USA).

CMC determination of fengycin

The reaction cell (1.456 ml) was filled with PBS buffer pH 7.4. The fengycin solution (50 µM) prepared also in PBS buffer was aspirated in the 300 µl syringe and introduced into the reaction cell in 40 successive 5 µl injections (after a first injection of 2 µl). Each fengycin addition took 10 seconds with an interval of 300 seconds between consecutive injections. During the titration, the content of the reaction cell was stirred continuously at 300 rpm. The heat effect due to the injection of PBS buffer into the PBS buffer was measured ($q_{(null)}$) and subtracted from the observed reaction heats ($q_{(obs)}$). The CMC value and its standard deviation were obtained from four independent measurements.

Interaction of fengycin with SC-mimicking multilamellar lipid vesicles

ITC measurements were performed by filling the reaction cell with a 5 mM suspension of MLVs and the injection syringe with a fengycin solution (ca. 700 µM) prepared in PBS buffer. The lipopeptide solution was added into the reaction cell in 50 successive 5 µl injections (after a first injection of 2 µl). The interval between the first 10 consecutive injections was 900 seconds, although it was 720 seconds for the next 20 and 400 seconds for the last 20 injections. In the beginning of the titration series, the reaction was not instantaneous but extended over about 10 min, so longer intervals were needed to reach the baseline after the reaction peak. During the titration, the content of the reaction cell was stirred continuously at 300 rpm. Measurements were made at 20, 32, 40 and 60 °C. The heat component due to the addition of the lipopeptide solution to the PBS buffer ($q_{(dil,fen)}$), the heat component due to the dilution of the lipid vesicle suspension (i.e. the heat resulting from the injection of buffer without lipopeptide into the vesicle suspension) ($q_{(ves,dil)}$) as well as the heat effect arising from the injection of PBS buffer into PBS buffer ($q_{(null)}$) were all measured in separate sets of experiments and subtracted from the observed reaction heats ($q_{(obs)}$) in order to calculate the enthalpy of the reaction:

$$q_{(reaction)} = q_{(obs)} - q_{(dil,fen)} - q_{(ves,dil)} - q_{(null)} \quad (1)$$

with:

$$q_{(dil,fen)} = q_{(dil,fen)obs} - q_{(null)} \quad (2)$$

$$q_{(ves,dil)} = q_{(ves,dil)obs} - q_{(null)} \quad (3)$$

Measurements were done at least in triplicate. A variation less than 15% was observed for the reaction enthalpy.

3. Results and Discussion

The first objective of the present study was to characterize both the micellization behaviour of fengycin and the thermotropic properties of the SC-mimicking model under investigation.

3.1. Fengycin micellization

The critical micelle concentration (CMC) corresponds to the lowest concentration of amphiphilic molecules that is required to form micelles. This parameter does reflect their surface-active properties. The association of free monomers into micelles in solution is a process strongly dependent on the chemical nature of the amphiphilic molecule as well as on environmental parameters such as ionic strength, temperature and pH [42].

The present study aimed first at determining a CMC value for fengycin since such information was not available in the literature. This experiment was done using the ITC technique. The heat flow vs. time profile resulting from the injection of 5 µl aliquots of a 50 µM fengycin solution into the reaction cell containing PBS buffer is shown in Figure 2A. The concentration of fengycin in the reaction cell increases in steps of 0.16 µM per injection. The first three injections cause an endothermic heat of about 1.2-1.3 µcal. As the titration continues, the heat adsorbed by the system at each injection progressively decreases to give constant, slightly exothermic values. These small exothermic peaks stem from a null-effect that was determined from injections of buffer solution into the reaction cell filled with buffer. The heat uptake accompanying each injection is obtained by integration of the heat flow peak after subtraction of the baseline and the heat effect measured from buffer-into-buffer injections (Figure 2B). The curve indicates a change from an initial endothermic process to an essentially athermic one. This curve shape is typically obtained for the disaggregation (demicellization) of amphiphilic compounds [42]. Thus, we ascribe the initial endothermic

step to the dilution and break up of fengycin micelles injected from the syringe into the buffer in the cell. Above a fengycin concentration of about 1.5 μM, added micelles are only diluted and give a negligible enthalpy change. The CMC is defined as the inflexion point in the enthalpy against concentration curve. It is obtained by calculating the first derivative of this curve. The CMC value of fengycin A C_{16} is 1.2 ± 0.1 μM (mean and standard deviation of four titration series in PBS buffer, pH 7.4, 32 °C). The enthalpy change (ΔH_{demic}) for the transfer of fengycin monomers from micellar aggregates to water can be estimated to be about + 4.3 ± 0.1 kcal/mol. Micelle formation corresponding to the reverse process consequently gives an enthalpy change of ΔH_{mic} = - 4.3 ± 0.1 kcal/mol at 32 °C in the PBS buffer at pH 7.4, meaning that this phenomenon is exothermic. The values obtained for both the CMC and ΔH_{mic} are of the order of magnitude that can be expected for the micelle formation of an ionic amphiphilic compound with a C_{16} hydrocarbon chain at high ionic strength [43]. The effect of the unusually bulky headgroup appears to be small. Moreover the nature of the process in the 1 μM concentration region which we ascribe to demicellization needs to be further studied by other methods. However, since the demicellization process occurs at a fengycin concentration lower than the concentration used in our ITC experiments involving MLVs, it can be neglected when analyzing our ITC data.

3.2. DSC characterisation of the SC-mimicking model

DSC has been widely used previously to characterize lipid thermal transitions in native human SC, in animal SC as well as in extracted SC lipids and SC-mimicking model membranes [44-56]. Samples prepared from native SC or isolated SC lipids have a very complex composition including about 250 different ceramides accounting for all combinations of sphingosine bases and fatty acids of different chain lengths [56,57]. Moreover, for native SC, the lipid fraction constitutes only one part of the whole SC matrix,

and the thermal transitions in these complex cutaneous samples are often very broad and not well-resolved. Consequently, the interpretation of the phase transitions observed from isolated SC lipids as well as SC-mimicking model mixtures can only be done if additional structural information obtained from techniques such as small-angle (SAXD) and wide angle X-ray diffraction (WAXD) techniques, Fourier-transformed infrared spectroscopy (FTIR), NMR and Raman spectroscopy are available [55].

In the present work, DSC was used to fingerprint the lipid system under investigation and to define the most appropriate temperatures to study the molecular interactions of fengycin with our specific SC-mimicking model membrane. It did not aim at fully characterizing the different phase transitions occurring in our lipid system.

The DSC thermograms obtained in the absence of fengycin are presented in Figure 3. Repeated DSC measurements gave reproducible DSC traces, which indicate that steady-state conditions were reached before starting the measurements. At least seven well-defined endothermic peaks (marked with Arabic numbers) can be identified. The DSC thermograms exhibit similar features as those obtained in previous studies [26,53,54,58-63], although direct quantitative comparisons cannot be made due to the specific composition of our model membrane.

The major thermal transition peaks, denoted 4 and 5 in Figure 3, occur at 40 - 60 °C. Based on information from previous studies [26,53,54,58-64], we assume that the pair of endotherms observed between 40 and 60 °C most likely corresponds to transitions between more-ordered phases and less-ordered fluid phases. Consequently, in this temperature range, more-ordered solid domains (crystalline or gel phase) coexist with less ordered fluid phase, and the proportion of fluid phase increases with temperature. In this co-existence, the fluid phase is believed to ensure cohesion between the solid domains [54,65].

Above the two main phase transitions (i.e. at temperatures higher than 60 °C), one disordered liquid crystalline phase composed of ceramide, fatty acid and some cholesterol most likely prevails. This phase likely co-exists with a liquid ordered lamellar phase rich in cholesterol that has similar properties as the lipid microdomains [66] composed of a mixture of saturated sphingolipids and cholesterol in the plasma membrane in cells. This assumption is supported by experimental observations of a liquid ordered phase in e.g. fatty acid/cholesterol [67] and ceramide/fatty acid/cholesterol [68,69] systems.

Below the two main phase transitions (i.e. at temperatures up to 40 °C), mixtures of ceramide-rich and fatty acid-rich solid phases probably co-exist. Some cholesterol is likely incorporated in these phases, and the remaining cholesterol likely presents an additional crystalline or amorphous phase as previously described for different model SC lipid systems [26,60,63]. The presence of an additional overlapping pair of endotherms below 40 °C may indicate that two different ordered phases coexist at temperatures close to the physiological ones. Such behaviour was proposed by Plasencia and her colleagues who performed DSC and Laurdan Generalized Polarization measurements on lipid dispersions composed of extracted human SC lipids [53]. These authors suggested that the presence of a phase transition close to physiological temperatures may have major implications for the skin barrier function.

3.3. Interactions of fengycin with SC-mimicking models

ITC measurements

Based on the DSC fingerprint of our SC-mimicking model membrane, four temperatures (20, 32, 40 and 60 °C) were selected to further study the thermodynamic changes associated with the interaction of fengycin with SC-mimicking MLVs: (i) 40 and 60 °C were chosen as they correspond to temperatures just below and above the main solid-fluid transitions; (ii) 20 °C was selected as it corresponds to temperature below the first

overlapping pair of endotherms. At this temperature, our MLVs are expected to form solid phases with a close-packed lateral lipid arrangement; (iii) As the skin temperature normally lies around 32 °C, this temperature was also used in our ITC measurements.

Figure 4A shows an example of the raw data from ITC measurements obtained from a series of 5 µl injections of a micellar solution of fengycin (ca. 700 µM) into a 5 mM suspension of SC-mimicking MLVs at 32 °C. The total concentration of fengycin (free in solution and/or bound to the lipid vesicles) in the reaction cell increases from ~3.5 µM after the second injection to ~115 µM at the end of the 50 injection sequence. The measured enthalpy changes refer to the interaction of fengycin micelles with the vesicles. The negative heats of reaction indicate the presence of large exothermic interactions between the components, (Figure 4A). The amplitude of the exothermic peaks decreases as the titration continues and becomes more or less constant after about 30 injections. At this stage, additional injections of fengycin do not produce any additional heat of reaction as the small exothermic peaks probably stem from null-effects. The enthalpy changes calculated per mole of added fengycin at 32 °C are plotted against fengycin concentration in Figure 4B. In the beginning, the interaction is strongly exothermic (being about - 13 kcal/mol), indicating that fengycin induces significant changes in the vesicles. The reaction becomes steadily less exothermic up to a fengycin concentration of 55 µmol/l where the curve again becomes much steeper, indicating a change in the way fengycin interacts with the lipid vesicles. The change of slope could also arise from the fact that fengycin has solubilized the first layer of the MLVs and then interacts with the next one. Above a fengycin concentration of 80 µmol/l (corresponding to a fengycin-to-lipid molar ratio above 1.6 mol %), only small and constant heat effects are observed, suggesting that no additional interaction between fengycin and SC-mimicking MLVs occurs.

The enthalpy curves resulting from the interaction between fengycin and SC-mimicking MLVs at 20 and 40 °C show similar features to the curve obtained at 32 °C (Figure 5). The main difference is observed in the beginning of the titration series. At 20 °C, the initial enthalpy changes are slightly less exothermic compared to the enthalpy changes at 32 °C, while at 40 °C the initial phase is much more exothermic being about -18 kcal/mol. The increase of temperature to 60 °C causes a radical change in the shape of the enthalpy versus concentration curve and the enthalpy changes observed at low fengycin concentration are highly endothermic (Figure 5). After the first injection, the enthalpy values increase to become almost constant at about 7 kcal/mol up to a fengycin concentration of 30 µmol/l. Then they decrease quite rapidly up to a concentration of 40 µmol/l and after that the enthalpies decrease slowly before becoming constant up to the highest concentration of the measurements.

At temperatures up to 40 °C, fengycin induces changes in the lipid structures that are exothermic. Extrapolations of the linear parts of the titration curves at low fengycin concentrations to the y-intercept give estimates of the enthalpy changes ΔH^0 associated with the initial binding of fengycin to the MLVs of -12.4, -13.3 and -18.8 kcal/mol at 20, 32 and 40 °C, respectively. The exothermic character implies the formation of a more solid structure, and hence, a condensing effect of fengycin.

At 60 °C, fengycin interacts with SC-mimicking lipid vesicles in the fluid state. The interaction enthalpy up to about 30 µmol/l fengycin is endothermic in contrast to the strong exothermic enthalpies observed at temperatures of 40 °C and below. The endothermic interaction enthalpies could arise from "melting" of remaining solid domains enriched in ceramide, which without fengycin melt at higher temperatures, e.g. transition 7 in Figure 3. Considering the low fengycin concentration and the complex lipid assembly in multilamellar vesicles, probably only the outermost layer of the MLV is affected by fengycin. It is likely

that fengycin preferentially locates at interfaces between lipid domains and thereby stabilizes the domain structure as a line-active agent in a similar way as previously been shown for, e.g., cholesterol [70], some proteins [71,72], peptides [73], and surfactin [42,74], another lipopeptide class produced by *Bacillus subtilis* strains.

Changes of lipid phases induced by fengycin may not be the only cause for the variation of the interaction enthalpies with temperature. Other molecular events such as lipopeptide aggregation at the vesicle surface, lipopeptide reorganization within the external lipid bilayers of the MLVs or changes in the peptide-lipid surface hydration may also contribute to the observed enthalpy variations, and we are not able to distinguish between those from the present calorimetric measurements. However, the magnitude of the enthalpy changes related to the latter effects is most likely much smaller compared to the enthalpy changes related to structural changes in lipid bilayers caused by lipopeptide interaction.

DSC measurements

To investigate the effect of fengycin on the phase transitions in our SC-mimicking model membrane, DSC scans were run on samples taken from the titration calorimeter once the experiments were completed. Thermograms observed for the solutions obtained from the ITC experiments at 20, 32 and 40 °C are very similar to the DSC curve without fengycin, see Figure 6. The main difference is a significant decrease in the intensity of transition 1. This transition peak is indeed missing when MLVs are titrated with fengycin at 20 °C and is clearly reduced after titration at 32 and 40 °C. MLVs titration with fengycin at 60 °C strongly alters the DSC thermogram. In contrast to lower temperatures, transition 1 is not affected by the addition of fengycin at this high temperature, although the intensity of transition 2 is notably reduced. Transitions 4 and 5 are also strongly affected and the ΔH value reduced by about 40 %. Transitions 6 and 7 are not longer observed.

The DSC data are in good agreement with the ITC results. The absence of phase transitions above 60 °C for the 60 °C ITC sample indicates that fengycin has a stronger effect on lipid vesicles in a fluid state than in a more solid state. The ability of lipopeptides to disturb lipid packing in other lipid model membranes (monolayers, supported bilayers and vesicles) has been demonstrated in previous studies [74-78]. Based on the ITC results, we propose that the interaction of fengycin with SC-mimicking MLVs in a fluid state (i.e. above 60 °C) is responsible for the "melting" of remaining solid domains enriched in high melting lipids such as ceramides. The absence of phase transitions above 60 °C in the DSC thermogram of the 60 °C ITC sample is in accordance with this hypothesis. More drastic changes in the structure of the lipid bilayers, such as a progressive disruption of the outermost bilayers of MLVs and formation of mixed micelles, could also be induced by fengycin at temperatures above 60 °C. However, such processes cannot be deduced from our ITC and DSC measurements and further investigations using for example cryo-TEM, X-ray diffraction and/or neutron scattering techniques need to be performed. Below the main solid-fluid transitions (up to 40 °C), the assumption of a fengycin-induced lipid ordering could explain the significant decrease of transition 1 observed at these temperatures.

4. Conclusion

Thermodynamic changes associated with the interaction of fengycin with SC-mimicking model membranes have been studied by means of two calorimetric techniques, ITC and DSC.

The nature of the molecular interactions strongly depends on the lipid phase behaviour of the vesicles. At lower temperatures, i.e. below the main solid–fluid transitions of pure MLVs, we suggest that fengycin promotes the ordering of the alkyl chains of fatty acid and ceramide molecules. At higher temperatures i.e. above the main solid–fluid transitions of pure MLVs, we propose that fengycin induces the "melting" of remaining solid domains, which are enriched in high melting lipids such as ceramides. However, changes in the structure of the lipid vesicles such as a progressive and partial disruption of the outermost bilayers of MLVs and the subsequent formation of mixed micelles could also occur at these higher temperatures. Further investigations involving cryo-electron microscopy and light scattering experiments are currently in progress in order to better understand the specific action of fengycin at temperatures below and above the main solid–fluid phase transitions.

In accordance with our previous study [24], we can suggest that cholesterol likely plays an important role in the interaction of fengycin with SC-mimicking lipid vesicles by favouring the partition of fengycin into the cholesterol-rich phase of our specific MLVs. Below the main solid-fluid transitions, both fengycin and cholesterol could be located at the frontier of solid domains made of ceramide and fatty acid molecules, thereby stabilizing the domain structure and the lipid bilayers. Above the main solid-fluid transitions, as cholesterol is incorporated in one unique fluid lamellar phase containing also ceramide and fatty acid, it might facilitate the interaction of fengycin with the outermost bilayers of MLVs and consequently might favour their progressive solubilization and the formation of mixed micelles.

By demonstrating the potential of fengycin to interact with SC-mimicking model membrane, we can assume that this lipopeptide is able to overcome the physical barrier of SC and can exert its antifungal activity where filamentous fungi involved in dermatomycoses proliferate.

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Figure captions

Figure 1. Chemical structure of (A) fengycin A with a *β*-hydroxy fatty acid chain of 16 carbon atoms. (Solid line-circles indicate the chemical functions that can be ionised or protonated depending on the pH. Dotted line-circle shows the lactone link between the hydroxyl group of the D-tyrosine and the carboxyl group of the L-isoleucine residue), (B) N-lignoceroyl-D-sphingosine (C_{24}-Ceramide 2), (C) cholesterol (Chol) and (D) tetracosanoic acid (C_{24}-fatty acid).

Figure 2. A. Heat flow (µcal/sec) versus time (min) profile resulting from injection of 5 µl aliquots of fengycin A C_{16} solution (50 µM) into the reaction cell containing PBS buffer (pH 7.4) at 32 °C. B. Reaction enthalpy obtained from peak areas in the heat flow curves against fengycin concentration.

Figure 3. DSC thermogram (first heating scan) of four independent suspensions of SC-mimicking multilamellar lipid vesicles.

Figure 4. A. Heat flow (µcal/sec) versus time (min) profile resulting from injection of 5 µl aliquots of solution of fengycin A C_{16} (ca. 700 µM) into the reaction cell containing a 5 mM suspension of SC-mimicking multilamellar lipid vesicles at 32 °C. B. Reaction enthalpy against total fengycin concentration.

Figure 5. Enthalpy changes for the reaction between fengycin and SC-mimicking multilamellar lipid vesicles at 20, 32, 40 and 60 °C as functions of total fengycin concentration.

Figure 6. Representative DSC thermograms (first heating scan) of 5 mM SC-mimicking multilamellar vesicles suspensions after ITC titration experiments with fengycin at 20, 32, 40 and 60 °C (four thermograms at the bottom). A representative DSC thermogram (first heating scan) of 5 mM suspensions of SC-mimicking multilamellar lipid vesicles without fengycin

has been added for comparison (thermogram at the top). Three independent samples were measured and show highly reproducible thermograms in both the transition maxima and the size of the peaks.

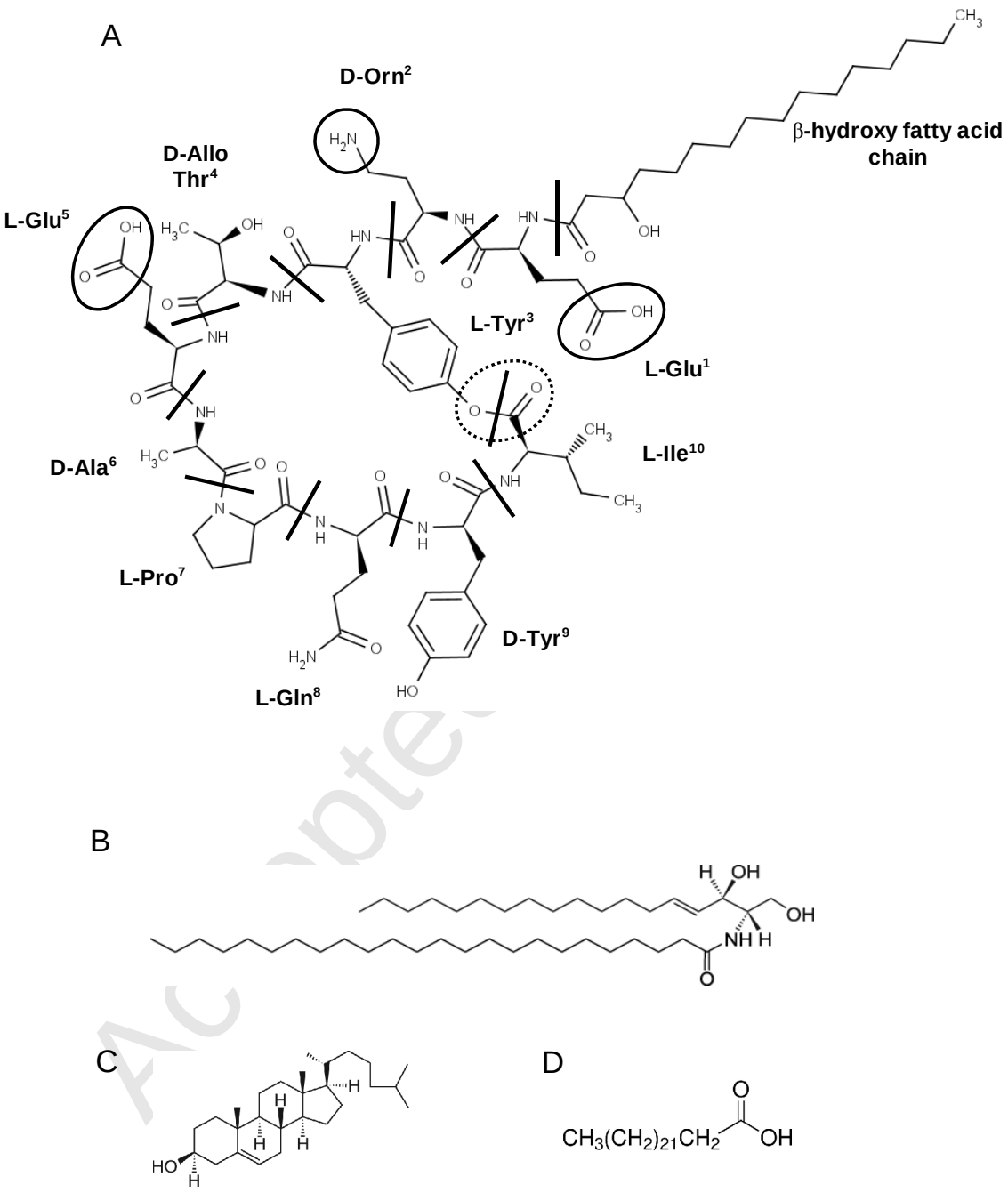


Figure 1

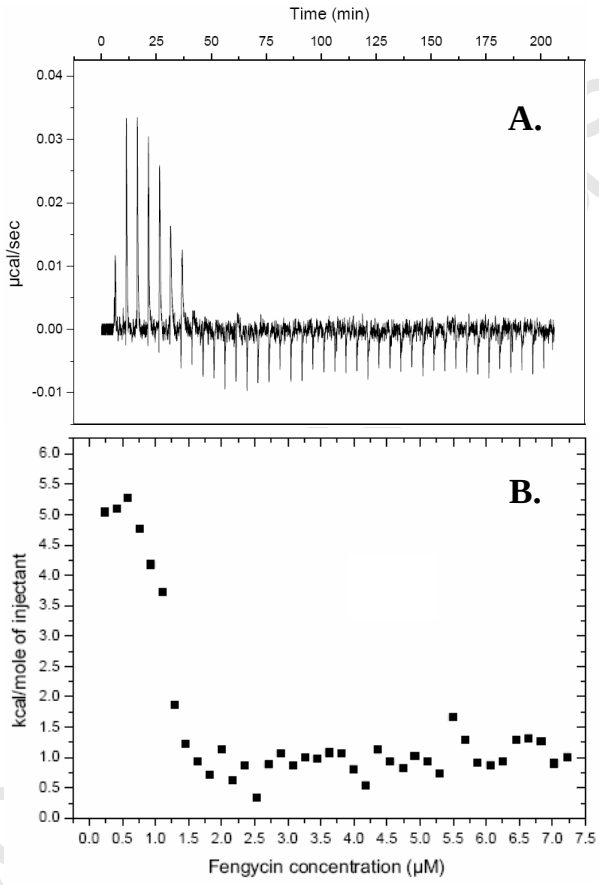


Figure 2

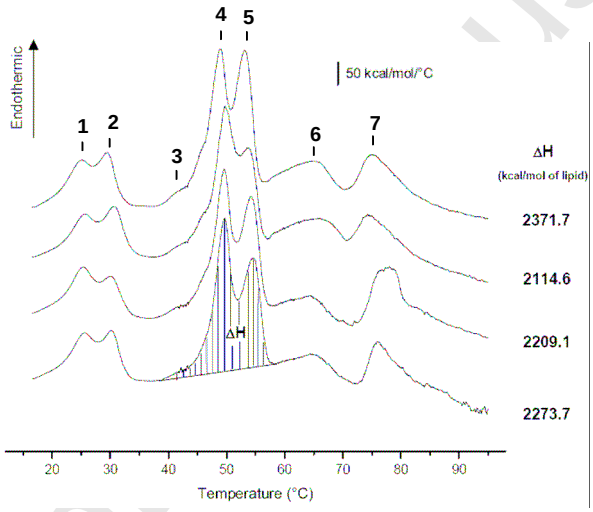


Figure 3

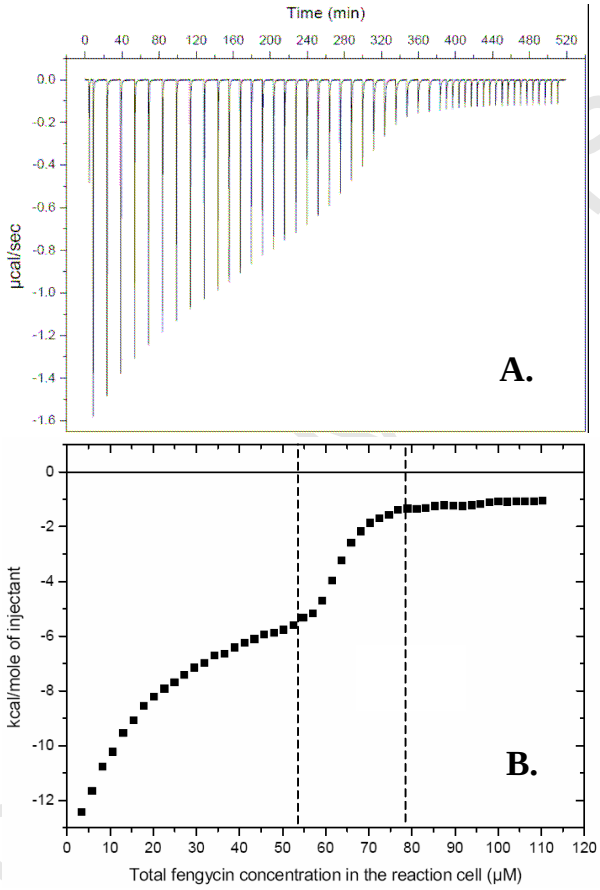


Figure 4

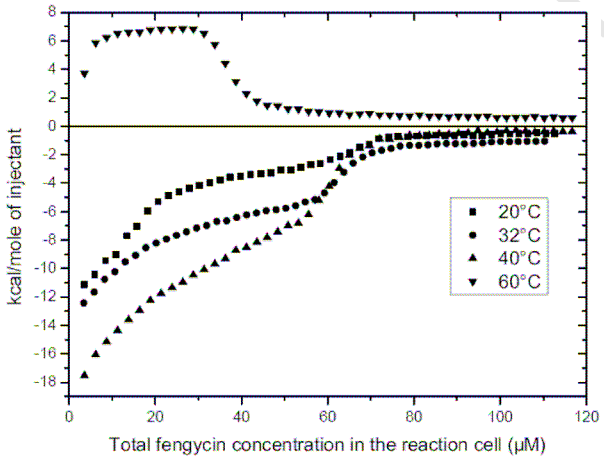


Figure 5

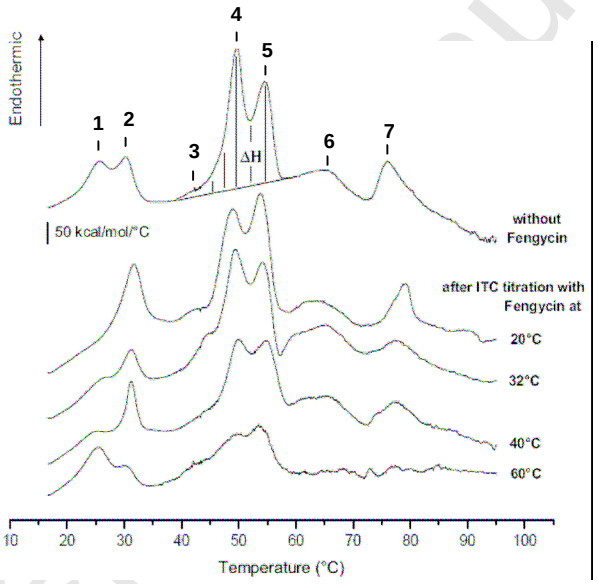


Figure 6

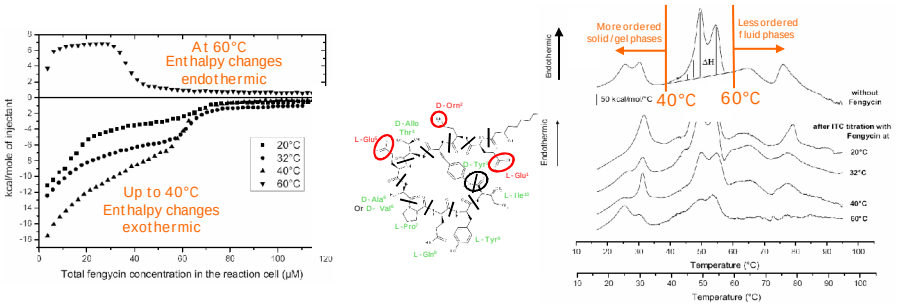
Highlights (Eeman et al.)

-Changes in lipid phase behaviour considerably affected the interaction of fengycin

-Below main solid–fluid transitions, fengycin promotes the ordering of bilayer

-Above main solid–fluid transitions it induces the melting of remaining solid domains

Graphical abstract (Eeman et al.)



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Fengycin has a stronger effect on M