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Effect of cholesterol on the interaction of the amphibian antimicrobial peptide DD K with liposomes

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ABSTRACT

DD K is an antimicrobial peptide previously isolated from the skin of the amphibian *Phyllomedusa distincta*. The effect of cholesterol on synthetic DD K binding to egg lecithin liposomes was investigated by intrinsic fluorescence of tryptophan residue, measurements of kinetics of 5(6)-carboxyfluorescein (CF) leakage, dynamic light scattering and isothermal titration microcalorimetry. An 8 nm blue shift of tryptophan maximum emission fluorescence was observed when DD K was in the presence of lecithin liposomes compared to the value observed for liposomes containing 43 mol% cholesterol. The rate and the extent of CF release were also significantly reduced by the presence of cholesterol. Dynamic light scattering showed that lecithin liposome size increase from 115 to 140 nm when titrated with DD K but addition of cholesterol reduces the liposome size increments. Isothermal titration microcalorimetry studies showed that DD K binding both to liposomes containing cholesterol as to liposomes devoid of it is more entropically than enthalpically favored. Nevertheless, the peptide concentration necessary to furnish an adjustable titration curve is much higher for liposomes containing cholesterol at 43 mol% (2 mmol L^{-1}) than in its absence (93 μmol L^{-1}). Apparent binding constant values were 2160 and 10,000 L mol^{-1}, respectively. The whole data indicate that DD K binding to phosphatidylcholine liposomes is significantly affected by cholesterol, which contributes to explain the low hemolytic activity of the peptide.

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1. Introduction

Antimicrobial peptides have been found to be essential for the innate immune defense of different organisms and they are amply found in the hemolymph and hemocytes of arthropods and molluscs [11,13,17,19,23,27,29] as well as in mammalian gastrointestinal mucosa [45] and skin secretions of amphibians [7,30,31,32]. Bacteria also produce antimicrobial peptides, the so-called bacteriocins, which prevent the growth of closely related species [12,14]. Antibiotic peptides are usually amphipathic and cationic, most of them capable of causing membrane disruption.

The dermaseptins are a class of peptides produced by the skin granular glands of anuran of the *Phyllomedusa* genera [24]. They have from 25 to 33 amino acid residues and their mechanism of action is probably through membrane disruption. The dermaseptins are cationic and amphipathic peptides that present a random-coil structure in aqueous media but become helicoidal in a membrane environment or in membrane–mimetic systems [4]. The Brazilian frog *Phyllomedusa distincta* secretes a plethora of antimicrobial peptides including the DD K, DD L and DD M dermaseptins. Among these peptides, DD K is the most active against both Gram-positive and Gram-negative bacteria and it is also interesting as a prototype antimicrobial drug since it has low hemolytic activity. DD K is a peptide of 33 amino acid residues with the following sequence: H-GLWSKIKAAG-KEAAKAAGKAALNAVSEAV-NH_2 [4]. Considering also that dermaseptins are anti-Trypanosome molecules [8], it is relevant to obtain new information on the mechanisms of action of these antimicrobial peptides. Details of the mode of dermaseptins association with membranes are unknown. Generally, pore formation by the barrel-stave model occurs at low peptide to phospholipid ratio while its action by the carpet model or by detergent effects occurs at higher peptide concentration [5]. Investigations of the peptide-to-phospholipid active range can be useful to provide insights into the antimicrobial peptide mechanisms. Thus, physical-chemical methods, such as conductance measurements, surface plasmon resonance (SPR), atomic force microscopy (AFM), isothermal titration microcalorimetry (ITC) and measurements of leakage of a fluorescence probe can give information that, combined to experiments of solid-state NMR, furnish information about peptide mechanism and orientation in phospholipid bilayers [6,25,35]. Some of these studies provide evidence that dermaseptins can lead to pore formation of defined size, in contradistinction to a detergent-like mechanism [15]. However, more information is necessary to define the mechanisms of action of dermaseptins on membranes.

Abraham et al. [1,2] used ITC to investigate the binding of the cyclic peptide Gramicidin, and its analogue GS14dK4 to liposomes of different phospholipid composition. The effect of cholesterol as a membrane component was also investigated. The authors showed that Gramicidin interacts with phosphatidylglycerol anionic liposomes with affinity one order of magnitude higher than with phosphatidylcholine (PC) zwiterionic liposomes. Since Gramicidin is a highly hemolytic peptide, it is expected that it can strongly interact with membranes with or without cholesterol. This is in agreement with results of Abraham et al. [1], which showed that the free energy of interaction of Gramicidin with PC liposomes is almost unaffected by the presence of cholesterol. Nevertheless, a good antimicrobial peptide must be selective to bacterial membranes [26] and thus its action has to be affected by cholesterol.

Concerning the application of antimicrobial peptides as drug prototypes, peptides with low hemolytic activity such as DD K deserve to be more fully investigated. These molecules are usually highly cationic and amphipathic [25]. Nevertheless, it has been described that there is an upper limit for the number of positive charges in antimicrobial peptides, since molecules containing more than seven-charged residues may become able to cross lipid bilayers without disrupting those membranes [18,39,40]. Thus, these polyarginine peptides are more adequate as vectors for carrying molecular or nanoparticle moieties instead of being antimicrobial. The differentiation between antimicrobial and vector peptide is not always straightforward, for instance Buforin is an example of a histone-like cell-penetrating peptide with antimicrobial activity [28].

It is amply known that cationic antimicrobial peptide interact more strongly with liposomes containing a high amount of anionic phospholipids than with mimetic membranes essentially containing zwitterionic phospholipids such as PC [6]. Nevertheless, antimicrobial peptides are usually still capable of interacting to and of disrupting liposomes constituted mainly of zwitterionic phospholipids. Thus, the effect of cholesterol upon the action of antimicrobial peptides with model membranes is relevant for understanding its interaction with bacterial membranes and for predicting its side effect upon mammalian cells [16]. In this study, the main focus is to investigate the effect of cholesterol upon the interaction of the dermaseptin, DD K, with egg lecithin liposomes. Isothermal titration microcalorimetry (ITC), dynamic light scattering (DLS), leakage of 5(6)-carboxyfluorescein (CF) from liposomes and intrinsic fluorescence of tryptophan residue were employed as physical–chemical methods for the investigation.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine, PC, was prepared as described before [37]. Cholesterol was purchased from Sigma–Aldrich and used after recrystallization from ethanol. 5(6)-Carboxyfluorescein, CF, (Sigma–Aldrich), was transformed in its sodium salt and purified as described in the literature [42].

2.2. Peptide synthesis, purification and characterization

DD K, with amidated C-terminus, was synthesized by the solid-phase approach on a Rink amide resin (0.61 mmol g^{-1}) by using the F_{moc}/*t*-butyl strategy [10]. Couplings were performed with *N*,*N*′-diisopropylcarbodiimide/1-hydroxybenzotriazole in *N*,*N*-dimethylformamide (DIC/HOBt) for 60–120 min. Cleavage and final deprotection were conducted

with trifluoroacetic acid (TFA): diisopropylsilane:ethanedithiol (90:5:5, v:v:v) for 90 min at room temperature. Peptide was precipitated with isopropyl ether, extracted with water and lyophilized. Purification was conducted by reversed-phase on a Vydac C18 column (220 mm × 10 mm) with 3 mL min^{-1} flow rate and detected at 220 nm. The elution occurred by using the following solutions: 0.1% (v/v) TFA aqueous solution (A) and 0.08% (v/v) TFA in acetonitrile, ACN (B). Percentage of B varied from 0 to 40 during 40 min. Fractions contained the main product were pooled, lyophilized and the purity and identity of DD K was confirmed by ESI-MS (Q-TOF, Micromass).

2.3. Entrapment of CF in liposomes (large unilamellar vesicles, LUVs)

Liposomes prepared only with PC will be named as PC-LUVs and those containing cholesterol as LUVs-Chol. LUVs were prepared as follows: a dichloromethane solution of PC, with or without cholesterol, was added to a tube and the solvent was evaporated with a nitrogen stream, which furnished a thin film. The film was maintained under vacuum for at least 1 h to remove the residual solvent and then hydrated with a buffer containing 10 mmol L^{-1} Tris–HCl, pH 8.0 and 50 mmol L^{-1} CF. The LUVs were prepared by submitting the suspension to extrusion (11 times) through two 100 nm polycarbonate membranes in a LiposoFast extrusion system (Avestin Inc., Ottawa, Canada). Free CF was removed by passing 1 mL of the extruded LUVs in a Sephadex-G25 column (1.2 cm × 20 cm) eluted with 10 mmol L^{-1} Tris–HCl, pH 8.0 containing 300 mmol L^{-1} NaCl. The LUVs were collected at the Vo. The PC content of the eluted LUVs was determined by phosphorous assay as described in literature [33,38]. The cholesterol percentage, % cholesterol, was calculated on molar basis.

2.4. Dye leakage measurements

CF in high concentration solutions as that used in these experiments is self-quenched. When CF contained in the internal liposome compartment is released to the external media by peptide or surfactant addition, the fluorescence of the diluted CF increases. Aliquots of LUVs (5 μL) were added to a fluorescence cuvette containing 3 mL of the same buffer used for the Sephadex-G25 column elution. The increase of CF fluorescence as a function of time at 25 °C was recorded continuously in a Hitachi F-2000 Fluorescence Spectrophotometer (λ_{ex} = 490 nm and λ_{em} = 512 nm). At the end of each experiment, total CF fluorescence was determined by the addition of 25 μL of 10% (w/v) Triton X-100. The percentage of CF leakage, % leakage, was determined by using Eq. (1), as previously described in literature [3]:

$$\text{Leakage}\,(\%) = \frac{I_{(t)} - I_0}{I_T - I_0} \times 100 \quad (1)$$

where $I_{(t)}$ is the fluorescence intensity on time (t), I_0 is the fluorescence intensity before peptide addition and I_T is the fluorescence intensity after Triton X-100 addition.

2.5. Observed rate constants calculations

The observed rate constant of CF release, k_{obs}, was calculated by using a single-exponential fitting of the recorded data of fluorescence intensity as a function of time.

2.6. Dynamic light scattering

A Malvern Zetasizer nano ZS particle analyzer using polyethylene square cells was used to measure the average hydrodynamic diameter of pure and peptide-bound LUVs. The solutions were subjected to scattering by a monochromatic light (10 mW He–Ne laser, wavelength 632.4 nm) and the scattered light intensity was measured at an angle of 90°. The hydrodynamic diameters are the average of five independent measurements, each one obtained as the mean of 20 counts. A solution of 1.0 mmol L^{-1} PC-LUVs in 10 mmol L^{-1} Tris–HCl buffer, pH 8.0, containing 200 mmol L^{-1} NaCl, was titrated with 1 mmol L^{-1} DD K prepared in the same buffer. The same experiment was also performed with 1.0 mmol L^{-1} LUVs containing cholesterol (at 12 mol%, 21 mol% and 43 mol%), LUVs-Chol. Titrations were conducted by addition of 50 aliquots (10 μL each) of the titrating solution. Before DLS titrations, the solutions were filtered through 0.45 μm millipore filters to eliminate any particulate material.

2.7. Intrinsic tryptophan fluorescence

The fluorescence spectra of DD K were recorded from 290 to 500 nm, with excitation at 280 nm, at 298.15 K, in a Hitachi F-2000 Spectrofluorometer, using a quartz cuvette (1 mL). Different volumes (from 1.5 to 4.5 μL) of the LUVs (10 mmol L^{-1} 1 Tris–HCl buffer, pH 8.0, 300 mmol L^{-1} NaCl) were added to

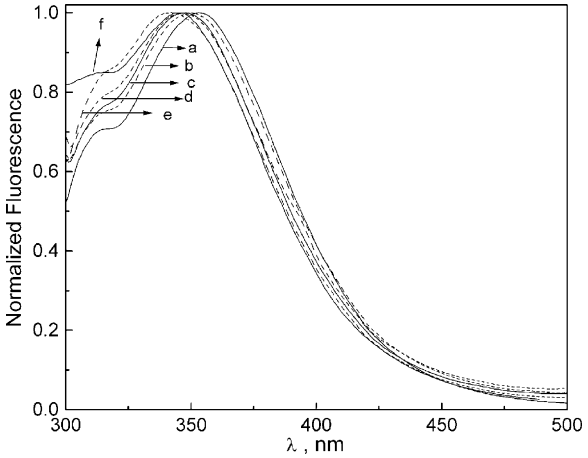

Fig. 1 – Effect of LUVs on the intrinsic tryptophan fluorescence emission spectra of DD K. λ_{ex} = 290 nm, DD K = 6.9 × 10^{-6} mol/L. LUVs were prepared in 10 mmol L^{-1} Tris–HCl buffer, pH 8.0 containing 300 mmol L^{-1} NaCl. LUVs were added to a 1 mL of DD K solution (6.9 μmol L^{-1}) prepared in the same buffer, under stirring. (a) Aqueous media; (b) PC-LUV; (c) 12 mol% cholesterol; (d) 21 mol% cholesterol; (e) 29 mol% cholesterol; (f) 43 mol% cholesterol. [PC] used in each spectrum are shown in Table 1.

Table 1 – Effect of [PC-LUVs] and [LUVs-Chol] on DD K[a] emission λ_{max}, [DD K] = 6.9×10^{-6} mol L^{-1}

0 mol% Cholesterol		12 mol% Cholesterol		27 mol% Cholesterol		29 mol% cholesterol		43 mol% cholesterol	
[PC] (mol L^{-1})	λ_{max} (nm)	[PC] (mol L^{-1})	λ_{max} (nm)	[PC] (mol L^{-1})	λ_{max} (nm)	[PC] (mol L^{-1})	λ_{max} (nm)	[PC] (mol L^{-1})	λ_{max} (nm)
0	353	–	–	–	–	–	–	–	
4.5×10^{-6}	347	2.8×10^{-6}	351	2.5×10^{-6}	350	1.6×10^{-6}	351	0.96×10^{-6}	350
8.9×10^{-6}	342	5.7×10^{-6}	348	5.1×10^{-6}	350	3.3×10^{-6}	351	1.92×10^{-6}	350
1.3×10^{-5}	342	8.5×10^{-6}	348	7.6×10^{-6}	346	4.9×10^{-6}	350	2.88×10^{-6}	350

1 mL of DD K solution (6.9 μmol L^{-1}) prepared in the same buffer, under stirring. Experiments were conducted with LUVs devoid of cholesterol and with LUVs prepared with 12%, 21%, 29% and 43 mol% cholesterol.

2.8. *Isothermal titration calorimetry*

Isothermal titration calorimetric (ITC) was carried out with one repetition with a VP-ITC microcalorimeter (Microcal LCC, Northampton, MA). Previously, the ITC instrument was electrically and chemically calibrated accordingly to MicroCal manual operation [41]. All the solutions employed in the experiment were previously degasified under vacuum (140 mbar) during 8 min. Each titration experiment consisted of 51 successive injections (a first 1 μL injection was discarded to eliminate diffusion effects of material from the syringe to the calorimeter cell and was followed by fifty injections of 5 μL) of 20 mmol L^{-1} LUVs suspended in 10 mmol L^{-1} Tris–HCl, pH 8.0 containing 300 mmol L^{-1} NaCl, at 298.15 K with 1.448 mL of 0.1 mmol L^{-1} DD K (prepared in the same buffer) into the reaction cell. The injection time was 2 s and the interval between the injections was 240 s. For the titration with LUVs containing cholesterol, LUVs-Chol, at 43 mol%, it was used a 2 mmol L^{-1} DD K solution. The heat of dilution was determined in control experiments by injecting the corresponding vesicle suspension or peptide solution into the buffer solution. The heats of dilution were subtracted from the heats determined in the corresponding peptide-to-lipid binding experiments. The overall binding enthalpy and the binding isotherm were obtained from these peptide-to-lipid binding experiments by using standard procedures as described by Wieprecht and Seelig [43]. In this study, since there is no direct experimental evidence to establish the transmembrane distribution of peptide molecules, it was used the total lipid concentration [33] in the estimation of the degree of binding and the determination of the thermodynamic binding parameters [1,2]. The raw data were analyzed by the software Microcal Origin 5.0 for ITC, supplied with the microcalorimeter.

3. Results

Previous studies showed, by various physical–chemical techniques, the effect of phospholipid variation upon the interaction of DD K and other dermaseptins with biomimetic membranes. Nevertheless, the effect of cholesterol was not studied [36]. Thus, in the present work the effect of cholesterol content on DD K binding to LUVs was investigated by intrinsic fluorescence of Trp residue, measurements of kinetics of CF

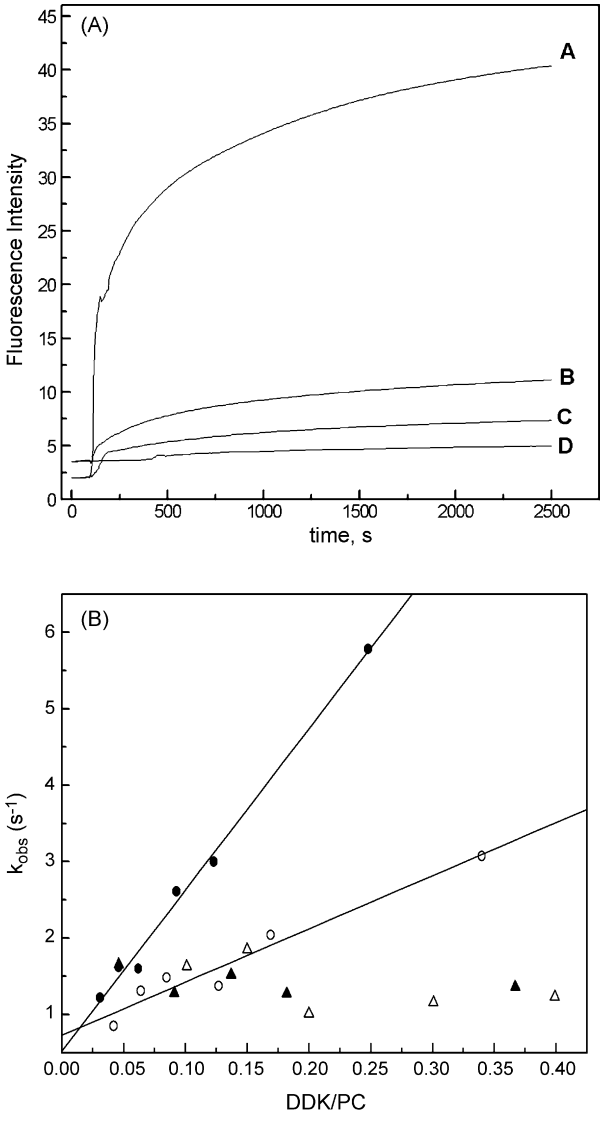

Fig. 2 – Kinetics of CF release from LUVs. (A) A-PC-LUVs, B-LUVs-Chol 12 mol%, C-LUVs-Chol 29 mol%, D-LUVs-Chol 43 mol%. (B) Observed rate constants, k_{obs}, of CF release vs. DD K/PC ratio: (●) PC-LUVs; (○) LUVs-Chol: 12 mol%; (▲) 29 mol%; (△) 43 mol%. LUVs (5 μL), in 10 mmol L^{-1} Tris–HCl buffer, pH 8.0 containing 300 mmol L^{-1} NaCl, were added to a fluorescence cuvette containing 3 mL of the same buffer. The fluorescence of released CF as a function of time at 25 °C was recorded in a Hitachi F-2000 Fluorescence Spectrophotometer (λ_{ex} = 490 nm and λ_{em} = 512 nm). At the end of each experiment, total CF fluorescence was determined by the addition of 25 μL of 10% (w/v) Triton X-100.

Table 2 – Effect of [DD K] on k_{obs} for the leakage of CF from PC-LUVs and LUVs-Chol

[DD K] (μmol L^{-1})	0 mol% cholesterol[a]		12 mol% cholesterol[b]		21 mol% cholesterol[c]		29 mol% cholesterol[d]		43 mol% cholesterol[e]	
	[DD K]/[PC]	k_{obs} (s^{-1})	[DD K]/[PC]	k_{obs} (s^{-1})	[DD K]/[PC]	k_{obs} (s^{-1})	[DD K]/[PC]	k_{obs} (s^{-1})	[DD K]/[PC]	k_{obs} (s^{-1})
0.30	0.031	1.22×10^{-3}	0.042	8.50×10^{-4}	0.101	1.63×10^{-3}	0.046	1.65×10^{-3}	0.050	0
0.45	0.046	1.62×10^{-3}	0.064	1.31×10^{-3}	0.150	1.85×10^{-3}	0.069	3.50×10^{-4}	0.075	0
0.60	0.062	1.60×10^{-3}	0.085	1.48×10^{-3}	0.200	1.01×10^{-3}	0.091	1.28×10^{-3}	0.100	0
0.90	0.093	2.61×10^{-3}	0.127	1.37×10^{-3}	0.301	1.16×10^{-3}	0.137	1.52×10^{-3}	0.150	0
1.19	0.123	3.00×10^{-3}	0.169	2.04×10^{-3}	0.399	1.23×10^{-3}	0.182	1.27×10^{-3}	0.198	0
2.38	0.248	5.78×10^{-3}	0.340	3.07×10^{-3}	0.804	1.75×10^{-3}	0.367	1.36×10^{-3}	0.396	0

[a] [PC] = 9.7×10^{-6} mol L^{-1}.
[b] [PC] = 7.1×10^{-6} mol L^{-1}.
[c] [PC] = 3.0×10^{-6} mol L^{-1}.
[d] [PC] = 6.5×10^{-6} mol L^{-1}.
[e] [PC] = 6.0×10^{-6} mol L^{-1}.

leakage, DLS and ITC. Cholesterol content in the LUVs varied by increments up to 43 mol% so that the larger cholesterol concentration employed was close to the typical for Chol/phospholipids molar ratio (0.84) found in erythrocytes [20].

Peptide synthesis was conducted by the F_{moc} solid-phase strategy with no special difficulties by using DIC/HOBt as coupling additives. Recouplings were conducted for the residues Ile-28, Trp-31, Leu-32, and Gly-33. The peptide was easily purified by RP-HPLC and its identity was confirmed by ESI-MS.

Fig. 1 shows the tryptophan (Trp) fluorescence spectra of DD K, which has a maximum emission at 353 nm in aqueous media. The shoulder visible at lower wavelengths is due to buffer Raman scattering [22]. When DD K is titrated with PC-LUVs, a significant blue spectral shift was observed, from 353 to 342 nm (Table 1). The addition of cholesterol to the LUVs decreases the blue shift of the DD K fluorescence spectra. The Trp maximum emissions, λ_{max}, in the presence of LUVs-Chol containing 12, 21, 29 and 43 mol% cholesterol, are 348, 346, 350 and 350 nm, respectively (Table 1), the last two being close to the value observed in aqueous media. Moreover, the results suggest that 29 mol% of cholesterol is enough to furnish a saturation effect related to the interaction of the DD K with these membranes. Comparison of DD K fluorescence spectra in LUVs-Chol, 43 mol% cholesterol, with PC-LUVs, indicates that the peptide interacts differently with those vesicles. The shift of Trp fluorescence emission spectra can be related to the environment polarity [22]. The spectral blue shift of 11 nm for DD K in the presence of PC-LUVs compared to the spectrum observed in aqueous media indicates that the DD K is partially inserted into the vesicles, which is in agreement with the idea that tryptophan has a preference for the interface regions of the LUVs [44]. Tryptophan insertion in less polar region such as the bilayer interior would give rise to larger blue-shift values. Thus, DD K is able to interact with phosphatidylcholine zwitterionic vesicles and the presence of cholesterol seems to make the peptide insertion in the membrane more difficult or it occurs at a site where tryptophan residue is more exposed to the aqueous media [46].

The rate of CF leakage from the vesicles was calculated by the increase of fluorescence emission as a function of time. CF is self-quenched at high concentrations such as when the molecule is entrapped into liposomes. The data were analyzed as a first-order process and a rate constant, k_{obs}, was calculated for each run. The kinetics of CF release from PC-LUVs and LUVs-Chol with 12, 29 and 43 mol% cholesterol are presented in Fig. 2 and the rate constants are shown in Table 2. The CF leakage from LUVs without peptide addition is negligible during the experimental time. The addition of DD K to PC-LUVs and LUVs-Chol leads to CF release (Fig. 2A). For PC-LUV increments in DD K/PC ratios leads to a linear increase in the rate of CF leakage (k_{obs}), Fig. 2B. In LUVs-Chol, 12 mol%, there is also a linear function of k_{obs} with DD K/PC ratio but the slope for the plot the k_{obs} vs. DD K/PC molar ratio is lower (almost three times) than the value obtained without cholesterol. At 29 mol% cholesterol, no dependence is observed between k_{obs} and DD K/PC molar ratio. For PC-LUVs,

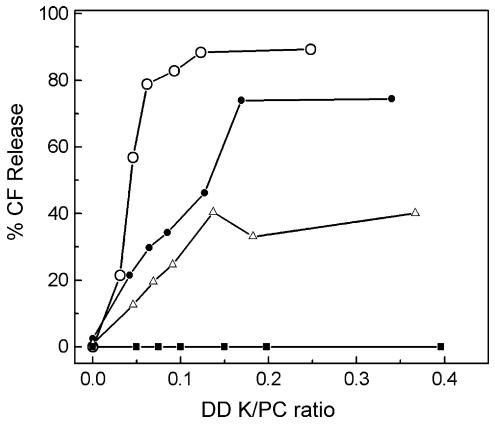

Fig. 3 – Percentage of CF leakage as function of [DD K]. Measurements were done at 2000 s. (○) PC-LUVs, (●) LUVs-Chol 12 mol% and (△) LUVs-Chol 43 mol%. LUVs (5 μL), in 10 mmol L^{-1} Tris–HCl buffer, pH 8.0 containing 300 mmol L^{-1} NaCl, were added to a fluorescence cuvette containing 3 mL of the same buffer. The fluorescence of released CF as a function of time at 25 °C was recorded in a Hitachi F-2000 Fluorescence Spectrophotometer (λ_{ex} = 490 nm and λ_{em} = 512 nm). Total CF fluorescence was determined by the addition of 25 μL of 10% (w/v) Triton X-100.

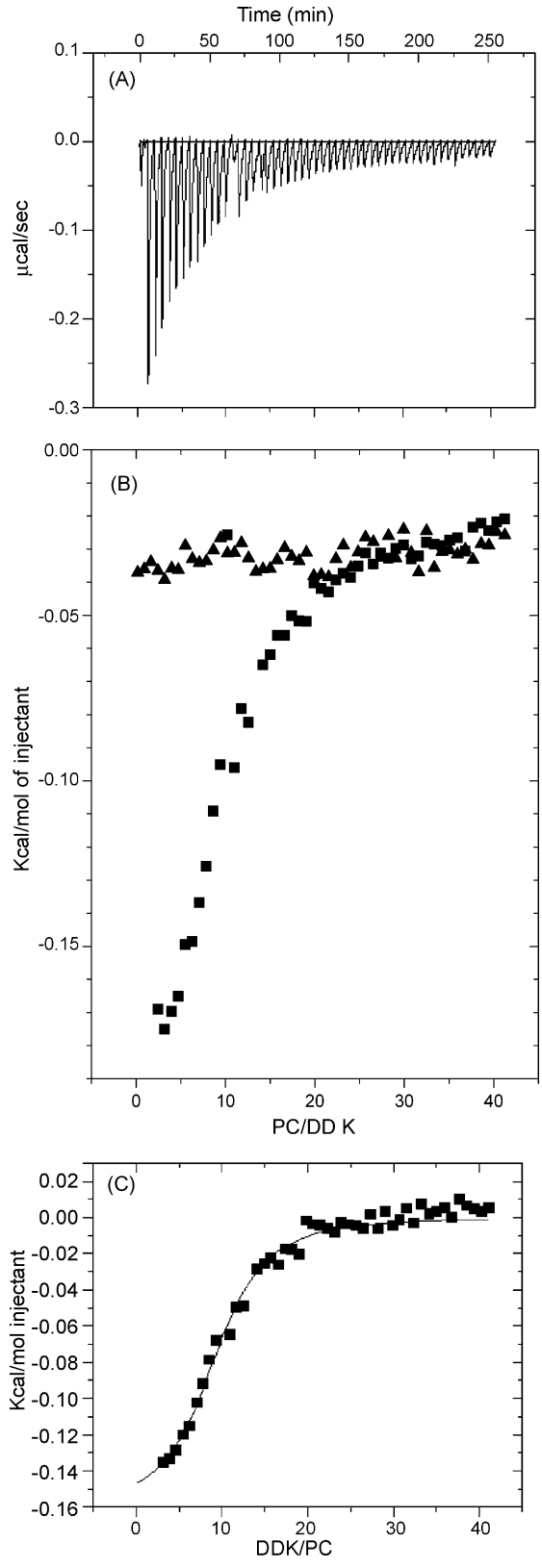

Fig. 4 – Isothermal titration calorimetric of DD K (9.3×10^{-5} mol L^{-1} in 10 mmol L^{-1} Tris–HCl, pH 8.0 containing 300 mmol L^{-1} NaCl) with PC-LUVs (2.0×10^{-4} mol L^{-1} stock solution in 10 mmol L^{-1} Tris–HCl, pH 8.0 containing 300 mmol L^{-1} NaCl). (A) The heat flow for each DD K injection as a function of time (raw data). (B) The binding (■) and dilution heats of PC LUVs (▲). The enthalpy as function of the DD K-to-PC molar ratio. (C) The enthalpy graph as a function of DD K-to-PC molar ratio. Baseline was corrected and dilution heat was subtracted from each experimental point.

at DDK/PC ratio of 0.031, the k_{obs} is 1.22×10^{-3} s^{-1} (Table 2). To obtain the same rate constant with LUVs-Chol, 29 mol% cholesterol, a DD K/PC ratio of 0.367 was necessary. The k_{obs} for LUVs-Chol, 43 mol%, could not be calculated because in the experimental conditions used the kinetics of CF release is too slow (Table 2).

The percentage of CF release (%CF release), after 2000s, was determined by using Eq. (1) (see Section 2) and the values were plotted as a function of DD K/PC molar ratio (Fig. 3). In all cases, an increment of %CF release was observed with an increase in DD K/PC molar ratio, reaching a plateau at higher DD K/PC, except for LUVs containing 43 mol% cholesterol where the percentage of CF release, in all DD K/PC molar ratios, was too small to be quantified (Fig. 3). As can be seen in Fig. 3, the %CF release in LUVs-Chol, at a fixed time, was always smaller than in PC-LUVs.

The interactions between DD K and PC-LUVs or LUVs-Chol were also analyzed by isothermal titration calorimetry (ITC). This technique has been extensively used for the characterization of peptide binding to small molecules, proteins and liposomes [9,43], by supplying all the apparent thermodynamic parameters of the interaction (ΔG, ΔH, the binding constant K and the stoichiometric coefficient, n) in a unique experiment.

To avoid the preparation of very concentrated peptide solution and possible aggregation, titration was always performed with the peptide solution in the calorimeter cell. Fig. 4A presents the raw data for the titration of a 93 µmol L^{-1} solution of DD K with 20 mmol L^{-1} PC-LUVs. Each peak in Fig. 4A corresponds to an addition of 5 µL of PC-LUVs into the cell. In Fig. 4B the enthalpy calculated for each injection of LUVs is plotted vs. PC/DD K ratio as well as the dilution heats of PC-LUVs added to the buffer solution without DD K. In Fig. 4C the heat binding of DD K to LUVs (shown in Fig. 4B) are subtracted from the dilution data and are presented as a function of PC-to-DD K molar ratio. The binding isotherm is shown in Fig. 4C and is calculated by non-linear curve fitting using the model of equivalent binding sites. The calculated thermodynamic parameters (ΔG, ΔH, the apparent binding constant, K_{app}, and the stoichiometric coefficient, n, for the interaction of DDK with PC LUVs without and with cholesterol (43 mol%) are shown in Table 3.

For PC-LUVs, the data show that n is approximately 10, thus the supramolecular structure formed by peptide binding to LUVs has 10 lipids for each peptide molecule (Table 3). This interaction probably occurs by partial peptide incorporation inside the LUVs bilayers, as it has been shown by a blue shift of tryptophan fluorescence (Fig. 1) and by the increase of the vesicle size observed by the DLS experiments (see below). The ITC data also showed that both the enthalpic and the entropic component favor the peptide binding to the PC-LUVs bilayers, although the entropic term is at least 30 times greater than the enthalpic contribution (Table 1).

Table 3 – Thermodynamic parameters for the interaction of DD K with PC-LUVs and LUVs-Chol at 43 mol%

LUVs	n	K_{app} (L mol^{-1})	ΔG (cal mol^{-1})	ΔH (cal mol^{-1})	$T\Delta S$ (cal mol^{-1})
PC-LUVs	9.7 ± 0.3	10,000	−5470	-162.8 ± 7.7	5307
LUVs-Chol	1.36 ± 0.01	2160	−4546	$-1{,}478.0 \pm 28$	3068

A DD K solution (93 μmol L^{-1}) titrated with 20 mmol L^{-1} LUVs-Chol, 43 mol% cholesterol did not show any significant binding and no thermodynamic parameters could be obtained, as shown by the lack of difference between the heats for the binding of DD K to LUVs-Chol and the dilution heats of LUVs-Chol (Fig. 5B). Thus, the binding of the peptide seems to be much weaker to LUVs-Chol than to PC-LUVs. In order to obtain the thermodynamic parameters for the interaction between DD K and bilayers containing cholesterol, it was necessary to use a higher peptide-to-PC molar ratio (2 mmol L^{-1} DD K to 20 mmol L^{-1} PC). The heat change in this binding, after each injection of LUVs to DD K solution, is shown in Fig. 5A. When the peptide concentration in the microcalorimeter cell was increased to 2 mmol L^{-1}, and DD K was titrated with LUVs-Chol containing 43 mol% cholesterol, the binding was observed (Fig. 5A). In Fig. 5B the heats of binding of DD K (at 2 mmol L^{-1}) to LUVs-Chol can be compared to the values obtained for 93 μmol L^{-1} DD K and to the dilution heat for LUVs-Chol. In Fig. 5C the heat involved in DD K/LUVs binding subtracted from the dilution heats are presented. The binding isotherm, determined by non-linear curve fitting, using the model of equivalent binding sites, is presented Fig. 5C. The high concentration of the DD K necessary to achieve the binding to LUVs-Chol suggests that the action of the peptide upon biological membranes containing cholesterol may be difficult in physiological conditions. The thermodynamic parameters calculated from these data are shown in Table 3.

The stoichiometric coefficient (n) determined for the interaction of DD K with LUVs-Chol was $n = 1$ (Table 3), significantly lower when compared to the value obtained for PC-LUVs ($n = 10$). The apparent binding constant (K_{app}) obtained for the association of DD K with LUVs-Chol, 43 mol%, was five times smaller ($K_{app} = 2.2 \times 10^3$ L mol^{-1}) than the value for PC-LUVs ($K_{app} = 1.0 \times 10^4$ L mol^{-1}). Nevertheless, it must be pointed out that the DD K binding to the LUVs-Chol ($\Delta H = -1478$ cal mol^{-1}) is more favored by the enthalpic component than its binding to PC-LUVs ($\Delta H = -162.8$ cal mol^{-1}). The entropic component ($T\Delta S$) for the peptide binding is of the same magnitude order both for LUVs-Chol and for PC-LUVs, and the values are 3068 and 5307 cal mol^{-1}, respectively.

In Fig. 6 the effect of DD K upon the hydrodynamic diameter (D_h) of LUVs is shown. Addition of aliquots of DD K to a LUV solution leads to an increase of D_h until a plateau is reached. For PC-LUVs a significant binding and incorporation of peptide into the bilayer occurs since the vesicles diameter increases from 115 up to 141 nm. Those maximum values are obtained at peptide-to-PC molar ratio of 0.2. Thus the maximal binding of peptide into the bilayers corresponds to one DD K per five phospholipid molecules. When the LUVs-Chol containing 12 or 21 mol% cholesterol, the vesicle D_h increases from 115 to 130 and 122 nm, respectively and more DD K is necessary to reach the plateau. In LUVs-Chol containing 43 mol% cholesterol, the hydrodynamic diameter variation of the LUVs is negligible (Fig. 6), indicating that DD K does not incorporate into the bilayer. This is in agreement with tryptophan fluorescence data that shows that the indole moiety is probably in an aqueous medium when DD K is in the presence of LUVs-Chol containing a high amount of cholesterol.

4. Discussion

Monte Carlo simulation studies of the effect of cholesterol on PC LUVs showed that it causes a reduction of the density of the head group at the interfacial region of the bilayer and an increase in the package of the phospholipid tails in the middle of the bilayer [21]. Thus it is generally expected that the insertion of hydrophobic moiety of peptides will be more favorable in PC-LUVs than in LUVs-Chol. Peptides less effective in the binding to LUVs-Chol could be selected as good candidates for antibacterial or even anti-parasitic drug [26].

All the physical–chemical data obtained in this report clearly indicate that DD K interaction with mimetic PC membranes is affected by cholesterol. Fluorescence spectroscopy and dynamic light scattering experiments showed that insertion of the peptide in the bilayer is significantly reduced by cholesterol. ITC studies showed that cholesterol reduces DD K apparent binding constant and affects its stoichiometry. Finally, the extent of leakage of CF incorporated into PC-LUVs was reduced by cholesterol addition.

The blue shift (11 nm compared to DD K in aqueous media) observed when DD K binds to PC-LUVs (Fig. 1) indicates that Trp, the third residue of DD K, is located in a moderately non-polar medium in the liposome without cholesterol. In the presence of high [PC-LUVs], DD K λ_{max} changes to 342 nm. When the indole moiety of Trp is in a very hydrophobic environment, the emission λ_{max} reaches values up to 330 nm [22]. Thus, one can assume that Trp residue in DD K is located in a region near the interface, in accordance to literature studies [44]. It is important to point out that the 11 nm blue shift was a little larger than the value obtained for DD K binding to phosphatidylethanolamine vesicles [36]. Monte Carlo simulations suggest that the lipid head group and the interface bilayer region are more hydrated in cholesterol rich membranes than in membranes without cholesterol [44]. Thus one could assume that the Trp residue in DD K is still present at the interface region in LUVs-Chol bilayers. Nevertheless, the non-variation of the hydrodynamic radius for LUVs containing 43 mol% cholesterol shows that there is no relevant peptide insertion into the bilayer and probably there occurs a transient binding that do not significantly affect LUVs-Chol structure. Thus, fluorescence spectroscopy and dynamic light scattering data show that DD K insertion in the hydrophobic membrane milieu is greatly affected by cholesterol in concentrations typically found in mammalian cells [20]. Previous studies showed that DD K is able to increase the sizes of partly anionic

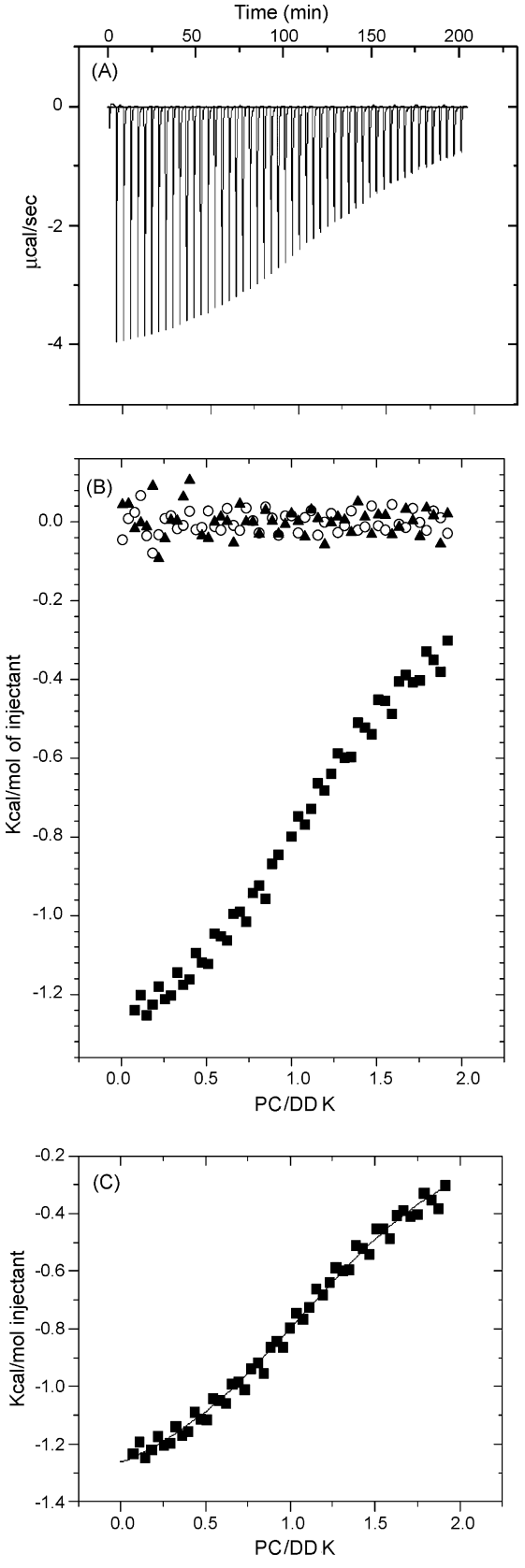

Fig. 5 – (A) The heat flow as a function of the time (raw data) for each DDK injection (at 2.0 mmol L^{-1} in 10 mmol L^{-1} Tris–HCl, pH 8.0 containing 300 mmol L^{-1} NaCl) titrated with LUVs-Chol at 43 mol% (2.0×10^{-4} mol L^{-1} stock solution in 10 mmol L^{-1} Tris–HCl, pH 8.0 containing 300 mmol L^{-1} NaCl). (B) Isothermal titration calorimetric of the peptide DD K at 0.1 mmol L^{-1} (○) or at 2.0 mmol L^{-1} (■) with LUVs-Chol (2.0×10^{-4} mol L^{-1}). Baseline was corrected and the dilution heat (▲) was subtracted from each experimental point. (C) The enthalpy plot as a function of peptide-to-lipid molar ratio for peptide at 2.0 mmol L^{-1}.

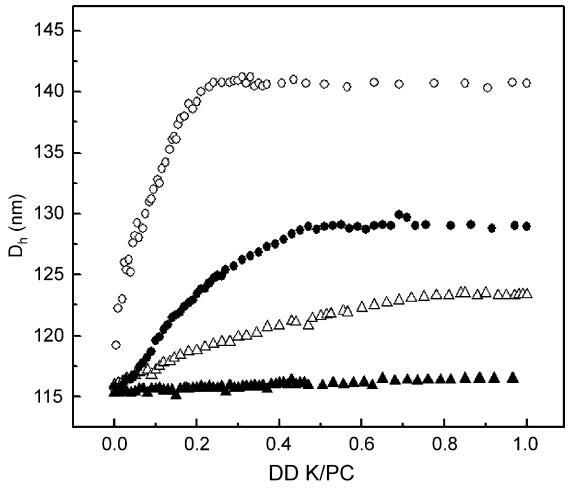

Fig. 6 – Hydrodynamic diameters of LUVs as a function of the [DD K/PC] molar ratio. [DD K] = 1 mmol L^{-1}, in 10 mmol L^{-1} Tris–HCl buffer, pH 8.0 and 200 mmol L^{-1} NaCl, titrated with 1 mmol L^{-1} LUVs prepared in the same buffer. (○) PC-LUVs, (●) LUVs-Chol (12 mol%), (△) LUVs-Chol (21 mol%), (▲) LUVs-Chol (43 mol%).

LUVs from 100 up to 125 nm. Nevertheless, the larger vesicles are not stable since time course studies by atomic force microscopy showed a vesicle disruption after about 1 h [36]. This is in contradistinction to our DLS studies that indicates that DD K is probably able to incorporate into PC-LUVs, which leads to significant increase of vesicle size.

ITC data (Figs. 4 and 5, Table 3) showed that interaction of DD K with PC liposomes is both entropically as enthalpically favored, independently of the presence of cholesterol. The entropic component is of the same magnitude for PC-LUVs or LUVs-Chol but the values are a little smaller in the LUVs-Chol.

Unpublished data from our group showed that DD K is almost an amphipathic helix from residues 6 up to 33. Thus, desolvation of the hydrophobic face and its insertion in the bilayer may be an important contribution to the entropic component, through the gain of translational and rotational entropy of water molecules [34]. Modification of the bilayer fluidity caused by interaction with the peptide may also add to the entropic component. Since the insertion of peptides in the hydrophobic bilayer milieu is more difficult in the presence of cholesterol, it is expected that peptide binding to LUVs containing cholesterol would have a less favorable entropic component. As previously discussed, data obtained from intrinsic Trp fluorescence and from DLS experiments also support this explanation.

The enthalpic component for the binding of DD K to liposomes containing cholesterol is one magnitude order

larger than the enthalpy of DD K binding to PC liposomes. This can be at least partly explained by a higher contribution of electrostatic interactions between the positive charges of DD K and the negative charges of phosphate group of PC. The more hydrated and polar interface of the liposomes containing cholesterol may facilitate the superficial binding of DD K. Nevertheless, since the entropic component is yet predominant, hydrophobic effect caused by liberation of water molecules from the peptide and bilayer surfaces may still be an important phenomenon. It is relevant to point out that a very high amount of peptide (a 2 mmol L^{-1} solution) was necessary to furnish the calorimetric titration data for LUVs-Chol, which indicates that DD K binding to cholesterol-containing membrane would be very weak in physiological conditions. The DD K concentration (93 μmol L^{-1}) employed for the titration with PC-LUVs were also well above the MIC range reported for this peptide [4]. Thus, the absence of negatively charged lipids disfavors DD K binding to membranes but the effect of cholesterol seems to be even more pronounced.

CF leakage (Figs. 2 and 3) together with tryptophan fluorescence data (Fig. 1) indicates a weaker interaction of the antimicrobial peptide with LUVs-Chol. Thus, DD K is capable of strongly interacting with neutral PC-LUVs and the insertion of cholesterol reduces membrane perturbation and CF leakage. Nevertheless, it must be pointed out that even with 43 mol% cholesterol there is still considerable CF leakage promoted by DD K (Figs. 2 and 3). Thus, the low hemolytic activity of DD K may be partly due to the presence of cholesterol in red blood cell membranes but the high amount of protein in these membranes may reduce even more the DD K incorporation.

The effect of DD K over the CF release in PC-LUVs can be analyzed as an equilibrium system where each vesicle can accommodate one to several monomers of the peptide. If the association equilibrium between the LUVs and DD K is rapid with respect to the rate of CF release, the k_{obs} calculated would reflect the intrinsic rate constant, k', of the release step of CF from LUVs and the plot of k_{obs} vs. DD K/PC would be linear. This is what is observed in Fig. 2B for the interaction of DD K with PC-LUVs. However, if the equilibrium of association were slower than k', there would be no linear relationship between k_{obs} and DD K/PC ratio. This behavior is observed in LUVs-Chol, 29 and 43 mol%, respectively. This slower association is attributed to the cholesterol effect on the membrane that would make more difficult the pore formation by DD K. Considering that increments of vesicle size for LUVs-Chol, at 29 and 43 mol%, is small or negligible, pore formation may be transient in these LUVs. The difference between the association of DD K with PC-LUVs and LUVs-Chol can also be seen in the fluorescence spectra, as discussed above.

The DD K structure in the presence of the membrane–mimetic solvent 2,2,2-trifluoroethanol and its orientation upon membrane insertion have been investigated by liquid-phase and by solid-state NMR and will be published elsewhere. Thus, details on this antimicrobial peptide interaction with membranes are being elucidated both at the structural and at the functional level. This study showed that it is important to investigate not only the effect of variation of membrane phospholipid composition but also the consequence upon antimicrobial peptide action of increasing amounts of cholesterol in phospholipid mimetic membranes.

As mammalian cells have a high content of cholesterol and variable protein content [20], DD K can be a prototype for an antimicrobial drug. Nevertheless, changes in its sequence and structure are necessary to improve its selectivity for membranes devoid of cholesterol.

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REFERENCES

[1] Abraham T, Lewis RNAH, Hodges RS, McElhaney RN. Isothermal titration calorimetry studies of the binding of the antimicrobial peptide Gramicidin S to phospholipid bilayer membranes. Biochemistry 2005;44:11279–85.
[2] Abraham T, Lewis RNAH, Hodges RS, McElhaney RN. Isothermal titration calorimetry studies of the binding of a rationally designed analogue of the antimicrobial peptide Gramicidin S to phospholipid bilayer membranes. Biochemistry 2005;44:2103–12.
[3] Alvarez C, Casallanovo F, Shida CS, Nogueira LV, Martinez D, Tejuca M, et al. Binding of sea anemone pore-forming toxins sticholysins I and II to interfaces—modulation of conformation and activity, and lipid–protein interaction. Chem Phys Lipids 2003;122:97–105.
[4] Batista CVF, Rosendo da Silva L, Sebben A, Scaloni A, Ferrara L, Paiva GR, et al. Antimicrobial peptides from the Brazilian frog *Phyllomedusa distincta*. Peptides 1999;20:679–86.
[5] Bechinger B. Structure and function of membrane-lytic peptides. Crit Rev Plant Sci 2004;23:1–22.
[6] Bechinger B, Aisenbrey C, Bertani P. The alignment, structure and dynamics of membrane-associated polypeptides by solid-state NMR spectroscopy. Biochim Biophys Acta 2004;1666:190–204.
[7] Brand GD, Leite JRSA, Mandela SMS, Mesquita DA, Silva LP, Prates MV, et al. Novel dermaseptins from *Phyllomedusa hypochondrialis* (Amphibia). Biochem Biophys Res Commun 2006;343:739–46.
[8] Brand GD, Leite JRSA, Silva LP, Albuquerque S, Prates MV, Azevedo RB, et al. Dermaseptins from *Phyllomedusa oreades* and *Phyllomedusa distincta*: anti-*Trypanosoma cruzi* activity without cytotoxicity to mammalian cells. J Biol Chem 2002;277:49332–40.
[9] Brockhaus M, Ganz P, Huber W, Bohrmann B, Loetscher H-R, Seelig J. Thermodynamic studies on the interaction of antibodies with β-amyloid peptide. J Phys Chem B 2007;111:1238–43.
[10] Chan WC, White PD. F_{moc} solid phase peptide synthesis. In: A practical approach. Oxford: Oxford University Press; 2000.

[11] Charlet M, Chernysh S, Philippe H, Hetru C, Hoffmann JA, Bulet P. Innate immunity. Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. J Biol Chem 1996;271:21808–13.
[12] Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for foods. Nat Rev 2005;3:777–88.
[13] Cytryńska M, Mak P, Zdybicka-Barabas A, Suder P, Jakubowicz T. Purification and characterization of eight peptides from *Galleria mellonella* immune hemolymph. Peptides 2007;28:533–46.
[14] Drider D, Fimland G, Héchard Y, McMullen LM, Prévost H. The continuing story of class IIa bacteriocins. Microbiol Mol Biol Rev 2006;70:564–82.
[15] Duclohier H. Bilayer lipid composition modulates the activity of dermaseptins, polycationic antimicrobial peptides. Eur Biophys J 2006;35:401–9.
[16] Egashira M, Gorbenko G, Tanaka M, Saito H, Molotkovsky J, Nakano M, et al. Cholesterol modulates interaction between an amphipathic class A peptide, Ac-18A-NH2, and phosphatidylcholine bilayers. Biochemistry 2002;41:4165–72.
[17] Fogaça AC, Almeida IC, Eberlin MN, Tanaka AS, Bulet P, Daffre S, et al. a novel antimicrobial peptide from the hemocytes of the cattle tick *Boophilus microplus* with inhibitory activity against serine proteases. Peptides 2006;27:667–74.
[18] Futaki S. Membrane-permeable arginine-rich peptides and the translocation mechanisms. Adv Drug Deliv Rev 2005;57:547–58.
[19] Hancock REW, Sahl H-G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol 2006;24:1551–7.
[20] Jain M, Wagner R. Introduction to biological membranes, 1st ed., USA: John Wiley & Sons; 1980.
[21] Jedlovsky P, Mezei M. Effect of cholesterol on the properties of phospholipid membranes. 1. Structural features. J Phys Chem B 2003;107:5311–21.
[22] Lakowicz JR. Principles of fluorescence spectroscopy. New York: Springer-Verlag; 2006.
[23] Lamberty M, Ades S, Uttenweiler-Joseph S, Brookhart G, Bushey D, Hoffmann JA, et al. Insect immunity. Isolation from the lepidopteran *Heliotis virescens* of a novel insect defensin with potent antifungal activity. J Biol Chem 1999;274:9320–6.
[24] Leite JR, Brand GD, Silva LP, Kückelhaus SA, Bento WR, Araújo AL, Martins GR, Lazzari AM, Bloch Jr C. Dermaseptins from *Phyllomedusa oreades* and *Phyllomedusa distincta*: secondary structure, antimicrobial activity, and mammalian cell toxicity. Comp Biochem Physiol A Mol Integr Physiol; in press. doi:10.1016/j.cbpa.2007.03.01.
[25] Lequin O, Ladram A, Chabbert L, Bruston F, Convert O, Vanhoye D, et al. Dermaseptin S9, an a-helical antimicrobial peptide with a hydrophobic core and cationic termini. Biochemistry 2006;45:468–80.
[26] Martins RM, Sforça ML, Amino R, Juliano MA, Oyama S, Juliano L, et al. Lytic activity and structural differences of amphipathic peptides derived from trialysin. Biochemistry 2006;45:1765–74.
[27] Osaki T, Omotezako M, Nagayama R, Hirata M, Iwanaga S, Kasahara J, et al. Horseshoe crab hemocyte-derived antimicrobial polypeptides tachystatins with sequence similarity to spider neurotoxins. J Biol Chem 1999;274:26172–8.
[28] Park CB, Yi KS, Matsuzaki K, Kim MS, Kim SC. Structure–activity analysis of Buforin II, a histone H2A-derived antimicrobial peptide: the proline hinge is responsible for the cell-penetrating ability of Buforin II. Proc Natl Acad Sci USA 2000;97:3245–50.
[29] Powers JP, Hancock REW. The relationship between peptide structure and antibacterial activity. Peptides 2003;24:1681–91.
[30] Prates MV, Sforça ML, Regis WCB, Leite JRSA, Silva LP, Pertinhez TA, et al. The NMR-derived solution structure of a new cationic antimicrobial peptide from the skin secretion of the anuran *Hyla punctata*. J Biol Chem 2004;279:13018–26.
[31] Pukala TL, Bowie JH, Maselli VM, Musgrave IF, Tyler MJ. Host-defense peptides from the glandular secretions of amphibians: structure and activity. Nat Prod Rep 2006;23:368–93.
[32] Rinaldi AC. Antimicrobial peptides from amphibian skin: an expanding scenario. Curr Opin Chem Biol 2002;6:799–804.
[33] Rouser G, Fleischer S, Yamamoto A. Two-dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 1970;5:494–6.
[34] Seelig J. Thermodynamics of lipid–peptide interactions. Biochim Biophys Acta 2004;1666:40–50.
[35] Shaw JE, Alattia J-R, Verity JE, Privé GG, Yip CM. Mechanisms of antimicrobial peptide action: studies of indolicidin assembly at model membrane interfaces by in situ atomic force microscopy. J Struct Biol 2006;154:42–58.
[36] Silva LP, Leite, JRSA, Brand GD, Regis WB, Tedesco AC, Azevedo RB, Freitas SM, Bloch Jr. C. Dermaseptins from *Phyllomedusa oreades* and *Phyllomedusa distincta*: Liposomes fusion and/or lysis investigated by fluorescence and atomic force microscopy. Comp Biochem Physiol A; in press. doi:10.1016/j.cbpa.2007.02.031.
[37] Singleton WS, Gray MS, Brown ML, White JL. Chromatographically homogeneous lecithin from egg phospholipids. J Am Oil Chem Soc 1965;42:53–8.
[38] Stewart JCM. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal Biochem 1980;104:10–4.
[39] Thorén PEG, Persson D, Karlsson M, Nordén B. The antennapedia peptide penetratin translocates across lipid bilayers the first direct observation. FEBS Lett 2000;482:265–8.
[40] Thorén PEG, Persson D, Lincoln P, Nordén B. Membrane destabilizing properties of cell-penetrating peptides. Biophys Chem 2005;114:169–79.
[41] Turnbull WB, Daranas AH. On the value of c: can low affinity systems be studied by isothermal titration calorimetry? J Am Chem Soc 2003;125:14859–66.
[42] Weinstein JN, Yoshikami S, Henkart P, Blumenthal R, Hagins WA. Liposome–cell interaction: transfer and intracellular release of a trapped fluorescent marker. Science 1977;195:489–92.
[43] Wieprecht T, Seelig J. Isothermal titration calorimetry for studying interactions between peptides and lipid membranes. Curr Top Membr 2002;52:31–56.
[44] Yau WM, Wimley WC, Gawrisch K, White SH. The preference of tryptophan for membrane interfaces. Biochemistry 1998;37:14713–8.
[45] Zasloff M. Antimicrobial peptides of multicellular organisms. Nature 2002;415:389–95.
[46] Zhao H, Kinnunen PKJ. Binding of the antimicrobial peptide temporin L to liposomes assessed by Trp fluorescence. J Biol Chem 2002;277:25170–7.