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# Thermodynamics of the interactions of tryptophan-rich cathelicidin antimicrobial peptides with model and natural membranes

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**Abstract**

Tritrpticin and indolicidin are short 13-residue tryptophan-rich antimicrobial peptides that hold potential as future alternatives for antibiotics. Isothermal titration calorimetry (ITC) has been applied as the main tool in this study to investigate the thermodynamics of the interaction of these two cathelicidin peptides as well as five tritrpticin analogs with large unilamellar vesicles (LUVs), representing model and natural anionic membranes. The anionic LUVs were composed of (a) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine/1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPE/POPG) (7:3) and (b) natural *E. coli* polar lipid extract. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was used to make model zwitterionic membranes. Binding isotherms were obtained to characterize the antimicrobial peptide binding to the LUVs, which then allowed for calculation of the thermodynamic parameters of the interaction. All peptides exhibited substantially stronger binding to anionic POPE/POPG and *E. coli* membrane systems than to the zwitterionic POPC system due to strong electrostatic attractions between the highly positively charged peptides and the negatively charged membrane surface, and results with tritrpticin derivatives further revealed the effects of various amino acid substitutions on membrane binding. No significant improvement was observed upon increasing the Tritrp peptide charge from +4 to +5. Replacement of Arg residues with Lys did not substantially change peptide binding to anionic vesicles but moderately decreased the binding to zwitterionic LUVs. Pro to Ala substitutions in tritrpticin, allowing the peptide to adopt an α-helical structure, resulted in a significant increase of the binding to both anionic and zwitterionic vesicles and therefore reduced the selectivity for bacterial and mammalian membranes. In contrast, substitution of Trp with other aromatic amino acids significantly decreased the peptide's ability to bind to anionic LUVs and essentially eliminated binding to zwitterionic LUVs. The ITC results were consistent with the outcome of fluorescence spectroscopy membrane binding and perturbation studies. Overall, our work showed that a natural *E. coli* polar lipid extract as a bacterial membrane model was advantageous compared to the simpler and more widely used POPE/POPG lipid system.


*Keywords:* Antimicrobial peptide; ITC; Indolicidin; Lipid vesicle; Tritrpticin

## 1. Introduction

Extensive use of conventional antibiotics over the last two decades has resulted in the emergence of new strains of bacteria that are resistant to most, if not all, available antibiotics [1,2]. Antimicrobial peptides have recently gained much attention because of their ability to overcome such resistance and have emerged as a potential new class of antibacterial agents [1,3–6]. Antimicrobial peptides are part of the innate immune system of all living organisms, including vertebrates, insects, bacteria and plants [1,3–5]. They exhibit significant activity against a broad spectrum of microbial organisms [4]. However, in addition to their high antimicrobial potency, these peptides often possess strong

*Abbreviations:* POPC, 1-palmitoyl-2oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2oleoyl-sn-glycero-3-phosphoglycerol; ePC, egg α-phosphatidylcholine; ePE, egg α-phosphatidylethanolamine; ePG, egg α-phosphatidylglycerol; ITC, isothermal titration calorimetry; LUVs, large unilamellar vesicles

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hemolytic and general cytotoxic activity towards eukaryotic cells [3,5,7,8]. Extensive research is underway to elucidate the mechanisms of antimicrobial peptide action in order to develop new analogs, which would preserve the high antimicrobial potency of the natural peptides but would lack the undesired activity towards eukaryotic cells [1–8].

In the present work we have studied the thermodynamics of the interaction of two antimicrobial peptides, indolicidin and tritrpticin (along with several of its analogs), with two model systems representing bacterial and mammalian cellular membranes, as well as with natural *E. coli* membranes utilizing high-sensitivity isothermal titration calorimetry (ITC). ITC can directly measure minute enthalpy changes accompanying the binding of a peptide to a membrane [9]. Moreover, unlike spectroscopic techniques, ITC allows simultaneous measurement of the reaction enthalpy $\Delta H$ and the binding constant $K$, thus providing full thermodynamic characterization of this binding event [9]. ITC has been previously successfully applied to study the interaction of antimicrobial peptides with model lipid systems [9–13]. Here, to our knowledge, we present the first ITC study of antimicrobial peptide interaction with lipids extracted from a natural bacterial source.

Fluorescence spectroscopy was used to complement the ITC results by studying the change in environment of the peptides' tryptophan side chains in different lipid environments as well as to study their membrane-disruptive properties with the anionic membrane systems.

Both indolicidin and tritrpticin consist of 13 residues (Table 1) and belong to the structurally diverse cathelicidin family of antimicrobial peptides, which are synthesized as larger precursor molecules in the bone marrow of mammals [14]. Indolicidin was first isolated from the cytoplasmic granules of bovine neutrophils [15]. Tritrpticin is thought to be released from a cathelicidin found in porcine leukocytes [16]. Both indolicidin and tritrpticin have a broad spectrum of antimicrobial activity [15,16], but they also exhibit relatively high hemolytic activity [17,18]. Both peptides have a high content of Trp (39% in indolicidin and 23% in tritrpticin); as well, they have abundant positively charged Arg/Lys residues (23% and 31%, respectively). It was shown by NMR [19,20] and other spectroscopic techniques [21,22] that tritrpticin adopts a well-defined amphipathic turn–turn secondary structure and indolicidin acquires a more open turn–turn "boat-like" structure in a membrane-mimetic environment (organic solvents or dodecylphosphocholine micelles). Significant progress has been made in understanding the role of each amino acid residue on the activity of indolicidin [23,24], however, less is known in this respect about tritrpticin [21,25,26]. In the present work we have investigated the effect of different amino acid substitutions in tritrpticin (Table 1) on the thermodynamics of the peptide–membrane interactions. Tritrp1 has an amidated C-terminus, which increases the peptide charge from +4 to +5. It is considered that a high positive charge is vital for the antimicrobial action of a peptide because it facilitates the initial binding to the negatively charged surface of bacterial membranes [3,5]. All the other tritrpticin analogs were designed from Tritrp1 by the substitution of various amino acids. Tritrp2 has all its Arg residues replaced by Lys, which preserves the total peptide charge but allows investigating the importance of the superior hydrogen bonding potential of the Arg side chain. In Tritrp3 both helix-breaking Pro residues were replaced by helix-stabilizing Ala residues [17,24,26], which enables this peptide, unlike the others, to adopt a stable α-helical structure in membrane-mimetic environments [21,26]. The presence of an α-helical structure in antimicrobial peptides is generally considered to facilitate their interaction with membranes thus assisting in membrane lysis [3,5]. Tritrp4 and Tritrp6 peptides have their Trp residues replaced by Tyr and Phe, respectively. These changes were introduced since tryptophan residues were suggested to play an important role in the hemolytic activity of tritrpticin [17,26]. Moreover Trp residues can play a unique role in peptide and protein interactions with membranes because of the preference of the indole side chain to insert at the membrane interface [27].

Table 1
Sequences and net charges for the peptides used in the study

| Peptide | Sequence | Net charge |
|---|---|---|
| Indolicidin | ILPWKWPWWPWRR-$NH_2$ | +4 |
| Tritrpticin | VRRFPWWWPFLRR-$COO^-$ | +4 |
| Tritrp1 | VRRFPWWWPFLRR-**$NH_2$** | +5 |
| Tritrp2 | V**KK**FPWWWPFL**KK-$NH_2$** | +5 |
| Tritrp3 | VRRF**A**WWW**A**FLRR-**$NH_2$** | +5 |
| Tritrp4 | VRRFP**YYY**PFLRR-**$NH_2$** | +5 |
| Tritrp6 | VRRFP**FFF**PFLRR-**$NH_2$** | +5 |

The substitutions made in the tritrpticin analogs are indicated in bold.

## 2. Materials and methods

All peptides were purchased at >95% purity from Anaspec (San Jose, CA). All lipids and the lipid extrusion apparatus were purchased from Avanti Polar Lipids (Alabaster, AL). All organic solvents and salts were purchased from Fisher Scientific (Ottawa, ON, Canada). Double distilled water was used in all experiments.

### 2.1. Sample preparation for ITC

A stock solution of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was prepared at a concentration of 10 mg/mL by dissolving the appropriate amount of dry lipids in a chloroform/methanol mixture (2:1) in a glass bottle, whereas 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) and *E. coli* polar lipid extract were received in solution at 10 mg/mL dissolved in chloroform. To closely mimic bacterial membrane composition, POPE/POPG mixtures were prepared at a 7:3 molar ratio [6], respectively, by mixing appropriate volumes of POPE and POPG chloroform stock solutions that were subsequently evaporated under a gentle flow of argon to form a lipid film. Trace amounts of the organic solvents were removed by placing the samples under vacuum overnight. Multilamellar lipid vesicles were formed by hydrating the dry lipid films with appropriate amount of HEPES buffer (10 mM HEPES, 100 mM NaCl, pH 7.0) at room temperature and by vortexing for several minutes until the entire lipid film was removed from the walls of the glass bottle and a homogenous suspension was formed. In order to obtain large unilamellar vesicles (LUVs), the multilamellar vesicle suspension was freeze-thawed 4–5 times in liquid nitrogen and then extruded 21 times through a Mini-Extruder equipped with a polycarbonate membrane filter with 100 nm pore diameter. The lipid concentration of the final LUV suspensions was determined by the Ames phosphate assay [28].

Peptide stock solutions were prepared by dissolving appropriate amounts of dry peptides in HEPES buffer. Exact peptide concentrations were determined by UV absorption at 280 nm using extinction coefficients obtained by the ProtParam tool (http://www.expasy.ch/tools/protparam.html).

### 2.2. ITC measurements

The ITC data were acquired using a Microcal VP-ITC calorimeter (Microcal, Northampton, MA) with a reaction cell volume of 1.415 mL. Directly prior to the measurements peptide solutions were degassed under vacuum for 5 min. The peptide solution (10–50 μM, depending on the peptide affinity to the lipid vesicles) was placed in the calorimeter cell. The LUV suspension (10–14 mM; 500 μL) was placed in the titration syringe and injected in aliquots of 15 μL (the first injection was 3 μL) with 360 s intervals between the individual injections. To account for the heat of dilution, control experiments were completed by titrating lipid vesicles into a buffered solution in the absence of peptide. All experiments were carried out at 37 °C. Data acquisition and analysis were performed using Microcal Origin software (v.7.0). The "One Set of Sites" binding model, provided with the software, was used. Enthalpy changes $\Delta H$ were calculated from the sum of heats of reaction. Changes in free energies and entropies were calculated using the equation:

$$\Delta G = \Delta H - T\Delta S \quad (1)$$

### 2.3. Tryptophan fluorescence measurements

Fluorescence measurements were done with a Varian Cary Eclipse fluorimeter equipped with a Peltier temperature control attachment to keep each measurement at 37 °C. Sample volumes were 2 mL using Tris buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4).

Blue shift and acrylamide quenching experiments were performed as previously described [29]. Tryptophan fluorescence scans for indolicidin, tritrpticin, Tritrp1, Tritrp2 and Tritrp3 were taken with excitation settings at 280 nm with a slit width of 5 nm and emission detection from 300 to 400 nm with a slit width of 5 nm. Data could not be acquired for Tritrp4 and Tritrp6 due to their substituted tryptophan residues. Measurements were made for 1 μM peptide in the following environments: buffer alone, 30 μM egg α-phosphatidylethanolamine:egg α-phosphatidylglycerol (ePE:ePG at 1:1) LUVs, 30 μM *E. coli* polar lipid extract LUVs, and 30 μM egg α-phosphatidylcholine (ePC) LUVs.

Acrylamide quenching titrations were done by incremental additions of 4.0 M stock solution to a final acrylamide concentration of 0.05 M. Stern–Volmer ($K_{sv}$) constants [30] were determined from:

$$F_0/F = 1 + K_{sv}[Q] \quad (2)$$

where $F_0$ is the fluorescence intensity prior to acrylamide addition, $F$ is the fluorescence intensity at a certain concentration of acrylamide, $[Q]$.

### 2.4. Calcein leakage assays

Calcein leakage assays were performed using the peptides and *E. coli* polar lipid extract LUVs as previously described [26]. Briefly, calcein-loaded vesicles were prepared by vortexing the dried lipid films in Tris buffer containing 70 mM calcein. Following standard LUV preparation as previously described, extravesicular calcein was removed by gel filtration using a Sephadex G-50 Superfine column loaded with calcein-free Tris buffer. Calcein-loaded LUVs were detected by light scattering at 400 nm.

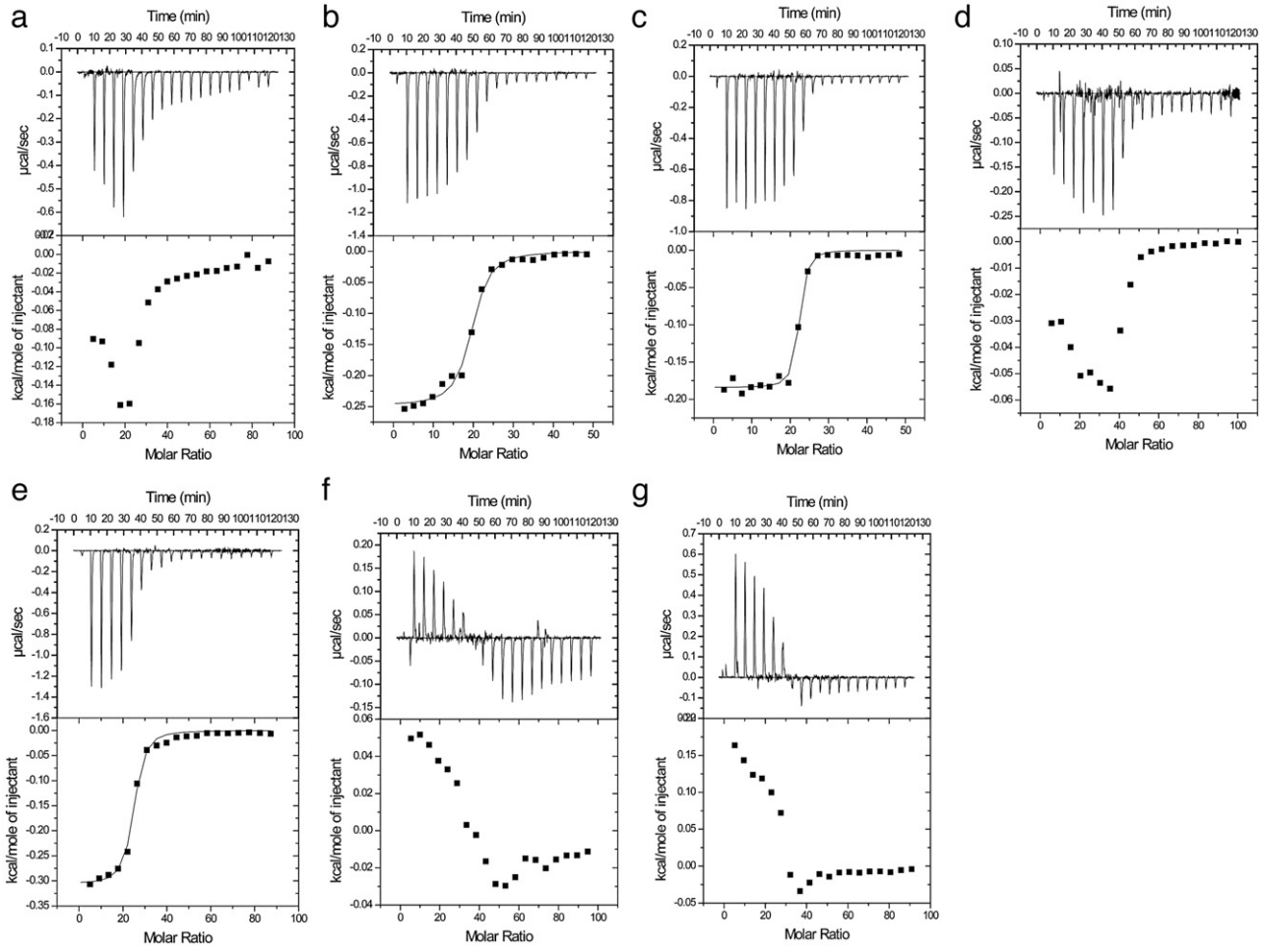


Fig. 1. Titration calorimetry of tryptophan-rich antimicrobial peptides with POPE/POPG (7:3) LUVs at 37 °C: a) indolicidin; b) tritrpticin; c) Tritrp1; d) Tritrp2; e) Tritrp3; f) Tritrp4; g) Tritrp6. The lower curve in each case represents the heat of reaction (measured by peak integration) as a function of the lipid/peptide molar ratio. The solid line is the best fit to the experimental data using the one site binding model.

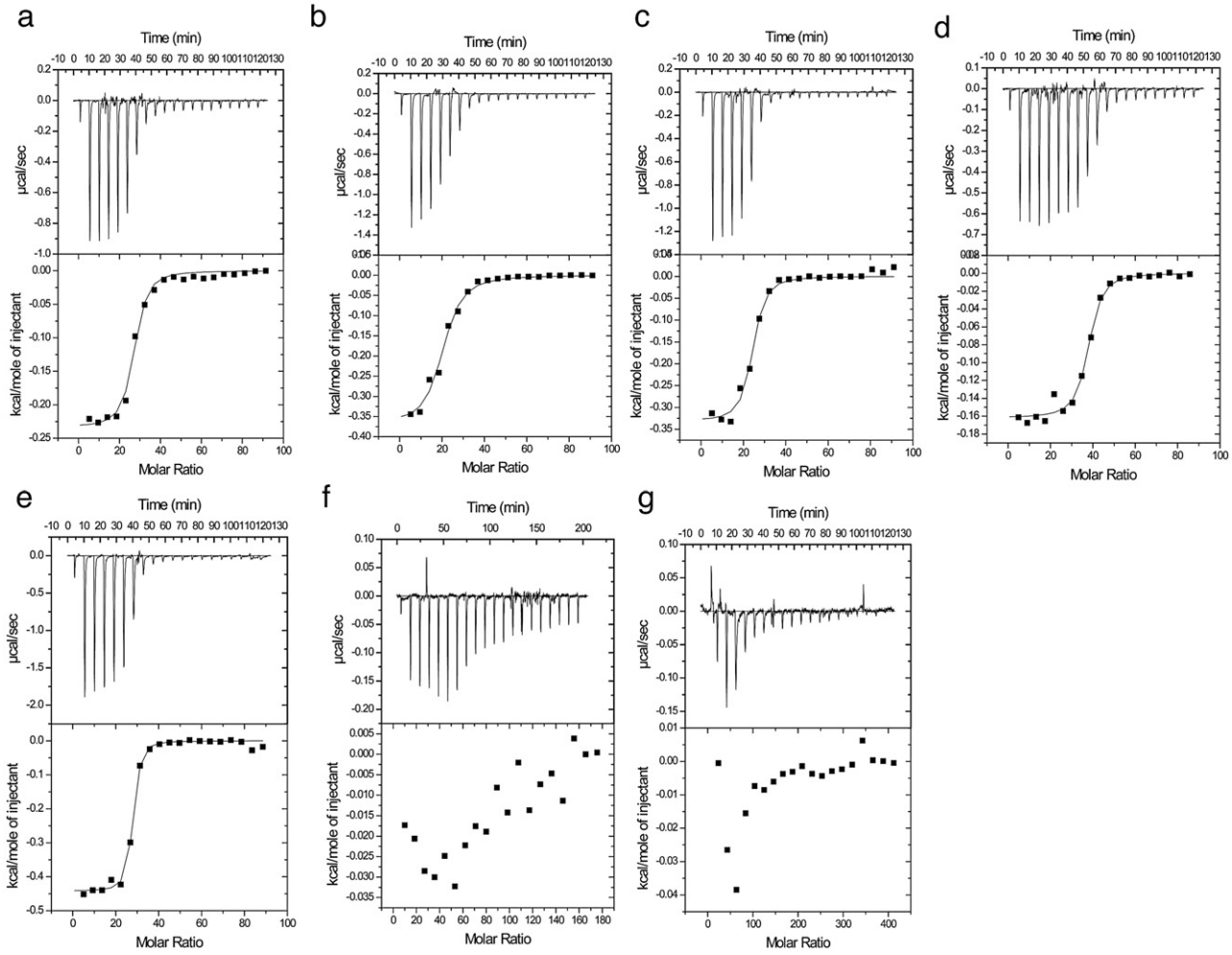


Fig. 2. Titration calorimetry of tryptophan-rich antimicrobial peptides with *E. coli* polar lipid LUVs at 37 °C: a) indolicidin; b) tritrpticin; c) Tritrp1; d) Tritrp2; e) Tritrp3; f) Tritrp4; g) Tritrp6. The lower curve in each case represents the heat of reaction (measured by peak integration) as a function of the lipid/peptide molar ratio. The solid line is the best fit to the experimental data using the one site binding model.

The fluorescence of calcein was recorded over time using an excitation wavelength of 490 nm with a slit width of 5 nm and emission wavelength recorded at 520 nm with a slit width of 5 nm. Calcein-encapsulated LUVs were diluted in Tris buffer to a final phospholipid concentration of 10 μM for the 10:1 lipid:peptide leakage run. After 1 min of baseline acquisition, the peptide was added to a final concentration of 1 μM, whereupon the fluorescence of the self-quenching calcein increased according to the extent of leakage caused by the peptide addition over a period of 10 min. The percent leakage was calculated against 100% leakage conditions being set by the resulting fluorescence after addition of 1% Triton X-100 detergent for full solubilization. For leakage runs with lipid:peptide ratios greater than 10:1, the concentration of calcein-encapsulated LUVs was kept at 10 μM and calcein-free LUVs were added as required. Each leakage assay was performed in triplicate.

## 3. Results

### 3.1. Peptide binding to anionic membranes

Phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are the major lipid components in many prokaryotic cellular membranes [1]. Therefore, we have chosen a PE/PG lipid mixture (7:3) as a relevant model of a bacterial membrane. We have also used natural *E. coli* polar lipid extract as an even more biologically relevant lipid system to model a bacterial membrane, which is composed of 67% PE, 23% PG, and 10% cardiolipin and contains a mixture of fatty acid tails [31].

Figs. 1 and 2 show ITC traces obtained by titrating either POPE/POPG or *E. coli* LUVs into solutions containing antimicrobial peptides at 37 °C. For most peptides, each injection produced an exothermic heat of reaction which decreased with consecutive injections. However, in some cases more complicated ITC traces were obtained. Indolicidin and Tritrp2 interacting with POPE/POPG displayed an initial increase of the exothermic heat of reaction with the number of injections, which was then followed by a decrease of the heat of reaction (Fig. 1a and d). Similar behaviour was displayed by Tritrp4 and Tritrp6 interacting with *E. coli* polar lipids (Fig. 2f and g). Interactions of Tritrp4 and Tritrp6 with POPE/POPG resulted in an initial endothermic reaction, which was followed by an exothermic reaction after several injections (Fig. 1f and g). Similar complex appearances of ITC traces have been shown for other peptides and proteins interacting with lipid vesicles and were interpreted as the result of other processes occurring along with the binding process. The superimposed reactions produce

Table 2
Thermodynamic parameters for tritrpticin and its derivatives binding to POPE/POPG (7:3) large unilamellar vesicles (LUVs) at 37 °C

| Peptide | $\Delta H$ (kcal/mol) | $\Delta G$ (kcal/mol) | $\Delta S$ (cal mol$^{-1}$ k$^{-1}$) | $T\Delta S$ (kcal/mol) | $K$ (mol$^{-1}$) |
|---|---|---|---|---|---|
| Tritrpticin | −4.67 | −8.73 | 13.1 | 4.06 | $1.44 \times 10^6$ |
| Tritrp1 | −3.92 | −10.0 | 19.6 | 6.08 | $1.08 \times 10^7$ |
| Tritrp3 | −7.12 | −9.23 | 6.81 | 2.11 | $3.19 \times 10^6$ |

different heats that cannot be resolved by the instrument, which can only detect the overall heat changes [10,32]. The nature of these other processes may vary and was tentatively assigned to pore formation [32], to changes in the lipid phase properties during the titration [10] or to initial peptide aggregation. Regardless of the nature of the second reaction, its superposition with the binding reaction makes it impossible to derive binding parameters for indolicidin and Tritrp2 interacting with POPE/POPG and for Tritrp4 and Tritrp6 interacting with both lipid systems. Thus, only qualitative conclusions can be made in these cases. The initial injections for indolicidin binding to POPE/POPG membrane show large heat values, while binding of Tritrp4 is associated with smaller enthalpy changes (Fig. 1). The poorly saturable binding process under our experimental conditions points to a relatively low affinity of Tritrp4 and Tritrp6 for *E. coli* polar lipids (compare molar ratio scales for these two peptides with the other peptides in Fig. 2).

The shape of the ITC traces for all peptides bound to POPE/POPG and *E. coli* polar lipids is generally similar and suggests a common mechanism of interaction with both anionic lipid systems for all peptides, with the exception of Tritrp4 and Tritrp6.

Thermodynamic parameters for the peptides displaying only a single binding reaction are summarized in Table 2 for POPE/POPG and in Table 3 for the *E. coli* polar lipids. The binding of tritrpticin and Tritrp1 to POPE/POPG membrane is characterized by comparable contributions from the enthalpy ($\Delta H$) and entropy ($T\Delta S$) components to the free energy change that accompanies the binding process (Table 2). In contrast, Tritrp3 binding to POPE/POPG vesicles is mostly an enthalpy-driven process ($\Delta H$ is −7.12 kcal/mol while $T\Delta S$ is only 2.11 kcal/mol). The binding of all three peptides to POPE/POPG membranes is entropically favourable, which is characterized by a positive entropy change, albeit less so for Tritrp3 when compared to tritrpticin and Tritrp1. All three peptides displayed a large negative change in free energy (~9–10 kcal/mol) and large binding constants ($10^6$–$10^7$) indicating strong binding and high affinity for POPE/POPG vesicles.

The binding of the peptides shown in Table 3 to LUVs made from *E. coli* polar lipids, unlike binding to POPE/POPG, is strongly enthalpy-driven, as seen from the considerably larger values of $\Delta H$ compared to $T\Delta S$. Only indolicidin and Tritrp2 showed significant entropy contributions to the free energy change. The reduced heat of the binding for these two peptides was compensated by more favourable entropic interactions and resulted in similar overall free energy values. The large negative values of $\Delta G$ and the large binding constants suggest strong binding of all peptides to *E. coli* polar lipid vesicles. The strongest binding and highest affinity for this matrix are shown by Tritrp3, as indicated by the largest negative $\Delta G$ and highest binding constant. Interestingly, this is also the only peptide that displayed a negative value of $T\Delta S$. This unfavourable entropy change is most likely due to the conformational change of the Tritrp3 peptide from an unordered structure in aqueous solution to a highly ordered α-helix upon interaction with the membrane. Nevertheless, although the entropy component is counteracting the enthalpy component of $\Delta G$ for Tritrp3, the overall free energy change is still large due to the compensating heat of reaction. The other peptides exhibited entropically favourable binding processes which contribute to the free energy change.

### 3.2. Peptide binding to a zwitterionic membrane

Since the zwitterionic phosphatidylcholines (PCs) are among the most abundant phospholipid classes in eukaryotic cellular membranes [1,33] POPC was chosen as a model to investigate the thermodynamics of antimicrobial peptide interaction with such membranes.

The ITC traces obtained by titrating POPC LUVs into solutions containing antimicrobial peptides at 37 °C are shown in Fig. 3 and the thermodynamic parameters of the binding process are summarized in Table 4. It is obvious that the observed ITC traces for POPC are drastically different compared to peptides binding to POPE/POPG and *E. coli* polar lipids (compare Fig. 3 with Figs. 1 and 2), indicating large differences in the peptide interaction with the zwitterionic membranes compared to anionic systems. For all peptides except Tritrp4 and Tritrp6, each injection produced an exothermic heat of reaction which decreased with consecutive injections. For the latter two peptides, the heat of the exothermic reaction was virtually independent of the injection number, which suggests that it is due to very weak binding of the peptides to the bilayer (Fig. 3f and g) [10]. This made it impossible to obtain reliable thermodynamic parameters for these peptides and therefore they are not included in Table 4. While the exothermic heat of reaction decreased with each consecutive injection for the other peptides, the process reached saturation over the range of experimentally accessible concentrations only for indolicidin and Tritrp3 (Fig. 3a and e), suggesting stronger binding for these peptides and relatively weak binding for tritrpticin, Tritrp1 and Tritrp2.

The binding of all peptides to POPC is enthalpy-driven, indicated by the larger values of $\Delta H$ compared to $T\Delta S$ (Table 4). However, negative values of $T\Delta S$ point out that the interaction

Table 3
Thermodynamic parameters for indolicidin, tritrpticin and its derivatives binding to *E. coli* polar lipid large unilamellar vesicles (LUVs) at 37 °C

| Peptide | $\Delta H$ (kcal/mol) | $\Delta G$ (kcal/mol) | $\Delta S$ (cal mol$^{-1}$ k$^{-1}$) | $T\Delta S$ (kcal/mol) | $K$ (mol$^{-1}$) |
|---|---|---|---|---|---|
| Indolicidin | −5.96 | −9.12 | 10.2 | 3.16 | $2.75 \times 10^6$ |
| Tritrpticin | −7.20 | −8.28 | 3.47 | 1.08 | $6.80 \times 10^5$ |
| Tritrp1 | −7.52 | −9.06 | 4.97 | 1.54 | $2.44 \times 10^6$ |
| Tritrp2 | −5.87 | −9.53 | 11.8 | 3.66 | $5.13 \times 10^6$ |
| Tritrp3 | −11.5 | −10.0 | −4.88 | −1.51 | $1.17 \times 10^7$ |

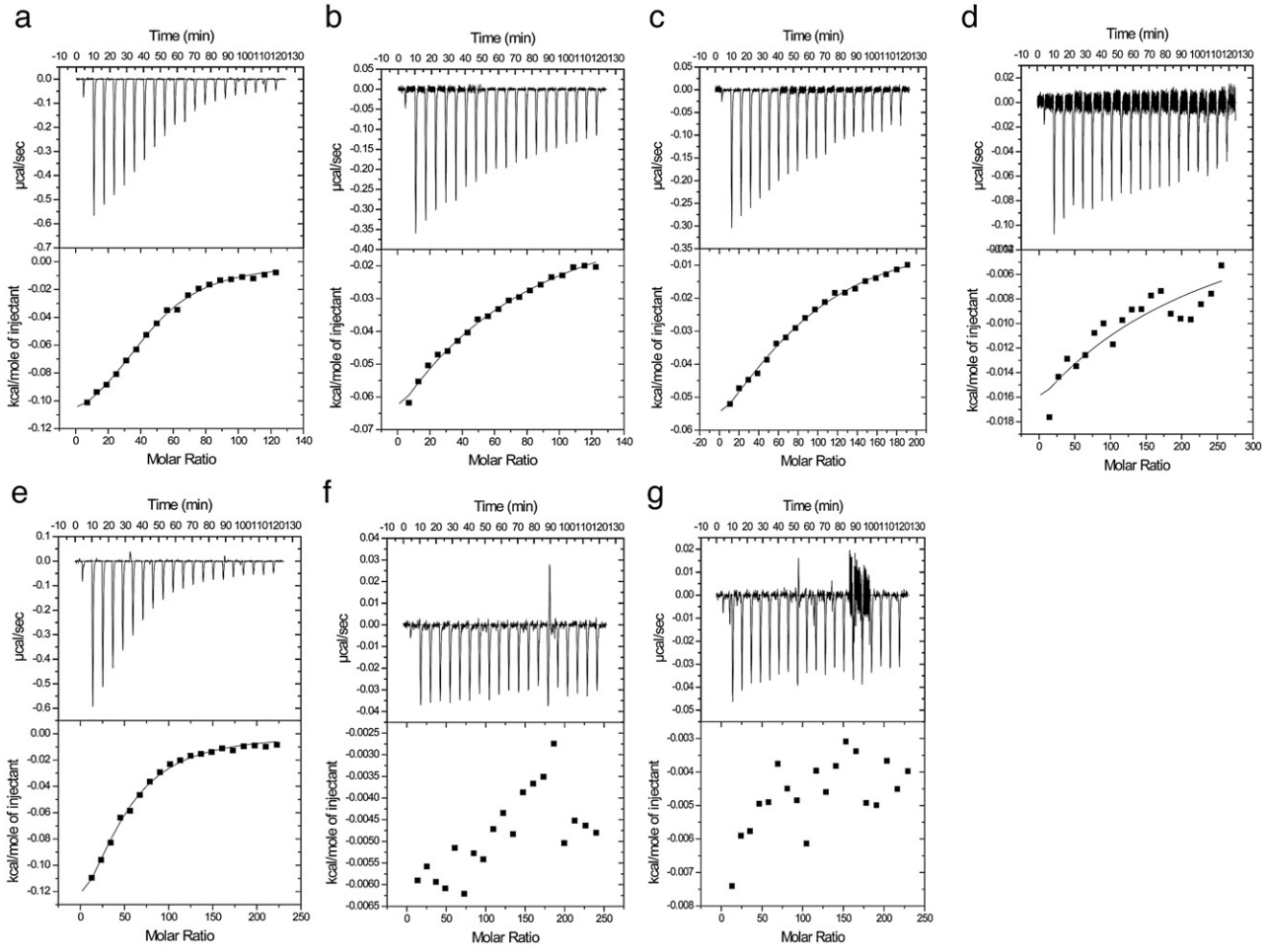


Fig. 3. Titration calorimetry of tryptophan-rich antimicrobial peptides with POPC LUVs at 37 °C: a) indolicidin; b) tritrpticin; c) Tritrp1; d) Tritrp2; e) Tritrp3; f) Tritrp4; g) Tritrp6. The lower curve in each case represents the heat of reaction (measured by peak integration) as a function of the lipid/peptide molar ratio. The solid line is the best fit to the experimental data using the one site binding model.

of all peptides with POPC, with the exception of indolicidin, is entropically unfavourable in contrast to the results obtained with the POPE/POPG and *E. coli* lipid matrices. In spite of similar values of $\Delta H$ for peptide binding to zwitterionic and anionic membranes (compare Tables 2, 3 and 4), due to the compensating enthalpy and entropy components of $\Delta G$, the resulting free energy change of peptide binding to POPC is relatively low compared to the values obtained for the interaction with POPE/POPG and *E. coli* lipids. The binding constants are also fairly low, on the order of $10^3$–$10^4$ for most peptides and only indolicidin and Tritrp3 demonstrate a higher affinity for POPC LUVs with a binding constant on the order of $10^5$.

### 3.3. Binding isotherms for peptide interaction with LUVs

Additional information about peptide binding to lipid vesicles can be obtained from binding isotherms that were calculated using previously described procedures [34]. As we do not have any direct indication that the peptides are penetrating deep into the bilayer, only the lipids in the outer layer of LUVs (50% of the total lipid) were considered [34]. The binding isotherms were calculated only for those peptides which exhibited single and clear binding reactions and for which all thermodynamic parameters could be reliably determined. The isotherms are shown in Fig. 4 and represent the function of the molar ratio of the bound peptide per lipid ($X_b$) versus the equilibrium concentration of the free peptide in solution ($C_f$).

The character of the binding isotherms for anionic POPE/POPG and *E. coli* membranes differs significantly from those for zwitterionic POPC membranes again pointing at different binding characteristics.

The binding isotherms for POPE/POPG and *E. coli* polar lipids exhibit a sharp change in the slope suggesting a complex

Table 4
Thermodynamic parameters for indolicidin, tritrpticin and its derivatives binding to POPC large unilamellar vesicles (LUVs) at 37 °C

| Peptide | $\Delta H$ (kcal/mol) | $\Delta G$ (kcal/mol) | $\Delta S$ (cal mol$^{-1}$ k$^{-1}$) | $T\Delta S$ (kcal/mol) | $K$ (mol$^{-1}$) |
|---|---|---|---|---|---|
| Indolicidin | −5.77 | −7.40 | 5.27 | 1.63 | $1.66\times10^5$ |
| Tritrpticin | −11.0 | −5.77 | −16.7 | −5.18 | $1.19\times10^4$ |
| Tritrp1 | −7.96 | −6.89 | −3.45 | −1.07 | $7.19\times10^4$ |
| Tritrp2 | −11.4 | −5.42 | −19.2 | −5.95 | $6.72\times10^3$ |
| Tritrp3 | −9.28 | −7.13 | −6.95 | −2.16 | $1.05\times10^5$ |

binding process exhibiting negative cooperativity. This means that the peptides bound to a vesicle will lower the vesicle affinity for the remaining free peptides in solution. The sharp slope of the curve also indicates a very high cooperativity for this process.

At low free peptide concentration the amount of bound peptide increases instantly with a slope close to 90°. This indicates that all free peptides at these concentrations are immediately bound to the lipid. It can be suggested that the major driving force for binding at low peptide concentrations is electrostatic attraction of the highly positively charged peptides to the negatively charged membrane surface. However at a certain threshold concentration of free peptide the slope of the binding isotherm sharply changes to values close to 0°. This indicates that increasing the free peptide concentration above this threshold level does not increase the amount of peptides bound to lipids and binding no longer takes place. The most probable reason for this would be saturation of the membrane surface with bound peptides, which would change the membrane surface charge from negative to neutral or even positive due to the high positive charge of the bound peptides [9]. This would result in repulsion of the free peptides in solution from the saturated membrane surface and thus significantly decrease the binding affinity for the remaining free peptides, and eventually limit the binding process. This explanation is confirmed when the binding isotherms for tritrpticin (charge +4) and Tritrp1 (charge +5) to anionic vesicles are compared. In the former case, the second part of the binding isotherm has a small slope, indicating that much weaker binding continues with increase of the free peptide concentration above the threshold level. In the latter case, the slope of the second part of the isotherm is eliminated, showing that no more binding occurs as repulsion between bound and free peptides is stronger. Also, the degree of binding $X_b$ is slightly higher in the former case. The stoichiometry obtained from the ITC traces suggests that membrane saturation with peptides, at which the slope of the binding isotherms drastically changes, occurs at lipid/peptide ratio of about 25/1 for most peptides (Figs. 1–3).

The binding isotherms for zwitterionic POPC are almost linear or show gradual changes in the slope. The linear behaviour of the binding isotherm was also seen for uncharged peptides [34] and indicates that electrostatic interactions do not play an important role in the peptide binding to this membrane. The gradual changes in slope suggest a simple adhesion process in a non-cooperative manner [35]. Therefore, we could assume that binding of all the peptides to the zwitterionic POPC is driven mainly by hydrophobic interactions and not by electrostatics. The relatively small slope of the isotherms and the small fraction of bound peptide are also indicative of the relatively low affinity of these peptides for POPC.

### 3.4. Burial of the tryptophan side chains

As a complement to the ITC results, fluorescence spectroscopy was used to study the environment of the Trp residues in the peptides when bound to membrane bilayers. When the indole

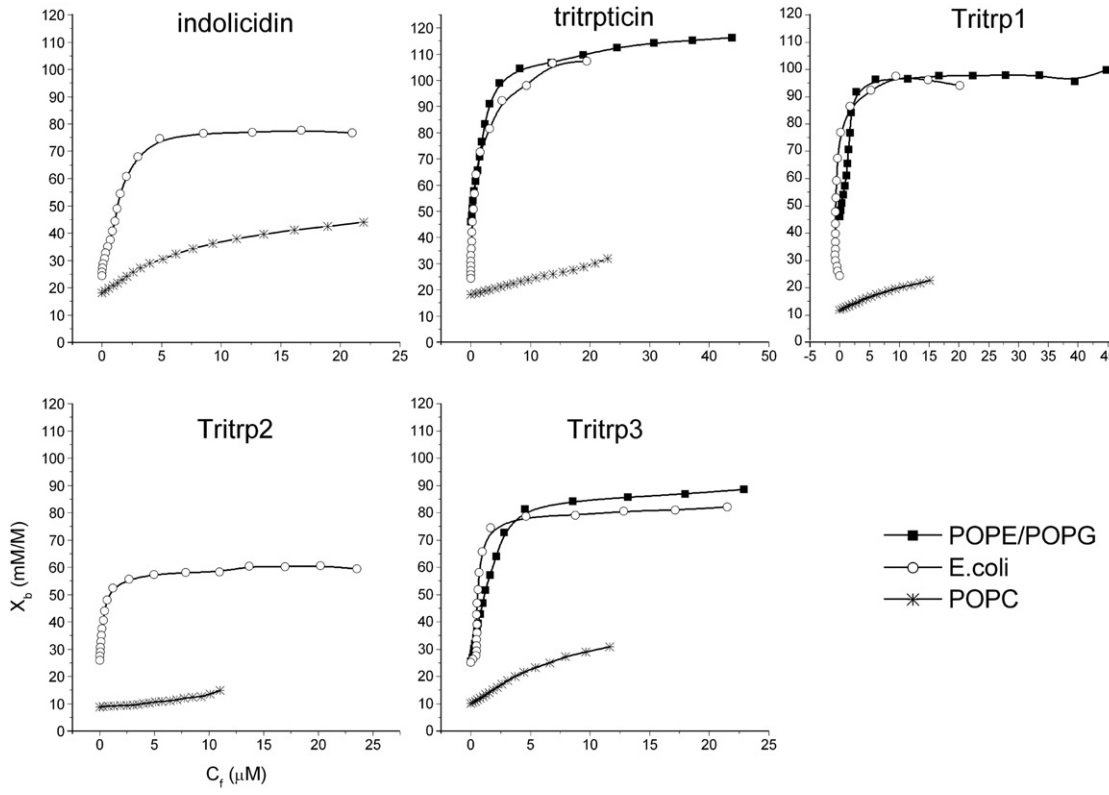


Fig. 4. Binding isotherms for tryptophan-rich antimicrobial peptide interaction with POPE/POPG (7:3) (■), *E. coli* polar lipids (○) and POPC (✳) LUVs at 37 °C. $C_f$ is the equilibrium concentration of the free peptide in solution. $X_b$ is the molar ratio of the bound peptide per lipid.

Table 5
Blue shifts in tryptophan maximum emission wavelengths ($\lambda$, nm) for indolicidin, tritrpticin and its derivatives in the presence of large unilamellar vesicles (LUVs) of different lipid compositions at 37 °C

| Peptide | ePE/ePG (1:1) | *E. coli* polar lipid extract | ePC |
|---|---|---|---|
| Indolicidin | 1 | 3 | 4 |
| Tritrpticin | 10 | 11 | 4 |
| Tritrp1 | 12 | 12 | 5 |
| Tritrp2 | 9 | 10 | 3 |
| Tritrp3 | 9 | 11 | 8 |

side chain of a tryptophan residue moves to a more nonpolar environment as it does when it undergoes a buffer-to-lipid transition, the maximum emission wavelength shifts to lower values. These blue shifts for the Trp-containing peptides in the presence of ePE/ePG (1:1), *E. coli* polar lipid extract, and ePC LUVs are shown in Table 5. The shifts for indolicidin are generally smaller than for tritrpticin and its derivatives. This is probably because the three Trp residues in tritrpticin are surrounded by two Phe residues. The more hydrophobic Phe side chains would help to increase the Trp residues' exposure to the nonpolar lipid tail core of the bilayers. The five Trp residues in indolicidin do not have these hydrophobic amino acid neighbors, and so they would reside more in the interfacial region of the bilayer, where the indole side chain is preferentially located [27]. Comparing tritrpticin, Tritrp1, Tritrp2 and Tritrp3 the shifts are greater with ePE/ePG and *E. coli* polar lipid extract LUVs than with ePC. This generally agrees with the binding constants calculated from the ITC data. Tritrp3 has a significantly greater shift in ePC vesicles than the other peptides, and this is reflected in its larger binding constant with POPC LUVs as well.

The extent of solvent exposure of the tryptophan side chains can also be measured indirectly with titrations of the soluble quencher acrylamide. The Stern–Volmer constants ($K_{sv}$) calculated from these titrations (shown in Table 6) indicate the degree of burial of the Trp residues in the lipid membranes. Overall, the lower $K_{sv}$'s for the peptides with ePE/ePG and *E. coli* polar lipid LUVs compared to ePC LUVs correspond well with the binding constants as determined by ITC. The only exception is the high $K_{sv}$ for Tritrp2 binding to ePE/ePG, for which the thermodynamic parameters could not be calculated from the ITC data.

The $K_{sv}$ for indolicidin in the presence of ePC LUVs is significantly lower than for the other peptides, confirming the tighter affinity calculated from ITC. Though the blue shift data indicate that the Trp side chains of indolicidin are not embedded deep into the nonpolar region of ePE/ePG and *E. coli* polar lipid vesicle membranes, the low amount of acrylamide quenching found in these cases indicates that the Trp residues of the peptide are still well protected from solvent exposure. As well, the higher affinities for both tritrpticin and Tritrp1 binding to POPE/POPG vesicles compared to *E. coli* polar lipid vesicles from the ITC data are reflected in the lower degree of Trp quenching in the presence of ePE/ePG vesicles. Altogether, the acrylamide quenching results show that the burial of the Trp residues provides a reasonable approximation of the binding affinity between these peptides to the vesicles.

Table 6
Stern–Volmer constants ($K_{sv}$, $M^{-1}$) from acrylamide quenching experiments for indolicidin, tritrpticin and its derivatives in the presence of buffer and large unilamellar vesicles (LUVs) of different lipid compositions at 37 °C

| Peptide | Buffer | ePE/ePG (1:1) | *E. coli* polar lipid extract | ePC |
|---|---|---|---|---|
| Indolicidin | 24.0 | 11.2 | 10.1 | 15.6 |
| Tritrpticin | 25.7 | 12.5 | 16.3 | 23.2 |
| Tritrp1 | 22.5 | 9.6 | 16.2 | 21.3 |
| Tritrp2 | 25.1 | 23.5 | 17.7 | 21.9 |
| Tritrp3 | 26.7 | 15.3 | 8.8 | 24.3 |

### 3.5. Intravesicular leakage from anionic membranes

Calcein leakage assays were performed to investigate the membrane-disruptive properties of the peptides on the more biologically relevant *E. coli* polar lipid extract membranes compared to ePE/ePG membranes (Fig. 5). Results for the latter membrane model system have been previously published [26]. For both anionic LUVs, the relative leakage profiles of the peptides agree well with each other. Tritrp3 induces the most leakage, followed closely by Tritrp1 and tritrpticin. The Trp-substituted tritrpticin derivatives, Tritrp4 and Tritrp6, remain low leakage inducing peptides. The major difference in the results

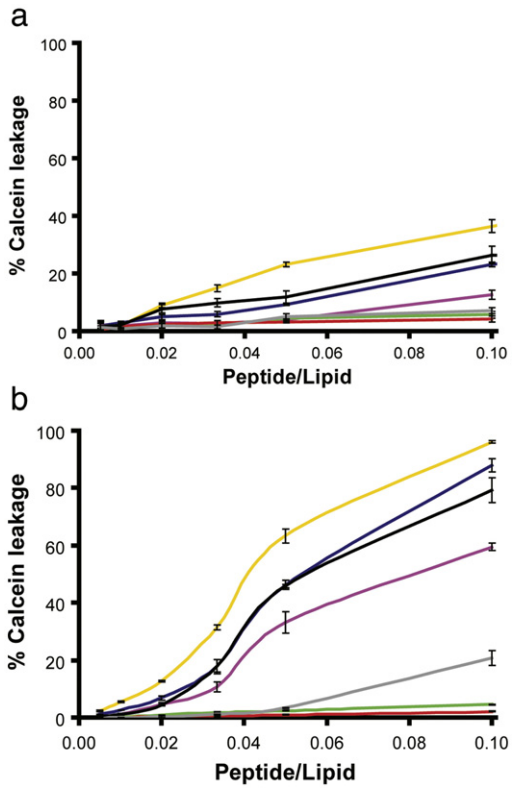


Fig. 5. Calcein leakage assay results caused by the peptides on LUVs composed of *E. coli* polar lipid extract (a). Previously published profiles for LUVs composed of 1:1 ePE:ePG are shown in panel (b) for comparison [26]. Peptides are as follows: tritrpticin (black), indolicidin (grey), Tritrp1 (dark blue), Tritrp2 (pink), Tritrp3 (yellow), Tritrp4 (green), and Tritrp6 (red). Assays were performed in triplicate.

between the *E. coli* polar lipid and ePE/ePG LUVs is that with the former, there is a major decrease in the overall amount of leakage caused by the peptides at the lipid:peptide ratios assayed. This suggests that the complexity of the *E. coli* polar lipid membrane makes it less susceptible to perturbation compared to the model ePE/ePG membrane.

## 4. Discussion

The results obtained in the present work show that all the peptides under study exhibited significantly higher affinity and stronger binding to anionic vesicles as compared to zwitterionic LUVs. Similar results have been reported in ITC studies of other cationic antimicrobial peptides, e.g. puroindolineA [36], combi-1 [37], bovine lactoferricin derived peptides [12], human lysozyme derived peptides [35], PGLa [32], and gramicidin S [11]. A considerable preference in peptide binding to anionic vs zwitterionic membranes most probably originates from the strong electrostatic interaction between the highly positively charged peptides and negatively charged membrane surface of the bacterial matrix [11,32]. Additionally, the fluorescence spectroscopy results suggest the possibility of some penetration into the hydrophobic part of the membrane.

Detailed analysis of the ITC data allows estimating the effect of each amino acid substitution in tritrpticin on the binding parameters. All peptides can be arranged in the following order according to their affinity for anionic LUVs: Tritrp3 > tritrpticin ≈ Tritrp1 > indolicidin ≈ Tritrp2 > Tritrp4 ≈ Tritrp6 and in the following order according to their affinity to zwitterionic LUVs: Tritrp3 ≈ indolicidin > tritrpticin ≈ Tritrp1 > Tritrp2 > Tritrp6 ≈ Tritrp4. These relative affinities were also supported by the Trp side chain fluorescence quenching results as well as conclusions reached by a recent DSC study of the interactions of these peptides with mammalian and bacterial model membranes [38].

The extra charge in Tritrp1 compared to tritrpticin (Table 1) did not substantially change the ITC traces recorded upon interaction with both anionic and zwitterionic LUVs (Figs. 1–3). Therefore, the thermodynamic parameters and the binding isotherms are very similar for both tritrpticin and Tritrp1. Their tryptophan fluorescence and calcein leakage profiles further support the similar binding and effect that the peptides have on the membranes, respectively. This shows that the overall increase of the peptide's positive charge does not improve its binding to anionic membranes and, as can be expected and recently shown with DSC results [38], does not greatly influence its interaction with the zwitterionic membranes. Similar antimicrobial and hemolytic activities for both of these peptides were also reported previously [26]. In fact, a significant increase in peptide charge might even hamper its overall binding capabilities as such peptides would introduce higher positive charge to the membrane at very low peptide/lipid ratios and prevent further peptide binding by surface charge neutralization and subsequent electrostatic repulsion. As discussed above, this trend was indeed noticed when the binding isotherms of tritrpticin and Tritrp1 were compared.

Substitution of Arg residues by Lys in Tritrp2 resulted in similar strength of binding to *E. coli* polar lipids when compared to Tritrp1. However, the binding isotherm shows slightly reduced affinity for *E. coli* lipid LUVs. The amino acid substitution had a more pronounced effect on Tritrp2 binding to zwitterionic vesicles and resulted in a moderate decrease in the peptide affinity for POPC. The stronger effect of the Arg to Lys substitution on binding to zwitterionic LUVs compared to anionic systems points to the importance of hydrophobic interactions and the nature of the Arg residue, e.g. a bulkier side chain group and superior hydrogen bonding potential. These data are in agreement with the DSC results [38] and with biological activity data [26], whereby the antimicrobial activity of Tritrp2 was comparable to Tritrp1 but Tritrp2 exhibited a much lower hemolytic activity.

The substitution of helix-breaking Pro residues by Ala in Tritrp3 enabled the peptide to adopt a stable α-helical structure [21,26]. This structural change is most probably responsible for Tritrp3 being one of the strongest interacting and membrane-disruptive peptides with both anionic and zwitterionic LUVs. In agreement with the present data, the DSC results showed a large distortion of the lipid packing induced by Tritrp3 in both anionic and zwitterionic membranes [38]. Moreover, high antimicrobial and hemolytic activity and low peptide selectivity was also demonstrated by biological activity assays [26].

The substitution of the Trp residues by Tyr in Tritrp4 or Phe in Tritrp6 did not change the peptide conformation drastically [21,26], however it significantly decreased the peptide affinity for anionic LUVs and virtually eliminated their binding to zwitterionic vesicles. This suggests that Trp is vital for the binding of these peptides to membranes because of its preferred positioning at the lipid-water interface region [27]. The ITC and fluorescence results are in agreement with the DSC data [38], but they do not correlate well with the biological activity results, which indeed show very low hemolytic activity but reasonable antimicrobial activity of both Tritrp4 and Tritrp6. This suggests that the ability to bind to the cellular membrane is not the only factor influencing the antimicrobial activity for these two peptides [26].

A comparison of the ITC data for indolicidin and tritrpticin shows that the former peptide exhibits a similar or a lower affinity for anionic membranes but a higher affinity for zwitterionic LUVs. In fact, its binding to zwitterionic vesicles is as strong as the binding of Tritrp3. Similar results were obtained by DSC [38] and also correlate well with the biological activity data [39], pointing at a reduced membrane selectivity for indolicidin.

To our knowledge we present here the first ITC study of antimicrobial peptide interactions with vesicles prepared from a natural *E. coli* polar lipid extract. Simpler defined lipid systems, such as POPG and POPE/POPG mixtures, have been widely used in previous ITC experiments, and they are considered good mimetic models for bacterial membranes. However, it will eventually be necessary to enhance the complexity of the model systems to mimic biological membranes in a more realistic manner and to establish more standardized screening conditions that would allow comparing data from different groups more readily. Our data show that despite the general agreement of the conclusions about the peptide interaction with both POPE/

POPG and *E. coli* polar lipids, the ITC traces for the interactions of a number of peptides with both lipid systems are different. While indolicidin and Tritrp2 interacting with POPE/POPG LUVs exhibited a superposition of several processes in addition to binding, they showed a clear single binding process for interaction with *E. coli* lipid LUVs, pointing at certain differences in the peptide binding of indolicidin and Tritrp2 binding to both bacterial model systems. Furthermore, Tritrp4 and Tritrp6 showed very complex multi-process endothermic/exothermic ITC traces upon interaction with POPE/POPG, with relatively high endothermic heat of reaction for Tritrp6, which is difficult to rationalize and ascribe to a particular process such as binding, peptide aggregation, or pore formation. ITC traces for Tritrp4 and Tritrp6 interacting with *E. coli* lipids clearly displayed relatively weak interaction with the lipid membranes. As well, the less complex binding profiles for tritrpticin, Tritrp1 and Tritrp3 also showed slightly lowered affinities for *E. coli* lipids compared to POPE/POPG membranes. The calcein leakage results also support the idea that the mixture of lipids in the *E. coli* membrane weakens the peptide interactions by showing decreased susceptibility to peptide-induced perturbation compared to the two-lipid model system. Therefore, while the simpler POPE/POPG lipid mixture can in general be used in the first approximation to model bacterial membranes, the actual *E. coli* polar lipid extract can provide more detailed and realistic information about peptide binding to bacterial membranes.

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