

Complex electrostatic effects on the selectivity of membrane-permeabilizing cyclic lipopeptides

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ABSTRACT Cyclic lipopeptides (CLiPs) have many biological functions, including the selective permeabilization of target membranes, and technical and medical applications. We studied the anionic CLiP viscosin from *Pseudomonas* along with a neutral analog, pseudodesmin A, and the cationic viscosin-E2K to better understand electrostatic effects on target selectivity. Calcein leakage from liposomes of anionic phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) is measured in comparison with net-neutral phosphatidylcholine by time-resolved fluorescence. By contrast to the typical selectivity of cationic peptides against anionic membranes, we find viscosin more active against PG/PE at 30 μ M lipid than viscosin-E2K. At very low lipid concentration, the selectivity is reversed. An equi-activity analysis reveals the reciprocal partition coefficients, $1/K$, and the CLiP-to-lipid mole ratio within the membrane as leakage after 1 h reaches 50%, R_e^{50} . As expected, $1/K$ to PG/PE is much lower (higher affinity) for viscosin-E2K (3 μ M) than viscosin (15 μ M). However, the local damage to the PG/PE membrane caused by a viscosin molecule is much stronger than that of viscosin-E2K. This can be explained by the strong membrane expansion due to PG/viscosin repulsion inducing asymmetry stress between the two leaflets and, ultimately, transient limited leakage at $R_e^{50} = 0.08$. PG/viscosin-E2K attraction opposes expansion and leakage starts only as the PG charges in the outer leaflet are essentially compensated by the cationic peptide ($R_e^{50} = 0.32$). In the high-lipid regime (at lipid concentrations $c_L \gg 1/K$), virtually all CLiP is membrane bound anyway and R_e^{50} governs selectivity, favoring viscosin. In the low-lipid regime at $c_L \ll 1/K$, virtually all CLiP is in solution, $1/K$ becomes important and the “cation attacks anionic membrane” selectivity gets restored. Overall, activity and selectivity data can only properly be interpreted if the lipid regime is known and predictions for other lipid concentrations or cell counts require knowledge of $1/K$ and R_e^{50} .

SIGNIFICANCE The study demonstrates that the electrostatic selectivity of membrane-active peptides is much more complex than the simple rule of thumb that cationic peptides act selectively against negatively charged, bacterial membranes. Instead, this selectivity can be reversed and changes with the absolute lipid concentration that is present in the assay. Opposite charge enhances membrane partitioning but may also inhibit the permeabilizing activity of the membrane-bound peptide simultaneously. While the first effect may dominate selectivity in the low-lipid regime, the latter governs the high-lipid regime. Understanding this concept is crucial for the interpretation of leakage and minimum inhibitory concentrations activity data and hence, helps a great deal to support a more rational design of new, highly active, and selective membrane-active peptides.

INTRODUCTION

Cyclic lipopeptides (CLiPs) targeting the cell membrane, like other antimicrobial peptides, have been discussed as po-

tential new antimicrobial drugs to fight infections of bacterial, viral, or fungal origin, and cancer for decades (1–6). Nevertheless, with rare exceptions like daptomycin (e.g., Cubicin) or polymyxins (e.g., Colistin, Neosporin), their medical application is still failing on a large scale due to lacking stability, selectivity, or activity; toxic side effects; solubility issues; and inexplicable modes of action (7). However, the situation is different for their usage as biocontrol agents, replacing unselective and environmentally

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harmful pesticides (8–10). Several already-registered microbial plant protection products such as Serenade ASO (*Bacillus subtilis*, S: QST 713), DiPel DF (*Bacillus thuringiensis* subspecies *kurstaki* ABTS-351 (strain HD-1)), or Cedemon (*Pseudomonas chlororaphis* MA 342) (11) successfully exploit the plant-beneficial ability of biodegradable, membrane-active cyclic lipopeptides by acting as biosurfactants. Attacking membranes of plant pests or inducing plant immune responses are general modes of action of these substances (12,13).

CLiPs are secondary metabolites produced by bacteria, e.g., *Bacillus*, *Streptomyces*, *Paenibacillus*, or *Pseudomonas* (4,14), and synthesized via non-ribosomal peptide synthetases. This synthesis pathway enables a wealth of structural diversity in terms of number and type of amino acids (L/D configuration, proteinogenic/non-proteinogenic, modifications) making them less vulnerable by peptidases, as well as in the properties of the fatty acid moiety (degree of unsaturation, carbon chain length). They possess an amphiphilic oligopeptide structure, which is partially cyclized via an ester bond, attached to a fatty acid moiety at its N terminus (2,15–20). Recent progress in the total synthesis of such CLiPs (21–29) allowed us to tackle the issue of electrostatic effects on lipid-CLiP-interactions by comparing the three almost identical CLiPs viscosin, pseudodesmin A, and viscosin-E2K (see Fig. 1 and section “materials” for details). These CLiPs differ primarily with respect to their amino acids at position two of the peptide sequence, resulting in different net charges at pH 7.4. Viscosin is negatively charged, pseudodesmin A is neutral, and viscosin-E2K is positively charged. Viscosin and pseudodesmin A are two natural CLiPs of the viscosin group produced by *Pseudomonas*. Viscosin-E2K is a synthetic analog of viscosin in which glutamic acid (E) is exchanged for lysine (K) and better mimics, with its cationic charge and partially hydrophobic residues, the properties of typical antibacterial AMPs (30–32).

Most antimicrobial peptides acting selectively against bacteria are cationic. Their selectivity is explained by the

electrostatic attraction to the anionic lipids present in the outer leaflet of bacterial membranes, whereas mammalian or plant membranes expose rather neutral cell surfaces. In contrast, the hydrophobicity of an antimicrobial peptide promotes overall membrane partitioning but does not contribute to selectivity (32,33).

Up to now, most studies focused on dose-response curves with different model membranes or cells at arbitrary concentrations to elucidate CLiP selectivity and activity. However, there are cases where a more detailed characterization of the system is essential to avoid misleading conclusions. Here, we demonstrate an example of very unusual and counterintuitive electrostatic targeting: an anionic cyclic lipopeptide that is more active against an anionic lipid membrane than its cationic analog. Furthermore, this selectivity is switched as the lipid concentration used in the assay is lowered from standard, 30 μ M, to 1 μ M.

This potentially counterintuitive finding can be explained considering that membrane partitioning governs the activity of any membrane-active compound only in a regime of low absolute lipid concentration; i.e., well below the membrane dissociation constant of the CLiP. Note that the active CLiP concentration in this regime, where only a minor fraction of the CLiP is actually localized within the membrane, is independent of the actual lipid concentration and, hence, not equivalent to a characteristic lipid-to-CLiP ratio. As described for liposome leakage (34), membrane receptor binding of hydrophobic or amphiphilic ligands (35) and, in great detail, for the inoculum effect on the minimum inhibitory concentrations (MICs) of membrane-active peptides (36,37), partitioning loses its selectivity effect in a high-lipid regime where most of the compound is membrane bound anyway. Moreover, changing the lipid concentration does not simply shift critical concentrations but reverts electrostatic selectivity. This suggests that electrostatics affect partitioning and the local activity of the membrane-bound peptide oppositely additionally to changing lipid concentration regimes.

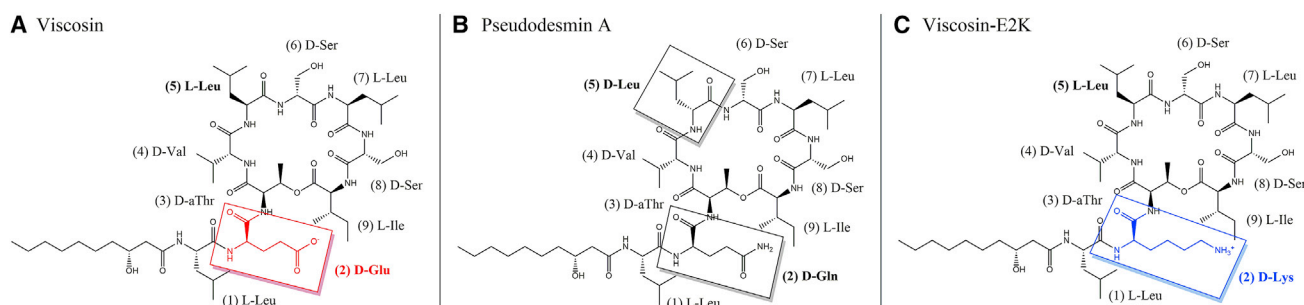


FIGURE 1 Structure and peptide sequence of viscosin (A), pseudodesmin A (B), and viscosin-E2K (C). Differences between the three CLiPs are highlighted by colored rectangular frames. This color code is used in all subsequent figures. Red stands for viscosin, black for the neutral pseudodesmin A, and blue for viscosin-E2K. Furthermore, the configurational change of leucine at position 5 between pseudodesmin A and viscosin or viscosin-E2K is highlighted in bold print. To see this figure in color, go online.

MATERIALS AND METHODS

Materials

The phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was kindly provided by Lipoid (Ludwigshafen, Germany). The phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and the phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) sodium salt (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). We chose liposomes of a mixture of POPG and POPE in a 7:3 mole ratio to mimic the highly negatively charged membrane of gram-positive bacteria (due to high POPG and cardiolipin proportions) but also reflecting the reduced amount of POPE in their membrane compared with many gram-negative bacteria of interest (38,39). POPC liposomes were used as a negative control lacking a significant overall surface charge.

Ammonium molybdate tetra-hydrate, calcein, ethylenediaminetetraacetic acid (EDTA), Fiske-Subbarow reducer, hydrogen peroxide solution 30 wt %, and Ludox HS-40 colloidal silica were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chloroform, dimethyl sulfoxide (DMSO), NaCl, NaOH, HCl and Tris(hydroxymethyl)aminomethane (Tris) were purchased from Carl Roth (Karlruhe, Germany). K₂HPO₄ was bought from VWR (Leuven, Belgium). Ultrapure water used for the preparation of all solutions was prepared by the Arium pro system (Sartorius, Göttingen, Germany).

The cyclic lipopeptides viscosin and pseudodesmin A were extracted as previously described (18). The viscosin-E2K analog was obtained using the total synthesis approach developed for viscosin and other *Pseudomonas* lipopeptides as previously described (27,29). All cyclic lipopeptides were solubilized in DMSO and quantified by NMR spectroscopy (ERETIC methodology based on PULCON as described by Wider and Dreier (40)). Table 1 shows the amino acid sequence of the cyclic lipopeptides and Fig. 1 illustrates their structure.

Liposome preparation

POPC or POPG/POPE (7:3 mole ratio) liposomes encapsulating the self-quenching fluorescence dye calcein (calcein-LUV) were prepared according to an adapted procedure of the thin lipid film hydration method followed by extrusion (41,42). Doing so, lipids were dissolved in chloroform to prepare suitable lipid stock solutions and subsequently pipetted in desired ratios into 1.5-mL high-pressure liquid chromatography vials (VWR International, Darmstadt, Germany) utilizing positive displacement pipettes (Eppendorf, Hamburg, Germany). Chloroform was removed via vacuum centrifugation at 36°C (RVC 2-18 CDplus, Martin Christ, Osterode am Harz, Germany) and lipid films were placed under high vacuum overnight. Next, vials with lipid films were weighed to double-check pipetting accuracy (Sartorius R180D analytical balances by Sartorius, Göttingen, Germany), the contained air replaced by inert argon gas, and vials sealed with parafilm. Lipid films were stored at −20°C or immediately used for the preparation of large unilamellar liposomes (LUV) loaded with the self-quenching fluorescence dye calcein.

To this end, lipid films were hydrated with 70 mM calcein buffer (70 mM calcein, 10 mM Tris, 0.5 mM EDTA, pH 7.4) at room temperature and four freeze-thaw cycles (dry ice and 50°C water bath) were performed. The liposome dispersion was extruded through a 200-nm polycarbonate membrane (Whatman Nuclepore) (30×) and then through a 100-nm (51×) polycarbonate membrane (Whatman Nuclepore) with a LiposoFast hand extruder (Avestin,

Ottawa, Ontario, Canada). To eliminate free calcein, the external calcein buffer was exchanged for isotonic Tris buffer (10 mM Tris, 110 mM NaCl, 0.5 mM EDTA, pH 7.4) using a PD-10 desalting column (GE Healthcare, Little Chalfont, UK). The Z-average of the hydrodynamic diameter (170–140 nm) and the polydispersity index (<0.1) were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Panalytical, Worcestershire, UK). The final phospholipid concentration was determined according to the Bartlett assay (43) by the colorimetric determination of inorganic phosphate. The calcein-LUVs were stored at room temperature to avoid phase transition.

Quantification of calcein leakage by TCSPC

Calcein release from calcein-LUVs (i.e., membrane permeabilization) triggered by the CLiP viscosin, pseudodesmin A, or viscosin-E2K was measured via time-correlated single-photon counting (TCSPC) with a FluoTime 100 spectrometer (PicoQuant, Berlin, Germany) as described (44,45). The fundamentals of TCSPC are discussed comprehensively elsewhere (46).

Briefly, the instrumental setup consisted of a 467-nm pulsed-laser diode (LDH-P-C-470, PicoQuant, Berlin, Germany) operated by a PDL 800-D laser driver (pulse width, 20 ps; repetition rate, 20 MHz) for excitation. Emitted photons were detected at >530 nm (OG530 longpass filter included) by a PMA 175-N detector (PicoQuant, Berlin, Germany) and attenuators set to ensure a detection rate of <200 kHz (1% of excitation pulses). The fluorescence decay of the fluorophore calcein was acquired for 30 s (resolution of 25 ps per bin). The instrument response function (IRF) was measured using a Ludox HS-40 scattering standard and deconvoluted with bi- or triexponential decay functions to fit the experimental data.

Due to the self-quenching properties of calcein, the fluorescence lifetime of calcein depends on its concentration. The presence of two distinct calcein populations with different local dye concentrations was assumed for data evaluation. The first calcein population is represented by the calcein entrapped inside the liposomes (i.e., high local calcein concentration) and characterized by a shorter fluorescence lifetime due to collisional and static quenching effects. At our experimental conditions (70 mM calcein buffer), samples with no calcein release yielded lifetimes of ≈0.4 ns for this population. The second calcein population is the free calcein (i.e., less concentrated) displaying a longer fluorescence lifetime due to low or no self-quenching effects. In our case, full calcein leakage (i.e., 100% membrane permeabilization) corresponded to a calcein concentration of about 5 μM and thus to a fluorescence lifetime of ≈4 ns for this free population, whereas partially diluted entrapped calcein yielded intermediate lifetimes between 0.4 and 4 ns. Obviously, it is a marked simplification to assume that there are only two calcein populations present in a sample, as the calcein-LUVs are most likely to leak to varying extents. However, the free biexponential data modeling yielded the best and most stable fits over triexponential data evaluation. Therefore, the fluorescence lifetime decays were fitted biexponentially according to Eq. 1, including deconvolution of the IRF with the FluoFit software by PicoQuant (Berlin, Germany), and goodness of fit was judged by calculating reduced χ^2 .

$$B(t) = B_E \cdot e^{-\frac{t}{\tau_E}} + B_F \cdot e^{-\frac{t}{\tau_F}} \quad (1)$$

The fitted pre-exponential factors B_E and B_F correlate with the amount of entrapped and free calcein, and the corresponding fluorescence lifetimes are

TABLE 1 Amino Acid Sequence of the Cyclic Lipopeptides

CLiP	FA				Amino Acid Sequence					
Viscosin	β-OH C ₁₀	L-L	D-E*	D-aT	D-V	L-L*	D-S	L-L	D-S	L-I
Pseudodesmin A	β-OH C ₁₀	L-L	D-Q*	D-aT	D-V	D-L*	D-S	L-L	D-S	L-I
Viscosin-E2K	β-OH C ₁₀	L-L	D-K*	D-aT	D-V	L-L*	D-S	L-L	D-S	L-I

Asterisks (*) indicate changes in the sequence.

denoted by τ_F and τ_F , respectively. Therefore, we may quantify the amount of released calcein, denoted as calcein leakage L , upon addition of the membrane-active CLiP to the sample by the following Eq. 2:

$$L = \frac{B_F - B_{F0}}{B_F - B_{F0} + Q_{Stat} \cdot B_E} \quad (2)$$

B_{F0} represents the amount of free calcein in a given batch that is found in samples without any leakage due to incomplete removal of free calcein by size exclusion chromatography. The self-quenching properties of calcein are mostly determined by dynamic (collisional) quenching as the Stern-Volmer constant of static quenching with $K_S \approx 0.005 \text{ mM}^{-1}$ is significantly lower compared with that for collisional quenching, $K_D \approx 0.13 \text{ mM}^{-1}$ (44). Static quenching is considered by a small empirical correction factor $Q_{Stat} = 1.2 \pm 0.2$ (44).

Sample preparation for leakage experiments with viscosin and viscosin-E2K followed a lipid-into-peptide mixing protocol (34,47–49). If applicable, this protocol is preferred since it avoids the possibility of irreversible binding or leakage effects due to transient, higher peptide concentrations in the process of mixing of liposome and peptide samples. To be precise, Tris buffer and CLiP were pipetted into disposable fluorescence cuvettes (Sarstedt, Nümbrecht, Germany), mixed, and small volumes of a calcein-LUV suspension were subsequently added to the peptide solution.

Sample preparation for leakage experiments with pseudodesmin A had to be adjusted to a reversed, peptide-into-lipid mixing protocol due to its low solubility in buffer, as described (18,45). Here, buffer and calcein-LUV were pipetted and mixed in disposable fluorescence cuvettes first, followed by the addition of pseudodesmin A to the lipid dispersion. This protocol requires a validation because it does not a priori eliminate the possibility of transient local peptide concentration differences, which might cause enhanced leakage or equilibrate only slowly once the peptide is bound anywhere. Fortunately, for pseudodesmin, this validation excluding mixing errors has been carried out already in a previous study (45), showing leakage errors due to slow peptide redistribution (occurring on a minute timescale (<30 min)) to be of maximum $\pm 10\%$.

Performing an equi-activity analysis

The procedure is described in detail elsewhere (41,44,45,50,51). Briefly, calcein-LUVs (POPC or PG/PE) at varying lipid concentrations of 30, 100, 200, and 300 μM were incubated with respective amounts of membrane-active CLiP (i.e., either viscosin, pseudodesmin A, or viscosin-E2K). Doing so, the mass of lipid was kept constant for all samples, only the amount of buffer and CLiP, respectively, were adapted to create different lipid and peptide concentrations during the incubation time. All samples were incubated under the exclusion of light in disposable fluorescence cuvettes (Sarstedt, Nümbrecht, Germany) on a rotating shaker (400 rpm, 25°C) for 1 h. Subsequently, buffer was added to each sample to reach a total volume of 1.4 mL prior to TCSPC measurement, resulting in a consistent final lipid concentration of 30 μM , also rendering calcein concentration and turbidity effects constant for all measurements. Moreover, a sample series containing only 1 μM lipid in 1.4 mL was investigated without top-up containing only a third of lipid and of the fluorophore calcein.

The data are then analyzed via an equi-activity analysis determining universal leakage curves and partitioning isotherms. The following five steps briefly summarize the procedure.

First, calcein leakage for each lipid concentration series is plotted versus its corresponding CLiP concentration, just like in standard dose-response curves. Second, the CLiP amount needed to trigger a certain leakage (L) at a certain lipid concentration (c_L), can be read from the plot by interpolation. If there is no third compartment where peptide could be localized (e.g., precipitates), the mass balance of the peptide requires the total peptide, c_P , to represent the sum of the free peptide, c_P^{aq} , and the membrane-bound peptide, c_P^b with $c_P^b = R_e \cdot c_L$:

$$c_P(L) = R_e(L) \cdot c_L + c_P^{aq}(L) \quad (3)$$

R_e describes the effective molar ratio of peptide per lipid within the membrane.

The argument (L) reminds one that, in the equi-activity plot, all data pairs c_P, c_L of one line were selected to correspond to the same leakage, L . Under the fairly robust assumption that leakage is a property of the membrane composition, i.e., each L within the sensitive range, say 10%–80%, corresponds unequivocally to an (unknown but specific) R_e , Eq. 3 represents a straight line with a slope R_e and ordinate intercept c_P^{aq} for any L selected.

Third, plotting leakage as a function of R_e yields a universal leakage curve describing a compound's local damage contribution to the overall membrane permeabilization independently of its partitioning behavior. Fourth, the y intercept of the linear regression yields the concentration of the not-membrane-bound CLiP (c_P^{aq}) and can be used to calculate the apparent membrane-water partition coefficient, K :

$$K = \frac{c_P^b}{c_L \cdot c_P^{aq}} = \frac{R_e}{c_P^{aq}} \quad (4)$$

Of the several definitions of partition coefficients (52), the most suitable one for this study is the apparent mole-ratio-based value (see Eq. 4) in a reciprocal form, which practically represents a dissociation constant in a concentration unit. That means, at the lipid concentration c_L that is equal to K^{-1} , just half of the overall peptide is membrane bound ($c_P^b = 0.5 c_P$). Additionally, at $c_L \gg K^{-1}$, virtually all peptide resides in the membrane.

Table 2 provides an overview of all variables and their meaning.

Cumulative leakage kinetics

TCSPC measurements were carried out to quantify CLiP-induced calcein leakage of neutral POPC calcein-LUV or negative PG/PE calcein-LUV over a time period of 24 h.

The final lipid concentration in all leakage kinetics samples was 30 μM . All samples were kept on a rotary shaker (400 rpm; 25°C), always protected from light. TCSPC measurements were performed immediately upon addition of calcein-LUV and after 10 min, 30 min, 1 h, 2 h, and 24 h. For controls, samples containing only buffer and calcein-LUV but no CLiP were prepared. The final DMSO concentration never exceeded 3 vol %. Reference experiments (tested DMSO concentrations from 1–20 vol %) indicated that ≤ 5 vol % DMSO did not affect calcein leakage significantly.

TABLE 2 List of all Variables Used in Equi-Activity Analysis

Variable	Unit	Meaning
c_P	μM	total peptide concentration
c_P^b	μM	concentration of membrane-bound peptide
c_P^{aq}	μM	concentration of free, not membrane-bound peptide in aqueous solution
L	%	calcein leakage: fraction of dye having leaked out after a defined time
$c_P(L)$	μM	total concentration of peptide needed to trigger a given leakage L
c_L	μM	lipid concentration
R_e		effective molar ratio of membrane-bound peptide per membrane lipid
R_e^{50}		R_e to cause 50% leakage
K	μM^{-1}	apparent membrane-water partitioning coefficient
K^{-1}	μM	reciprocal of K representing a dissociation constant

RESULTS

Counterintuitive dose-response curves

First, we recorded typical dose-response curves of the neutral CLiP pseudodesmin A, and of its anionic (viscosin) and cationic (viscosin-E2K) analogs against PG/PE calcein-LUV (Fig. 2 A and B), whose net surface charge (i.e., what the membrane-active CLiP “encounters” first) is negative. PG/PE calcein-LUV represent a basic model for the plasma membrane of gram-positive bacteria. The experiments were carried out at two fixed lipid concentrations, 30 μM and 1 μM , after 1-h incubation time. As the main focus in this research is to elucidate the influence of charge of a membrane-active compound on its membrane activity, we additionally included simple POPC calcein-LUV, presenting an

overall neutral membrane surface, as controls (Fig. 2 C and D).

Surprisingly, Fig. 2 A reveals that anionic PG/PE membranes are preferably attacked and damaged by the anionic peptide viscosin over the cationic peptide analog viscosin-E2K at 30 μM lipid. The trivial explanation that the E2K-exchange renders the molecule generally inactive, for example by a change in structure, is ruled out by the finding that viscosin-E2K is, in fact, the most active analog in the control experiment with zwitterionic POPC liposomes (Fig. 2 C). Instead, it appears that, in this case, electrostatic attraction has an inhibitory effect, whereas electrostatic repulsion as in the viscosin-PG/PE system boosts activity.

Moreover, the data in Fig. 2 A and B show that changing the lipid concentration of the anionic PG/PE LUV does not

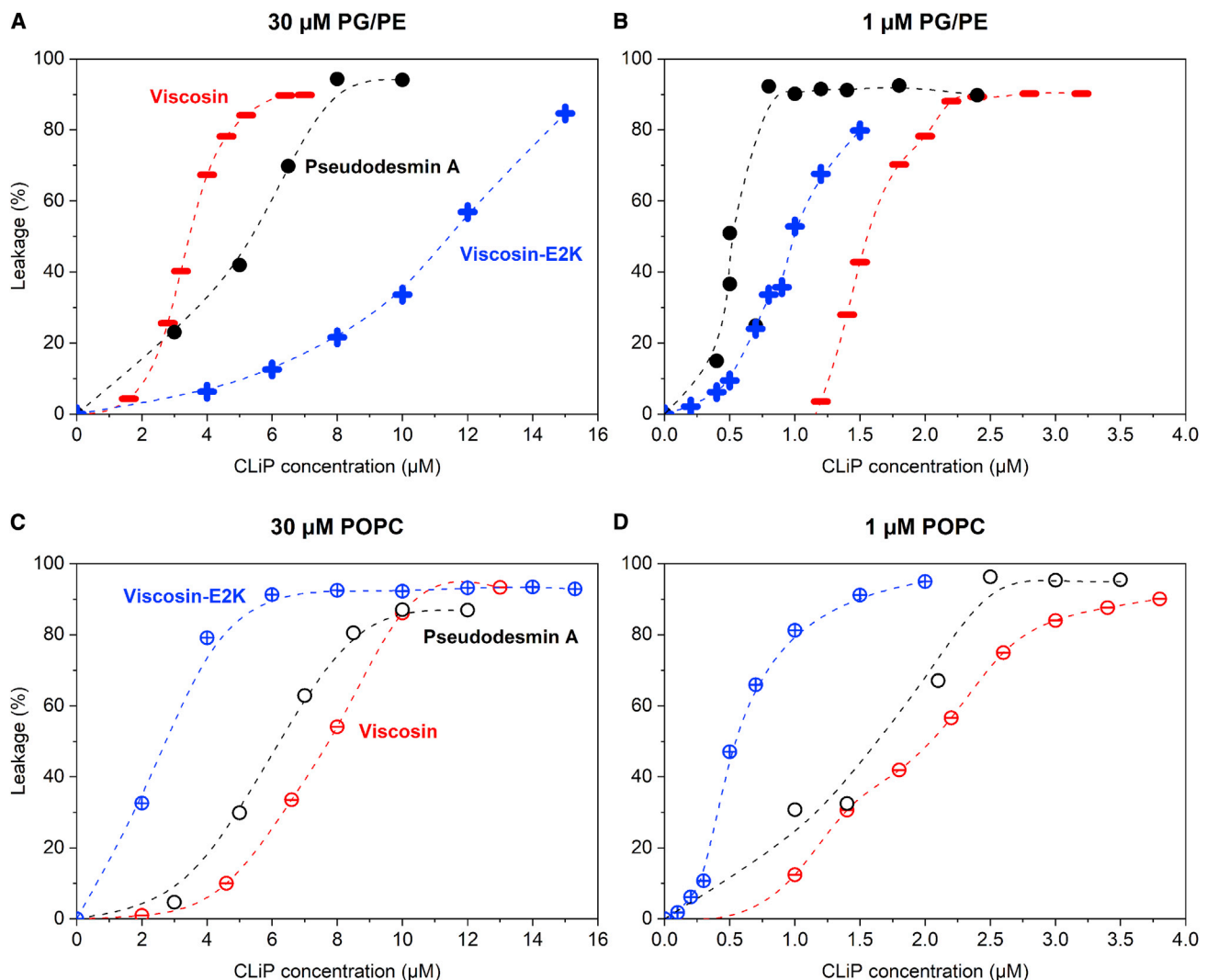


FIGURE 2 Calcein leakage as percentage shown as a function of increasing CLiP concentrations (μM) after 1-h incubation time of 30 μM and 1 μM PG/PE calcein-LUV (A and B) and of 30 μM and 1 μM POPC calcein-LUV (C and D). Red minuses represent viscosin-triggered (negatively charged), black circles pseudodesmin A-triggered (neutral), and blue crosses viscosin-E2K-triggered (positively charged) calcein leakage in PG/PE calcein-LUV, respectively. Symbols (red minuses, open black circles, and blue pluses) in circles represent CLiP-triggered calcein leakage in POPC calcein-LUV, respectively. Lines are to guide the eye. To see this figure in color, go online.

simply shift the dose-response curves along the concentration scale, as would be expected and is demonstrated by the POPC control (Fig. 2 C and D), but switches their order. Although, at 1 μM lipid, the cationic peptide viscosin-E2K is more active against the anionic membrane as one tends to expect, this selectivity is reversed at 30 μM .

The membrane activity of the neutral compound pseudodesmin A remains virtually the same against both lipid bilayers at 30 μM but is more active against PG/PE membranes at 1 μM .

So what interactions are responsible for a CLiP's membrane activity and selectivity? Merely increased partitioning with the target bilayer due to electrostatic attraction forces or hydrophobicity is not enough, as demonstrated by the membrane activity data of these three almost identical CLiPs.

Equi-activity analysis to separate partitioning from local activity effects

The widespread assumption that membranes containing anionic lipids such as POPG are preferably permeabilized by cationic peptides is of course based on the effect of electrostatic attraction; i.e., a stronger partitioning of the peptide from the aqueous solution into the membrane.

To consider partitioning effects explicitly, we have run more experiments at additional lipid concentrations and performed an equi-activity analysis (see [supporting material](#)). This procedure provides the 1) active peptide contents within the membrane (i.e., after eliminating partitioning effects, see next section), and 2) the partitioning isotherm of the peptide into the membrane, as long as partitioning is not too strong and aqueous peptide concentrations can be quantified well.

Partitioning isotherms

Fig. 3 shows the resulting membrane dissociation isotherms of the three CLiP analogs with negatively charged PG/PE calcein-LUV (A) and neutral POPC calcein-LUV as control (B). For proper statistics, only truly independent points are shown, typically the ones corresponding to 20%, 40%, 60%, and 80% leakage. As is expected for compounds with very high membrane affinity (49), i.e., aqueous concentrations of a few micromolar or less at given leakage levels, the equi-activity fits (see [supporting material](#)) yield R_e with very low, but c_p^{aq} with very large, errors (see Y error bars in Fig. 3).

Generally, one should expect such an isotherm to show a linear range at low effective mole ratios of membrane-bound peptide per lipid, R_e (i.e., dilute peptide in membrane). The slopes of straight lines through the origin represent K^{-1} . At high R_e , the slopes may increase due to electrostatic repulsion of the peptide by pre-bound peptide or from a general destabilization of the membrane; the latter effect also

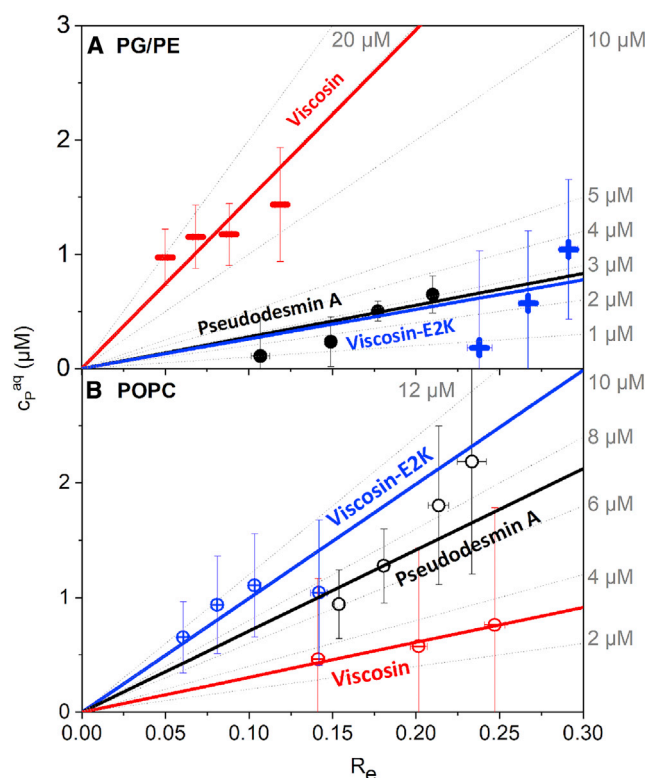


FIGURE 3 Membrane partitioning of viscosin (red minuses), pseudodesmin A (black circles), and viscosin-E2K (blue crosses) with negatively charged PG/PE calcein-LUV (A) and neutral POPC calcein-LUV (B, symbols in circles). Error bars for the CLiP-to-lipid mole ratio in the membrane, R_e , and the concentration of the non-membrane-bound CLiP, c_p^{aq} (μM), are standard errors of the equi-activity analysis. Note that c_p^{aq} (μM) are very small (<3 μM) and therefore error bars appear large. On the contrary, error bars for R_e are very small and therefore hardly visible. The error-weighted, linear fits yield K^{-1} as shown in Table 3. Gray dotted lines depict c_p^{aq} and R_e at arbitrarily chosen K^{-1} values to make errors of equi-activity analysis tangible in the sense of their significance for K^{-1} . To see this figure in color, go online.

applying to non-ionic amphiphiles (52). As long as electrostatic effects govern the shape of the isotherm, this can be modeled quantitatively in terms of the Gouy-Chapman model and the Stern equation (53–56). For this study, such a fit was neither needed nor realistic, and so we simply focus on estimates of the initial slopes.

To begin with, let us have a closer look at Fig. 3 A depicting the partitioning behavior of viscosin, viscosin-E2K, and pseudodesmin A with the PG/PE calcein-LUV. Table 3 lists corresponding K^{-1} values. The presence of anionic lipid enhances membrane binding of the cationic viscosin-E2K substantially, illustrated by the decrease in K^{-1} from $9 \pm 1 \mu\text{M}$ for POPC to $2.2 \pm 0.8 \mu\text{M}$ for PG/PE. The membrane insertion of the anionic viscosin (K^{-1} increased from 3.0 ± 0.1 to $15 \pm 2 \mu\text{M}$) is opposed by the anionic lipid.

The neutral pseudodesmin A showed intermediate K^{-1} with a somewhat preferred binding to charged membranes, which can at least qualitatively be explained by the fact that insertion of the peptide reduces PG-PG contacts and

TABLE 3 Membrane Partitioning Described by the Reciprocal Apparent Mole-Ratio Partitioning Coefficient K^{-1} (μM)

Type of Vesicle	K^{-1} (μM)		
	Viscosin (–)	Pseudodesmin A	Viscosin-E2K (+)
PG/PE calcein-LUV	15 ± 2	2.2 ± 0.5	2.2 ± 0.8
POPC calcein-LUV	3.0 ± 0.1	7.8 ± 0.6	9 ± 1

Errors are standard errors of the error-weighted fits of partition isotherms in Fig. 3 at $R_e < 0.2$ (except for viscosin-E2K-PG/PE: $R_e < 0.3$). To relate K^{-1} errors to individual error bars of measured $c_p^{\text{aq}}(R_e)$, see grid lines in Fig. 3.

lowers the charge density of the PG/PE membrane, thereby relaxing electrostatic repulsion between the lipids. All these findings are therefore perfectly in line with common expectations for electrostatic interactions.

The systematic differences between K^{-1} of the different peptides for POPC are expected to result from minor differences in the primary and secondary structure of the peptides, their positioning within the membrane, and their effect on neighboring lipids.

Universal leakage curves

The equi-activity analysis also produces a so-called universal leakage curve. It visualizes membrane leakage as a function of the local content of membrane-inserted peptide per lipid in the membrane (mole ratio R_e), and, hence, it is independent of partitioning effects. To give one example, a CLiP-to-lipid mole ratio of $R_e^{50} = 0.1$ means that 10 membrane-bound CLiP molecules per 100 lipids are needed to cause 50% membrane leakage of the vesicle after a defined time. The term “universal” indicates that this curve (and, e.g., R_e^{50}) does not depend on the lipid concentration used in the assay. The universal leakage curves of POPC calcein-LUV and PG/PE calcein-LUV are shown in Fig. 4 and the R_e^{50} values for each system are given in Table 4.

First of all, it is striking that CLiP charge has opposing effects on CLiP partitioning and CLiP-triggered local membrane damage. The open-circled plus symbols in Fig. 4 indicate that, in the absence of electrostatic interactions between net-charged lipids and peptides, i.e., for POPC, viscosin-E2K needs the lowest content in the membrane to initiate leakage, followed by the neutral pseudodesmin and the anionic viscosin. Strong electrostatic lipid-CLiP repulsion as for viscosin-PG boosts the permeabilizing activity of membrane-inserted CLiP (bold red minus signs). Electrostatic attraction between viscosin-E2K and PG substantially inhibits activity (bold blue plus signs). The value of $R_e^{50} = 0.32$, needed for viscosin-E2K to finally leak PG/PE 7:3 membranes, converts (as long as all CLiP remains in the outer leaflet) to a charge ratio of viscosin-E2K:PG of 0.91. This suggests that viscosin-E2K remains essentially inactive until the membrane surface eventually loses its negative surface potential.

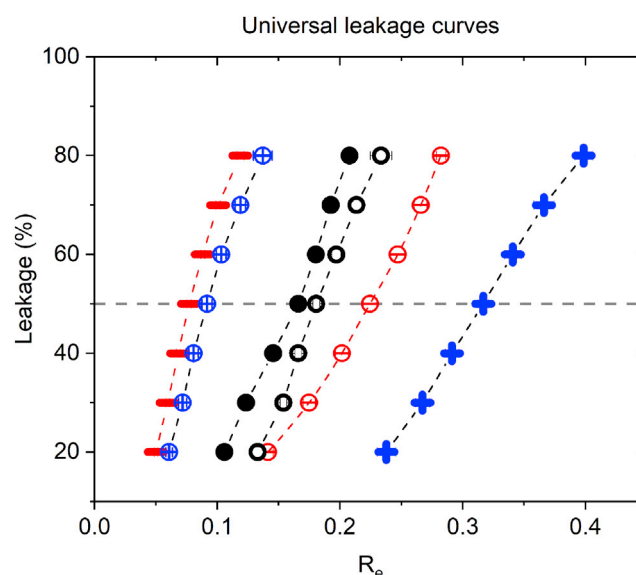


FIGURE 4 The universal leakage curves depict CLiP-induced membrane leakage (%) of the negatively charged PG/PE calcein-LUV (viscosin, red minuses; pseudodesmin A, filled black circles; viscosin-E2K, blue pluses) and of the neutral POPC calcein-LUV (viscosin, red minuses in circles; pseudodesmin A, open black circles; viscosin-E2K, blue pluses in circles) as a function of the respective CLiP-to-lipid ratio in the membrane, R_e . Error bars for R_e are standard errors derived by the equi-activity analysis and hardly visible. Lines are to guide the eye. To see this figure in color, go online.

Leakage kinetics

Different leakage kinetics have been assigned to principal modes of action (57–61). Therefore, we tracked calcein leakage of POPC calcein-LUV and PG/PE calcein-LUV induced by viscosin, pseudodesmin A, and viscosin-E2K for 24 h. To facilitate data analysis in terms of charge impact, we chose a set lipid concentration of 30 μM for all leakage kinetics experiments.

The key outcome of this experiment is the presence of fast but limited leakage (i.e., a fraction of dye leaking out within a few minutes or less, often within the dead time of our experiment), whereas the other, significant fraction of dye is leaking at a much slower rate, by a different process.

Let us now examine 1) the fast but limited and 2) the slow but probably continuous contributions to the traces collected in Fig. 5. Insertion of, for example, 4 μM anionic viscosin into anionic PG/PE membranes (A) causes 66% leakage within 30 min. After 1 day (1400 min), leakage has hardly increased any further, reaching 71%. In the absence of electrostatic repulsion between the CLiP and a net-negative lipid, 4.6 μM viscosin (D) causes only 8% leakage after 30 min but the slow progress increasing leakage to 15% within the following 23 h is slightly stronger than in (A). Similar observations are obtained at other concentrations, implying that the electrostatic repulsion within the membrane enhances, in particular, the transient, limited leakage

TABLE 4 R_e^{50} Values Derived by Equi-Activity Analysis after 1 h of Incubation Time

Type of Vesicle	R_e^{50} Values		
	Viscosin (–)	Pseudodesmin A	Viscosin-E2K (+)
PG/PE calcein-LUV	0.08	0.17	0.32
POPC calcein-LUV	0.22	0.18	0.09

The standard errors of the equi-activity fit are all below 0.01.

component (1). This is supported by the finding that, in the case where neither CLiP nor lipid carry any net charges (pseudodesmin A and POPC membrane, E), the contribution of the fast component at a comparable concentration of 5 μM (blue line at 24% after 30 min) to the long-term leakage (60% after 1400 min) gets even lower. Considering the trace for 6 μM viscosin-E2K in PG/PE membranes, electrostatic attraction appears to inhibit fast, transient leakage even more strongly than slow leakage. Summarizing, the long-term kinetic data appear to be consistent in indicating that the effects of electrostatics on the local damage of the CLiPs within the membranes, as quantified by R_e^{50} , result primarily from the fast and limited leakage mechanism. The principal leakage kinetics triggered by the negative CLiP viscosin seen in Fig. 5 A and D also apply to experiments carried out at 1 μM (see Fig. S13 in supporting mate-

rial), where we had found selectivity effects to be altered (see Fig. 2 A and B).

DISCUSSION

Fundamental differences between the high- and low-lipid regimes

We have demonstrated that the selective action of a given CLiP against a certain membrane may depend on 1) partitioning and 2) local damage, and that, in the case of viscosin and its analogs interacting with PG/PE membranes, strong partitioning (low K^{-1}) of a CLiP was accompanied by weak local damage (high R_e^{50}) and vice versa. The balance between these two effects depends on the lipid concentration, as demonstrated in Fig. 2 A and B by the reversed membrane activity of viscosin and viscosin-E2K at 1 μM and 30 μM lipid. The dose-response curve at 1 μM was found to be more affected by the electrostatic repulsion of viscosin and attraction of viscosin-E2K (i.e., partitioning effects) to the anionic PG lipid in the membrane than at 30 μM lipid.

This phenomenon can quantitatively be explained by the influence of the two parameters, K^{-1} and R_e^{50} , on the active peptide concentration causing 50% leakage, c_p^{50} , after

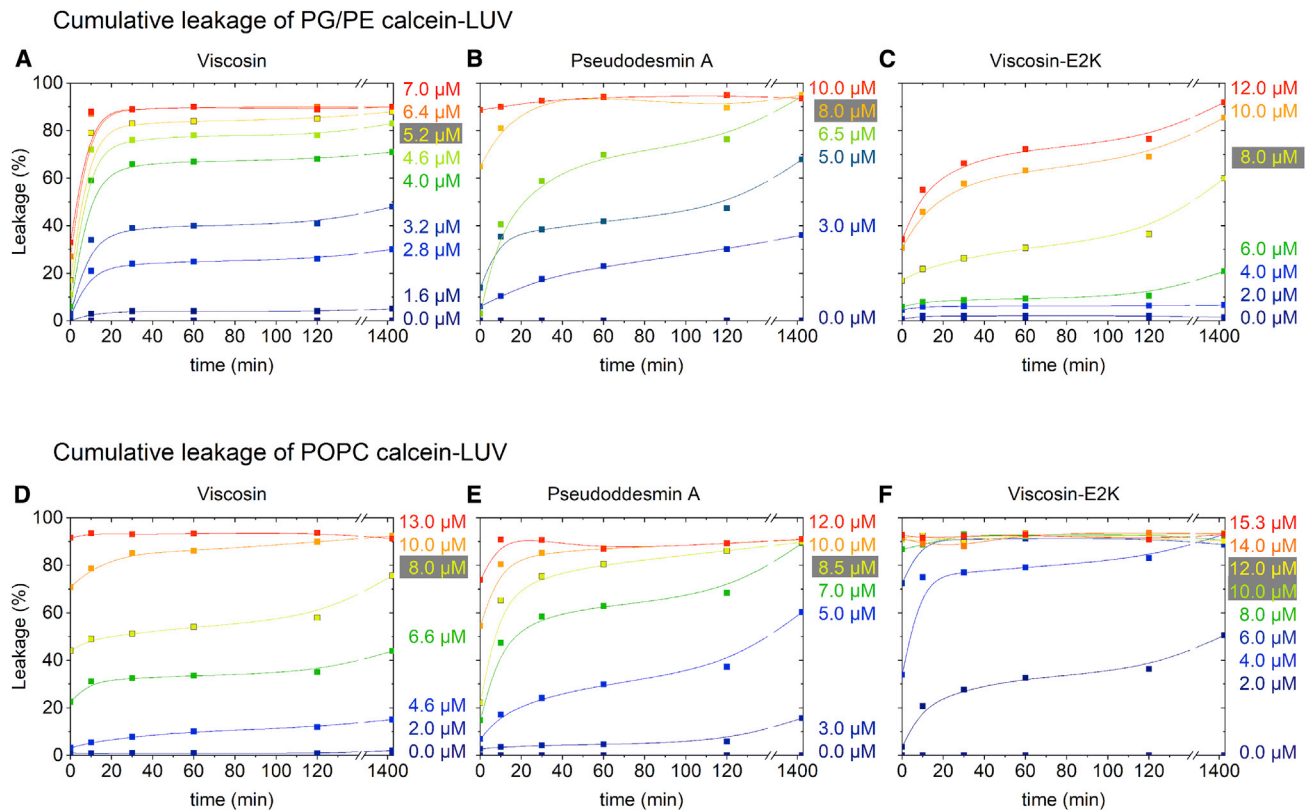


FIGURE 5 The upper row shows cumulative leakage of 30 μM PG/PE calcein-LUV as a function of time triggered by viscosin (A), pseudodesmin A (B), and viscosin-E2K (C). The lower row shows cumulative leakage of 30 μM POPC calcein-LUV as a function of time triggered again by viscosin (D), pseudodesmin A (E), and viscosin-E2K (F). Color code of lines represent tested CLiP concentrations (0 μM CLiP, purple line; highest concentration, red line). Lines are to guide the eye.

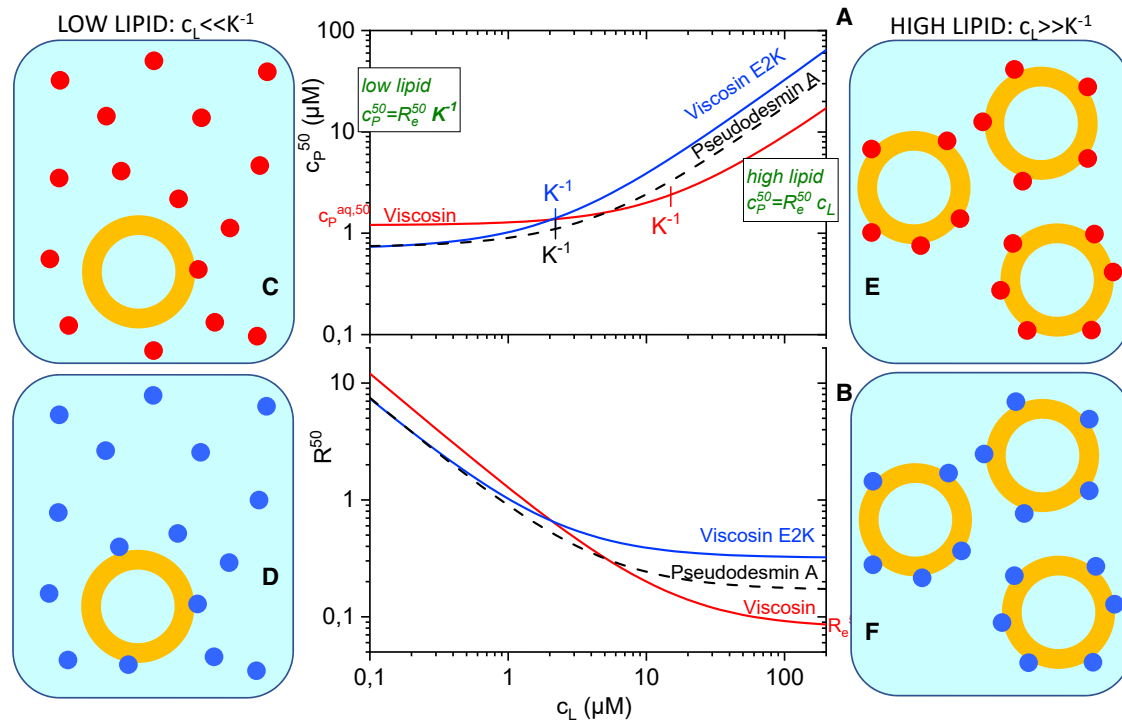


FIGURE 6 Effect of changing lipid concentration on membrane-active peptide concentration, c_P^{50} (A), and on corresponding total CLiP-to-lipid mole ratios inducing 50% leakage, R^{50} (B), as a function of the lipid concentration, c_L . Simulated for the parameters in Tables 1 and 2. Note that y and x axis are both on a double logarithmic scale. Vertical bars in (A) indicate K^{-1} values. The cartoons illustrate that, in the low-lipid regime, virtually all peptide is in solution and the local concentration of cationic peptide (blue dots, D) in anionic membranes (orange circles) is larger than that of anionic peptides (red dots, C) due to electrostatic attraction. In the high-lipid regime, virtually all peptide is membrane bound anyway and differences between the affinities of cationic and anionic peptides have no effect on membrane composition (E and F). Further detail is not required; specific numbers and dimensions are not meaningful.

standard incubation for 1 h. To recall, R_e^{50} describes the necessary ratio of membrane-bound peptide molecules per lipid in the membrane to cause 50% vesicle leakage.

$$c_P^{50} = R_e^{50} \cdot K^{-1} \cdot \left(1 + \frac{c_L}{K^{-1}}\right) \quad (5)$$

Eq. 5 is a transformation of Eq. 3 and describes the linear relationship, to which we refer to as equi-activity line, for the common peptide activity to induce 50% leakage after 1 h.

For discussing the effects of changing the lipid concentration over orders of magnitude on 1) partitioning (K^{-1}) and 2) local damage (R_e^{50}), we plotted the simulated c_P^{50} as a function of the lipid concentration, c_L , on a double logarithmic scale in Fig. 6 A. Fig. 6 B shows the corresponding total CLiP-to-lipid mole ratios inducing 50% leakage, R^{50} , after 1 h, with $R^{50} = c_P^{50} / c_L$, as a function of c_L . The curves illustrate the reversal of selectivity with increasing lipid concentration: viscosin (red curve) is least active (highest c_P^{50}) at low lipid concentrations.

The mass balance (Eq. 3) requires that all peptide is either in aqueous solution (superscript *aq*) or membrane bound (superscript *b*). With the definition of K^{-1} given by Eq. 4 used to eliminate either c_P^b or c_P^{aq} , it yields:

$$c_P = c_P^{aq} \cdot \left(1 + \frac{c_L}{K^{-1}}\right) = c_P^b \cdot \left(1 + \frac{K^{-1}}{c_L}\right) \quad (6)$$

As explained for model membrane leakage (34), ligand binding to membrane receptors (35), and the MIC assays of antimicrobials (36,37,62), we may distinguish three characteristic lipid concentration ranges: 1) the low-, 2) the high-, and 3) the intermediate-lipid regime.

1. The low-lipid regime: $c_L \ll K^{-1}$, see cartoons Fig. 6 C and D. This applies at lipid concentrations well below the K^{-1} of a given CLiP so that $c_L / K^{-1} \ll 1$. For this case, Eq. 6 implies that $c_P \approx c_P^{aq}$, meaning that virtually all peptide is in aqueous solution and the membrane-bound fraction of the peptide is negligible. In other words, since there are so few target membranes, they do not significantly compete for the peptide but each liposome binds the peptide to a characteristic R_e , which solely depends on the strength of peptide partitioning K^{-1} , independently of the presence of other liposomes (i.e., of c_L). The equi-activity line in Eq. 5 then becomes:

$$c_L \ll K^{-1} \rightarrow c_P^{50} \approx R_e^{50} \cdot K^{-1} \quad (7)$$

Thus, the active, total CLiP concentration (specified, for example, as c_P^{50}) approaches a constant value that scales linearly with K^{-1} . In this range, preferred partitioning renders the cationic viscosin-E2K more active against PG/PE membranes than the negative viscosin, as indicated by the blue line being lower than the red line in Fig. 6 A for low c_L and illustrated by the cartoons Fig. 6 C and D.

2. The high-lipid regime: $c_L \gg K^{-1}$, see cartoons Fig. 6 E and F. Then, $c_L/K^{-1} \gg 1$ and, in this case, Eq. 6 implies that $c_P \approx c_P^b$ and that $c_P^{aq} \approx 0$. Thus, virtually all peptide is membrane bound and a further increase in membrane affinity would not have any effect on membrane permeabilization. The equi-activity line in Eq. 5 in this case becomes:

$$c_L \gg K^{-1} \rightarrow c_P^{50} \approx R_e^{50} \cdot c_L \quad (8)$$

and $R_e^{50} \approx R_e^{50}$. Since c_P^{50} (and R_e^{50}) in this regime do not depend on K^{-1} , electrostatic attraction of a cationic peptide to an anionic membrane or the repulsion of an anionic peptide do not affect leakage; i.e., compound selectivity due to partitioning is lost at high lipid concentrations (see cartoons Fig. 6 E and F). Only the local damage imposed by a membrane-bound peptide (represented by R_e^{50} or, more generally, the universal leakage curve) controls selectivity. This is the reason for the, at first glance, counterintuitive selectivity of the CLiPs viscosin and viscosin-E2K seen at 30 μ M (Fig. 2 A).

3. The intermediate-lipid regime: $c_L \approx K^{-1}$. This applies if c_L is within the same order of magnitude as K^{-1} ; i.e., to the curves in Fig. 6 in the vicinity of the vertical bars. Given practicable protocols for leakage measurements (typically, $c_L \geq 10 \mu$ M) and MIC assays (5×10^5 colony forming units per milliliter according to EUCAST guidelines) and common values of K^{-1} , this must be considered a common case.

The effect of electrostatics on the local leaking activity of a membrane-permeabilizing peptide

The reversal of peptide selectivity for negative PG/PE membranes in the high-lipid regime where the effect of partitioning becomes negligible implies that electrostatic interactions with anionic PG render the membrane-bound, countercharged viscosin-E2K substantially less active to leak the membrane. This effect of electrostatics is visualized in Fig. 7, showing the ratio between R_e^{50} against PG/PE (electrostatic lipid-CLiP interactions) and against PC (no such interactions). R ratios were calculated on the basis of the data in Table 4. For viscosin, the local action is boosted about threefold (to 36% of the R_e^{50}) compared with that in POPC, and, for viscosin-E2K, it is inhibited by about the same ratio. For R_e^{50} of the neutral pseudodesmin A, it

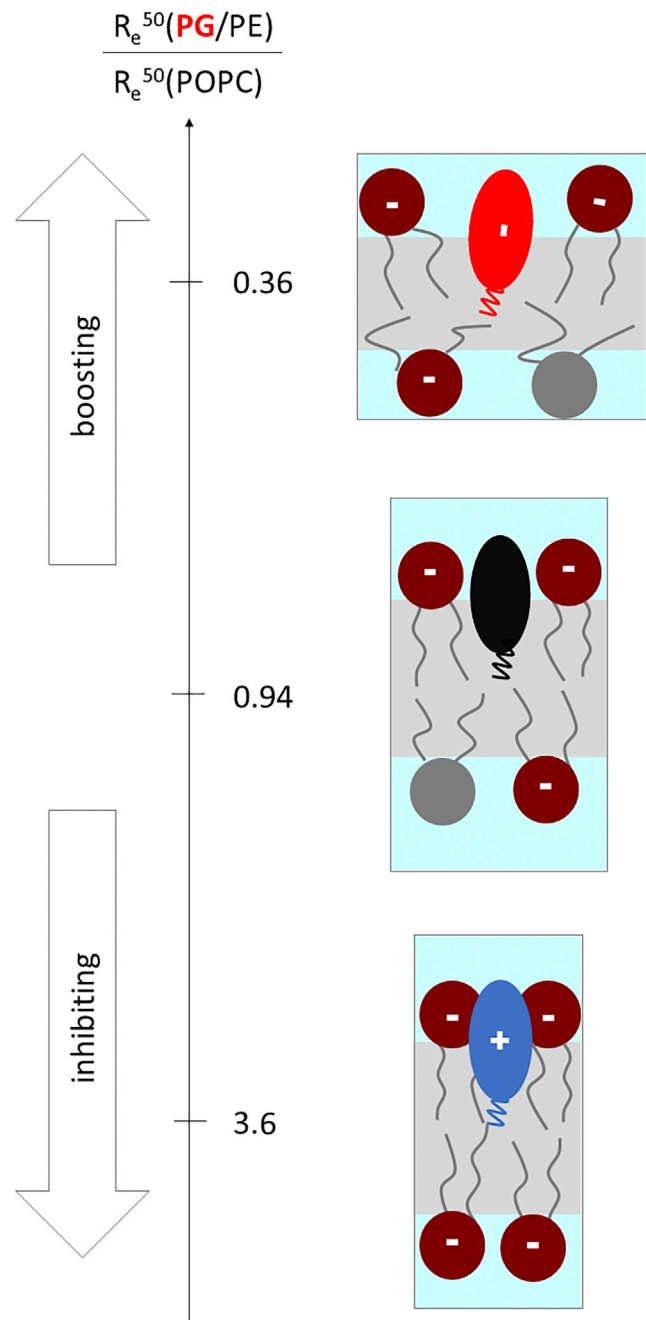


FIGURE 7 The effect of electrostatic lipid-CLiP interactions on the leakage-inducing activity of bound CLiP as quantified in terms of the ratio between the 50%-leaking mole fractions in anionic and neutral membranes, $R_e^{50}(\text{PG/PE})/R_e^{50}(\text{POPC})$. Calculations are based on the data in Table 4; see also lipid-induced shifts in universal leakage curves in Figure 4. The minus signs in the cartoons represent the negative charges of the peptide or lipid. The negative CLiP viscosin is shown in red, the positive viscosin E2K in blue, and the neutral pseudodesmin A in black. A larger area expansion by the peptide causes larger asymmetry stress.

does not matter whether the membrane contains anionic lipid or not (ratio ≈ 1).

The most prominent, and probably relevant, mode of action of the peptide shows fast yet limited leakage, which

is generally accepted to indicate a mode of action based on asymmetry stress (60). Alternative explanations are ruled out in a separate section below. As illustrated by the cartoons in Fig. 7, asymmetry stress builds up as the outer leaflet is expanded by asymmetrically inserting molecules, and the inner layer gets stretched to match this expansion. As the stress reaches a critical level depending on the stability of the membrane, the latter undergoes a transient rupture that causes a translocation of lipid and peptide to the underpopulated inner leaflet, along with a transient leakage of the membrane. Hence, R_e^{50} needed for this mode of action depends on 1) the amount of stress induced per molecule, and 2) on the threshold stress needed to activate leakage (45,63,64). The stress itself is trivially related to the area increase of the leaflet per molecule inserted and, as depicted, stronger for a peptide repelling its neighbors than for one attracting them. Furthermore, we may also speculate (45) that a tightly packed, very cohesive membrane as for viscosin-E2K/PG can tolerate more asymmetry stress before rupturing, an effect that would further increase R_e^{50} .

On longer time scales (hours) and at higher CLiP concentrations, other modes of action can come into play, observed as slow, apparently unlimited leakage processes. Such phenomena can arise from toroidal pores or pore-forming oligomers of the CLiP. Both mechanisms would probably also be affected by electrostatic lipid-CLiP interactions, but since limited leakage dominates here and gets even more dominant in the charged membranes, we will not discuss these in more detail.

The proper way of specifying leakage-inducing peptide concentrations

The above discussion answers the question regarding the quantity that should be used to specify the leakage-inducing concentration and represent, practically, the “canonical” abscissa for dose-response curves. Some authors assume that the effect of varying lipid concentrations can generally be limited by normalizing active concentrations to the lipid, specifying, e.g., the total peptide-to-lipid mole ratio inducing 50% leakage, R^{50} . Inspection of Fig. 6 reveals that this only applies in the high-lipid regime, where R^{50} indeed becomes a characteristic constant, independently of the lipid concentration. In the low-lipid regime, normalization is misleading and the characteristic property of a peptide is the absolute concentration at 50% leakage, c_P^{50} . If the dissociation constant of the peptide is unknown and, hence, it is not a priori known whether an assay is carried out under high- or low-lipid conditions, an experiment should be repeated at sufficiently different c_L showing whether c_P^{50} or R^{50} is conserved. In the general case, providing R_e^{50} and K^{-1} gives a complete description and permits predicting R^{50} and c_P^{50} at any c_L selected.

Overall, it should be emphasized that, since K^{-1} depends on the CLiP, the lipid, and the aqueous medium,

comparing results for different peptides or membranes and interpreting in vitro data in view of biological systems may suffer from the cases representing different regimes. For example, the bacterial load in clinical infections may vary over several orders of magnitude (37). This has also been demonstrated in cell viability studies finding that the active concentration remains a constant for low-cell-density experiments but the MIC significantly changes (up to 100 times) with increasing inoculum density (i.e., high c_L) (36,37,62).

The mode of action responsible for limited leakage in the light of the lipid regimes

As for many other AMPs studied before, we have observed fast leakage of a limited fraction of the dye (see Fig. 5). Different scenarios have been considered to account for this phenomenon:

- 1) Inactive fraction: technically, a fraction of peptide might become unavailable for leakage of a particular liposome because it is practically irreversibly bound (slow off-rate) to another one, precipitated, micellized or whatever.
- 2) Dilution from outer to inner leaflet: Matsuzaki and co-workers (57) have shown that limited leakage could result if leakage needs a threshold value of peptide in the outer leaflet (e.g., in order to assemble to a pore-forming oligomer). Then, peptide redistribution to the inner leaflet after some leakage, or, simply, after some time (65), could render the local content subcritical for continued leakage.
- 3) Leakage by asymmetry stress: the initial preferential insertion of the peptide into the outer bilayer leaflet causes an imbalance between the optimal areas of the outer (expanded) and inner (unchanged) leaflets, resulting in mechanical stress and, finally, transient leakage that disseminates the asymmetry.

An inactive fraction as in 1) would, in particular, be feasible for a peptide-into-lipid titration as done here for pseudodesmin A, where liposomes encountering the added peptide stock first could bind considerably more than their equilibrium share of peptide, leak accordingly, and potentially retain this peptide. For pseudodesmin A (45) (and earlier for fengycin (66)), this has been ruled out by testing, for example, the effect of administering the peptide in several consecutive steps. Recalling the discussion of the lipid regimes above, we should add that irreversible binding should essentially be limited to the high-lipid regime since, at low lipid, virtually no peptide is bound (and could thus be immobilized) in the first place. A consistent equi-activity curve reaching into the intermediate- and/or low-lipid regime rules out such hidden peptides playing a substantial role.

The scenario 2 of peptide concentration in the outer leaflet being diluted below a threshold by peptide

redistribution to the inner leaflet must be limited to the high-lipid regime. In the low-lipid regime, the peptide flipping to the inner leaflet would simply be replaced by peptide binding from the aqueous solution, so that the local content in the outer leaflet would not decrease. The finding of limited leakage at $c_L = 1 \mu\text{M}$ for viscosin/PG, which is clearly in the low-lipid regime ($K^{-1} \approx 15 \mu\text{M}$), rules out scenario 2 for this system.

Of all the modes of action and phenomena that were described to potentially allow for limited leakage, only the one based on asymmetry stress as described above can explain all observations made here.

CONCLUSIONS

Electrostatic attraction between a membrane-active peptide and the membrane lipid enhances membrane affinity (reducing the membrane dissociation constant K^{-1}) but may, as in the example of viscosin-E2K and POPG studied here, inhibit the permeabilizing activity per membrane-bound peptide.

It is crucial for the interpretation of leakage (and antimicrobial activity) data to distinguish between low-lipid, intermediate-lipid, and high-lipid regimes. In the high-lipid regime at $c_L \gg K^{-1}$, virtually all peptide is membrane bound anyway and partitioning loses its selectivity effect. This accounts for the counterintuitive observation that the anionic viscosin is more active against anionic membranes than its cationic analog viscosin-E2K at $30 \mu\text{M}$. In the low-lipid regime, selectivity is governed by both partitioning and local membrane damage.

Electrostatic repulsion in a membrane leaflet increases the effective lateral area required by the polar groups and, hence, it increases asymmetry stress (causing transient leakage) and the spontaneous curvature within a leaflet (promoting equilibrium leakage). Electrostatic interactions may also interfere with the self-association of peptides, which might be important for their permeabilizing activity.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

SUPPORTING MATERIAL

Supporting material can be found online at <https://doi.org/10.1016/j.bpj.2022.07.033>.

AUTHOR CONTRIBUTIONS

J.S. performed the experiments, analyzed the data, and wrote the first draft of the manuscript. Y.V. synthesized viscosin E2K under the supervision of A.M. and N.G. Y.V. extracted pseudodesmin A and viscosin from natural

sources. All authors jointly designed the research, discussed the results, and finalized the manuscript.

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This study is dedicated to Klaus Gawrisch, highlighting his great contributions to membrane biophysics and to the friendly, open-minded, and constructive spirit of our scientific community. H.H. thanks Klaus for having been a key mentor throughout his scientific career and a dear friend. We celebrate you whenever we refer to your results, ideas, and initiatives and at each sushi dinner (be it for the PUFA or not).

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Pirri, G., A. Giuliani, ..., A. Rinaldi. 2009. Lipopeptides as anti-infectives: a practical perspective. *Open Life Sci.* 4:258–273.
2. Raaijmakers, J. M., I. De Bruijn, ..., M. Ongena. 2010. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiol. Rev.* 34:1037–1062.
3. Mandal, S. M., A. E. A. D. Barbosa, and O. L. Franco. 2013. Lipopeptides in microbial infection control: scope and reality for industry. *Bio-technol. Adv.* 31:338–345.
4. Patel, S., S. Ahmed, and J. S. Eswari. 2015. Therapeutic cyclic lipopeptides mining from microbes: latest strides and hurdles. *World J. Microbiol. Biotechnol.* 31:1177–1193.
5. Cochrane, S. A., and J. C. Vederas. 2016. Lipopeptides from *Bacillus* and *Paenibacillus* spp.: a gold mine of antibiotic candidates. *Med. Res. Rev.* 36:4–31.
6. Cardoso, P., H. Glossop, ..., C. Valery. 2021. Molecular engineering of antimicrobial peptides: microbial targets, peptide motifs and translation opportunities. *Biophys. Rev.* 13:35–69.
7. Koo, H. B., and J. Seo. 2019. Antimicrobial peptides under clinical investigation. *Pept. Sci.* 111:e24122.
8. Malviya, D., P. K. Sahu, ..., G. P. Brahmaprakash. 2020. Lesson from ecotoxicity: revisiting the microbial lipopeptides for the management of emerging diseases for crop protection. *Int. J. Environ. Res. Public Health.* 17:1434.
9. Crouzet, J., A. Arguelles-Arias, ..., S. Dorey. 2020. Biosurfactants in plant protection against diseases: rhamnolipids and lipopeptides case study. *Front. Bioeng. Biotechnol.* 8:1014.
10. Blake, C., M. N. Christensen, and Á. T. Kovács. 2021. Molecular aspects of plant growth promotion and protection by *Bacillus subtilis*. *Mol. Plant Microbe Interact.* 34:15–25.
11. EPPO. Databases of Registered PPPs.
12. Ma, Z., M. Ongena, and M. Höfte. 2017. The cyclic lipopeptide orfamide induces systemic resistance in rice to *Cochliobolus miyabeanus* but not to *Magnaporthe oryzae*. *Plant Cell Rep.* 36:1731–1746.

13. Pršić, J., and M. Ongena. 2020. Elicitors of plant immunity triggered by beneficial bacteria. *Front. Plant Sci.* 11:594530.
14. Girard, L., M. Höfte, and R. De Mot. 2020. Lipopeptide families at the interface between pathogenic and beneficial *Pseudomonas*-plant interactions. *Crit. Rev. Microbiol.* 46:397–419.
15. Raaijmakers, J. M., I. de Bruijn, and M. J. D. de Kock. 2006. Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: diversity, activity, biosynthesis, and regulation. *Mol. Plant Microbe Interact.* 19:699–710.
16. Ongena, M., and P. Jacques. 2008. Bacillus lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol.* 16:115–125.
17. Schneider, T., A. Müller, ..., H. Gross. 2014. Cyclic lipopeptides as antibacterial agents – potent antibiotic activity mediated by intriguing mode of actions. *Int. J. Med. Microbiol.* 304:37–43.
18. Geudens, N., M. N. Nasir, ..., M. Deleu. 2017. Membrane interactions of natural cyclic lipodepsipeptides of the viscosin group. *Biochim. Biophys. Acta. Biomembr.* 1859:331–339.
19. Geudens, N., and J. C. Martins. 2018. Cyclic lipodepsipeptides from *Pseudomonas* spp. – biological Swiss-army knives. *Front. Microbiol.* 9:1867.
20. Götz, S., and P. Stallforth. 2020. Structure, properties, and biological functions of nonribosomal lipopeptides from pseudomonads. *Nat. Prod. Rep.* 37:29–54.
21. Saini, H. S., B. E. Barragán-Huerta, ..., R. M. Maier. 2008. Efficient purification of the biosurfactant viscosin from *Pseudomonas libanensis* strain M9-3 and its physicochemical and biological properties. *J. Nat. Prod.* 71:1011–1015.
22. Sinnaeve, D., C. Michaux, ..., J. C. Martins. 2009. Structure and X-ray conformation of pseudodesmins A and B, two new cyclic lipodepsipeptides from *Pseudomonas* bacteria. *Tetrahedron.* 65:4173–4181.
23. Sinnaeve, D., P. M. S. Hendrickx, J. C. Martins, ..., 2009. The solution structure and self-association properties of the cyclic lipodepsipeptide pseudodesmin A support its pore-forming potential. *Chem. Eur. J.* 15:12653–12662.
24. Crowet, J.-M., D. Sinnaeve, ..., L. Lins. 2019. Molecular model for the self-assembly of the cyclic lipodepsipeptide pseudodesmin A. *J. Phys. Chem. B.* 123:8916–8922.
25. Sinnaeve, D., M.-A. Delsuc, ..., B. Kieffer. 2012. Insight into peptide self-assembly from anisotropic rotational diffusion derived from ¹³C NMR relaxation. *Chem. Sci.* 3:1284–1292.
26. Geudens, N., B. Kovács, ..., J. C. Martins. 2019. Conformation and dynamics of the cyclic lipopeptide viscosinamide at the water-lipid interface. *Molecules.* 24:E2257.
27. De Vleeschouwer, M., D. Sinnaeve, ..., A. Madder. 2014. Rapid total synthesis of cyclic lipodepsipeptides as a premise to investigate their self-assembly and biological activity. *Chemistry* 20:7766–7775.
28. De Vleeschouwer, M., T. Van Kersavond, ..., A. Madder. 2020. Identification of the molecular determinants involved in antimicrobial activity of pseudodesmin A, a cyclic lipopeptide from the viscosin group. *Front. Microbiol.* 11:646.
29. De Roo, V., Y. Verleysen, J. C. Martins, ..., 2022. An NMR fingerprint matching approach for the identification and structural re-evaluation of *Pseudomonas* lipopeptides. *Microbiol. Spectr.* <https://doi.org/10.1101/2022.01.07.475420>.
30. Brown, K. L., and R. E. W. Hancock. 2006. Cationic host defense (antimicrobial) peptides. *Curr. Opin. Immunol.* 18:24–30.
31. Bhattacharjya, S., and A. Ramamoorthy. 2009. Multifunctional host defense peptides: functional and mechanistic insights from NMR structures of potent antimicrobial peptides. *FEBS J.* 276:6465–6473.
32. Nguyen, L. T., E. F. Haney, and H. J. Vogel. 2011. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol.* 29:464–472.
33. Lohner, K. 2009. New strategies for novel antibiotics: peptides targeting bacterial cell membranes. *Gen. Physiol. Biophys.* 28:105–116.
34. Heerklotz, H., and J. Seelig. 2007. Leakage and lysis of lipid membranes induced by the lipopeptide surfactin. *Eur. Biophys. J.* 36:305–314.
35. Heerklotz, H., and S. Keller. 2013. How membrane partitioning modulates receptor activation: parallel versus serial effects of hydrophobic ligands. *Biophys. J.* 105:2607–2610.
36. Savini, F., V. Luca, ..., L. Stella. 2017. Cell-density dependence of host-defense peptide activity and selectivity in the presence of host cells. *ACS Chem. Biol.* 12:52–56.
37. Loffredo, M. R., F. Savini, ..., L. Stella. 2021. Inoculum effect of antimicrobial peptides. *Proc. Natl. Acad. Sci. USA.* 118. e2014364118.
38. Lohner, K., and E. J. Prenner. 1999. Differential scanning calorimetry and X-ray diffraction studies of the specificity of the interaction of antimicrobial peptides with membrane-mimetic systems. *Biochim. Biophys. Acta.* 1462:141–156.
39. Malanovic, N., and K. Lohner. 2016. Gram-positive bacterial cell envelopes: the impact on the activity of antimicrobial peptides. *Biochim. Biophys. Acta.* 1858:936–946.
40. Wider, G., and L. Dreier. 2006. Measuring protein concentrations by NMR spectroscopy. *J. Am. Chem. Soc.* 128:2571–2576.
41. Fan, H. Y., M. Nazari, ..., H. Heerklotz. 2014. Utilizing zeta potential measurements to study the effective charge, membrane partitioning, and membrane permeation of the lipopeptide surfactin. *Biochim. Biophys. Acta.* 1838:2306–2312.
42. Zhang, H. 2017. Thin-film hydration followed by extrusion method for liposome preparation. In *Liposomes: Methods and Protocols*. G. G. M. D'Souza, ed. Springer, pp. 17–22.
43. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466–468.
44. Patel, H., C. Tscheka, and H. Heerklotz. 2009. Characterizing vesicle leakage by fluorescence lifetime measurements. *Soft Matter.* 5:2849–2851.
45. Steigenberger, J., Y. Verleysen, ..., H. Heerklotz. 2021. The optimal lipid chain length of a membrane-permeabilizing lipopeptide results from the balance of membrane partitioning and local damage. *Front. Microbiol.* 12:669709.
46. Lakowicz, J. R. 2006. Principles of Fluorescence Spectroscopy, Third edition. Springer.
47. Wierprecht, T., M. Beyermann, and J. Seelig. 1999. Binding of antibacterial magainin peptides to electrically neutral membranes: thermodynamics and structure. *Biochemistry.* 38:10377–10387.
48. Coraiola, M., P. Lo Cantore, ..., M. Dalla Serra. 2006. WLIP and tolaasin I, lipodepsipeptides from *Pseudomonas reactans* and *Pseudomonas tolaasii*, permeabilise model membranes. *Biochim. Biophys. Acta.* 1758:1713–1722.
49. Fiedler, S., and H. Heerklotz. 2015. Vesicle leakage reflects the target selectivity of antimicrobial lipopeptides from *Bacillus subtilis*. *Biophys. J.* 109:2079–2089.
50. Encinas, M. V., and E. A. Lissi. 1982. Evaluation of partition constants in compartmentalised systems from fluorescence quenching data. *Chem. Phys. Lett.* 91:55–57.
51. de la Maza, A., and J. L. Parra. 1997. Solubilizing effects caused by the nonionic surfactant dodecylmaltoside in phosphatidylcholine liposomes. *Biophys. J.* 72:1668–1675.
52. Heerklotz, H. 2008. Interactions of surfactants with lipid membranes. *Q. Rev. Biophys.* 41:205–264.
53. McLaughlin, S. 1989. The electrostatic properties of membranes. *Annu. Rev. Biophys. Biophys. Chem.* 18:113–136.
54. Beschiaschvili, G., and J. Seelig. 1992. Peptide binding to lipid bilayers. Nonclassical hydrophobic effect and membrane-induced pK shifts. *Biochemistry.* 31:10044–10053.
55. Keller, S., H. Heerklotz, and A. Blume. 2006. Monitoring lipid membrane translocation of sodium dodecyl sulfate by isothermal titration calorimetry. *J. Am. Chem. Soc.* 128:1279–1286.

56. McLaughlin, S., and H. Harary. 1976. The hydrophobic adsorption of charged molecules to bilayer membranes: a test of the applicability of the Stern equation. *Biochemistry*. 15:1941–1948.
57. Matsuzaki, K., O. Murase, and K. Miyajima. 1995. Kinetics of pore formation by an antimicrobial peptide, magainin 2, in phospholipid bilayers. *Biochemistry*. 34:12553–12559.
58. Andersson, A., J. Danielsson, ..., L. Mäler. 2007. Kinetic models for peptide-induced leakage from vesicles and cells. *Eur. Biophys. J.* 36:621–635.
59. Almeida, P. F., and A. Pokorny. 2009. Mechanisms of antimicrobial, cytolytic, and cell-penetrating peptides: from kinetics to thermodynamics. *Biochemistry*. 48:8083–8093.
60. Guha, S., J. Ghimire, ..., W. C. Wimley. 2019. Mechanistic landscape of membrane-permeabilizing peptides. *Chem. Rev.* 119:6040–6085.
61. Wimley, W. C., and K. Hristova. 2019. The mechanism of membrane permeabilization by peptides: still an enigma. *Aust. J. Chem.* 73:96–103.
62. Schefter, B. R., S. Nourbakhsh, ..., B.-Y. Ha. 2021. Modeling cell selectivity of antimicrobial peptides: How is the selectivity influenced by intracellular peptide uptake and cell density. *Front. Med. Technol.* 3:626481.
63. Marrink, S. J., A. H. de Vries, and D. P. Tieleman. 2009. Lipids on the move: simulations of membrane pores, domains, stalks and curves. *Biochim. Biophys. Acta*. 1788:149–168.
64. Esteban-Martín, S., H. J. Risselada, ..., S. J. Marrink. 2009. Stability of asymmetric lipid bilayers assessed by molecular dynamics simulations. *J. Am. Chem. Soc.* 131:15194–15202.
65. Dietel, L., L. Kalie, and H. Heerklotz. 2020. Lipid scrambling induced by membrane-active substances. *Biophys. J.* 119:767–779.
66. Patel, H., C. Tscheka, ..., H. Heerklotz. 2011. All-or-none membrane permeabilization by fengycin-type lipopeptides from *Bacillus subtilis* QST713. *Biochim. Biophys. Acta*. 1808:2000–2008.