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# Determining the mechanism of membrane permeabilizing peptides: Identification of potent, equilibrium pore-formers

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ABSTRACT

To enable selection and characterization of highly potent pore-forming peptides, we developed a set of novel assays to probe 1) the potency of peptide pores at very low peptide concentration; 2) the presence or absence of pores in membranes after equilibration; 3) the interbilayer exchangeability of pore-forming peptides; and 4) the degree to which pore-forming peptides disrupt the bilayer organization at equilibrium. Here, we use these assays to characterize, in parallel, six membrane-permeabilizing peptides belonging to multiple classes. We tested the antimicrobial peptides LL37 and dermaseptin S1, the well-known natural lytic peptides melittin and alamethicin, and the very potent lentivirus lytic peptides LLP1 and LLP2 from the cytoplasmic domain of HIV GP41. The assays verified that that the antimicrobial peptides are not potent pore formers, and form only transient permeabilization pathways in bilayers which are not detectable at equilibrium. The other peptides are far more potent and form pores that are still detectable in vesicles after many hours. Among the peptides studies, alamethicin is unique in that it is very potent, readily exchanges between vesicles, and disturbs the local bilayer structure even at very low concentration. The equally potent LLP peptides do not exchange readily and do not perturb the bilayer at equilibrium. Comparison of these classes of pore forming peptides in parallel using the set of assays we developed demonstrates our ability to detect differences in their mechanism of action. Importantly, these assays will be very useful in high-throughput screening where highly potent pore-forming peptides can be selected based on their mechanism of action.



## 1. Introduction

Peptides that self-assemble into pores or channels across membranes have potential utility as biosensor platforms [1,2], targetable cytotoxins [3,4], chemo-sensitizing agents [5], and exogenous ion channels, as suggested for the treatment of cystic fibrosis, for example [6]. In order to obtain peptides with specific properties, one must be able to design them *de novo*, rationally engineer known pore-forming peptides, or select peptides with the desired properties from peptide libraries. For example, designing an ideal biosensor peptide would require the identification of a peptide that self-assembles into long-lived, transmembrane pores that remain open and stably inserted in membranes [7,8]. While there are many membrane permeabilizing peptides and a range of mechanisms by which such peptides can act [9-12], there are few, if any, that are known to self-assemble into long-lived multimeric, transmembrane pores. Most pore forming peptides are in dynamic equilibrium between free and bound states and/or between monomeric and oligomeric states in the membrane [13,14]. Therefore, the design or selection of a stable pore-forming peptide would be a significant advance in bioengineering. But there are barriers to this goal arising, in part, from the knowledge gap in the fundamental principles of folding and assembly of peptide pores in membranes. Furthermore, the lack of simple analytical tools for characterizing pore-forming peptides at very low peptide concentrations (≤0.1 mol% peptide) where a useful biosensor would be active has hindered the design and selection of peptides with such properties.

A long-term goal of our work is to discover peptides that assemble into such long-lived pores in membranes by selecting them from combinatorial peptide libraries using high-throughput methods [15-18]. For this purpose we have designed a novel set of analytical tools that can be used to help identify such peptides by measuring four properties at very low peptide to lipid ratio: 1) the overall potency of a peptide pore-former in lipid vesicles; 2) the continued existence (or absence) of peptide pores at equilibrium; 3) the interbilayer exchangeability of pore-forming peptides; and 4) the degree to which pore forming peptides disrupt the bilayer organization at equilibrium. A membrane pore could theoretically be detectable at concentrations as low as to 1–2 pores (i.e. 4–20 peptides) per vesicle. Furthermore, a long-lived pore that could potentially be useful as a biosensor will, by definition, remain active in a membrane at equilibrium, will not perturb the lipid organization, and will not exchange in and out of bilayers on the time scale of at least a few hours.

In the work presented here, we use a set of analytical tools we developed to characterize, in parallel, the behavior of six known α-helical,

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membrane-permeabilizing peptides in synthetic lipid vesicles. The test peptides consist of cationic host-defense antimicrobial peptides as well as amphipathic α-helical pore forming peptides, including the well-known peptides, alamethicin and melittin. The experiments, performed at peptide concentrations as low as 50 peptides/vesicle, reveal observable differences in the behavior of the different classes of pore-forming peptides, and demonstrate ways in which one could select for particular mechanisms of action in high-throughput screens. The cationic antimicrobial host defense peptides are inactive at low peptide to lipid ratios, and show little or no evidence of equilibrium pores at any concentration. The lytic helical peptides, on the other hand, form explicit pores in vesicles that can be detected at equilibrium, even at very low peptide concentration. While it is known that there are multiple mechanisms of membrane permeabilization by peptides, members of multiple classes have rarely been studied in parallel using the same techniques. In the parallel studies described here, we demonstrate that the assays we describe here can readily distinguish between different mechanisms of peptide pore formation in membranes, and can be used to select peptides from libraries that act via particular mechanisms to permeabilize membranes.

## 2. Materials and methods

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) and 1,2, dioleoyl-sn-3-glycero-phosphoethanolamine-N-(7-nitro-2-1,3-benxodiazol-4-yl) (NBD-PE) were purchased from Avanti Polar Lipids. Alamethicin, melittin, dermaseptin S1, dipicolinic acid, $TbCl_3$, and reduced triton-X 100 were from purchased from Sigma-Aldrich Chemical Co. LL-37 was purchased from Innovagen. NBD-labeled lysolipids were synthesized for the flip-flop assay. Briefly, NBD-succinamidyl ester was coupled to lyso-phosphatidylethanolamine (lyso-PE) and purified by thin layer chromatography (TLC). The NBD-labeled lysolipid end product was confirmed by MALDI-MS. With the exception of alamethicin, peptide stocks were stored in $H_2O$ or buffer. Concentrated alamethicin stocks were stored in methanol and were diluted into buffer for all experiments.

### 2.1. Liposome preparation

Lipid aliquots in chloroform were vacuum dried overnight, suspended in buffer and freeze–thawed twenty times. Large unilamellar vesicles (LUVs) were formed by extruding the lipid suspension ten times through two stacked 100 nm nucleopore polycarbonate filters [19]. For the *two step* assay, vesicles were prepared with 1 mol% headgroup-labeled diacyl NBD-PE. The buffer consisted of 50 mM $TbCl_3$, 100 mM sodium citrate, and 10 mM TES at pH 7.2. After the extrusion process, liposomes were separated from external terbium via gel filtration chromatography [16,17] using a solution containing 300 mM NaCl to balance the ionic strength of $TbCl_3$. The buffer for all experiments contained 300 mM NaCl and 10 mM TES, pH 7.2. For leakage assays 50–100 μM of DPA was added to the buffer. For flip-flop experiments, the NBD-labeled lyso-lipids were dried together with POPC and POPG at 1 mol% NBD-lysoPE. Acceptor vesicles in the flip-flop assay were prepared with 1 mol% N-lissamine rhodamine B-labeled-DOPE as a collisional quencher of NBD-lysolipid. Lipid concentration was determined by the Bartlett assay.

### 2.2. Two step assay

The *two step* assay was designed to measure, in the same vesicles, membrane permeabilization and long-term access to vesicle interior through equilibrium peptide pores. Large unilamellar vesicles made from POPC or from 90/10 POPC/POPG containing entrapped terbium and 1% NBD-labeled diacyl phospholipids were incubated for at least 8 hours with peptide and external dipicolinic acid in 96-well microplates. Peptide concentration was 1 μM and terbium/NBD vesicles were always at 200 μM final concentration. To change the stringency (i.e. the overall P:L ratio) liposomes without $Tb^{3+}$ or NBD were added. Wells with liposomes only were used as negative controls, and wells with liposomes plus 0.1% final concentration of reduced Triton-X 100 detergent were used as positive controls. After incubating for at least 8 hours to allow the system to reach equilibrium, terbium leakage was measured, followed by measurements of interior access by dithionite quenching of NBD. Measurements were performed on a Synergy-2 Biotek microplate reader. DPA–Terbium complex fluorescence was detected with an excitation filter of 284/11 (band pass maximum/half-width) and emission filter of 530/25, using a xenon flash lamp with 250 μs delay and 1 ms retention time. Sensitivity was adjusted so that the positive controls' fluorescence was roughly 30% of the instrument maximum. The percentage of terbium released from samples was determined using the equation:

$$\%\ \text{Leakage} = \left(\frac{F_{\text{sample}} - F_{\text{background}}}{F_{\text{Triton}} - F_{\text{background}}}\right) \times 100\%$$

Where $F_{\text{sample}}$ is the fluorescence intensity of the sample, $F_{\text{background}}$ is the fluorescence intensity of lipids only as the negative control, and $F_{\text{Triton}}$ is the fluorescence intensity of a separate sample lysed with triton-X detergent as the positive control. In the second step of the assay, the initial fluorescence intensity of the NBD-labeled lipids was recorded using 480/25 excitation and 530/25 emission filters. Sodium dithionite (10 μL of a freshly prepared at 0.6 M in 1.0 M sodium phosphate, pH 10.0 for each experiment) was added to each well and then the fluorescence was measured every 5 min for up to 70 min. NBD fluorescence was rapidly quenched by dithionite with a half time of 5–10 min. Intensity reached a plateau by 30–60 min. In the presence of Triton X-100, the NBD intensity was quenched completely. The percent of NBD quenched was calculated using the equation:

$$\%\,\text{Quenched} = \left(1 - \frac{F_{\text{final}} - F_{\text{background}}}{F_{\text{Initial}} - F_{\text{background}}}\right) \times 100\%$$

### 2.3. Flip-flop Assay

Transbilayer lipid movement (flip-flop) was detected by a modification of an assay that is described in detail elsewhere [20]. Briefly, we assessed flip-flop in POPC vesicles by monitoring the exchange of NBD-lysolipid between vesicles. NBD-lysolipids on the outer monolayer of a vesicle exchange rapidly between vesicles through the aqueous phase. Inner monolayer lipids are not normally exchangeable into other vesicles due to very slow equilibration between monolayers. Thus, normally only about half the lysolipid is exchangeable. If a peptide or other molecule perturbs the bilayer enough to induce rapid transbilayer lipid exchange, then all of the NBD-lysolipid will be exchangeable. Here we performed flip-flop experiments to examine the degree of bilayer disruption by pore-forming peptides at equilibrium. The fluorescence of NBD-lysolipid was measured on a SLM-Aminco fluorescence spectrometer with excitation and emission wavelengths at 464 nm and 532 nm respectively. All assays were at P:L = 0.001 with 800 nM peptide, 160 μM POPC LUVs symmetrically labeled with lyso-NBD and 640 μM non-fluorescent POPC LUVs. After equilibration with peptide, fluorescence was monitored for 2–5 min at the initial conditions, and then 250 μM rhodamine-labeled POPC liposomes were added and the fluorescence was recorded for 10 min. Exchange of the NBD-lysolipid in the outer monolayer into the rhodamine vesicles caused a decrease in fluorescence due to quenching. As a positive control for flip-flop (i.e. exchange of outer and inner monolayer NBD-lysolipid) we added 0.6 nmol alamethicin (P:L = 0.006) at about 10 min and recorded an additional 15 min of data. Previous

work with the assay showed that this concentration of alamethicin induces rapid flip-flop immediately upon addition to liposomes [20,21].

### 2.4. Incremental leakage assay

The terbium-leakage assay described above was modified to address whether a peptide's pore-forming activity involves a dynamic process of peptide equilibration into and out of membranes or whether the peptide pores, once formed, are stably inserted into membranes. To examine this, the *incremental* assay was implemented to detect pore-forming activity in sequential additions of liposomes. Vesicles (9:1 POPC:POPG) with entrapped terbium were added to the same peptide solution in three separate additions. The lipid concentration was constant at P:L = 0.001 (1:1000) for each increment except for melittin which was studied at P:L = 0.005 (1:200) for each increment, due to its lower activity. Peptides were allowed at least 8 hour incubation before each measurement. With each volume of liposomes added, the leakage activity was determined using the following equation:

$$\%\ \text{Leakage}^{i} = \left(\frac{F^{i}_{\text{sample}} - F^{i-1}_{\text{sample}} - F^{i}_{\text{background}}}{F^{i}_{\text{Triton}} - F^{i-1}_{\text{Triton}} - F^{i}_{\text{background}}}\right) \times 100\%$$

$F_i$ is the fluorescence of sample well with $i$ lipid additions, $F_{i-1}$ is the fluorescence of same sample with $i-1$ lipid additions (i.e. before the $i$th addition). $F_{\text{Triton}}$ is the value of a detergent lysed control sample for the *I or the i − 1* lipid addition, measured in a different sample. $F_{\text{background}}$ is the background intensity from the wells of liposomes only of the $i$th lipid addition. All experiments were performed in triplicate and averaged.

### 2.5. Serial dilution assay

The pore-forming activity of peptides requires them to partition into membranes and disrupt the lipid bilayer. In the simplest model of pore-formation, the peptide activity should depend on how much peptide is bound to membrane per unit area, i.e. bound peptide to lipid ratio ($P_B$:L). Experimentally, one should be able to use the mole fraction partition coefficient ($K_X$) and the P:L ratio to ascertain the critical bound peptide-to-lipid ratio (P:$L_B$) for alamethicin activity. We used the serial dilution assay to test this idea. In this assay, alamethicin was added to terbium containing POPC/POPG (90/10) LUVs in 96-well microplates. Each column had a fixed total P:L ratio, but from the top to bottom rows had decreasing overall peptide and overall lipid concentrations. The intensity for complete leakage was determined by adding Triton X-100 to control wells containing the appropriate amount of lipid vesicles. As a control for loss of peptide at low concentration due to adhesion to the wells of the 96 well plates, we repeated the serial dilution assays in the presence of bovine serum albumin (BSA), which blocks peptide binding sites, and found no difference in the results.

## 3. Results

### 3.1. The peptides studied

The goals of this work were to develop a set of novel assays that will enable us to explore the detailed mechanism of peptide pore-formation and to select peptides with particular mechanisms of action from peptide libraries. Here we use the assays that we developed to characterize, in parallel, six α-helical, membrane permeabilizing peptides that cover a range of proposed mechanisms of action. First we used two well-characterized antimicrobial host-defense peptides; human LL37 and the tree frog peptide dermaseptin S1. We and others have suggested that this class of peptides permeabilize membranes by causing transient bilayer destabilization without formation of explicit pores [10,12]. We also studied four lytic helical peptides that have been proposed to form explicit pores in membranes: Alamethicin, a fungal peptaibol [13,22], melittin, the lytic peptide form European Honey Bee venom [23], and the lentivirus lytic peptides LLP1 and LLP2 from the cytoplasmic domain of HIV GP41 which have been shown to be highly potent pore-formers [24]. The chemical and structural properties of the six peptides studied in this work are given in Table 1.

### 3.2. Potent, equilibrium peptide pores in vesicles: The two step assay

For this work, we developed the orthogonal, *two step assay* which can be used to independently assess the potency of a pore-forming peptide in lipid vesicles (step one) and the continued presence of peptide pores at equilibrium (step two). The two measurements are made sequentially in the same vesicles. The second step is important because it distinguishes true pore-forming peptides from interfacially-active peptides, such as antimicrobial peptides [10,25], which often cause leakage only transiently (i.e. only for a short time immediately after addition to vesicles). The second step does not distinguish between stable, long-lived pores and dynamic pores that fluctuate in and out of existence with short lifetimes. However it does assess whether or not a leakage pathway (of any type) exists in the membrane at equilibrium. For the purposes of these experiments we define equilibrium as at least 8 hours after addition of peptide to vesicles. Given the fact that binding, leakage and structure transitions in vesicles usually take place on time scales of tens of minutes after addition [26,27] this is a reasonable definition. In the first step of the assay, peptide-induced leakage from vesicles is assessed using the binary terbium/dipicolinic acid assay we have described elsewhere [17,18]. Vesicles have entrapped terbium ($Tb^{3+}$) which can form a luminescent complex with the aromatic chelator dipicolinic acid (DPA), present in the external solution; but only if the membranes are permeabilized. In the second step of the assay, access to lipid headgroups on the inner monolayer of the same vesicles is assessed *after equilibration* by measuring the ability of the membrane-impermeant reducing agent dithionite (M.W. 128 Da) to chemically quench the dye NBD linked to lipids that are symmetrically distributed on the inner and outer leaflets of the bilayer vesicle. In vesicles without peptide or without pores, only dye-labeled lipids exposed on the outer leaflet (~55% of the total lipid) can be quenched. Quenching of the interior NBD lipids after equilibration can occur only if a peptide pore allows dithionite access to inside of the vesicle, or if a peptide disturbs the bilayer enough to allow transbilayer equilibration (flip-flop) of NBD-lipids to the outer monolayer.

In Fig. 1 we show the results of *two step* assays performed on the six membrane-permeabilizing peptides studied. For all measurements peptide concentrations were constant at 1 μM and the lipid concentration was varied to achieve different peptide to lipid (P:L) ratios as shown. We used vesicles made from zwitterionic POPC lipids and vesicles that also contain 10 mol% anionic POPG. Differences between the two lipid compositions were minor. The two antimicrobial peptides, LL37 and dermaseptin S1 caused measurable leakage only at the highest P:L ratios studied and allowed essentially no access to the vesicle interior at equilibrium. The only exception was dermaseptin S1 at the highest P:L studied, P:L = 0.02 (1:50), and only in PG-containing vesicles. Under these conditions high leakage and high access to NBD lipids were observed. However, at this high P:L ratio, detergent-like, physical disruption of vesicle architecture is possible [28], which would also allow access to interior NBD-lipids. In support of this idea, dermaseptin S1, at P:L = 0.005 (1:200), causes nearly 80% leakage while allowing no access to the vesicle interior at equilibrium. Equilibrium pores are not required for the permeabilizing activity of dermaseptin or LL37.

**Table 1**
Properties and sequences of the peptides studied.

| Peptide | Organism | Length (aa) | Charges Pos/Neg | 2° Structure | Amino acid sequence |
|---|---|---|---|---|---|
| LL37 | Human | 37 | +12/−6 | α | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES |
| Dermaseptin S1 | Tree Frog | 34 | +5/−2 | α | ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ |
| Melittin | Honey Bee | 26 | +6/0 | α | GIGAVLKVLTTGLPALISWIKRKRQQ-amide |
| LLP1 | HIV | 28 | +8/−3 | α | RVIEVVQGACRAIRHIPRRIRQGLERIL |
| LLP2 | HIV | 20 | +5/−3 | α | YHRLRDLLLIVTRIVELLGR |
| Alamethicin | fungus | 20 | +0/−0 | α | Ac-UPUAUAQUVUGLUPVUUQQF-OH (U = AiB) |

Properties of the peptides studied in this work. Charges include the amino and carboxyl termini, where appropriate, but do not include histidine. All of the peptides have α-helical secondary structure when bound to membranes. Alamethicin is secreted as a heterogeneous mixture of closely related peptides. Commercial sources contain peptides either predominantly from the uncharged (Q18) group or mostly from the anionic (E18) group. The peptide we worked with here is the uncharged form with glutamine in the 18th position.

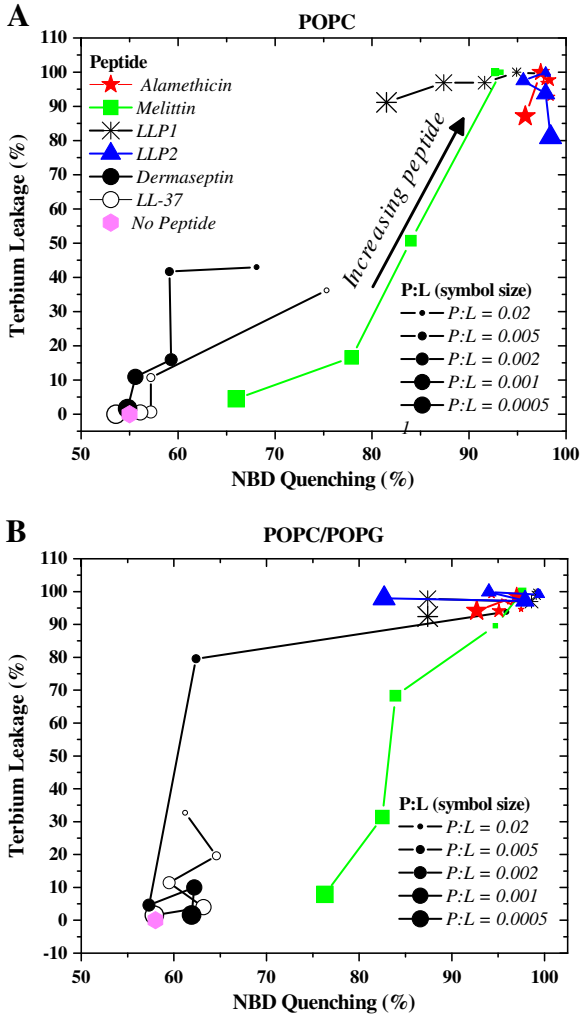


**Fig. 1.** The two step assay. The assay consists of orthogonal measurements of peptide-induced leakage from vesicles (step one) and access of a non-membrane permeable quencher, dithionite, to the vesicle interiors through peptide pores at equilibrium (step two). We assume that equilibration is reached by 8 hours. We performed *two step* assays with six peptides at P:L ranging from 0.02 to 0.0005 (1:50 to 1:2000). Larger points signify higher lipid concentration (lower P:L ratio). A: Two step assay using 100% POPC vesicles. B: *Two step* assay using 90:10 POPC:POPG vesicles. In each measurement 1 μM peptide was used along with vesicles with entrapped terbium which also contained 1 mol% dioleoylphosphatidylethanolamine lipid labeled on the headgroup with the dye NBD. Different lipid concentrations were used to bring the total P:L ratio to one of the 5 values studied. After incubation, leakage of terbium was measured as described above, followed by quenching of accessible NBD by the addition freshly prepared dithionite. Vesicles treated with triton-X detergent were the positive controls for all experiments. Samples with liposomes only (no peptide) were used as negative controls. Vesicles alone (negative control) showed no detectable terbium leakage and had NBD-quenching around 55%, corresponding to the external NBD lipids.

At the other end of the activity spectrum, alamethicin and the LLP peptides showed potent, equilibrium pore-formation at all concentrations studied as evidenced by high leakage of terbium and high access of the quencher to interior lipids at equilibrium indicating equilibrium pores. This was true at P:L ratios as low as 0.0005 (1:2000) which is equivalent to only about 50 peptides per vesicle. In fact, alamethicin shows measurable pore-forming activity at P:L ratios as low as 0.0001 (1:10000 or 10 peptides per vesicle, see supplemental Figure S1). Melittin formed equilibrium pores in vesicles also, but only at P:L ratios of 0.005 (1:200) or higher. At P:L ratios ≤ 0.002 melittin's activity was diminished, suggesting that its monomer-oligomer (pore) equilibrium, under the conditions of these experiments, is shifted more towards peptide monomer or another inactive form relative to alamethicin and the LLP peptides. The elevated NBD quenching with low leakage that occurs with melittin at low P:L suggests that melittin-dependent bilayer disruption can increase the flip-flop of NBD lipids.

### 3.3. Bilayer disruption at low peptide concentration: the flip-flop assay

In the *two step* assay, NBD quenching greater than ~55% indicates either that the quencher dithionite has access to the vesicle interior through an equilibrium peptide pore or that the peptide disrupts the bilayer enough to allow the interior lipids to equilibrate across the membrane to the outer bilayer where they can be quenched. Because transbilayer equilibration of lipids is normally very slow (half time = days) [20,29] rapid transbilayer equilibration of lipids (half time < 1 hour) would require a substantial local disruption of bilayer packing and organization. A peptide that induces rapid flip-flop will probably be indistinguishable from one that forms a true pore because a disturbance to the bilayer packing that allows the translocation of zwitterionic lipid head groups would presumably also allow passage of other polar molecules. To assess if lipid flip-flop is occurring when pore forming peptides are present at equilibrium, we employed an equilibrium version of an assay that we described previously [20]. In this assay, the exchangeability of a dye-labeled lysolipid between vesicles is used to assess the rate of transbilayer equilibration. We have previously shown that alamethicin and other pore-forming peptides at high concentration cause rapid transbilayer equilibration of lipids immediately after addition to lipid vesicles [20] (see supplemental figure S2). In this work, we extended the measurements of flip-flop to vesicles containing low peptide concentrations (P:L = 0.001) that have been allowed to equilibrate before the assay.

We envision that a long-lived or stable pore, which might interact with the membrane in a way that resembles an integral membrane protein, would cause little disruption to the packing of the bilayer lipids, especially at very low concentration. On the other hand, a dynamic pore-forming peptide, such as one exchanging in and out of the bilayer, or acting through a toroidal or other disordered lipid pore [30] would be expected to catalyze flip-flop through local perturbation of the bilayer. All of the pore-forming peptides studied here caused lipid flip-flop at higher concentration, P:L > 0.005 (P:L > 1:200), immediately after addition to vesicles (supplemental Figure S2). However, after equilibration, at low P:L = 0.001 (1:1000) (Fig. 2) only alamethicin caused detectable lipid flip-flop. Neither melittin nor the LLP peptides caused any

measurable flip flop activity under these conditions. This result shows that alamethicin pores cause a significant and persistent local disruption of the bilayer architecture at equilibrium even at concentrations equivalent to only a few pores per vesicle. Melittin is not very active as a pore former at this concentration (Fig. 1), so the implications of the lack of flip-flop (in the presence of melittin) are not clear. On the other hand, the LLP peptides, like alamethicin, are very potent equilibrium pore formers at this concentration. Thus their lack of induced lipid flip-flop indicates that active LLP pores cause much less disruption of the bilayer than alamethicin pores under the same conditions.

### 3.4. Detection of exchanging pore-formers: the incremental assay

To be able to assess, in a high-throughput manner, the dynamic nature of peptide pore-formation in vesicles (on a timescale of minutes to hours) we developed the *incremental* leakage assay. This assay was used to test whether pore-forming peptides are stably embedded into lipid bilayers or if they can exchange between membranes and permeabilize additional vesicles. This is an important piece of information. For example, a peptide that is in dynamic equilibrium between bilayers would be far less attractive as the basis for a biosensor platform than a non-exchangeable peptide forming stably inserted or long-lived pores. On the other hand an exchangeable pore-former could make a more effective toxin or cytolytic agent. Thus we must be able to distinguish between non-exchangeable and exchangeable pores in a high throughput manner. This can be done with the incremental assay, which consists of a concentrated liposome solution with entrapped terbium and external DPA added sequentially, in three separate additions, to the same solution of peptide. Hours-long equilibration between additions is used to insure that we are examining equilibrium exchange. Because all the peptides studied here bind well to vesicles, there is little free peptide remaining in the aqueous phase after the first or second addition. Leakage from subsequent additions of vesicles requires that the peptides exchange between vesicles. In this *incremental* assay all the peptides are highly active against the first iteration of vesicles, but only alamethicin and melittin were very active against subsequent additions. The LLP peptides, which are extremely potent pore formers at the lipid concentrations used (see Fig. 1) would cause essentially 100% leakage overall if all three vesicle increments were added at once, but instead caused small amounts of leakage in subsequent vesicle additions. This result indicates that the LLP peptide pores do not readily exchange between vesicles on the timescale of several hours.

Our incremental assay results for alamethicin and melittin verify previous reports that they exchange between vesicles on a timescale of an hour or less [31]. Alamethicin showed 100% permeabilizing activity against three consecutive additions of vesicles at P:L = 0.001 (1:1000) (for each addition). Melittin also showed significant leakage from the second and third lipid additions at P:L = 1:200 as well (Fig. 3). Based on the measured partition coefficients (see supplemental data) the fraction of peptide bound to lipid was >95% with the initial lipid addition and higher for subsequent additions. Thus, the amount of alamethicin or melittin in solution available for direct partitioning into the second and third addition of vesicles is small. The halftime of leakage against the first increment of liposomes was ~15 min, and was ~30–45 min against the following increments. Nonetheless, significant leakage occurred after each increment of vesicles when alamethicin or melittin were used, indicating that they exchange between bilayers and form pores in new vesicles. We also showed that alamethicin can exchange between, and translocate across, the bilayers of multilamellar vesicles (MLVS, see supplemental

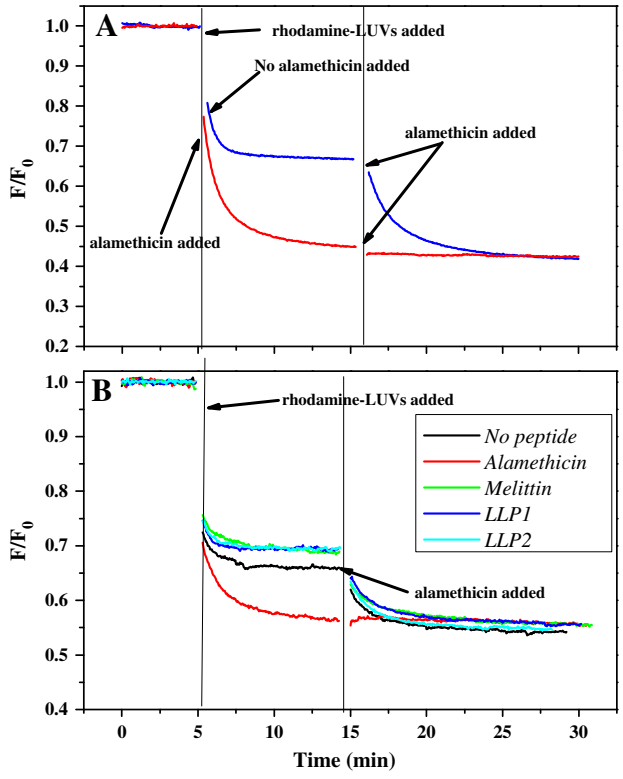


**Fig. 2.** The Flip-flop Assay. A: Example of the flip-flop assay in POPC vesicles using alamethicin. Vesicles containing 1 mol % NBD-labeled lysolipid are mixed an excess of vesicles containing 1 mol% rhodamine lipids, which quenches NBD fluorescence. The single chain NBD lyso-lipid can exchange rapidly from the outer monolayer of the donor vesicles into the rhodamine (acceptor) vesicles where its fluorescence is quenched. The diacyl rhodamine lipids do not exchange. The lysolipid on the inner monolayer does not readily equilibrate across the membrane unless the bilayer is perturbed. Alamethicin causes rapid transbilayer equilibration of lipids immediately upon addition [20]. Panel A contains control experiments which show NBD-lysolipid exchanging between vesicles in the presence and absence of alamethicin (added at 5 min). A second addition of alamethicin at 15 min allows complete equilibration of inner and outer monolayer lipids. B: Flip flop in POPC vesicles containing pore-forming peptides at equilibrium. Vesicles were pre-incubated for at least 8 hours with the four pore-forming peptides studied here, allowing the peptides to reach equilibrium. Then rhodamine-labeled vesicles were then added and exchange was monitored for 10 min. Addition of alamethicin at 15 min allowed all the available NBD lysolipid to exchange. All assays were performed at P:L = 0.001 (1:1000).

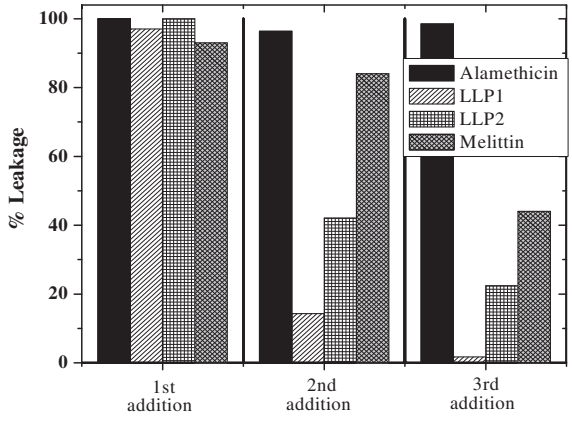


**Fig. 3.** The incremental assay. A single peptide solution of 1 μM is subjected to multiple additions of POPC/POPG (90/10) vesicles with entrapped $Tb^{3+}$ and external DPA. The leakage process for each addition of vesicles is allowed to incubate for at least 8 hours before the next addition to insure that we are examining equilibrium exchange. Each addition of vesicles is at P:L = 0.001 (1:1000) relative to the original peptide, except for the melittin experiment. Because melittin is less active overall we performed each lipid addition for melittin at P:L = 0.005 (1:200). Under these conditions, all four peptides would cause significant leakage if all increments were added at the same time. Significant leakage from the second or third additions of vesicles requires that the peptide be able to productively exchange between vesicles. Vesicles plus the detergent Triton-X 100 were used as a positive control and vesicles only as a negative control.

Figure S4), and that melittin does not. Translocation of melittin across bilayers is probably prevented by its highly cationic C-terminus.

At equilibrium in bilayers, melittin is predominantly oriented parallel to the membrane surface [23]. The transmembrane pore is thus a minor fraction of the total peptide. For this reason, it is not surprising that melittin exchanges between bilayers. It is interesting to note that alamethicin is predominantly in a transmembrane orientation in bilayers [32]. Yet it also exchanges readily between bilayers, suggesting that its interaction with the membrane generates an unstable membrane-spanning peptide-lipid structure from which the very hydrophobic alamethicin is able to exchange into the aqueous phase.

To further assess the mechanism of alamethicin exchange we repeated the incremental assay using vesicles containing 5 mol % PEG2000-headgroup conjugated lipids, which prevents close contact between vesicle bilayers [33]. The results were the same: alamethicin exchanged between vesicles and caused near complete leakage in multiple lipid additions (supplemental Figure S5A). Thus, alamethicin exchanges between vesicles through the aqueous phase, not by vesicle fusion or by contact-mediated transfer of peptide. Finally, we also used vesicles doped with fluorescent lipids to determine if lipid exchanges along with alamethicin (i.e. if the exchanging unit is a peptide/lipid micelle). We observed no lipid exchange between vesicles (supplemental Figure S5B). Alamethicin exchanges between vesicles in the form of free peptide or a peptide aggregate.

### 3.5. Threshold concentration for dynamic exchange: the serial dilution assay

To explore the nature of the exchanging unit of alamethicin we performed a serial dilution leakage assay [34]. We measured leakage from vesicles at a series of peptide concentrations (from 0.078 to 1.25 μM). For each peptide concentration, we used a set of fixed total P:L ratios ranging from 0.05 to 0.001 (1:20 to 1:1000). In Fig. 4A and B we show the observed leakage results for the alamethicin serial dilution experiments plotted in two different ways: versus total peptide concentration and versus total lipid concentration. In supplemental Figure S3 we show, for comparison, the results of a simple numerical simulation of a multimeric pore-forming peptide. Melittin has been shown to behave almost ideally in a serial dilution assay like this one [34]. While the experiments and simulations agree at low alamethicin concentrations (<400 nM), fundamental differences are apparent at higher alamethicin concentrations. Unexpectedly, the measured activity of alamethicin, as shown in Fig. 4B, appears to be mostly independent of overall peptide:lipid ratio and therefore is independent of peptide bound per lipid. For example the rightmost point of each curve in Fig. 4A corresponds to a P:L of 0.001( 1:1000). The strong binding of alamethicin, coupled with the same total P:L in this set of points leads to the prediction of similar leakage activity (assuming that leakage is dependent on bound peptide per lipid). Yet in the actual experiment, leakage in these points ranges from zero (at the lowest peptide concentration) to 100% (at the highest peptide concentration). Instead of depending, as expected, on the bound P:L ratio, leakage is more closely related to total peptide concentration. This can be seen in the family of leakage curves in Fig. 4B which all pass through a midpoint at a peptide concentration of about 3–400 nM peptide, with only a small dependence on P:L. (see also supplemental Fig. 3B). All of the leakage curves reach 100% leakage by 500 nM peptide, independent of total P:L.

At low alamethicin concentration, leakage decreases when lipid concentration is increased. This is expected, because the peptide bound per lipid ratio is reduced, and has been observed for other systems, including melittin [34]. Above about 400 nM, however, alamethicin appears to switch into a mode of "continuous" activity such that the amount of alamethicin present in a sample can permeabilize almost any amount of added lipid. Notice, for example, in Fig. 4A, the dramatic difference in behavior between 400 nM and ≥500 nM alamethicin. Above the critical alamethicin concentration (400 nM) the dilution of peptide with lipid does not significantly diminish net leakage activity. The simulations (supplemental Figure S3) do not predict this behavior. Most likely, the behavior of alamethicin above the threshold occurs because the peptide can sequentially permeabilize multiple vesicles by exchanging between them as an aggregate of peptide.

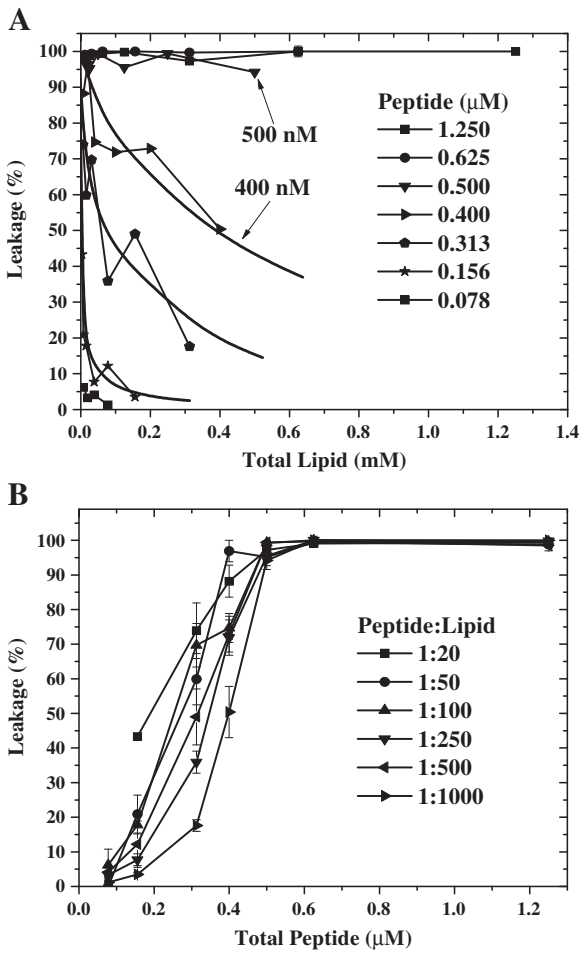


**Fig. 4.** Serial dilution assay. Alamethicin-induced leakage from POPC vesicles was measured at a set of peptide and lipid concentration corresponding to a set of six fixed total peptide:lipid ratios. The same data are plotted with different X-axes in panels A and B. A: Leakage is shown as a function of total lipid concentration. Each curve represents a set of experiments at the same peptide concentration. B: Leakage is shown as a function of total peptide concentration. Each curve represents a set of experiments at the same total peptide to lipid ratio.

## 4. Discussion

### 4.1. Novel tools for exploring the mechanism of pore-formation in vesicles

The analytical tools described here, especially the *two step* and *incremental* assays, can be used in a high-throughput format at very low peptide concentration to select for peptides that assemble into potent, non-exchanging equilibrium pores that would be suitable for biosensor applications, for example. Alternately they could be used to select for dynamic, exchanging pores that would, perhaps be highly potent cytotoxins. Although we performed the *two step* and *incremental* assays separately here, they can be combined into a single high-throughput assay. The novel *two step* assay provides a uniquely useful tool to study pore-forming peptides by allowing us to probe their potency and the functional state of the peptide pore after leakage

has taken place and after the peptide bilayer system has reached equilibration. We used the *two step* assay to verify that the antimicrobial peptides do not form equilibrium pores in vesicles, but instead cause short-lived, transient leakage events as we have discussed elsewhere [9,10]. It also showed that alamethicin and the LLP peptides are extremely potent and form equilibrium pores in lipid vesicles at P:L lower than P:L = 0.0005 (P:L = 1:2000 or 50 peptides per vesicle). These pores can be detected even after equilibration. The complementary *incremental* assay allows us to assess the dynamic nature of membrane binding and pore formation by peptides on time scale of minutes to hours, by assessing the exchangeability of pore forming peptides at equilibrium. This is an important consideration for biosensor design in which stable insertion is desired. The *flip flop* and *serial dilution* assays are not amenable to high throughput screening, but will be extremely useful, post-selection, to characterize peptide-induced perturbation of the bilayer architecture and to assess the mode of activity of pore forming peptides selected using the two-step and incremental assays.

### 4.2. Classes of membrane permeabilizing peptides

With the assays developed for this work it is possible to easily recognize at least three mechanistic classes of membrane permeabilizing peptides. Importantly for our future work, these techniques can also be used for high throughput screening to select for specific classes of membrane permeabilizing peptides. The antimicrobial peptides we studied here are not potent membrane-permeabilizing peptides and do not form equilibrium pores in membranes, even in anionic membranes. This is the typical behavior for many antimicrobial peptides [9,10,35] and is probably related to their need to be selective for bacterial versus eukaryotic membranes. Explicit equilibrium, transmembrane pores have been reported for LL37 [36], but we observe no evidence of transmembrane pores under any condition. *Alamethicin and melittin* are two of the most thoroughly studied pore forming peptides known. Their pore structure and the dynamic nature of their pore formation in membranes have been thoroughly described in the literature [13,22,23,37-40]. Here we used them to validate the assays developed for this work and to extend our description of their pore formation to low concentration, equilibrium conditions. Consistent with reports on alamethicin in the presence of a transmembrane potential [21,31] we found that alamethicin is especially dynamic, exchanging in and out of membranes and causing very significant disruption of lipid packing and bilayer architecture at equilibrium, even at P:L ratios as low as 1:1000, or 100 peptides per vesicle.

In terms of our goal of designing stably inserted peptide pores, alamethicin is an excellent example of the type of behavior we will have to *select against*. On the other hand, the lentivirus lytic peptides show the type of behavior we would be *selecting for* in a screen for stable pore formers. They are extremely potent, and create equilibrium pores in bilayers at low concentration. But unlike alamethicin, the LLP peptides do not exchange readily between bilayers and do not perturb the bilayer packing and organization at equilibrium. Additional focused studies are underway to examine the structure and detailed mechanism of the LLP pores in membranes.

## 5. Conclusions

Here we have described a set of novel experimental tools that we used to probe the character and dynamics of three classes of pore-forming peptides in lipid vesicles at low concentration, at equilibrium, and at time-scales that have not frequently been explored. With these techniques, we can easily distinguish between various classes of membrane-permeabilizing peptides (e.g. transient pores, dynamic equilibrium pores, stable equilibrium pores). Importantly for our long-term goals, these methods can be used to select for pore forming peptides belonging to any of the specific classes discussed here.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamem.2012.02.009.

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