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Lipid Composition-Dependent Membrane Fragmentation and Pore-Forming Mechanisms of Membrane Disruption by Pexiganan (MSI-78)

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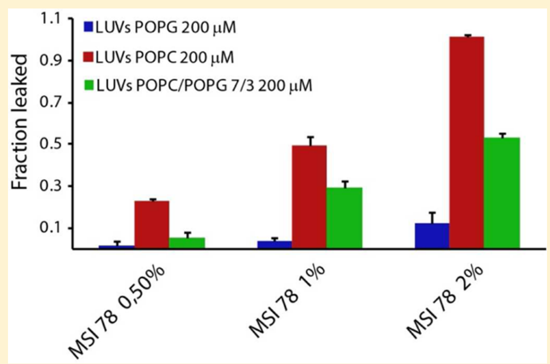
Supporting Information

ABSTRACT: The potency and selectivity of many antimicrobial peptides (AMPs) are correlated with their ability to interact with and disrupt the bacterial cell membrane. *In vitro* experiments using model membranes have been used to determine the mechanism of membrane disruption of AMPs. Because the mechanism of action of an AMP depends on the ability of the model membrane to accurately mimic the cell membrane, it is important to understand the effect of membrane composition. Anionic lipids that are present in the outer membrane of prokaryotes but are less common in eukaryotic membranes are usually thought to be key for the bacterial selectivity of AMPs. We show by fluorescence measurements of peptide-induced membrane permeabilization that the presence of anionic lipids at high concentrations can actually inhibit membrane disruption by the AMP MSI-78 (pexiganan), a representative of a large class of highly cationic AMPs. Paramagnetic quenching studies suggest MSI-78 is in a surface-associated inactive mode in anionic sodium dodecyl sulfate micelles but is in a deeply buried and presumably more active mode in zwitterionic dodecylphosphocholine micelles. Furthermore, a switch in mechanism occurs with lipid composition. Membrane fragmentation with MSI-78 can be observed in mixed vesicles containing both anionic and zwitterionic lipids but not in vesicles composed of a single lipid of either type. These findings suggest membrane affinity and membrane permeabilization are not always correlated, and additional effects that may be more reflective of the actual cellular environment can be seen as the complexity of the model membranes is increased.



Because of the rise of bacterial resistance to conventional small molecule antibiotics, there is considerable interest in developing novel antibiotics for the treatment of drug-resistant infection.[1] Antimicrobial peptides (AMPs), which exhibit broad-spectrum antibacterial activities, have been thought to have the potential to become the next generation of antibiotic compounds.[2,3] Most AMPs are believed to kill bacteria by directly interacting with the lipid components of the cell membrane.[4,5] Because the interactions of AMPs do not depend on any specific interactions with proteins, it is very difficult for bacteria to evolve resistance to AMPs.[5] Differences between bacterial and mammalian cell membranes are believed to be responsible for the selectivity of AMPs toward bacteria, and for this reason, there is significant fundamental interest in understanding how AMP−membrane interactions vary with lipid composition. In particular, the presence of anionic lipids in bacterial cell membranes and their absence in the outer leaflet of mammalian cell membranes are believed to play a major role in the selectivity of a cationic AMP.[6−9] A similar difference in anionic charge is also believed to be responsible for the selective targeting of AMPs to cancerous cells.[10] A full understanding of how lipid composition affects membrane disruption could aid in the design of more efficient AMPs and may also be useful for understanding the cell toxicity by other molecules that operate by similar mechanisms, such as the apoptotic proteins Bax[11,12] and tBid.[13]

While the electrostatic interaction between cationic AMPs and the anionic lipids in bacterial membranes has been shown to be a significant thermodynamic driving force for selectively binding to bacterial membranes,[14] the role of anionic lipids after the binding is less clear. In this study, we demonstrate the role of lipid charge on the mechanism of membrane disruption by MSI-78 (also known by its commerical name pexiganan, sequence GIGKFLKKAKKFGKAFVKILKK), an antimicrobial peptide developed for the treatment of diabetic foot ulcer infections.[15] MSI-78 exhibits a broad spectrum of potent antimicrobial activities against both Gram-negative and Gram-positive bacteria.[16] More fundamentally, MSI-78 has been used

Received: January 23, 2013
Revised: April 16, 2013
Published: April 16, 2013

dx.doi.org/10.1021/bi400087n | *Biochemistry* 2013, 52, 3254−3263

as a model for a large class of cationic, helical AMPs.[17] Along with many other AMPs,[18,19] MSI-78 has been shown to form nontraditional pores in which the membrane folds in on itself to create a lipid-lined toroidal pore.[20] In this study, we have investigated the effect of anionic lipids on the mechanism by which an AMP disrupts the lipid membrane. As discussed below, the mechanism of action of MSI-78 depends on the lipid composition, and the presence of an anionic lipid can completely alter the mechanism depending on the molar ratio of anionic lipid present in the membrane. Interestingly, results reported in this study show that membrane disruption is strongly suppressed in completely anionic lipids by the formation of an inactive membrane surface-associated state. Furthermore, vesicles containing both zwitterionic and anionic lipids are susceptible to membrane fragmentation by MSI-78, a membrane disruption mechanism that does not occur in membranes composed of either lipid alone.

■ MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine sodium salt (POPS), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(1′-*rac*-glycerol) sodium salt (POPG) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). 6-Carboxyfluorescein was purchased from Fluka (St. Louis, MO). MSI-78 was designed, synthesized, and donated by Genaera Corp.[16] Stock solutions of MSI-78 were prepared in Millipore water.

Preparation of Lipid Vesicles. Lipid solutions in $CHCl_3$ were dried under a stream of dry nitrogen gas and evaporated under high vacuum to dryness in a round-bottom flask. For dye leakage experiments, the resulting lipid film was hydrated with an appropriate amount of phosphate buffer [10 mM buffer and 100 mM NaCl (pH 7.4)] and dispersed by vigorous stirring to obtain multilamellar vesicles (MLVs). For NMR experiments, 8 mg of lipids was used to prepare MLVs in 200 μL of HEPES buffer [10 mM HEPES (pH 7.4) and 50 mM NaCl] using the procedure described above. To obtain large unilamellar vesicles (LUVs), the MLVs were extruded through polycarbonate filters (pore size of 100 nm for dye leakage or 1000 nm for solid-state NMR) (Nuclepore, Pleasanton, CA) mounted in a mini-extruder (Avestin Inc., Ottawa, ON) fitted with two 0.5 mL Hamilton gastight syringes (Hamilton, Reno, NV).[21] Samples were typically subjected to 23 passes through two filters in tandem. An odd number of passages were performed to avoid contamination of the sample by vesicles that have not passed through the first filter.

Dye Leakage Experiments. Dye leakage experiments were conducted by using 6-carboxyfluorescein-filled LUVs of POPC, POPG, or POPC and POPG (7:3 molar ratio) at a final concentration of 200 μM. Dye-filled LUVs were prepared by hydrating the dry lipid film with the buffer solution containing 6-carboxyfluorescein [10 mM phosphate and 70 mM 6-carboxyfluorescein (pH 7.4)] according to the procedure described above. The reduction in salt content in the dye-filled vesicles relative to the outside buffer ensures osmotic balance.[22] The nonencapsulated 6-carboxyfluorescein was removed by placing the solution containing LUVs on a Sephadex G50 gel exclusion column (Sigma-Aldrich, St. Louis, MO) and eluting with the final buffer [10 mM phosphate and 100 mM NaCl (pH 7.4)]. The first colored band containing the separated dye-containing vesicles was collected. Samples were prepared by diluting the dye-filled vesicle solution with buffer solution [10 mM phosphate buffer solution and 100 mM NaCl (pH 7.4)] to a final concentration of 200 μM.

Samples were prepared by adding 1, 2, or 4 μL of the MSI-78 stock solution (200 μM) to 200 μL of a 200 μM LUV solution, which produced samples with 0.5, 1, and 2% peptide-to-lipid mole percentages, respectively. Membrane disruption was quantified by detecting the increase in fluorescence emission intensity of 6-carboxyfluorescein due to its dilution (dequenching) in buffer as a consequence of membrane leakage. Time traces were recorded using an excitation wavelength λ_{ex} of 490 nm, an emission wavelength λ_{em} of 520, and excitation and emission slits of 1 nm. The fraction leaked was calculated by

$$\text{fraction leaked} = (I - I_0)/(I_{100} - I_0) \tag{1}$$

where I is the emission intensity of the sample, I_0 is the emission intensity obtained in the absence of the peptide (baseline control), and I_{100} is the emission intensity obtained after adding Triton X-100, a detergent, which acted as a positive control to give 100% leakage. All measures were taken in triplicate at 25 °C without stirring of the sample.

NMR Experiments. ^{31}P and ^{14}N NMR spectra were obtained from an Agilent/Varian 400 MHz solid-state NMR spectrometer using a 5 mm ^{1}H/X double-resonance magic angle spinning probe (Agilent/Varian). ^{31}P NMR experiments were performed using 35 kHz proton decoupling, a 90° pulse duration of 5 μs, and a recycle delay of 3 s. All ^{31}P NMR spectra were processed using 50 Hz line broadening referenced externally to phosphoric acid (0 ppm). ^{14}N NMR experiments were performed using a quadrupole-echo sequence (90°−τ−90°−τ with a τ of 60 μs) with 35 kHz proton decoupling by TPPM, a 90° pulse duration of 9 μs, and a recycle delay of 0.5 s. All ^{14}N NMR spectra were processed using 50 Hz line broadening. All experiments were performed at 37 °C.

^{31}P NMR spectra obtained from POPC LUVs of varying sizes are given in Figure S1 of the Supporting Information. Lamellar phase powder pattern spectra obtained from 1000 nm POPC LUVs suggest that these vesicles are stable and suitable for solid-state NMR measurements (Figure S1 of the Supporting Information). ^{31}P NMR spectra of LUVs without MSI-78 were first collected. An appropriate amount of MSI-78 from a stock solution in water was then added and the mixture gently shaken before the sample was placed back into the magnet for further measurements.

Paramagnetic Quenching NMR Experiments. The extent of the peptide's exposure to water when MSI-78 is bound to SDS or DPC micelles was measured by paramagnetic quenching of ^{1}H resonances by Mn^{2+} ions. The ^{1}H NMR spectra of 300 μM MSI-78 embedded in 30 mM deuterated SDS or deuterated DPC micelles [10 mM phosphate buffer and 100 mM NaCl (pH 7.4) at 25 °C] in the presence and absence of varying concentrations of $MnCl_2$ were acquired with 256 scans with a recycle delay of 2 s.

■ RESULTS

Membrane Permeabilization by MSI-78 Is Strongly Suppressed in Anionic Membranes. MSI-78 is a highly cationic peptide with a net charge of +9. The electrostatic interaction between the cationic peptide and anionic lipids in the cell membrane has been proposed to be a major factor for its bacterial selectivity. To better understand the role of anionic lipids in the events after membrane binding, we investigated the interaction of MSI-78 with both zwitterionic and anionic LUVs. To evaluate the ability of MSI-78 to disrupt membranes, we performed dye leakage experiments by using LUVs filled with 6-carboxyfluorescein (Figure 1 and Figure S2 of the Supporting Information). MSI-78 shows a concentration-dependent increase in apparent membrane permeabilizing activity with zwitterionic POPC vesicles, with complete dye leakage reached after 300 s at 1 mol % MSI-78. Surprisingly, in light of the highly cationic nature of MSI-78, the inclusion of an anionic POPG lipid decreased the amount of dye leakage relative to that of vesicles formed purely from POPC. A very small amount of dye leakage induced by MSI-78 was observed from LUVs containing only POPG, with a maximum of 10% leakage recorded at the highest peptide concentration used (2 mol %). LUVs containing a POPG/POPC mixture display an intermediate amount of dye leakage between that of pure POPC and that of pure POPG LUVs (Figure 1).

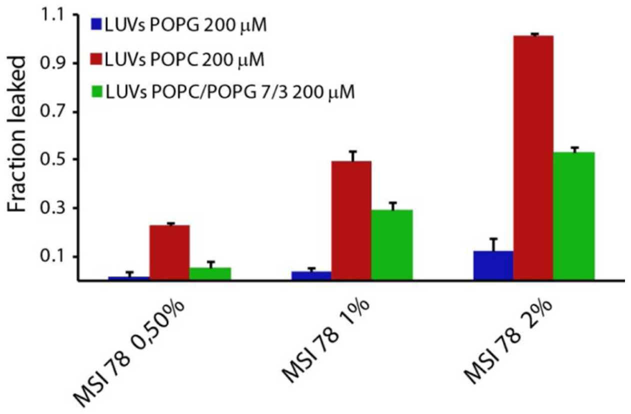


Figure 1. Extent of membrane permeabilization by MSI-78, which is higher in zwitterionic POPC than anionic POPG. Dye release after 300 s from 100 nm diameter LUVs composed of either POPG (blue), POPC (red), or a 7:3 POPC/POPG mixture (green) induced by MSI-78 at the indicated mole ratios. Measurements were performed in phosphate buffer [10 mM phosphate and 100 mM NaCl (pH 7.4)] at 25 °C. Error bars indicate the standard error of measurement (sem) for $n = 3$.

The absence of dye leakage in POPG LUVs is surprising in light of MSI-78's high affinity for anionic lipids relative zwitterionic ones.[23] While dye leakage is very commonly used as a measure of membrane permeabilization, not all pores are permissive for the passage of carboxyfluorescein.[24] In particular, MSI-78 is believed to disrupt membranes by a toroidal pore mechanism in which the bilayer is folded inward to create a lipid-lined pore. In this type of pore, the negatively charged POPG headgroups would create a region of strong negative electrostatic potential in the pore region, which may repel the negatively charged carboxyfluorescein molecule.[24,25] A toroidal pore in zwitterionic POPC bilayers will have a more complex electrostatic potential because of the presence of the positively charged choline headgroup near the surface and the negatively charged phosphate group further into the membrane. As such, the difference in electrostatic potential within the toroidal pore may create an ion selectivity effect for membrane permeabilization.[26]

To test this possibility, we measured the permeability of POPC, POPG, or 7:3 POPC/POPS LUVs to Mn^{2+} cations using ^{31}P NMR (Figure S3 of the Supporting Information).[24] The paramagnetic ion quenches the intensity of ^{31}P resonances of phosphate headgroups in its vicinity. In the absence of MSI-78, the addition of 470 μM $MnCl_2$ to 1000 nm POPG LUVs partially quenches the signal. This result is expected for an intact membrane, as Mn^{2+} can quench the outer leaflet but does not have access to the inner leaflet in the absence of pores. The addition of 2 mol % MSI-78 has virtually no effect on the ^{31}P NMR spectra, confirming the POPG LUVs remain intact after the addition of up to 2 mol % MSI-78. The absence of apparent membrane permeabilization of POPG LUVs in the dye leakage assay is therefore not likely the result of either the charge or size of the carboxyfluorescein molecule. As the concentration of MSI-78 is increased beyond 2 mol %, the observed ^{31}P signal intensity decreased, indicating the integrity of the bilayer has been compromised and Mn^{2+} is able to penetrate into the inner leaflet (Figure S3 of the Supporting Information). This increase in Mn^{2+} influx is correlated with lipid aggregation and sedimentation of the LUVs at these peptide concentrations (Figure S3b of the Supporting Information).

MSI-78 Is Buried More Deeply in Zwitterionic Micelles Than in Anionic Micelles. Evidence of a distinctly different binding mode in anionic versus cationic membranes was obtained from paramagnetic quenching experiments with MSI-78 bound to detergent micelles. Paramagnetic quenching experiments reveal the exposure of the peptide to solvent: if a residue is exposed on the surface of the micelle, the corresponding resonance is broadened, while if it is buried within the micelle, the quenching agent has little or no effect. ^1H NMR experiments were conducted with zwitterionic DPC (dodecylphophocholine) and anionic SDS (sodium dodecyl sulfate) detergent micelles containing MSI-78 to measure the depth of penetration of MSI-78 into each micelle using the paramagnetic Mn^{2+} ion. The degree of paramagentic quenching of MSI-78 in SDS micelles compared to DPC micelles therefore gives an approximation of the degree of penetration of MSI-78 in anionic and zwitterionic membranes, if the inherent differences in lipid packing between detergent micelles and lipid bilayers are kept in mind.

The spectra of MSI-78 are noticeably different in SDS than the spectra from DPC in the absence of Mn^{2+}. In particular, the resonances are noticeably broader, particularly in the aliphatic region. MSI-78 adopts similar amounts of helical secondary structure in DPC[27] and SDS[28] micelles. The strong electrostatic interaction between SDS and the cationic peptide could be a possible reason for the major differences in the spectra obtained from these micelles. In addition, MSI-78 is also known to form an antiparallel dimer in DPC micelles and DMPC bicelles.[27,28] A comparison of the REDOR difference spectra of MSI-78 and MSI-594, a homologous peptide that is monomeric in DPC with a shallower insertion, has indicated that the broadening effect may be due to exchange between the monomer and dimers in the intermediate kinetic regime.[27]

The addition of the paramagnetic quencher $MnCl_2$ for samples containing DPC micelles had a moderate line broadening effect on the peaks observed in the ^1H NMR spectra of MSI-78, with many resonances clearly visible even in the presence of a high concentration (800 μM) of Mn^{2+}. This finding is consistent with the penetration of side chains of MSI-78 into the hydrophobic region of the micelle with less exposure to the aqueous phase (Figure 2). In anionic SDS micelles, the paramagnetic quenching effect is more severe and several peaks are broadened and disappear at lower Mn^{2+} concentrations. This finding suggests that the side chains of MSI-78 are more exposed to solvent in anionic SDS than zwitterionic DPC.

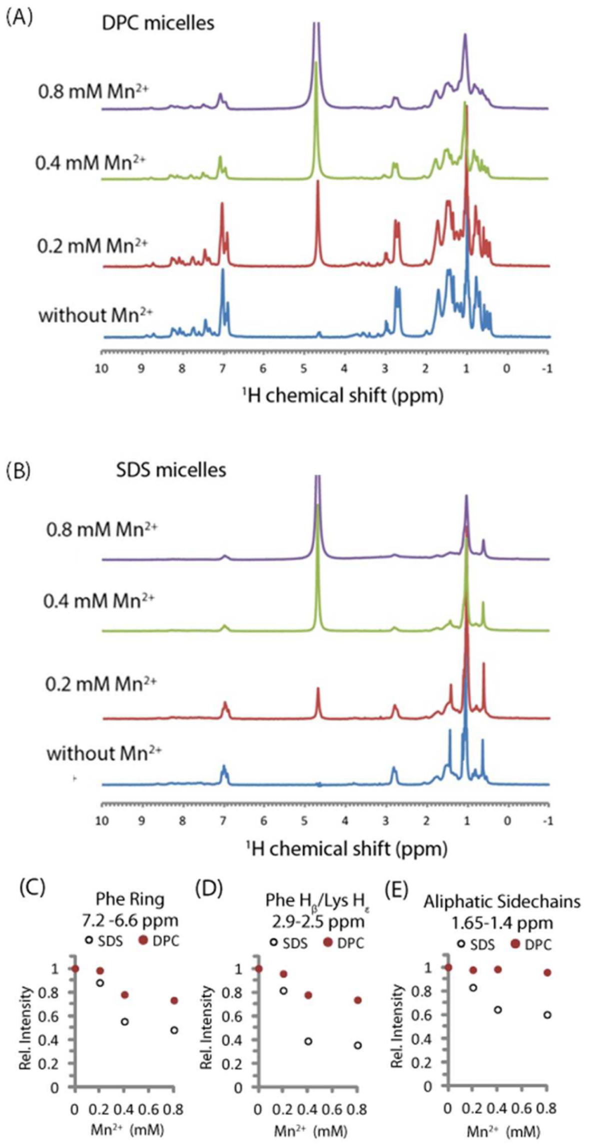


Figure 2. MSI-78 penetrates more deeply into zwitterionic detergent micelles than anionic ones. (A and B) Paramagnetic quenching by Mn^{2+} of 300 μM MSI-78 bound to 30 mM DPC (A) or SDS (B) micelles. (C−E) Paramagnetic quenching as a function of the Mn^{2+} concentration for the resolved resonances. Manganese has a moderate effect on MSI-78 in the presence of DPC micelles; the more severe effect in the presence of SDS is an indication of greater solvent exposure of MSI-78 when it is bound to the negatively charged micelles. All experiments were conducted in 10 mM phosphate buffer and 100 mM NaCl (pH 7.4) at 25 °C.

MSI-78 Does Not Disrupt the Lipid Bilayer Structure of LUVs Containing either Completely Anionic or Completely Zwitterionic Lipids. We next probed the phase structure of anionic and zwitterionic vesicles containing MSI-78 using static solid-state ^{31}P NMR experiments. The degree of ^{31}P chemical shift anisotropy is sensitive to the degree of motion of the lipid headgroup, which increases if the lamellar structure of the bilayer is disrupted. In particular, in the extreme case where the membrane is fragmented to small lipid aggregates, the chemical shift anisotropy is reduced to near zero by motional averaging due to rapid tumbling of fragments. Static ^{31}P NMR spectra of LUVs of POPC, POPS, and POPG containing various amounts of MSI-78 are shown in Figure 3. LUVs exhibited a ^{31}P chemical shift powder pattern with a span of 45 ppm (from −16 to 29 ppm) for POPC, 50 ppm (from −17 to 33 ppm) for POPS, and 34 ppm (from −10 to 24 ppm) for POPG in the absence of MSI-78. The inclusion of 2 or 4 mol % MSI-78 resulted in no significant changes in the ^{31}P spectra of POPC or POPS LUVs, although a slight broadening effect could be observed in POPG LUVs and POPC LUVs show an increase in the perpendicular (high field) edge relative to the parallel (low field) edge after the addition of MSI-78, consistent with deformation of the spherical LUVs to more ellipsoidal shapes.[29,30] The absence of significant changes in the ^{31}P spectra suggests that the presence of up to 4 mol % MSI-78 does not significantly alter the lipid bilayer structure of LUVs composed entirely of a single anionic or zwitterionic lipid. In particular, there is no evidence in the spectra of the formation of nonlamellar lipid structures like hexagonal phases, cubic phases, or micelles in vesicles containing MSI-78 and either purely anionic or purely zwitterionic lipids.

MSI-78 Fragments LUVs Containing a Mixture of Anionic and Zwitterionic Lipids. A significantly different result was obtained in vesicles containing a mixture of both anionic and zwitterionic lipids. Unlike LUVs containing exclusively either anionic and zwitterionic lipids, mixed vesicles of both anionic and zwitterionic lipids are susceptible to fragmentation of the bilayer to form small lipid peptide aggregates. In the absence of MSI-78, 7:3 POPC/POPS and 7:3 POPC/POPG LUVs show a lamellar phase ^{31}P chemical shift powder pattern spectrum similar to the ^{31}P spectra of LUVs containing either anionic or zwitterionic lipids alone (Figure 4). In the presence of MSI-78, most of the spectra closely resemble the control spectra without MSI-78. However, a narrow peak at 0 ppm is also seen in these samples, an indication of the partial fragmentation of the membrane into small aggregates of lipids. Fragmentation of the membrane was further confirmed by ^{14}N NMR experiments, which showed an isotropic peak for 7:3 POPC/POPS LUVs (Figure 4).

MSI-78 Does Not Cause Macroscopic Clustering of Anionic Lipids in Mixed Vesicles. The fragmentation of the membrane only in mixed vesicles is unusual and was investigated further by varying the POPC:POPG ratio (Figure 5). Membrane fragmentation was not detected in LUVs containing either very low (<20 mol %) or high (>50 mol %) percentages of POPG. We further analyzed the effect of membrane composition on the peptide-induced membrane fragmentation by separating the membrane fragments from the intact membranes by centrifuging 7:3 POPC/POPG LUVs containing 2 mol % MSI-78 at 4000 rpm for 10 min, a procedure that has been shown to completely sediment intact LUVs of this size (1000 nm in diameter) while leaving smaller lipid aggregates in the supernatant.[24,31] Enrichment of particular lipids in the supernatant is an indication that the formation of macroscopic domains of specific lipids is involved in membrane fragmentation. For example, similar techniques have been used to show phosphatidylethanolamine (PE) lipids are greatly enriched in the supernatant after the fragmentation of *Escherichia coli* membranes by cyclotides.[32]

^{31}P NMR experiments confirmed the presence of small fragmented membranes in the supernatant solution (Figure S4 of the Supporting Information). The supernatant and pellet solution was then lyophilized and dissolved in chloroform to easily resolve ^{31}P peaks of POPC from those of POPG (Figure 6). Comparison of the chemical shifts of POPC and POPG in each fraction suggests more peptide is bound per lipid in the

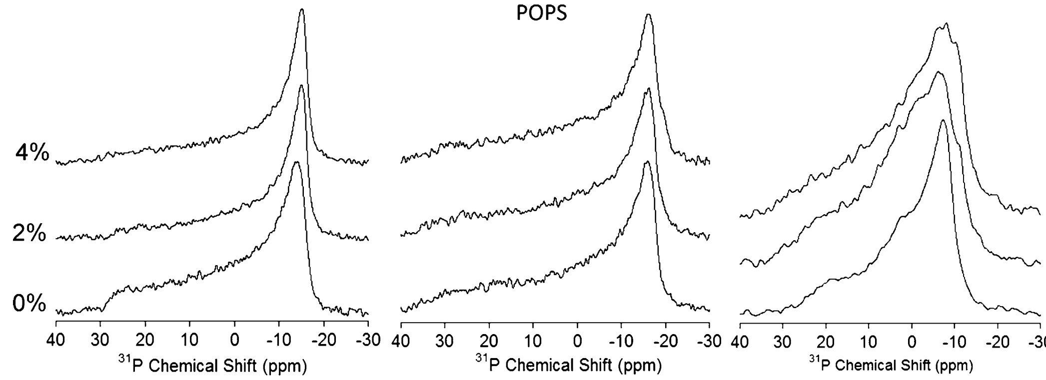


Figure 3. MSI-78 does not disorder the lipid bilayer structure composed entirely of anionic or zwitterionic lipids. ^{31}P NMR spectra of LUVs composed of zwitterionic POPC, anionic POPS, and anionic POPG containing 0, 2, and 4 mol % MSI-78 at 37 °C in 10 mM HEPES (pH 7.4) with 50 mM NaCl. The addition of up to 4% MSI-78 had little effect on the spectra of any of the samples.

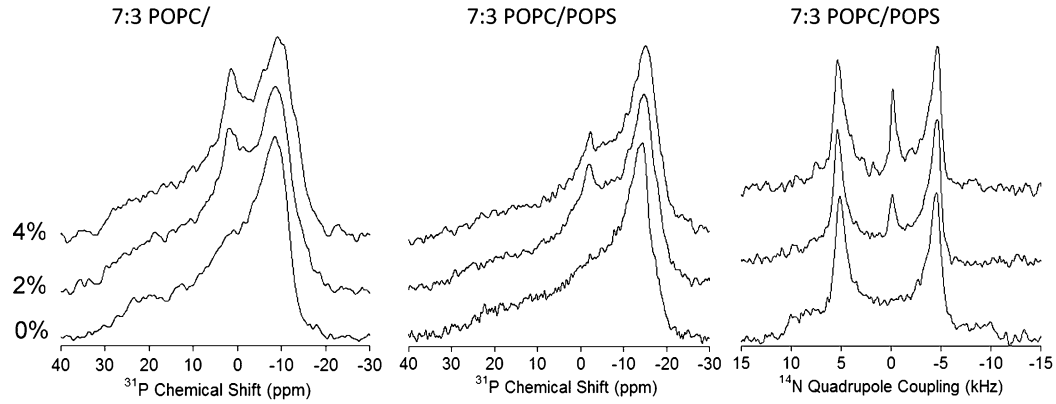


Figure 4. MSI-78 fragments membranes composed of a mixture of anionic or zwitterionic lipids. ^{31}P static and ^{14}N quadrupole coupling NMR spectra of 1000 nm LUVs composed of a mixture of anionic and zwitterionic lipids containing MSI-78 at 37 °C in 10 mM HEPES (pH 7.4) with 50 mM NaCl. The peak near 0 ppm results from the fragmentation of the membrane into small micelle-like aggregates or smaller vesicles.

supernatant fraction than in the pellet. The larger shift observed for the POPG resonance compared to that of POPC also suggests MSI-78 may have a stronger interaction with the POPG headgroup, although it is difficult to extrapolate the results from a cholorform solution to an intact membrane (Figure 6). However, the ratio of the intensities of the POPC and POPG resonances closely matches a control sample with a 7:3 POPC:POPG ratio directly dissolved in chloroform and is nearly identical to that of the pellet and supernatant solutions. The absence of a noticeable elevation in the levels of POPG in the fragmented portions of the membrane suggests MSI-78 does not cause large-scale clustering of anionic lipids in the membrane.

■ DISCUSSION

Several lines of evidence suggest membrane disruption by antimicrobial peptides is a multistep process with distinct binding,[33−36] oligomerization,[37−39] membrane insertion,[36,40,41] and pore formation[33−35,39] steps. It is clear from electrostatic considerations that a highly charged cationic peptide should bind anionic lipids more strongly than zwitterionic ones. While direct determination of the binding affinity is difficult under our conditions because of aggregation within the samples,[42] multiple studies under other conditions have shown higher binding affinity of MSI-78 and other cationic peptides for anionic lipids.[23,43−46] While the influence of lipid charge on membrane binding is well-established, the influence of anionic lipids on the other steps of the membrane disruption process is less clear.

For most AMPs, both membrane association and membrane permeabilization are stronger in anionic membranes.[7,8,43,45,46] MSI-78 is less effective at disrupting purely anionic vesicles than purely zwitterionic ones (Figure 1 and Figure S1 of the Supporting Information), suggesting membrane disruption by MSI-78 is not completely correlated with membrane binding affinity. Because of the complexity of the membrane disruption process of MSI-78 and the existence of several separate membrane disruption mechanisms, the exact reasons for this somewhat surprising result cannot be definitively established. However, several changes in the interactions of MSI-78 with membranes as the membrane composition changed were noted. First, MSI-78 penetrates deeper into the zwitterionic detergent DPC than the anionic detergent SDS, with the implication that MSI-78 also penetrates into zwitterionic lipid bilayers deeper than anionic bilayers (Figure 2). Second, large amounts of lipid aggregation are detectable to the naked eye in anionic (POPG) vesicles but not in zwitterionic (POPC) vesicles (Figure S3b of the Supporting Information). Finally, membrane fragmentation was noted in mixed vesicles of POPC and POPG but not in vesicles of either lipid alone (Figures 4−6 and Figure S4 of the Supporting Information).

Membrane Permeabilization Correlates with Membrane Penetration. The degree of membrane penetration may be a particularly important factor in determining the

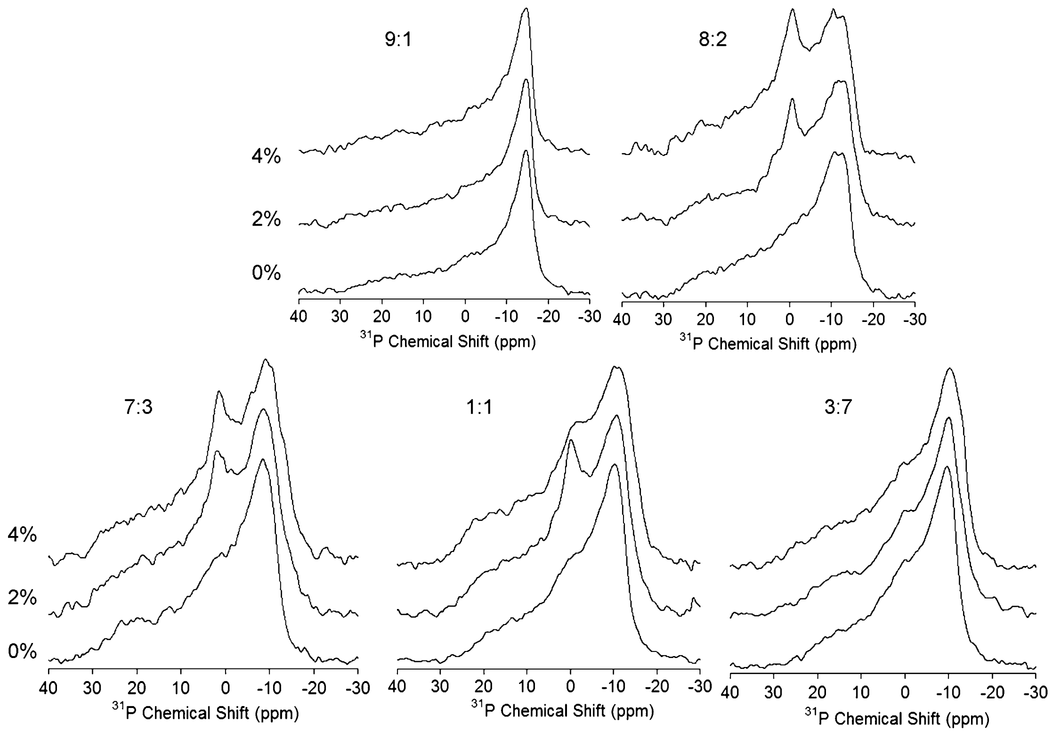


Figure 5. Membrane fragmentation by MSI-78 is dependent on the ratio of anionic to zwitterionic lipids. Effect of zwitterionic (POPC) to anionic (POPG) lipid ratio on the ^{31}P static NMR spectra of 1000 nm LUVs in the presence of MSI-78 at 37 °C in 10 mM HEPES (pH 7.4) with 50 mM NaCl. Peaks near 0 ppm, indicative of membrane fragmentation, are observed at an intermediate ratio of zwitterionic to anionic lipids. The ratio in the legend indicates the molar ratio of POPC to POPG.

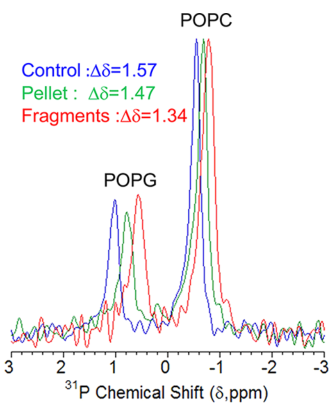


Figure 6. Fragmented membranes of POPC/POPG vesicles have the same composition as the overall membrane. ^{31}P NMR spectra of 7:3 POPC/POPG vesicles containing 2 mol % MSI-78 after centrifugation to remove the membrane fragments from the intact membrane. The sample was centrifuged and the pellet and the supernatant solution separated and lyophilized. Spectra of the pellet dissolved in chloroform (green) and supernatant (red) are shown along with the control (blue). ^{31}P NMR spectra of the supernatant and pellet before lyophilization can be found in Figure S4 of the Supporting Information. The ratio of intensities is the same as the control, suggesting that the MSI-78-induced membrane fragments have the same lipid composition as the overall membrane.

effectiveness of membrane permeabilization by MSI-78 and, by extension, other membrane binding peptides as well. Peptides that show only superficial penetration into the membrane frequently bind membranes without disrupting their integrity.[47] As a specific example, $PAP_{248-286}$ possesses a charge similar to that of MSI-78 but binds in a surface-associated state with little penetration into the membrane.[48,49] In this state, $PAP_{248-286}$ does not disorder or permeabilize membranes.[50] Other amyloid-forming peptides also have high membrane binding affinities and low membrane permeabilizing activities if bound in a surface-associated state.[51−54] Similar results have been obtained with nonprotein flexible polymers that also exhibit superficial penetration of the membrane.[55,56] These results suggest that while membrane binding affinity and membrane permeabilizing activity are frequently strongly correlated,[46] this correlation does not always hold.[57] Other factors besides membrane affinity, such as membrane penetration depth or hydrophobic matching, may influence membrane disruption by peptides.[58−60]

The differences in the relative penetration in anionic versus zwitterionic membranes may also explain the relative effectiveness of MSI-78 against each membrane. AMPs that are selective against anionic membranes frequently penetrate deeper into anionic membranes than zwitterionic ones.[61−65] Conversely, AMPs selective against zwitterionic membranes frequently show a greater depth of penetration into zwitterionic membranes. For example, both pardaxin and melittin can adopt a transbilayer orientation in zwitterionic membranes under specific conditions at high peptide:lipid ratios.[66−70] Under the same conditions, both pardaxin and mellitin will adopt a surface-associated orientation in anionic membranes.[66,67] Like MSI-78, significantly more of these peptides are needed to permeabilize POPG vesicles compared to POPC vesicles.[67,71] The mechanism for membrane disruption for both pardaxin and melittin also appears to change from the creation of small pores selective for the passage of ions to nonselective membrane lysis as the anionic content of the membrane increases.[66,67] In the case of pardaxin and mellitin, surface binding modes are associated with relatively inefficient membrane fragmentation, while deeply penetrating binding modes are associated with membrane disruption by a more efficient porelike mechanism.[67]

Evidence of a similar switch in binding mode in anionic and zwitterionic membranes can be seen in Figure 2 for MSI-78. Paramagnetic quenching experiments show MSI-78 binds closer to the surface in anionic detergents than zwitterionic detergents (Figure 2). This difference in membrane localization may have consequences for the mechanism of membrane disruption. MSI-78 is believed to disrupt membranes at low concentrations by a toroidal pore mechanism in which the membrane is folded inward by the peptide to create a lipid-lined toroidal pore.[20,72] The toroidal pore mechanism is dependent on the creation of curvature within the normally flat membrane.[19] The amount of curvature strain is dependent in turn on the depth of insertion of the peptide into the membrane.[73] The degree of antimicrobial activity for a series of cationic antimicrobial peptides has been shown to be positively correlated with the degree of membrane insertion; shallower insertion corresponds to lower antimicrobial activity.[7,74,75] This result has been supported by theoretical calculations of the bilayer curvature strain caused by the insertion of amphipathic helices, which show maximal curvature strain is obtained at an insertion depth of 40% of the monolayer thickness.[73] Deeper membrane penetration by MSI-78 in zwitterionic membranes is therefore likely to lead to increased curvature strain and stronger membrane permeabilization by the toroidal pore mechanism.

Probable Reorientation of MSI-78 in Anionic Membranes May Cause Lipid Aggregation. A difference in membrane binding topology may also be responsible for the large-scale lipid aggregation seen in anionic POPG vesicles (Figure S3b of the Supporting Information). MSI-78 is designed to bind as an amphipathic helix. In zwitterionic lipids, the high degree of membrane penetration suggests MSI-78 binds with the hydrophobic face facing into the membrane. This is confirmed by the paramagnetic quenching experiment, which shows the complete burial of the aliphatic side chains (Figure 2E, red circles) but only partial burial of the lysine side chains (Figure 2D, red circles). In SDS, both aliphatic (Figure 2E, black circles) and lysine (Figure 2D, black circles) are partially exposed to the solvent. The partial exposure of the lysine side chains in SDS may indicate a reorientation of the helix toward the membrane surface. The binding of MSI-78 to anionic POPG vesicles may also create a hydrophobic patch on the membrane surface in addition to reducing the extent of electrostatic repulsion between liposomes.[54] A similar effect has been seen in the cationic cell-penetrating peptide RL12. Like MSI-78, RL12 causes extensive lipid aggregation in anionic but not zwitterionic vesicles.[76] While lipid aggregation can promote membrane instability,[39,68,77,78] it may also lower the extent of dye leakage by limiting access of the peptide to the interior of the aggregate.[79] The exact contribution lipid aggregation makes to membrane disruption is difficult to determine using ensemble methods and will likely require a more focused study tracking leakage from individual vesicles within aggregates.[77,80]

Finally, we have shown that in mixed vesicles containing both anionic and zwitterionic lipids, the NMR data indicate the presence of two types of lipid structures in the samples in the presence of MSI-78: unperturbed LUVs and small aggregates formed by the fragmentation of the membrane (Figures 4 and 5). The existence of these fragmented membranes indicates an additional mechanism for membrane disruption that occurs in mixed vesicles containing both zwitterionic and anionic lipids that does not occur in vesicles composed of either lipid alone, even when the peptide is incorporated at relatively high peptide:lipid ratios (4%). In mixed vesicles, MSI-78 and other cationic AMPs cause partial lipid demixing; anionic lipids cluster around the peptide away from the bulk bilayer.[42,81,82] The ability of AMPs to cause lipid clustering is positively correlated with their antimicrobial activity.[81,82] The mechanism by which lipid clustering causes membrane disruption is not known.[42] Because membrane fragmentation is concentration-dependent,[83,84] one possibility is that MSI-78 is unevenly distributed throughout the bilayer with high local concentrations in the regions of the membrane enriched in anionic lipids. The local concentration in these regions may therefore exceed a threshold concentration for membrane disruption. However, the ^{31}P NMR data do not agree with this mechanism. First, the absence of membrane fragmentation at higher concentrations of MSI-78 in pure POPG bilayers suggests an increase in local concentration caused by lipid demixing is not likely to be the sole reason for membrane fragmentation (Figure 3). Second, ^{31}P NMR analysis shows the fragmented region of the bilayer has the same composition as the main bilayer (Figure 6), suggesting anionic lipids are only clustered in the immediate vicinity of the peptide and the fragmented membranes are substantially larger. Local clustering of this type will still create boundary defects around the vicinity of the cluster, creating a line tension that can lead to weakening of the membrane. Notably, phase-segregated membranes containing cholesterol are more sensitive to membrane disruption by MSI-78 and other antimicrobials than non-phase-segregated membranes containing cholesterol.[85−88] Boundary defects of this type have also been shown to play a prominent role in the binding and membrane permeabilization of other membrane disruptive proteins such as amyloidogenic peptides[89,90] and are likely to prove to be a fruitful avenue of research for increasing the potency and selectivity of antimicrobial peptides.[91]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{31}P NMR spectra of POPC LUVs of varying size, kinetics of MSI-78-induced release of dye from LUVs, Mn^{2+} quenching of POPG vesicles containing MSI-78, and ^{31}P NMR spectra of LUVs after centrifugation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This research was supported by National Institutes of Health (AI054515, GM095640, GM084018 to A.R.). D.-K.L. was partly supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0087836).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

AMP, antimicrobial peptide; NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine sodium salt; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) sodium salt; MLV, multilamellar vesicle; LUV, large unilamellar vesicle; TPPM, two-pulse phase modulation; DPC, dodecylphosphocholine; SDS, sodium dodecyl sulfate.

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