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Does cholesterol suppress the antimicrobial peptide induced disruption of lipid raft containing membranes?

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ABSTRACT

The activity of antimicrobial peptides has been shown to depend on the composition of the target cell membrane. The bacterial selectivity of most antimicrobial peptides has been attributed to the presence of abundant acidic phospholipids and the absence of cholesterol in bacterial membranes. The high amount of cholesterol present in eukaryotic cell membranes is thought to prevent peptide-induced membrane disruption by increasing the cohesion and stiffness of the lipid bilayer membrane. While the role of cholesterol on an antimicrobial peptide-induced membrane disrupting activity has been reported for simple, homogeneous lipid bilayer systems, it is not well understood for complex, heterogeneous lipid bilayers exhibiting phase separation (or "lipid rafts"). In this study, we show that cholesterol does not inhibit the disruption of raft-containing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine:1,2-dipalmitoyol-*sn*-glycero-3-phosphocholine model membranes by four different cationic antimicrobial peptides, MSI-78, MSI-594, MSI-367 and MSI-843 which permeabilize membranes. Conversely, the presence of cholesterol effectively inhibits the disruption of non-raft containing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine or 1,2-dipalmitoyol-*sn*-glycero-3-phosphocholine lipid bilayers, even for antimicrobial peptides that do not show a clear preference between the ordered gel and disordered liquid-crystalline phases. Our results show that the peptide selectivity is not only dependent on the lipid phase but also on the presence of phase separation in heterogeneous lipid systems.

1. Introduction

Antimicrobial peptides (AMPs) are small, highly cationic, amphipathic peptides known for their cell-selective membrane lytic activities [1–3]. Most AMPs, like magainins or cecropins, preferentially act on bacterial cells [4–6], yet others like melittin and gramicidins have been shown to interact with both bacteria and eukaryotic cells [5,7]. Bacterial selectivity is believed to be linked largely to the ability of antimicrobial peptides to discriminate between different membrane types [8,9]. For the majority of these peptides, their cationic nature accounts for the selective disruption of bacterial membranes, since bacterial membranes contain significantly more acidic phospholipids than eukaryotic membranes (~10–70% of the total, depending on the species) [10]. Furthermore, the distribution of lipids is non-uniform in eukaryotic cells, with the acidic lipids largely concentrated in the inner leaflet in eukaryotic membranes [11]. As such, studies considering the influence of lipids in the membrane targeting of antimicrobial peptides has largely focused on the role of negatively charged phospholipids. However, the action of these peptides is also dependent on membrane cholesterol levels, a component primarily found in eukaryotic membranes [12].

In most homogeneous lipid systems, cholesterol is known to increase membrane cohesion and mechanical stiffness [13,14]. The presence of membrane-stabilizing cholesterol has been shown to protect human erythrocytes from attack by magainin 2 [9,15]. Previous studies have also shown a protective effect of cholesterol on the membrane disrupting activity of other antimicrobial peptides such as pardaxin [16–19]. From these studies, it has been inferred that cholesterol plays an important role in the selective targeting of AMPs to bacterial membranes over eukaryotic ones [8]. However, both bacterial and eukaryotic cell membranes are actually complex mixtures of lipids whose physical properties vary non-linearly with the composition of the membrane. In particular, liquid ordered–liquid disordered (L_o–L_d) phase separation in eukaryotic membranes (i.e., the formation of "raft" domains) has been shown to play essential roles in the organization and activity of membrane proteins [20]. Few studies have systematically looked at membrane disruption by AMPs in such systems. An exception is two studies by the Almeida group, which systematically studied the membrane permeabilizing activity of δ-lysin in raft-like palmitoyl-2-oleoylphosphatidylcholine/cholesterol/sphingomyelin (POPC/Chol/SM) mixtures [21,22]. These studies showed that membrane permeabilization by δ-lysin occurs exclusively in the L_d phase in membranes with L_d–L_o phase segregation and that the localization of δ-lysin to the L_d phase results in greater membrane disruption than

Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyol-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; SM, sphingomyelin; Chol, cholesterol; AMP, antimicrobial peptide; LUV, large unilamellar vesicle; L_d, liquid-disordered; L_o, liquid-ordered; L_α, liquid-crystalline

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would be expected in the absence of phase segregation [22]. We generalize this important result to a diverse set of AMPs encompassing several membrane disruptive mechanisms, including AMPs that do not show a clear preference for either the gel or liquid crystalline phases. Our results further show that phase separation nullifies the effect of cholesterol against membrane disruption for all the AMPs tested. Importantly, we show that the formation of the L_o phase by cholesterol strongly inhibits membrane disruption even for AMPs that are active against the similarly ordered gel phase, implying that the resistance to membrane disruption of the L_o phase by AMPs is not simply a result of increased acyl chain packing or bilayer thickness.

2. Materials and methods

2.1. Materials

DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyol-*sn*-glycero-3-phosphocholine), and cholesterol were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and used without further purification or modification. Chloroform and methanol were purchased from Aldrich Chemical (Milwaukee, WI). Carboxyfluorescein (99%) was purchased from ACROS (Pittsburg, PA). All of the peptides were synthesized and donated by Genaera Corporation (Plymouth Meeting, PA).

2.2. Preparation of lipid vesicles

Stock solutions of DOPC (20 mg/mL), DPPC (20 mg/mL), and cholesterol (20 mg/mL) in chloroform were used to prepare a set of 16 samples with DOPC/DPPC/cholesterol molar ratios of 1/0/0, 80/0/20, 70/0/30, 60/0/40, 0/1/0, 0/80/20, 0/70/30, 0/60/40, 1/1/0, 40/40/20, 35/35/30, 30/30/40, 33/66/0, 26/53/20, 23/46/30, and 20/40/40. The concentration of total phospholipid (DOPC/DPPC) was held constant at an initial mixing concentration of 7 mM. The appropriate volumes of stock solution for each sample were mixed in a small, round-bottomed flask and the solvent was removed by evaporation over a gentle stream of dry nitrogen gas. Residual solvent was removed under vacuum overnight at room temperature. After the complete removal of solvents, the dry lipid films were hydrated at room temperature in the same small, round-bottomed flask with carboxyfluorescein dye at a concentration of 70 mM in 10 mM pH 7.5 sodium phosphate buffer without NaCl. The hydrated mixture was then carefully mixed by hand using a small glass rod and then transferred to a snap-cap centrifuge tube. To conclude the mixing process, samples were routinely subjected to five freeze–thaw cycles in which each mixture was frozen via liquid nitrogen submersion and then heated to 60 °C. The mixture was then kept at a constant 60 °C (well above the chain melting temperature of both lipids used) and was passed twenty-one times through a stainless steel extruder containing two nylon filters and a polycarbonate membrane containing 100 nm pores obtained from Fisher Scientific (Wayne, Mi) to produce a homogenous mixture of large unilamellar vesicles (LUVs). Non-encapsulated carboxyfluorescein was removed from the vesicle solution through size exclusion chromatography using a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden). LUV solution subsequently contained 10 mM sodium phosphate buffer and 100 mM NaCl at pH 7.5. This combination of buffers ensures that the osmotic strength is matched between the inside and outside of dye-filled LUVs, as determined by baseline leakage in the absence of peptide. Freshly prepared LUVs were used for each experiment.

2.3. Fluorescence experiments

Fluorescence readings were taken at an excitation wavelength of 493 nm and an emission wavelength of 518 nm. A baseline reading was taken on the solutions prior to the addition of peptide. Immediately after addition of the AMP, the fluorescence intensity was recorded for 900 s of interaction. The fluorescence signal given by the addition of peptide was then normalized by the addition of Triton X detergent, causing all vesicles present to release any remaining dye to obtain the total possible fluorescent signal.

3. Results and discussion

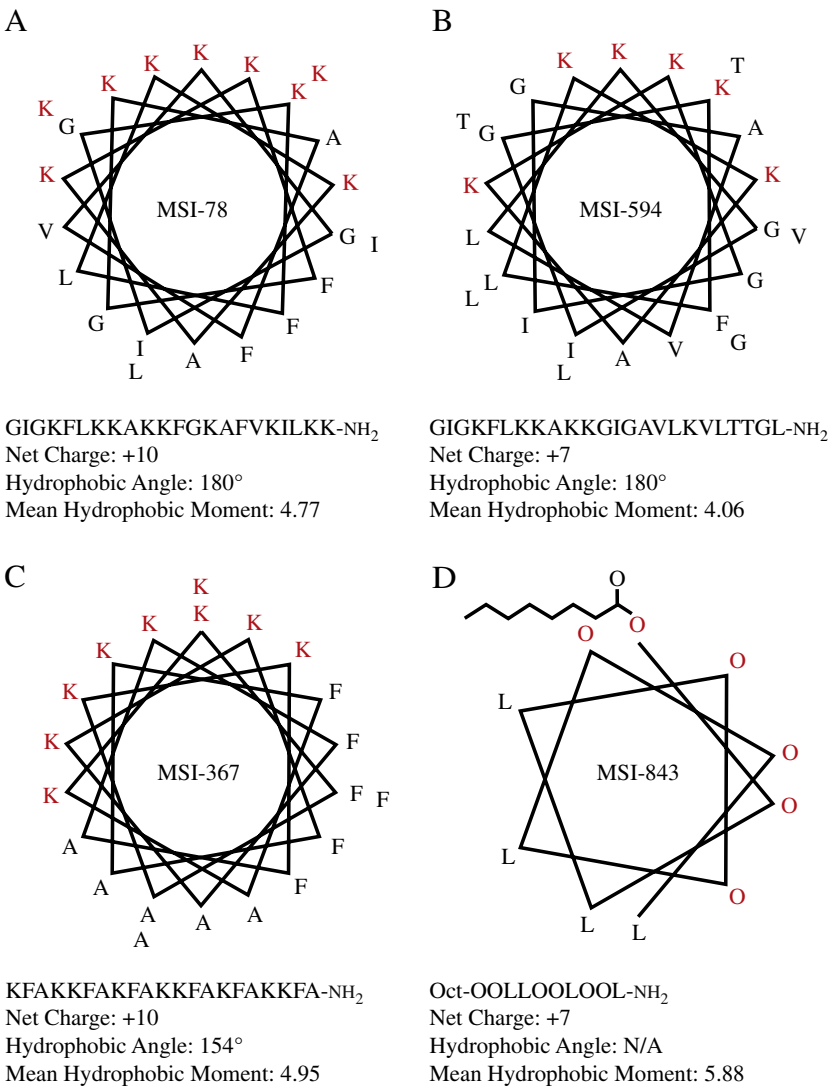
We have measured the effect of cholesterol on the membrane disrupting properties of four different antimicrobial peptides (MSI-78, MSI-594, MSI-843, and MSI-367) against both raft-containing membranes with liquid-disordered–liquid-ordered (L_d–L_o) phase separation (DOPC/DPPC/Chol) and non-raft membranes in the liquid crystalline (L_α) and gel phases (DOPC/Chol and DPPC/Chol). The phase boundaries of these systems have been extensively mapped at the temperature used in this study, 25 °C, by a number of groups [23–28]. The amino acid sequences and other properties of the AMPs used are given in Fig. 1. The antimicrobial peptides chosen are highly potent against a broad-spectrum of both Gram-positive and Gram-negative bacteria and encompass a diverse set of membrane permeabilizing mechanisms and peptide–lipid interactions, [29–35] including MSI-78 (pexiganin), which was in Phase II clinical trials for the treatment of diabetic foot ulcers. [36–41]

3.1. Membrane disruption by AMPs can be selective for either the gel or liquid crystalline phase in the absence of cholesterol

The release of the carboxyfluorescein dye from LUVs was measured as a function of time to test for differences in the extent of membrane disruption induced by MSI-78, MSI-594, MSI-843, and MSI-367 peptides in each of the 16 different lipid systems used in this study (Figs. 2 and 3). Carboxyfluorescein is self-quenched in intact LUVs at the high concentration (40 mM) used in the experiment. Disruption of the membrane by a peptide allows carboxyfluorescein to be released from LUVs, eliminating the self-quenching effect and therefore increasing the fluorescence.

Since cholesterol exerts a strong ordering effect on the acyl chain order of the bilayer [42], we began by examining the degree of membrane disruption by each AMP in LUVs that are either in the ordered gel phase (DPPC LUVs) or the more disordered liquid crystalline L_α phase (DOPC LUVs) (Fig. 3, filled and open circles respectively) [23]. Three of the four antimicrobial peptides used in this investigation clearly favor a specific lipid phase. Both MSI-78 and MSI-594 have a marked selectivity for disordered, liquid crystalline (L_α) phase lipids, as shown by the greater amounts of dye released in DOPC LUVs compared to DPPC LUVs (open compared to filled circles in Fig. 3A and B). This observation is supported by previous investigations showing that MSI-78 induces significant changes in bilayer structure that are indicative of toroidal pore formation [30,39,43–45]. We attribute a similar mechanism to MSI 594 because MSI-594 is a synthetic hybrid of MSI-78 (residues 1–11) and the bee venom toxin melittin (residues 12–24) and also shows a similar random coil to alpha-helix structural transition as MSI-78 [46]. Toroidal pore formation requires partial insertion of the peptide into the bilayer [31–33]. Such peptide insertion is dependent upon lipid order and packing, as demonstrated by the antimicrobial peptide protegrin-1 [47]. The greater degree of membrane disruption observed for MSI-78 and MSI-843 in liquid crystalline samples therefore matches expectations based on the energetics of peptide insertion, as peptide insertion is energetically more difficult in gel phase lipids.

Membrane disruption by MSI-843, however, is more favorable in gel phase lipids, as indicated by the greater amounts of dye released in DPPC liposomes compared to DOPC liposomes in the absence of cholesterol (filled circles compared to open circles in Fig. 3C). MSI-843 is a lipopeptide consisting of a single fully saturated chain of octanoic acid



GIGKFLKKAKKFGKAFVKILKK-NH_2
Net Charge: +10
Hydrophobic Angle: 180°
Mean Hydrophobic Moment: 4.77

GIGKFLKKAKKGIGAVLKVLTTGL-NH_2
Net Charge: +7
Hydrophobic Angle: 180°
Mean Hydrophobic Moment: 4.06

KFAKKFAKFAKKFAKFAKKFA-NH_2
Net Charge: +10
Hydrophobic Angle: 154°
Mean Hydrophobic Moment: 4.95

Oct-OOLLOOLOOL-NH_2
Net Charge: +7
Hydrophobic Angle: N/A
Mean Hydrophobic Moment: 5.88

Fig. 1. Helical wheel projections, amino acid sequences, and physical parameters of (A) MSI-78, (B) MSI-594, (C) MSI-367, and (D) MSI-843. MSI-78 is an analog of Magainin-2, and MSI-594 is a hybrid of MSI-78 and melittin, a bee venom toxin.

attached to a short helical stretch of the nonstandard amino acid ornithine [29]. Observing that the MSI-843 lipopeptide prefers the more hydrophobic, ordered phase is not surprising, as this behavior is common to other lipidated peptides [48]. MSI-367 does not appear to have a strong dependence on a specific phase, yet it has shown highly potent activity for each single-phase system. (Fig. 3D) This behavior could be because the peptide prefers to remain at the lipid–water interface [35], which is similar in both phases.

3.2. Cholesterol inhibits membrane disruption by AMPs in non-raft membranes

We next examined the effect of cholesterol on membrane disruption by AMPS in non-raft membranes (DOPC/Chol and DPPC/Chol, open and filled circles in Fig. 3, respectively). For all the AMPs tested, AMP induced membrane disruption in non-raft LUVs decreases as the cholesterol content is increased. However, the concentration dependence of the effect is different for DOPC and DPPC liposomes. For DOPC membranes, the effect is nonlinear with the cholesterol concentration, with a sharp decrease in membrane disruption occurring when the cholesterol concentration was increased above 20%. DOPC LUVs incorporating 20% cholesterol are only slightly more resistant to disruption than DOPC LUVs without cholesterol (<5% difference for each AMP) (Fig. 3 open circles). Only as the cholesterol content is increased beyond the 20% threshold is a sharp reduction in membrane disruption observed for all the AMPs tested (~45% at 30% cholesterol). This finding neatly corresponds with the known phase properties of DOPC cholesterol mixtures. Below a concentration threshold of 20% cholesterol, DOPC/Chol membranes exist as a mixture of the L_o and liquid crystalline (L_α) phases [23]. Above the 20% concentration threshold, DOPC/Chol liposomes exist purely in the L_o phase [23]. The existence of a sharp reduction in membrane disruption at 20% cholesterol suggests that while AMPs are excluded from the L_o phase, they are free to attack L_α domains on the remainder of the membrane [22]. Membrane disruption in DPPC liposomes, on the other hand, is strongly inhibited even by the incorporation of 20% cholesterol (Fig. 3, filled circles). This finding is also consistent with the known phase properties of DPPC/cholesterol membranes, which are almost entirely in the L_o phase at 20% cholesterol at 25 °C [22,42].

Resistance of the L_o phase to membrane disruption is observed even in the two AMPs (MSI 843 and MSI 367) that do not show a clear preference for the less tightly packed liquid crystalline L_α phase over the rigid gel phase (Fig. 3C and D). The properties of the L_o phase are in most respects intermediate between the liquid crystalline

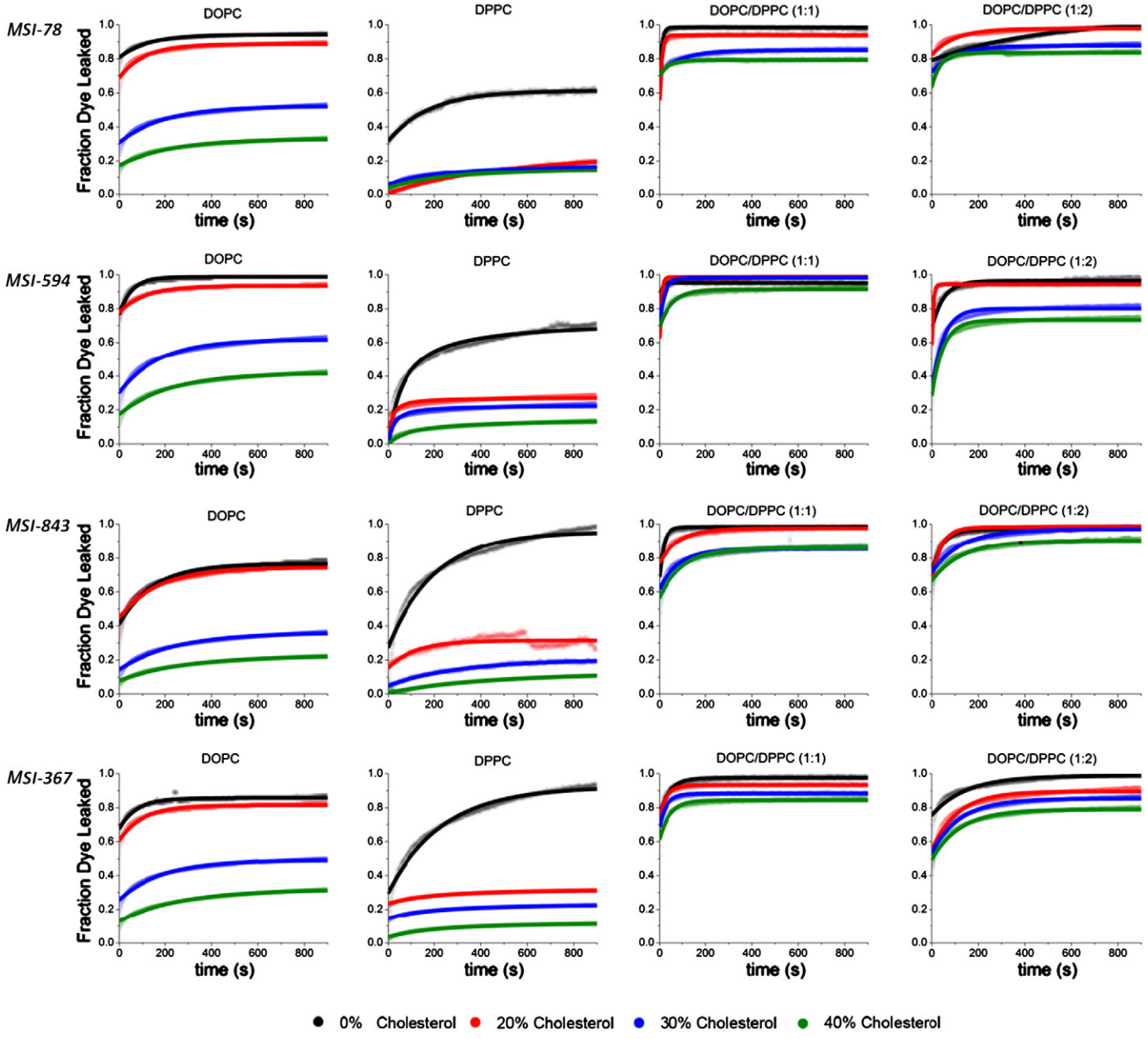


Fig. 2. Fluorescence time curves for the release of encapsulated carboxyfluorescein dye from 100 nm LUVs upon incubation with the indicated antimicrobial peptide. Each sample was maintained at a peptide/lipid ratio of 1/1000 and a temperature of 25 °C. Dye release is essentially complete after 900 s for all samples.

and gel phases, including bilayer thickness, acyl chain ordering, viscosity, and elastic modulus [14,49,50]. Based on these findings, it might be expected that membrane disruption in L_o phases should also be intermediate between the values found for the liquid crystalline L_α and gel phases. However, Fig. 3 suggests that this is clearly not the case. Membrane disruption for all the AMPs tested is clearly lower for the L_o phase than either the gel or liquid crystalline L_α phases. However, not all properties of the L_o phase are intermediate between the gel and liquid crystalline phases. Hydration in the interfacial region of the membrane is significantly lower in the L_o phase compared to the gel phase [51], most likely due to the formation of hydrogen bonds from the 3-OH group of cholesterol to the Sn2 and phosphate group of lipids [52]. This result suggests that cholesterol can compete with the peptide for electrostatic and hydrogen binding to the Sn2 and phosphate groups of the lipids.

3.3. Raft-domain containing LUVs are disrupted by AMPs irrespective of the presence of cholesterol

In non-raft membranes, cholesterol strongly inhibits membrane disruption at moderate concentrations for all the AMPs tested. However, this is not true for raft type mixtures of lipids in which phase separation is expected. We observed high fractions of dye release (80–100%) for all AMPs in both DOPC/DPPC (1/1) and DOPC/DPPC (1/2) systems when cholesterol was incorporated between 20 and 40 mol% (Fig. 3, open and filled squares), contrasting with the strongly attenuated membrane disruption in non-raft LUVs in this concentration range (Fig. 3, open and filled circles). Membrane disruption by AMPs in phase separated lipid systems does not decrease significantly with increasing membrane cholesterol content as it does for single-phase lipid systems. Instead, only a slight decrease is observed as the

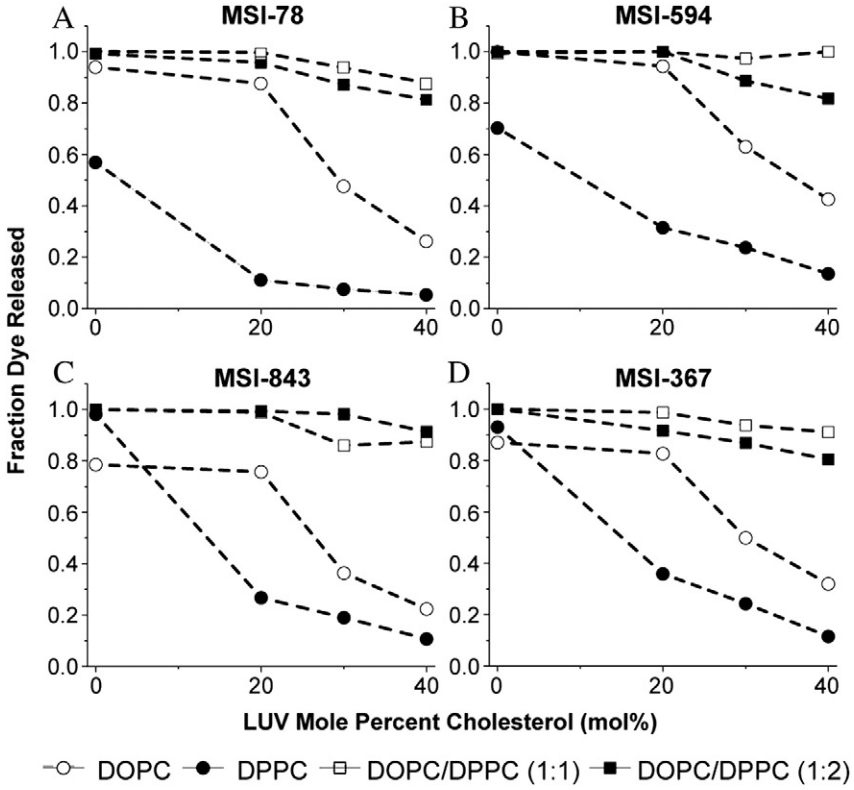


Fig. 3. Fraction of dye released after 900 s from model LUVs (DOPC open circles, DPPC filled circles, DOPC/DPPC (1/1) open squares; DOPC/DPPC (1/2) filled squares) as a function of the concentration of cholesterol present in the membrane when incubated with MSI-78, MSI-594, MSI-843, and MSI-367.

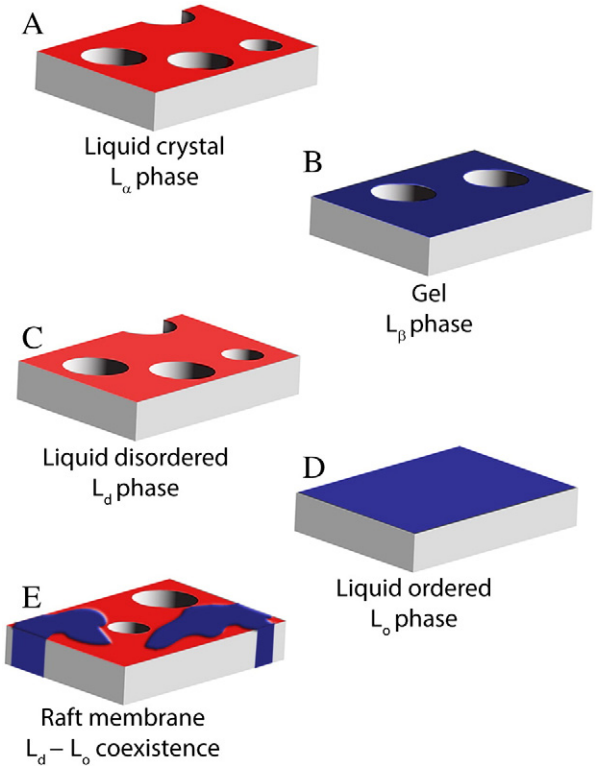


Fig. 4. Cartoon showing membrane disruption in liquid crystalline (L_α) (A), gel (B), liquid disordered (L_d) (C), and raft membranes with liquid disordered–liquid ordered coexistence (E), but not in membranes in the liquid ordered phase only (D).

cholesterol concentration is increased from 20 to 40% (5–15% change as opposed to 25–90% change). This finding is consistent with preferential targeting of AMP to L_α domains in systems with L_o and L_α coexistence (Fig. 4). We cannot exclude an additional effect from line tension at the L_o–L_α interface based on this data, yet we believe that the relatively minimal hydrophobic mismatch afforded by our lipid systems would not be supportive of such a model [53,54].

4. Conclusion

We have shown here the importance of lipid heterogeneity and phase on antimicrobial peptide selectivity. Most importantly, we show that the addition of cholesterol to membranes that have phase separation does not greatly inhibit peptide activity to the extent that it does for homogeneous lipid systems of either ordered or disordered lipids. We conclude that cholesterol's protective, membrane stabilizing effect does not occur to an appreciable level in lipid systems containing raft domains. These results not only provide insight into the processes by which MSI-78, MSI-594, MSI-843, and MSI-367 interact with lipid membranes, but they also broaden the understanding of the interactions involved in general antimicrobial peptide targeting. This information may also prove useful for the design of heterogeneous model membranes for the use of various peptide–lipid interactions.

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