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Functional Determinants of a Synthetic Vesicle Fusion System

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Abstract: Selective membrane mergers may be driven by small-molecule recognition between synthetic surface-displayed fusogens which bear vancomycin glycopeptide and its native binding target, D-Ala-D-Ala dipeptide. These recognition motifs are membrane anchored by antimicrobial peptide magainin II and a phosphatidylethanolamine lipid derivative, respectively. We report herein characterization of this synthetic membrane fusion reaction with regard to the following: effects of fusogen concentration, lipid composition, and membrane charge. Our findings indicate that these parameters are determinants of fusion rate, vesicle stability, peptide binding, catalytic fusion and membrane disruption during fusion. Notably, these data indicate the importance of coupling between molecular recognition and insertion for bilayer activation as well as the critical role of membrane subdomain formation for membrane fusion reactivity. These phenomena are general to lipid membrane chemistry, and therefore these findings provide a guideline for understanding more complex biomembrane systems.

Introduction

Experimental and theoretical studies[1,2] concur that membrane fusion proceeds through at least two steps: membrane docking and actual fusion, resulting in the mixing of membrane lipids and membrane-bound contents (Scheme 1). Fusion may occur upon close (1–2 nm)[2] docking of target membranes, driven by the binding of surface groups. Docking "strains" the surfaces, allowing lipids from the two membranes to mix and ultimately form a fusion pore connecting the two compartments. Insertion of a hydrophobic anchor into the lipid matrix can frustrate efficient lipid packing and activate the membrane toward noncovalent reactions such as lysis and fusion; these reactions are essentially lipidic[3] and are precisely controlled in Nature by molecular recognition events. Much of experimental data on selective membrane fusion has been gathered in studies of synaptic[4] vesicle fusion machinery as well as in viral fusion machinery.[5–7] Physical membrane deformation or insertion of a hydrophobic fusion peptide allows the formation of a high-energy intermediate nonbilayer lipid surface[8] that fuses with its target membrane when drawn into apposition by surface binding. Native membrane recognition elements are proteins, which in class I viral fusion[5] and synaptic vesicle fusion are coiled-coils. Helical bundle formation draws the membranes into apposition; this binding is thought to locally dehydrate and mechanically deform the membrane, lowering the activation energy for lipid mixing and fusion.[1] Notably, enveloped viruses such as HIV[6] and influenza[7] similarly employ coiled-coil recognition to guide fusion with the host membrane. That all known native membrane fusion is driven by protein recognition (and often with coiled coils), raises the question of whether recognition strategies between small molecules would also be fusion competent. Such a minimal fusogenic molecular system would be useful to determine the fundamental requirements for membrane fusion catalysis. A simple synthetic model system of specific fusion via molecular recognition would allow physical organic methods to be applied to rigorously probe the scope and limitations of controlled lipid membrane fusion. All known molecular recognition systems that induce selective fusion are derived from native fusogenic proteins that must be expressed; further, their fusogenic behavior is complex and modulated by other species in vivo.[9] Thus, detailed study of membrane fusion phenomena using native fusogenic systems as probes is complicated by limited control over chemical content and the participation of multiple proteins in the native system. Though there have been reports of small molecule fusogenic systems,[10,11] these systems lack controlled valency, defined partner selection and are not thoroughly characterized.

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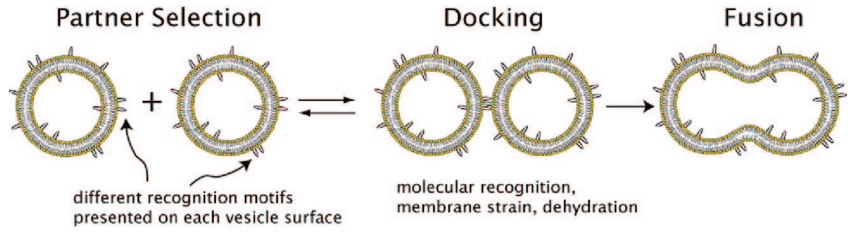
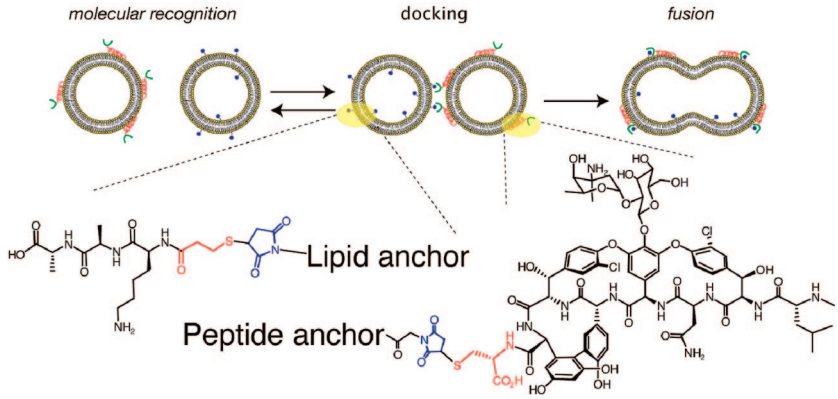
Scheme 1. Model of Molecular Recognition Guided Vesicle Fusion



Scheme 2. Selective Vesicle Fusion Guided by Vancomycin/ D-Ala-D-Ala Recognition



We have previously reported a well-defined chemical model system which has allowed us to examine the molecular determinants of membrane fusion.[12] We report here our detailed examination of the parameters that define membrane fusion behavior. Our designed fusion system incorporates the two functions of recognition and disruption as compactly as possible (Scheme 2).

We chose vancomycin glycopeptide and D-Ala-D-Ala dipeptide as a recognition pair to guide fusion and an antimicrobial peptide, magainin II, fused to vancomycin served both as a membrane anchor and a membrane disrupting module, while the D-Ala-D-Ala dipeptide was anchored using a POPE lipid derivative. Reaction of fusogen-functionalized vesicles as shown in Scheme 2 results in rapid vesicle aggregation and fusion; this can be completely suppressed by addition of underivatized vancomycin, which blocks all available surface D-Ala-D-Ala sites and therefore, membrane apposition. Vancomycin is an antibiotic of last resort that inhibits the transpeptidation step of peptidoglycan synthesis by binding to D-Ala-D-Ala dipeptide on lipid II, a key biosynthetic intermediate of the peptidoglycan cell wall of bacteria.[13] Recognition occurs via the formation of five hydrogen bonds between the two peptide backbones and the free *C*-terminus of the dipeptide (Figure 1). Due to its therapeutic importance, this micromolar dissociation binding event is very well studied.[13] Though only loosely structured, membrane ligand presentation results in increased binding avidity,[14] which can overcome surface repulsion energies and result in membrane apposition. The importance of vancomycin has led to a large body of literature on how it interacts with D-Ala-D-Ala as well as synthetic methodology for modification of the commercially available drug; we therefore judged the vancomycin-D-Ala-D-Ala recognition pair to be an ideal starting point for exploration of small-molecule triggered fusion.

Frog skin-derived antimicrobial peptide magainin II,[15,16] was used to anchor vancomycin to the membrane for reasons similar to our choice of recognition pair: magainin is a very well-studied peptide whose membrane binding mode is known, and it is also

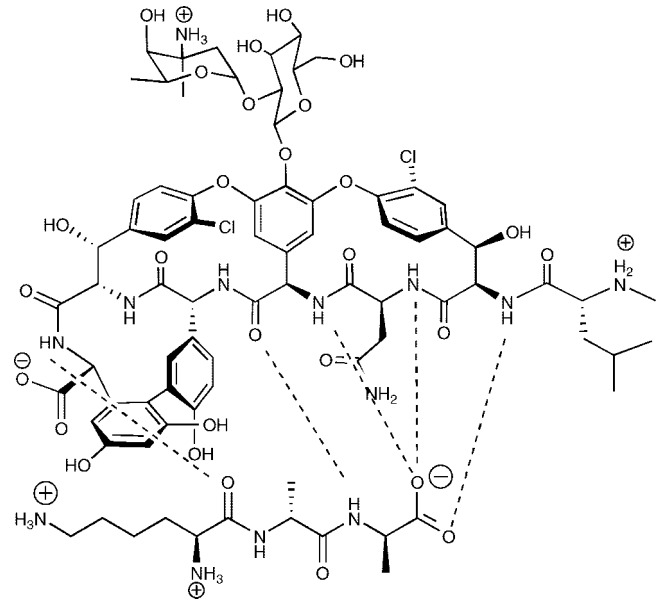


Figure 1. Vancomycin (top) binds to Lys-D-Ala-D-Ala (bottom) via five hydrogen bonds.

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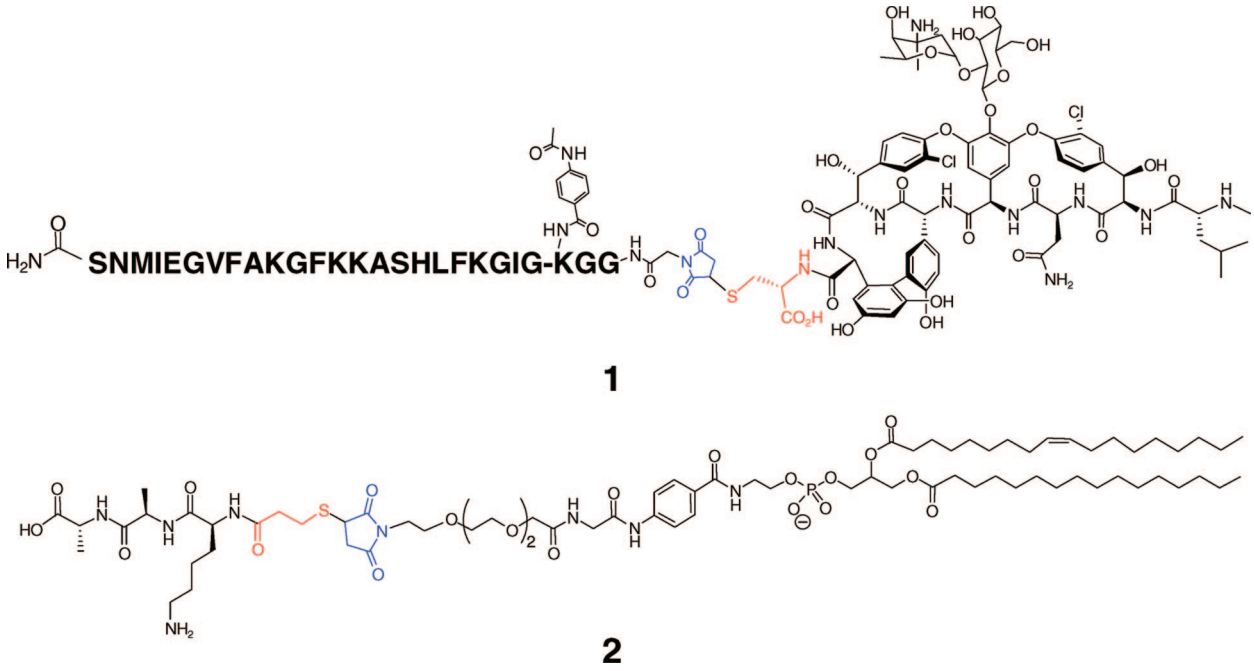
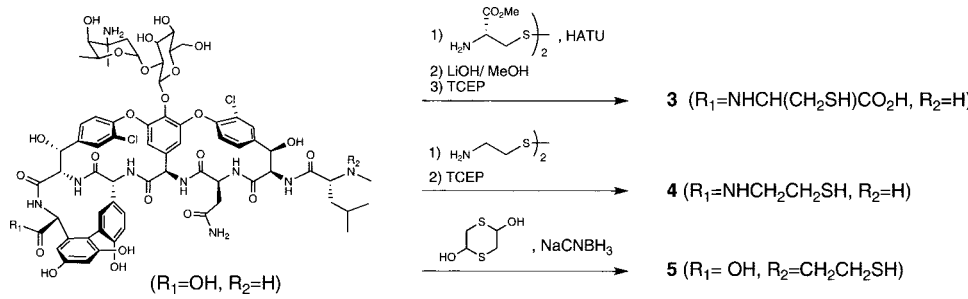


Figure 2. Synthetic fusogens **1** (vancomycin−magainin conjugate) and **2** (Lys-D-Ala-D-Ala phospholipid).

Scheme 3. Sulfhydryl Functionalization of Vancomycin



known to destabilize (perturb) membranes in a concentration dependent manner. Notably, experiments using vancomycin coupled to the relatively nonperturbative POPE lipid anchor instead of the magainin anchor resulted in liposome aggregation without fusion, suggesting that a more disruptive anchor is required to trigger lipid mixing and fusion. Lipid packing determines membrane strain,[17] thus disruption of hydrophobic packing *activates* membranes for noncovalent reactions such as fusion. While phospholipases can increase the rate of fusion by producing single-chain lipids that are hydrophobically mismatched with two-chain lipids,[18] *noncovalent* modification by peptide insertion can activate the bilayer for reaction in much the same way. Shallow insertion of a hydrophobic anchor such as a peptide into the top monolayer of a bilayer (Figure 2) can create negative membrane curvature,[19–21] which destabilizes the lamellar phase. Many antimicrobial (AMPs)[15,20,22] and viral fusion peptides[23] insert in this way and activate permeation and fusion by lowering the energy barrier to non-bilayer lipid phases. Permeation and lysis are dose dependent, and in low concentration AMPs may bind to membranes without lysis or fusion and with minimal permeability increases. Thus, natural product AMPs and viral fusion peptides are possible perturbative components of designed fusogenic systems if coupled to molecular recognition motifs. Magainin II binds selectively into the hydrophobic matrix of negatively charged membranes and perturbs lipid packing without vesicle fusion in the micromolar peptide concentration regime; there are possibly many other natural and synthetic peptides that could serve this role.[24] Thus, we have previously prepared synthetic fusogens **1** and **2** (Figure 2) and verified that they can indeed selectively induce fusion of vesicular membranes via specific molecular recognition of surface-displayed vancomycin and Kaa peptides.[12] We present herein detailed study of the parameters affecting membrane fusion in this system.

Results and Discussion

Synthesis of Vancomycin−Magainin Conjugates. Sulfhydryl functionality was installed on the vancomycin skeleton either at the N-terminus or at the C-terminus (Scheme 3) to allow Michael addition to maleimide-functionalized membrane anchors, which are readily prepared by solid-phase peptide

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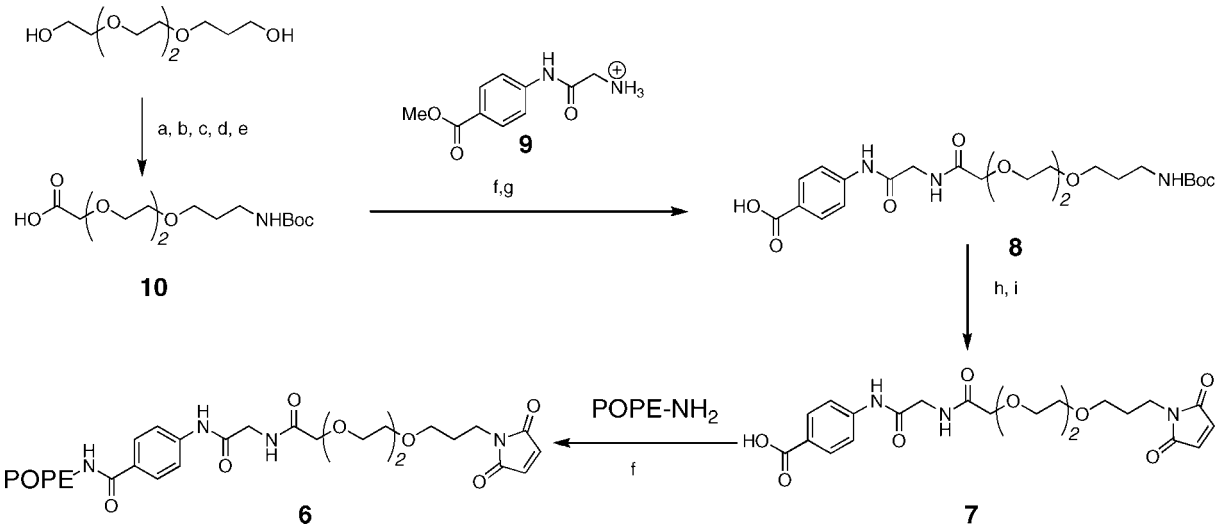
Scheme 4. Preparation of Linkers for Phospholipid Fusogen **2**[a]



[a] (*a*) MsCl, Et_3N, THF; (*b*) NaN_3, MeOH; (*c*) CrO_3, acetone; (*d*) $HS(CH_2)_2SH$, Et_3N, MeOH; (*e*) Boc_2O, KOH, THF, H_2O; (*f*) TBTU, DIEA, DMF; (*g*) LiOH, MeOH, H_2O; (*h*) TFA; (*i*) *N*-methoxy-carbonylmaleimide, Et_3N.

synthesis.[25,26] Reductive alkylation of vancomycin with 1,4-dithiane-2,5-diol yielded the tertiary *N*-terminal aminoethanethiol **5**. Alkylation of the vancosamine sugar was not observed; this was confirmed by acidic cleavage of the aminoglycoside to yield a single product by HPLC with mass of aglycon plus the thioethyl fragment. Glycopeptide was also derivatized by carboxyamidation at the *C*-terminus[27] with aminoethanedisulfide. Interestingly, the thioethyl derivatives of vancomycin **4** and **5** were highly membrane active against synthetic phosphocholine membranes, inducing rapid and irreversible lipid precipitation upon mixing. We hypothesized that the neutral adducts may be increasing the membrane interaction of vancomycin and thus chose to couple cysteine to the glycopeptide C-terminus by carboxamidation with cystine dimethylester. Saponification and disulfide reduction yielded a thiolated vancomycin derivative **3** that retains the native negative charge at the C-terminus. Gratifyingly, this derivative did not detectably induce vesicle aggregation, as judged by light-scattering measurements, and was successfully coupled to a synthetic magainin II derivative bearing an N-terminal maleimide to yield magainin–vancomycin conjugate **1**.

Synthesis of Lys-D-Ala-D-Ala−Phospholipid Derivatives. The binding partner to **1** was similarly prepared by coupling a synthetic mercaptopropionamide-capped tripeptide sequence from the lipid II peptide, Lys-D-Ala-D-Ala (Kaa) to a palmitoyl-oleoyl phosphatidyl ethanolamine (POPE) lipid derivative.[12] The POPE lipid anchor was functionalized with a PEG linker[25], bearing a terminal maleimide (Scheme 4). Both the magainin and POPE anchors bear an acetamidobenzamide (ABA) moiety as a UV label ($\varepsilon_{270} = 18{,}000\ M^{-1}cm^{-1}$) to determine concentration. While Lehn and co-workers have shown that long PEG linkers are necessary to induce fusion in LUVs upon metal complexation with tethered ligands,[11] we have used relatively short linkers to minimize the entropic cost of surface binding and to avoid possible complications from PEG-induced membrane activity.

With these maleimide-derivatized anchors in hand, preparation of peptide and lipid fusogens **1** and **2** was straightforward (Scheme 5). Mixing vancomycin−cysteinamide with the magainin anchor in water in a 1:1 ratio resulted in clean formation of the adduct upon mixing, which was purified on HPLC. Similarly, phospholipid derivative was reacted with the Kaa-mercaptopropionate in methanol with diisopropylethylamine to yield fusogen **2**, which was purified by HPLC.

Magainin−Vancomycin Conjugate Binding to Large Unilamellar Vesicles (LUVs). The binding of magainin to negatively charged membranes is well established,[2829] as is its inability to bind to neutral membranes. Our magainin−vancomycin conjugate is designed to bind to both negatively charged membranes and any membrane bearing Lys-D-Ala-D-Ala (Kaa) lipid fusogen **2**. To better understand the role of the peptide anchor in fusion, we set out to determine the binding constants of our magainin−vancomycin fusogen **1** to both negatively charged and Kaa-displaying vesicular membranes. Fusogen **1** is random coil (unfolded) in aqueous solution, but rapidly folds into an α-helix as judged by circular dichroism (CD) upon interaction with LUVs with 10% and 20% negative charge from palmitoyl-oleoyl phosphoglycerol (POPG) lipids.[19,20] While **1** did not fold with neutral phosphocholine (PC) LUVs, POPG LUVs induced a lipid-dependent coil−helix transition. There is clearly a strong electrostatic component for surface binding as the CD signal increases more intensely and rapidly with increasing POPG

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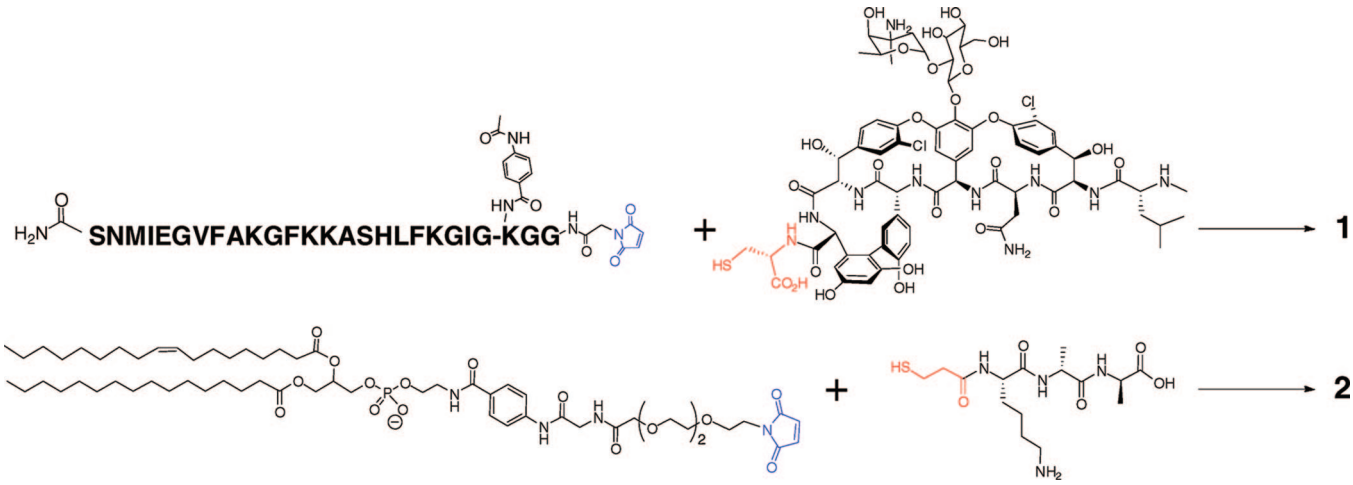
Scheme 5. Fragment Coupling to Produce Fusogens **1** and **2**



content in the LUV titration. The lipid dependence of helicity was analyzed using a surface-partitioning model,[30] in which the partitioning constant K_p is defined as:

$$K_p = \frac{C_b/C_l}{C_{p,free}}$$

where C_b, C_l, and $C_{p,\ free}$ are concentrations of surface-bound peptide, total lipid, and free peptide in solution, respectively. Thus, when surface concentration X_b (C_b/C_l) is plotted as a function of $C_{p,free}$, the slope yields the partitioning constant K_p:

$$X_b = K_p \times C_{p,free}$$

This model assumes that the partitioning constant does not change with bound peptide concentration, an assumption which breaks down when binding is driven by electrostatic interactions or specific lipid binding.[29,31] In these latter cases, the propensity for membrane partitioning decreases the more peptide is bound, due to either decreased surface potential from the positively charged peptide, or as a result of saturation of the surface binding sites. Thus, simple partitioning yields a linear X_b vs $C_{p,free}$ plot, while situations with both hydrophobic partitioning and a secondary interaction (electrostatics or specific recognition) exhibit nonlinear saturation behavior. In the nonlinear plot, the apparent partitioning constant may be calculated at each peptide concentration. We performed this analysis for the binding of magainin−vancomycin to vesicles bearing a 10% and 20% negative charge as well as neutral vesicles bearing 2% D-Ala-D-Ala lipid, using CD and isothermal titration calorimetry (ITC) to follow binding, respectively (Figure 3).

The 10% POPG membrane exhibits a roughly linear fractional binding plot, consistent with weak electrostatic binding, with an apparent K_p (slope) of 1300 M^{-1} (data not shown). As charge content increases to 20% POPG, the X_b plot becomes significantly nonlinear, indicative of a partitioning constant that decreases as more cationic peptide is bound. Notably, binding to the 2% Kaa membrane saturates at a surface concentration X_b of ~0.01 (1%), indicating two points: (1) the peptide fusogen **1** can only bind the Kaa on the *outer* monolayer of the vesicle, which has roughly half the total Kaa, and (2) binding is completely driven by specific Kaa lipid recognition by **1**. The 20% POPG surface has a higher binding capacity for fusogen **1**, saturating at ~3% surface concentration. Furthermore, fractional binding to the POPG surface is consistently higher than the Kaa surface at the same equilibrium free peptide (fusogen **1**) concentration. Though scatter noise from high lipid concentration in the CD titration prevents exploration of lower peptide−lipid ratios, the trend clearly indicates that partitioning into the 20% POPG membrane is favored over binding to the 1% Kaa membrane. For example, when the equilibrium concentration of peptide fusogen **1** is 1 μM in solution, the apparent partitioning constant the membrane is 7000 M^{-1} as compared to 18,000 M^{-1} for the POPG membrane. The difference in partitioning constants should widen further at lower free peptide concentrations. It is clear from this analysis that under the conditions of the fusion reaction, there is effectively no free peptide in solution; all is membrane-bound, and mostly on the POPG membrane. A 1% loading of fusogen **1** correlates to a peptide−lipid ratio of 0.01 on 20% POPG LUVs, which is completely bound (X_b = 0.01), whereas the same loading on a 1% Kaa membrane is only 70% bound (X_b = 0.007) (Figure 3). Importantly, although electrostatics drives peptide fusogen **1** partitioning more so than specific lipid recognition, the partitioning constants are still within the same order of magnitude, meaning that fusogenic peptide has significant affinity to both POPG and Kaa membranes; this is in line with expectations for a peptide that acts to drive apposition and fusion of these two surfaces.

Fusion Is Catalytic in Peptide Fusogen 1. We followed fusion using the same fusion assay as in previous studies;[12] NBD and rhodamine functionalized lipids were incorporated into LUVs with Kaa lipid **2** and this suspension was treated with POPG LUVs and peptide fusogen **1**; increase in NBD (donor) fluorescence was taken as a reporter of loss of FRET, membrane dilution and therefore fusion. In typical fusion reactions, we found that the rate of change in NBD fluorescence decreased and leveled off with time, suggesting that fusion also slows and then stops.[12] The observation that charge content greatly affects fusogen binding suggested that fusion rate should depend on surface charge differential between the two fusing LUVs. This is likely the reason why fusion slows and stops; the charge gradient between the two liposome populations erodes as fusion proceeds, leading to product inhibition and eventual halting of fusion as fusogen **1** binds preferentially to the product (fused) membrane which has both negative charge and Kaa lipid, forming a "cis" complex. Thus, we reasoned that a fusion system

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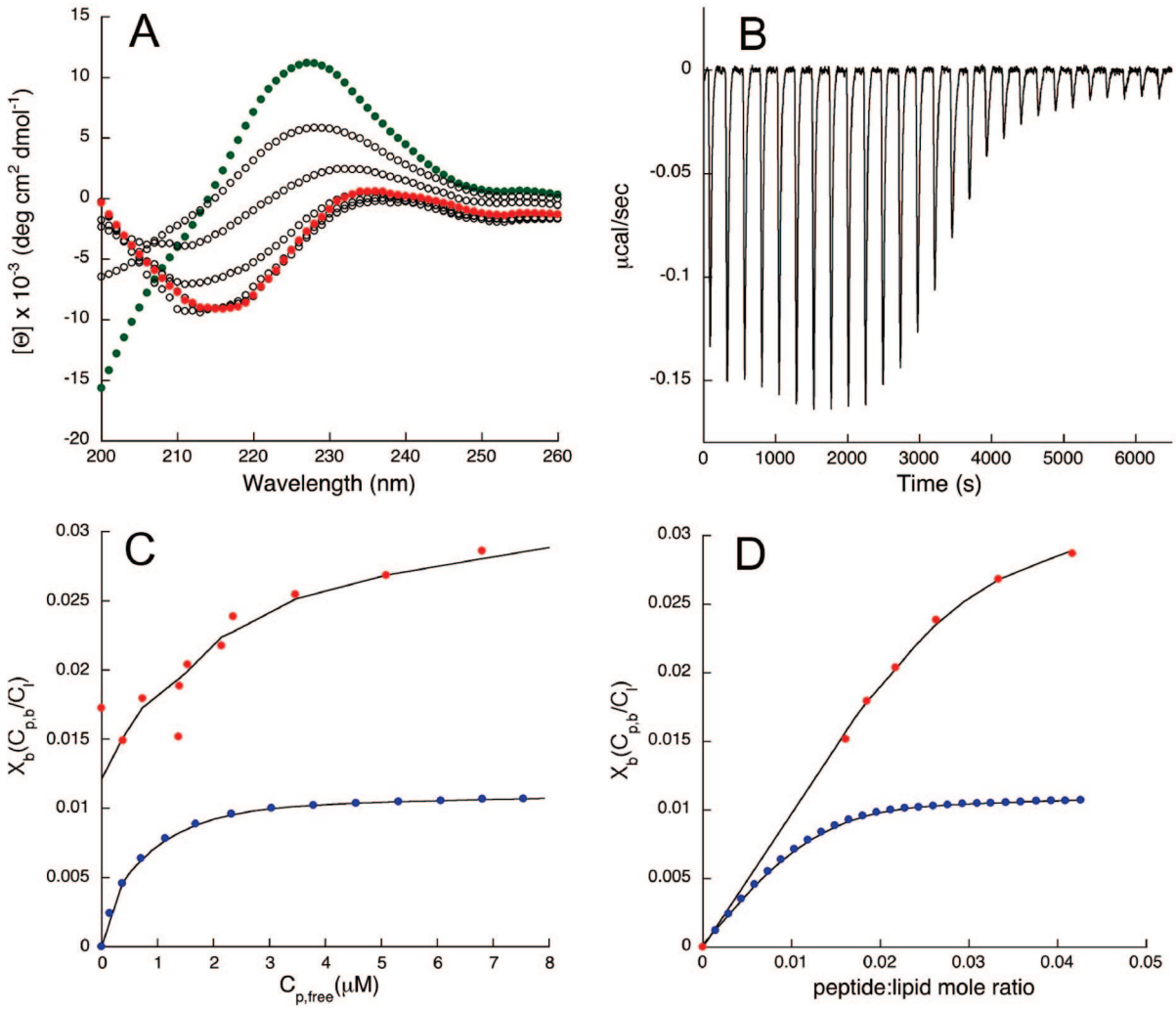
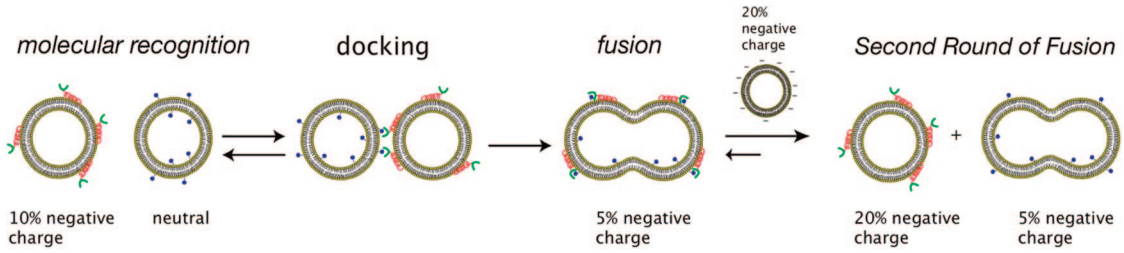


Figure 3. (A) Mean residue ellipticity of fusogen **1** with 20% POPG LUVs. CD scans decrease in ellipticity at 222 nm when total lipid/peptide mole ratio (L/P) varies from (green ○) zero to (red ○) 62; traces in between (○) represent L/P of 10, 20, 30, 42 and 50, respectively. (B) ITC trace of fusogen **1** into LUVs with 2% Kaa lipid **2**. (C) Fractional binding vs free peptide plots from peptide−lipid titration of fusogen **1** and 20% POPG LUVs followed by CD (red ○), and with 1% Kaa LUVs followed by ITC (blue ○). (D) As in (C), plotted as a function of peptide:lipid ratio. Smoothed curves are visual guides only.

Scheme 6. Charge Gradients Render Fusion Catalytic in Peptide Fusogen **1**



catalytic in **1** would be possible. One round of fusion between neutral and 10% POPG LUVs results in product liposomes with 5% POPG and unreacted 10% POPG LUVs. Addition of 20% POPG LUVs should competitively bind all **1** in the system as the partitioning constant is 1–2 orders of magnitude higher than to 10% POPG, permitting the formation of new "trans" complexes and a new round of fusion (Scheme 6).

FRET dilution experiments[32] confirm that this indeed happens (Figure 4). We allowed a fusion reaction with a 10% negative charge differential come to equilibrium and then charged the system with 1 equiv of 20% POPG LUVs, *without additional fusogen* **1**. This resulted in a second burst of fluorescence change, suggesting that new fusion active LUVs may be formed if the charge gradient is sufficient. The system showed a response to multiple injections of naked 20% POPG LUVs, with diminishing response as expected with the eroding charge gradient between product and unreacted LUVs. Thus, sequential rounds of fusion may be accomplished with this strategy; these

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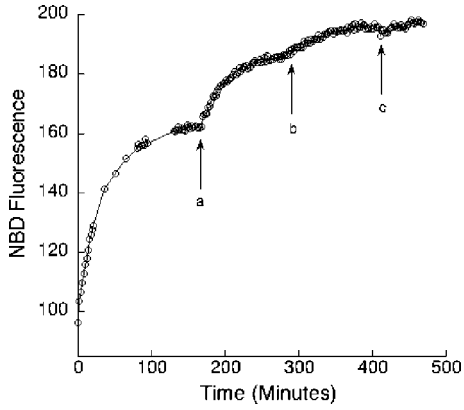

Figure 4. Fusion followed by FRET dilution assay. 4:1 donor (**1**/10% POPG) to acceptor (2% **2** in ePC) LUVs. One equivalent LUVs (20% POPG) added at (a) 168 min, (b) 289 min, and (c) 409 min.

characteristics indicate an artificial fusogenic system whose behavior is not only robust, but predictable.

Effects of Altering Charge Content and Ligand Concentration on Fusion Rate. To determine the effects of varying the relative affinity of peptide fusogen to either reacting membrane, fusion rate was measured as a function of charge content in the membrane as well as surface concentration of fusogen Kaa lipid **2**. Interestingly, analysis of initial rates indicates that there is a minimum concentration of ~0.5% Kaa lipid required before fusion will occur; after this "turn on" concentration, fusion rate increases steadily and ultimately levels off at higher Kaa lipid concentration (Figure 5). The dependence on surface fusogen concentration suggests there must be a minimum level of fusogen partitioning into the Kaa membrane before the membranes are activated enough to fuse. A similar experiment was undertaken by varying charge content in the apposing membrane. In this case, 10% POPG LUVs fused more slowly than 20% POPG, but above 20% negative charge, fusion rates decreased sharply with a significant change in rate profile (Figure 5). We speculate that more negatively charged membranes bind the magainin anchor of the peptide fusogen too tightly to allow the pendant glycopeptide to interact with its Kaa binding partner. Unlike vancomycin/Kaa lipid binding, magainin−bilayer interactions involve the entire length of the peptide anchor, and tightening this binding by increased electrostatic interactions may decrease steric accessibility to the Kaa reactant membrane. This does not appear to be an issue when Kaa lipid concentration is increased, possibly because the magainin−vancomycin conjugate **1** is only anchored in the membrane at a single point by glycopeptide−lipid recognition, with the magainin anchor likely partitioning weakly in the neutral PC membrane. These differences in the way fusogen **1** anchors to the two reacting membranes may have important functional consequences, as discussed below.

Peptide−Membrane Binding and Membrane Activation. Vancomycin binding facilitates insertion of the magainin anchor into a neutral egg PC membrane, as judged by the Kaa LUV-dependent CD spectrum, which shows peptide helicity increasing with lipid concentration (Figure 6). The amphipathic sequence of many AMPs favors insertion in the lipid matrix[33] although not all of these peptides (such as magainin) insert spontaneously, requiring assistance from electrostatic interactions. Lipid recognition could similarly provide the binding energy to drive spontaneous partitioning. These two different insertion pathways have strikingly different effects on membrane function.

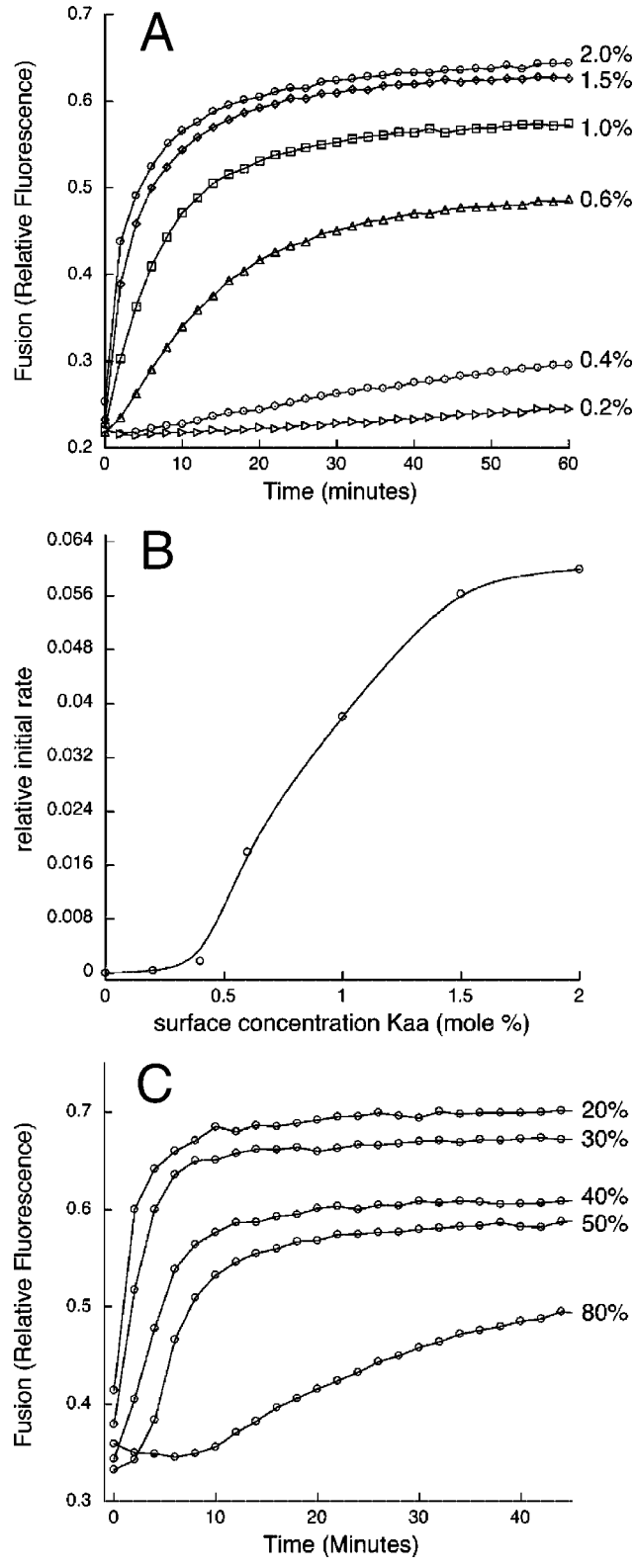

Figure 5. Fusion rates are represented by relative change in NBD fluorescence. (A) Fusion as a function of percentage Kaa lipid fusogen **2** in membrane. (B) Initial rates for fusion as function of Kaa surface concentration. (C) Fusion as a function of percentage of POPG in membrane.

We studied contents release upon fusogen **1** binding with vesicles that encapsulated dye at self-quenching concentration;[34,35] we noted that peptide fusogen **1** induces rapid dequenching (release) of contents from vesicles with 1% Kaa lipid fusogen

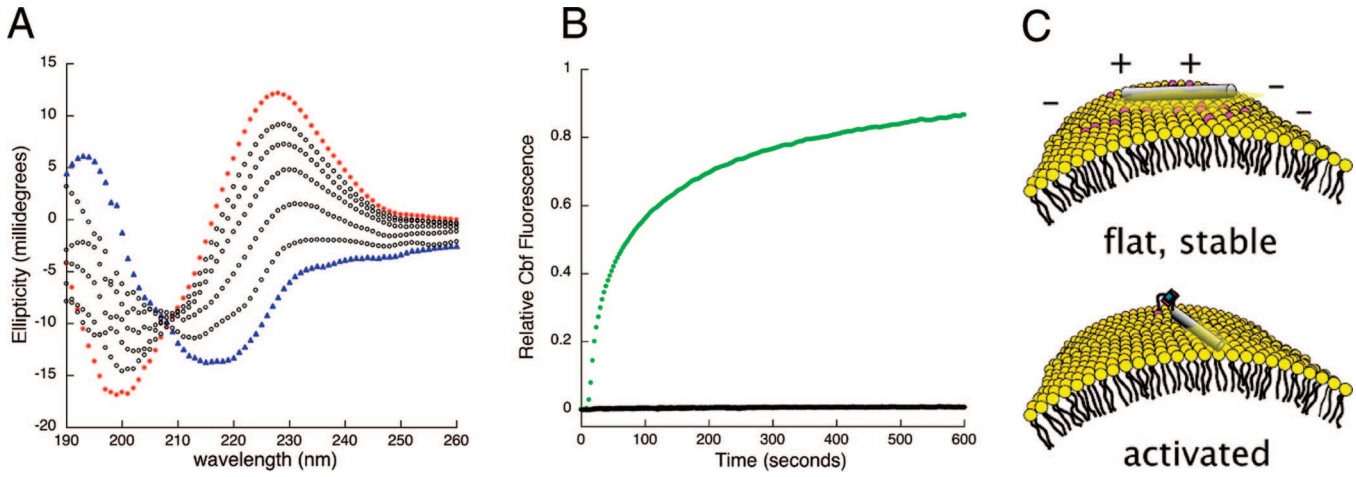

Figure 6. (A) CD monitoring of lipid to magainin−vancomycin (MV) titration. Two mole percent Kaa lipid in eggPC liposomes are titrated into the magainin−vancomycin conjugate. (red ●) lipid/peptide mole ratio = 0; (blue ▲) lipid/peptide mole ratio = 30; (○) lipid/peptide mole ratios in between 0 and 30, all traces decrease at 222 nm with ratio = 30 the lowest. (B) Dye release from liposomes upon treatment with MV at a peptide/lipid ratio of 1:139 and concentrations of 0.625 μM and 86.9 μM, respectively. Green trace = 2% Kaa lipid in eggPC, lower traces = 20% POPG in eggPC and 100% eggPC. (C) Model for how lipid headgroup binding can alter peptide insertion angle and result in more disruptive insertion.

Scheme 7. Possible Domain Interaction Leading to Enhanced Membrane Activity

2, whereas **1** binding to 20% POPG LUVs under similar conditions elicits no detectable contents release.[36] This indicates that peptide insertion driven by specific lipid headgroup recognition is much more disruptive than insertion of an amphipathic peptide into the membrane. Clearly, the physical parameters of insertion are different. While electrostatically driven binding results in roughly uniform insertion of the hydrophobic face of helical magainin, specific binding interaction with a lipid headgroup dictates that the binding terminus must remain out of the membrane while the other end is inserted (Figure 6). The latter mode would involve only partial insertion which could make it more difficult for the lipid matrix to compensate for lipid packing defects. This is similar in concept to the greater membrane activity of obliquely inserting peptides versus transmembrane inserted peptides. Transmembrane insertion merely compresses lipid packing, whereas oblique or partial insertion frustrates packing in the outer lipid monolayer only. This study supports the notion that specific lipid recognition[37] can dramatically alter the functional and physical consequences of peptide−membrane insertion by controlling peptide insertion; presumably, insertion angle and depth are important parameters. This finding lays the groundwork for future designs of membrane activators, including selective fusogenic and pore-forming systems.

Effect of Lipid Composition on Contents Leakage, Domain Formation, and Fusion Rate. One function of cholesterol in biomembranes is to fill in packing defects and stabilize unsaturated lipid membranes.[38] We hypothesized that peptide insertion was causing lipid matrix packing defects that resulted in permeability increases, and thus we incorporated cholesterol in reacting membranes in an effort to decrease leakage during fusion. Using the Cbf release assay as above, we discovered that cholesterol additives resulted in a minor decrease in leakage, but also slowed the fusion reaction. The latter does make sense, as the more rigid the membrane, the less reactive it should be, although the reason why cholesterol does not significantly decrease leakage is unclear. However, when dipalmitoyl phosphatidyl choline (DPPC) was added to the egg PC membrane, leakage decreased and fusion rates increased, in conjunction with increasing DPPC content (Figure 7). We interpret these results in the following way: the increased DPPC content causes the membrane to rigidify and thus decrease leakage, while increased fusion rate reflects domain formation in the membrane. It has been reported that mixtures of saturated and unsaturated lipids such as DPPC and POPC or POPG will phase separate,[39] resulting in separate fluid and gel-phase membrane domains.

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(36) (a) Previous experiments using an ANTS/DPX vesicle contents-mixing assay had indicated nonleaky fusion in our system, but a simple carboxyfluorscein (Cbf) dequenching assay indicated that contents release was rapid from both reacting vesicles. This may indicate that both leakage and transfer occur during fusion. In the ANTS/DPX assay, ANTS leakage results in an increase in fluorescence through dilution dequenching while transfer results in quenching by DPX (decrease). We found a nominal fluorescence decrease upon fusion, possibly a sum of two effects. (b) Ellens, H.; Bentz, J.; Szoka, F. C. *Biochemistry* **1985**, *24*, 3099–3106.
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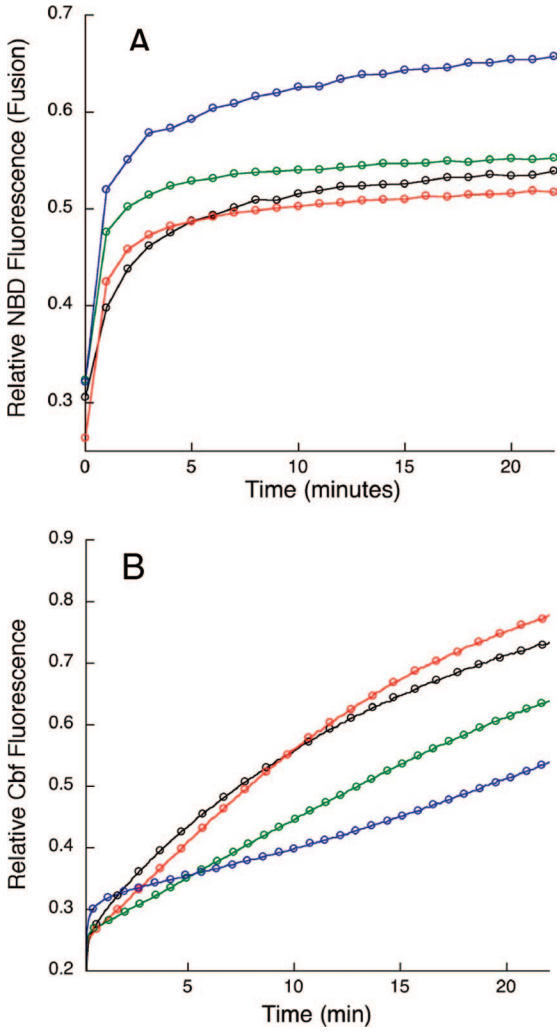

Figure 7. Fusogenic LUVs are mixed at $t = 0$. Donor LUVs preincubated with magainin−vancomycin conjugate and all have 20% POPG and vary in ePC/DPPC ratio: ○ = 80% ePC; ○ (red) = 60% ePC, 20% DPPC; ○ (green) = 40% ePC, 40% DPPC; ○ (blue) = 20% ePC, 60% DPPC. (A) *Fusion rate:* acceptor LUVs composed of 2% Kaa lipid, 1.5% NBD-PE, 1.5% Rh-lipid, 40% DPPC in ePC. (B) *Dye release:* acceptor LUVs composed of ePC/2% Kaa encapsulating Cbf at self-quenching concentration.

Cholesterol has the opposite effect, causing a mixing of domains that results in one liquid crystalline domain that is both strong and fluid, which is critical for biomembranes.[38] These biophysical properties of DPPC and cholesterol-containing membranes may be the underlying cause of the observed chemistry.

Domain formation in a POPG/DPPC/eggPC membrane would segregate the fluid (POPG, egg PC) from the gel (DPPC) and also would segregate the negative charge (POPG) from the gel. Therefore, binding of the magainin−vancomycin fusogen would occur on the negatively charged fluid phase island only (Scheme 7). The POPG domain would have higher charge density, and the membrane as a whole would have peptide binding directed to a surface subdomain instead of being uniformly distributed. This would increase both the density of ligands and degree of activation, thereby increasing the avidity and productivity of initial membrane apposition, resulting in faster fusion.

Conclusions

We have presented herein studies which identify the aspects of our designed membrane fusion system that are critical for reactivity. These findings indicate that membrane activation for fusion depends strongly on the way the fusogen anchors into the lipid matrix. "Single-point" attachment via specific lipid recognition by a peptide results in much more disruptive binding than "multipoint," as when the entire helical face is buried by electrostatically driven binding. This suggests that insertion depth and angle of peptide helices can determine membrane activity and further indicates that specific lipid recognition may generally enhance the membrane activity of all antimicrobial peptides. The presence of gel-phase lipids was found to significantly increase membrane fusion rate and decrease leakage, which provides support for the notion that membrane subdomains formed by lipid mismatch may serve to cluster lipids and fusogens in the bilayer, enhancing binding and function. While the functional role of such lipid rafts in biology remains contentious,[40] their physical existence in synthetic membranes is well established,[41] and this study suggests how lipid subdomains may play a *functional* role in a synthetic fusion system. Examination of this synthetic model system has yielded insights into both fusion and permeation processes, which are general for the lipidic phase of membrane function, and thus has relevance for these same processes in biological membranes.

Experimental Section

General Methods and Instrumentation. All synthetic transformations were carried out at room temperature and all measurements and fusion assays were thermostatted at 25 °C. Stock solutions of fusogens **1** and **2** were prepared and concentrations determined using acetamidobenzamide absorbance ($\varepsilon_{270} = 18{,}000$ $M^{-1}cm^{-1}$). Lipid fusogen **2** was dispersed in lipid films at 1.0–2.0 mol % while peptide fusogen **2** was added to preformed LUVs at 1 mol % concentration. Lipid film hydration was used to prepare a polydisperse population of multilamellar vesicles that were extruded through a 100-nm polycarbonate filter to obtain large unilamellar vesicles[34] (LUVs) with average diameter of 150 nm as judged by light-scattering measurements. LUVs were prepared using mixtures of neutral egg phosphocholine lipids (egg PC) and negatively charged phosphoglycerol (POPG) lipids. Fluorescence measurements were performed in a Perkin-Elmer LS-50B using a 3-mL cuvette or in 96-well plate format with a Spectramax M5 plate reader (Molecular Devices). Circular dichroism measurements were made with an Aviv CD spectrometer. Titration calorimetry experiments were run using a VP-ITC microcalorimeter. Peptides were synthesized on an Advanced Chemtech (Apex 396) automated peptide synthesizer using standard Fmoc chemistry and purified to homogeneity on reverse phase HPLC. Purified peptides and vancomycin derivatives were identified by ESI-MS or MALDI-MS, as were purified lipid derivatives, prepared as previously described.

Materials. Fluorescent lipids NBD-PE and Rh-DHPE were purchased from Invitrogen and used as provided by the manufacturer. Phospholipids POPG, DPPC, and egg PC were purchased from Avanti Polar Lipids. Amino acid derivatives were purchased from Advanced Chemtech and EMD Biosciences, vancomycin hydrochloride and other fine chemicals were purchased from Aldrich and used as provided.

Synthesis of Vancomycin-Cysteinamide 3. Vancomycin hydrochloride hydrate (75 mg, 50 μmol) was dissolved in 1 mL of DMSO and activated for 10 min with HATU (1 equiv, 19 mg) and DIEA (2 equiv, 13 mg) and then treated with cystine dimethyl ester dihydrochloride (4 equiv, 68 mg) and DIEA (8 equiv, 52 mg). The

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mixture was stirred for 6 h and then diluted with water and neutralized with 1 M HCl and purified on a C_{12} semiprep HPLC column using a gradient of 15–50% solvent B/solvent A over 40 min (solvent A = 1% acetonitrile, 0.01% TFA in water, solvent B = 90% acetonitrile, 0.007% TFA in water). The purified glycopeptide was lyophilized to give the cystine adduct in 66% yield based on vancomycin (MALDI-MS, calculated: 1699, found: 1722 ($M + Na^+$). Vancomycin-cystinedimethyl ester (17 mg, 10 μmol) was dissolved in water (1 mL), and lithium hydroxide (10 equiv, 2.4 mg) was added. The mixture was stirred for 30 min after nitrogen sparge then neutralized by 1 M HCl. Tris-carboxyethyl phosphine (6 mg, 2 equiv) was added, and pH was adjusted to be 7–8 with $NaHCO_3$. After stirring for 10 min, the reaction mixture was purified on a C-12 semiprep column with the same gradient used for the cystine adduct. Lyophilization yielded compound **3** in 85% yield. Molecular weight was verified by MALDI (calculated: 1551, found: 1574 (M + Na)).

Addition of Kaa Thiol to POPE Lipid Anchor. *S*-Trityl and *N*-Boc protected peptide ($TrtS(CH_2)_2CO_2$-Lys(Boc)-D-Ala-D-Ala-OH, 28 mg, 38 μmol) was dissolved in a minimum amount of TFA (200 μL), yielding a bright-yellow solution which was diluted with 2 mL of ethyl ether after 2 min. The suspension was centrifuged to obtain a white pellet. The supernatant was removed, and the pellet was resuspended in fresh ether and spun down twice more to remove TFA. The white precipitate was dissolved in degassed methanol and added directly to a methanol solution of POPE anchor **6** (30 mg, 25 μmol) with 6 equiv DIEA under argon. Reaction was followed by HPLC and purified to homogeneity on a C_4 column with a gradient of 70% solvent B to 100% solvent B in solvent A over 40 min (solvent A = 4.99% methanol, 95% H_2O, 0.1% formic acid; solvent B = 4.99% H_2O, 45% methanol, 50% isopropanol, 0.1% formic acid). Product mass was confirmed by electrospray mass spectroscopy (calculated: 1539, found: 1540.2).

Membrane Binding Experiments. All peptide−lipid titrations were performed using 10 mM Tris buffer, pH 7.4, 100 mM NaCl. Changes in signal upon interaction with lipid (ellipticity, heat) were normalized by the maximum change in signal for each experiment after background subtraction. The resulting normalized fractional change in signal at each step of the titration was taken as fractional binding. Each addition of titrant was followed by an equilibration time; for ITC this was taken as the time required for heat flow to return to baseline, and for CD equilibration time was 3 min.

Fusion and Release Experiments. All experiments were run in 10 mM Tris buffer, pH 7.4, 100 mM NaCl. A 5:1 ratio of POPG LUVs to egg PC LUVs containing NBD-PE and Rh-PE at 1.5 mole percent was used unless otherwise noted. Total lipid concentrations were typically to 312.5 μM (POPG LUVs) to 62.5 μM (ePC LUVs). In the charge dependence study (Figure 5C), a 9:1 ratio of POPG LUVs (562.5 μM) to ePC LUVs (62.5 μM) was used. Studies of the effects of DPPC on dye release during fusion (Figure 7B) were performed at a 1:1 LUV ratio (62.5 μM each).

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