Planar aggregation of the influenza viral fusion peptide alters membrane structure and hydration, promoting poration

To infect, enveloped viruses employ spike protein, spearheaded by its amphipathic fusion peptide (FP), that upon activation extends out from the viral surface to embed into the target cellular membrane. Here we report that synthesized influenza virus FPs are membrane active, generating pores in giant unilamellar vesicles (GUV), and thus potentially explain both influenza virus’ hemolytic activity and the liposome poration seen in cryo-electron tomography. Experimentally, FPs are heterogeneously distributed on the GUV at the time of poration. Consistent with this heterogeneous distribution, molecular dynamics (MD) simulations of asymmetric bilayers with different numbers of FPs in one leaflet show FP aggregation. At the center of FP aggregates, a profound change in the membrane structure results in thinning, higher water permeability, and curvature. Ultimately, a hybrid bilayer nanodomain forms with one lipidic leaflet and one peptidic leaflet. Membrane elastic theory predicts a reduced barrier to water pore formation when even a dimer of FPs thins the membrane as above, and the FPs of that dimer tilt, to continue the leaflet bending initiated by the hydrophobic mismatch between the FP dimer and the surrounding lipid.

For all enveloped viruses, one or more glycoproteins on the surface of the viral membrane mediate the fusion of the envelope with the cell membrane for transport of the viral genome to the target cell cytoplasm, bringing about infection. In the first electron microscopy visualizations of purified viral spike proteins from rabies, rubella, influenza, and other viruses, a striking similarity between spikes from different viruses was the assembly of these purified viral spike proteins into aggregates, termed rosettes, as their hydrophobic transmembrane domains (TMDs) aggregated. Most enveloped viruses enter their target cells via the endocytic pathway, where the viral envelope spike protein encounters acidic pH. For the influenza virus spike protein hemagglutinin (HA), acidic pH activation of HA is necessary and sufficient for triggering fusion of the viral envelope to a variety of target membranes, including receptor-doped phospholipid bilayers. In the absence of their TMDs, activation of isolated soluble ectodomains of HA led anew to fresh rosettes of those trimers. The N-terminal domain of HA2 is responsible for this second aggregation of HA ectodomains—it is a short amphiphilic N-terminal sequence that became known as the fusion peptide (FP). As with the first rosette of HA, this second rosette formation is considered a consequence of the hydrophobic effect: the hydrophobic surface formed by one side of the FP would avoid water via association with the
hydrophobic surface of another FP. The influenza FP comprises the N-terminal 21 amino acids of HA2, located within the HA ectodomain (at neutral pH), proximal to the HA trimer surface but near the TMD. At low pH the FP is found in the target membrane, as evidenced by hydrophobic photolabeling\textsuperscript{13,17}. The FP is required for infection in vivo and membrane fusion in vitro and is featured in all hypotheses on HA-mediated fusion\textsuperscript{5,7,9}. Since the FP is a highly conserved region of the influenza virus genome across many different subtypes of influenza virus\textsuperscript{10,22}, and a universal feature of enveloped viral fusion proteins, determining the FP’s role for infectivity and membrane fusion is critical to finding variant-independent immunogens and pan-viral therapeutics to ameliorate morbidity and mortality.

We recently studied influenza hemifusion intermediates in vitro with high-resolution cryo-electron tomography using a phase plate to enhance signal-to-noise by a factor of four\textsuperscript{22}. The cryo-tomograms of the FP is a highly conserved region of the influenza virus genome across many different subtypes of influenza virus\textsuperscript{10,22}, and a universal feature of enveloped viral fusion proteins, determining the FP’s role for infectivity and membrane fusion is critical to finding variant-independent immunogens and pan-viral therapeutics to ameliorate morbidity and mortality.

In MD simulations crafted to understand the chemistry by which FPs act, a third kind of rosette emerged: the aggregation of FP via their lateral side chains (not their hydrophobic surfaces) into FP rosettes. This aggregation is significantly increased by our data based on a tilting of FPs towards each other to further structure locally thinned the bilayer and significantly increased the probability of water entry. A new model is proposed to explain our data based on a tilting of FPs towards each other to further thin the remaining lipids immediately under even an FP dimer. For larger aggregates, this more hydrated, thinner membrane structure replaces the lipid bilayer in a small domain wherein a lipidic pore can form.

**Results**

**Experimental results:** fusion peptide is sufficient to porate GUV

Stable pore formation, detected as passive transport of the water-soluble, membrane-impermeable fluorescent dye Alexa 488 into (influx) or out (efflux) of GUV, was monitored by confocal microscopy.

**Influx:** GUV are immersed in Alexa 488 containing solution. Prior to FP addition, the interiors of GUV were dark. Upon addition of fluorescently labeled FP at neutral pH, FP bound to GUV (Fig. 1A, red). In fractions of the GUV (here defined as the leakage fraction), Alexa 488 accumulated inside the GUV changing the black interior to green (Fig. 1A, Influx). In other fractions of GUV, leakage was not detected and the absence of poration was deduced despite the binding of FP: no Alexa 488 influx was detected (Fig. 1A No Influx). The fluorescence signal increased until the signals detected inside ($F_{in}$) and outside ($F_{out}$) of the GUV were equal (Fig. 1B; representative examples; $n = 7$ from 2 independent GUV preparations); the influx ratio $\frac{F_{in}}{F_{out}} = 1$ is defined as 100% influx. GUV integrity was maintained during and after poration (the intensity and distribution of DiD lipid dye, labeling the GUV membrane, remained unchanged). Efflux: Poration was observed as a loss of GUV internal fluorescence with the efflux ratio, following normalization, $R = (F_{in} - F_{out})/(F_{in} + (t=0) - F_{out}) = 0$ defined as 100% efflux. POPC GUV encapsulating 3000 MW-\textsuperscript{a} Alexa 488-labeled dextran showed 100% efflux into a non-fluorescent bathing solution ($n = 2$ from 1 GUV preparation). FP-induced pores in POPC GUV were a) stable on time scales that allow equilibration of the probe with the external medium, and b) large enough to allow transport of both 3000 and 570.5 MW molecules. Thus, the poration of GUV seen upon acidification of attached influenza virus or viral-like particles...
Viral-induced pores were preferentially detected in target membranes containing low cholesterol (≤40 mol%)22,28, the dependence of FP-induced pores on lipid composition was tested. Target membranes comprising higher cholesterol concentrations hindered FP-induced pore formation. In the presence of FP, vesicle poration (Leakage %) of POPC GUV with varying cholesterol concentrations decreased with increasing cholesterol concentration, consistent with our hypothesis (Fig. 1C, n = 3 independent GUV preparations evaluating 50–100 GUV per experiment); leakage decreased from ~80 to 40% for cholesterol concentrations 30 and 50 mol% corresponding to leakage decreases of 34 and 48%, respectively, relative to 0 mol% cholesterol.

The ring structure of cholesterol confers rigidity to membranes and this property could explain the observed decreasing leakage with increasing cholesterol. To determine whether cholesterol-induced rigidity or if negative monolayer spontaneous curvature (MSC) of the GUV leaflet is a correlating parameter, GUV were prepared with the same MSC (−0.258 nm) as those of the highest cholesterol concentration used in this study (50 mol%), by replacing cholesterol with 24 mol% dioleoyl glycerol (DOG). The MSC of cholesterol, DOG, and POPC in POPC alone (see calculation of the mixture MSC are: −0.39, −0.99, and −0.22 nm, respectively) and use the commonly accepted approximation that the MSC of a lipid mixture is defined as a concentration weighted average of the component MSCs. In GUV did not exhibit statistically different leakage fractions from 50% cholesterol GUV (% Leakage) of POPC GUV (40.3 ± 8.2% for 50 mol% cholesterol compared to 49.3 ± 4.4% for 24 mol% DOG; mean ± SEM; n = 3, respectively; p = 0.39, 2-tailed equal variance T-test). These results are consistent with the hypothesis that leakage and MSC are correlated parameters, and lipids with negative MSC, e.g. cholesterol or DOG, hinder pore formation.

According to the classical theory29, lipids can be described by their effective molecular shapes: (i) conical lipids have larger tail than headgroup cross-sectional areas, which leads to negative MSC; (ii) inverted conical lipids have smaller tail than headgroup cross-sectional areas leading to positive MSC; and (iii) cylindrical lipids have zero MSC. Lipids with positive MSC (inverted cone) tend to form structures with positive geometrical curvature like the pore edge, thus enhancing membrane poration and inhibiting fusion. Lipids with negative MSC (cone) tend to form structures with negative geometrical curvature like fusion sites, thus inhibiting membrane poration and enhancing fusion.

To gain insight into the relative lifetime of the FP-induced pores with and without cholesterol, pore stability was monitored via the influx ratio, R, in the presence and absence of 50% cholesterol (Supplementary Fig. 1A). Pure POPC vesicles exhibited R close to 1 (≥80%, n = 14 of 17 reported, have filling ratios > 0.95), consistent with stable pores over a time sufficient for equilibration with the outside concentration to occur (tens of seconds, Fig. 1B). In the presence of cholesterol, R ranged from 0.1 to 0.6 (> 90%, n = 28 of 30 reported, have R < 0.6). The sub-maximal level of influx in these GUV and estimates of pore open time (Supplementary Fig. 1B) are consistent with a higher probability of pore closing in the presence of high cholesterol. Thus, FP-induced pore lifetime is shorter in cholesterol/POPC GUV than in POPC GUV.

To further confirm whether submaximal leakage indicates a pore closing event or a slowly flickering pore, a second marker (sulforhodamine B, SRB) was introduced 25 min later following the introduction of the first marker, Alexa 488. Since SRB and Alexa 488 have similar molecular weights (539 and 643 g/mol, respectively), if a pore allows entry of Alexa 488 then the same pore should allow influx of SRB. Consistent with this hypothesis, when introduced simultaneously, Alexa 488 and SRB have comparable levels of influx (Supplementary Fig. 2A). However, if an initially open pore closes, then the influx of SRB would be restricted. Supplementary Fig. 2B shows the influx of Alexa 488 (the first soluble marker) when incubated in the presence of the FP, but, in this example, SRB was excluded when introduced later, i.e., there was no influx. Hence, over this time scale, FP-induced pores are not stable in the presence of 50% cholesterol although they are stable in membranes without cholesterol.

Lastly, the question of FP aggregation was considered. Analyses of the distributions of FP in bulk solution and on the vesicle indicated that, in addition to an increase in the average amount of FP on the vesicle in time, the variability at the pixel scale (~500 nm) also increases in time. In all experiments, the distribution of pixels for the best fit intensity surface indicated the presence of pixels with larger intensity deviations compared to solution; the differences between the peptide in solution and on the GUV is not explained by the differences in intensity but is consistent with the appearance, in time, of a nonuniform or segregated distribution of FPs. Specifically, poration occurs with a characteristic time, 101 ± 22 sec, and normalized FP density, 2.7 ± 0.05, (mean ± SEM; n = 9, including both Alexa 488 and dextran experiments), both log-normally distributed, where the characteristic time represents the difference between the time leakage is first detected and the time the normalized FP fluorescence on the vesicles increases above the FP fluorescence in solution (see Fig. 9 and aggregation analyses in Methods). The combined deviations from n = 9 experiments taken around the estimated poration time are shown in Fig. 2. The intensity deviations for FP on the GUV were broader with extended tails, relative to the solution distribution, indicating that many pixels have higher than expected intensities under the null hypothesis that the distribution of FP on the vesicle is the same as the solution distribution. The differences in the distributions between the two distributions is significant (n = 16,200 for both distributions, alpha = 0.00001, Ansari-Bradley test). This observation is consistent with the aggregation of FP at the optical pixel scale. The pixels with higher-than-expected peptide intensities are ~10 times greater than the average intensity in solution. With a nominal voxel size of 1 × 1 × 2 μm and all peptides in the voxel contributing to the intensity measured in a nominal 1 × 1 μm2 pixel, a rough estimate of the number of peptides present at these “hot spots” is ~36,000. Assuming a uniform distribution at the level of a single voxel, the number of FPs, when scaled to the MD simulation...
considerable mixing over 21 μs in both systems. Of note, the most stable dimers are antiparallel, with the two N-terminal helices interacting with one another. Interactions between FPs are primarily mediated by direct FP–FP interactions, rather than water or ion bridges. This aggregation may be surprising given the net negative charge of these FPs. However, because even a single FP leads to local membrane deformations, clustering of FPs decreases the net energy of the FP-lipid boundary of the system which stabilizes clusters (see Theory section below). Aggregation in the 6 FP systems is much more limited (Supplementary Fig. 4), as consistent with the lower peptide concentration. A detailed examination of aggregation as a function of concentration will be reported in future work using coarse-grained models. 

The effect of the FP clustering on the membrane is profound: aggregates displace lipids in the upper (cis) leaflet, curve the trans leaflet (Fig. 3 and Supplementary Fig. 5), and sit much lower in the leaflet than an isolated FP (Supplementary Fig. 6). As shown in Fig. 5A for two snapshots of FP in POPC from the 10 FP simulation, even an antiparallel dimer can displace lipids, and thereby come in direct contact with the trans leaflet. This can be understood as follows: FP monomers are cradled by adjacent lipids, as has been observed previously for extended surface-associated amphipathic helices. In contrast, the inserted area of the dimers is too large to be covered by lipids in the same leaflet, and consequently the lipids are replaced by the peptide aggregate. The top two panels of Fig. 5B show views of the underside of the entire cis leaflet at t = 0 for both 10 FP bilayers. The hydrophobic undersides of the FP are only faintly visible indicating negligible lipid displacement. The bottom two panels of this figure show substantial displacement of lipids by 3 dimers in POPC at 17 μs, and by a hexamer in chol:POPC at 19 μs.

The structure of the helical hairpin also promotes dimerization and insertion. Specifically, irrespective of clustering or insertion depth, all FP in all simulations were seen as rotated from a reference state in which both N- and C-terminal helical components of the FP would be the same average distance from the bilayer midplane. This rotation was quantified by a roll angle ρ, (Supplementary Fig. 8), with the N-terminal helix of the hairpin beneath the C-terminal helix; similar rotations were reported by Brice and Lazaridis from simulations of a single FP in DMPC. The FP roll angle was larger in the chol:POPC membranes, with ρ > 36 ± 0.7° as compared to 31.6 ± 0.8° in pure POPC (mean ± SEM; p < 0.003, pooled t-test). The long axis of the peptides remained roughly parallel to the membrane surface, regardless of aggregation or membrane composition, though large (＞25°) positive or negative tilt angles are occasionally sampled (Supplementary Fig. 8, tilt angle).

This orientation of the FP differs from that of most amphipathic peptides, which consist of a single long alpha helix lying relatively flat on the bilayer surface. Partial vertical stacking of the two FP helices increases the peptides’ thickness projection along the bilayer normal, with the FP subtending a total thickness of -15 Å. However, the rolled orientation of the FP does not prevent water molecules from penetrating this sparse layer, unlike the lipid leaflet that they replace. This rolling and concomitant thickening along the z-axis allow the FP assembly to replace lipids in the cis leaflet more easily than a linear amphipathic peptide would, and with less hydrophobic mismatch at the cluster boundary. Consistent with the increased leaflet thickness of chol:POPC compared to pure POPC, the larger value of ρ allows the FP to span a longer distance along the z-axis. Movies of each of the system snapshots shown in Supplementary Fig. 3, demonstrating peptide tilt and roll, are included as Supplementary Movies 1-6.

Lipid displacement by the FP aggregate in turn leads to localized membrane thinning (Fig. 6 upper left). Thinning is most pronounced in the 10 FP chol:POPC system, which has the densest FP aggregates. The chol:POPC:10FP system thins by 4.5 Å in the region of the aggregate, to a bilayer hydrophobic thickness of 31.0 Å compared to 34.5 Å in the peptide-free case (Fig. 6). The POPC:10FP system thins by a more
modest 2 Å (from 27.5 Å to 25.4 Å). This combination of thinning and lipid displacement leads to substantial deformation of the trans lea
felt (Supplementary Fig. 3 and Supplementary Fig. 5), as the lea
felt bows towards the cis lea
felt, so that the terminal methyl groups of the trans lea
felt lipids can contact the hydrophobic underside of the FP aggre-
gate. As anticipated, the extent of this deformation becomes more
pronounced with increasing aggregate size (Supplementary Fig. 5). Because chol:POPC is a thicker bilayer than POPC and displays a
greater extent of FP-generated thinning, the explicit curvature of the
trans lea
felt is more prominent in these systems (Supplementary
Fig. 5). Also, the peptide rides higher into the aqueous media bathing
the cis lea
felt in chol:POPC when surrounded by lipid but inserts
deeper into the membrane to abut the trans methyl groups, so the
thinning itself is greater in absolute terms, and the lipid bounding the
FP has a greater deformation in chol:POPC. In the theory on FP
aggregation (below) this increased deformation creates a large line
tension around the FP cluster and explains the slight bias towards FP
dimer formation seen in chol:POPC (Fig. 3 and Supplementary Fig. 3).

These geometric changes modulate the spontaneous curvature of
each lea
felt in ways not well-described by the traditional theoretical
model of non-interacting FPs. The FP aggregate-dependent displace-
ment of lipids in the cis lea
felt, and the high degree of localized bilayer

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**Fig. 4 | Snapshots and clustering of microsecond time-scale MD simulations with 10 FP.** Snapshots are shown at times 0, 11 and 21 μs of the 21 μs simulation. Plots show the cluster composition at each microsecond, with the symbol representing the number of peptides in a given cluster. The peptide colors in the plot correspond to the colors in the snapshots. Source data are provided as a Source Data file.
thinning, suggest that bilayers with FP present would be more susceptible to pore formation.

While the conventional MD simulations presented above are not of sufficient length to generate pores or pre-pore configurations, they do yield information on water content. The free energies, \( F(z) \), for water in the FP-containing and FP-free POPC and chol:POPC bilayer systems are plotted in Fig. 6 (right) across the thickness of the bilayer. These profiles are obtained from the probability distributions of water (Eq. 6 in Methods) and are commonly termed potentials of mean force (PMF). All systems show low free energy in the well-hydrated headgroup region and a substantial free energy barrier in the hydrophobic hydrocarbon region. The barrier at the midplane (\( z = 0 \)) for pure POPC is characteristic of most homogenous bilayers; the metastable minimum at the midplane in chol:POPC results from chain ordering and is found in liquid-ordered phases. Addition of a single FP only slightly lowers the free energy barrier, while the aggregates observed in the 10 FP simulations substantially alter the energy landscape. The barrier height is reduced by approximately 1.6 \( k_B T \) for POPC:10FP, and the width at half-height by roughly 3 Å; the barrier height is relatively unchanged for chol:POPC:10FP but the width at half-height is reduced by 12 Å, indicating significant membrane thinning. Furthermore, the plateau region of the PMF around \( z = 0 \), which is characteristic of cholesterol-containing membranes, is absent in the 10 FP system. Aggregates of intermediate sizes from the 6 FP simulations have an analogous effect following the same trend as the extreme cases of 10 FP and FP-free — reduction of the barrier height and concomitant narrowing in the POPC membranes and narrowing with an associated shape change in chol:POPC. Since pore formation is presumed to be initiated by the formation of a water wire (see Theory section below), these reductions in the free energy for water can be taken as an indirect measure of the propensity of the aggregates to generate pores. The formation of hydrophilic pores is developed in the following section.

The free energy of water in each membrane can be related to the permeability by the inhomogeneous solubility diffusion (ISD) model (Eq. 7)\(^\text{(36)}\). Fig. 6 (lower left) plots the inverse resistances to water permeability \( 1/R \) evaluated from \( F(z) \) and the ISD model (Eq. 8). Since the permeability \( P \) is proportional to \( 1/R \), it is most instructive to compare values of \( 1/R \) between systems. The presence of FP aggregates leads to a ~7-fold increase in \( 1/R \) for the case of 10 FP in pure POPC, while the 10 FP aggregate has a more moderate ~3-fold increase for the chol:POPC membrane. Even the presence of a single FP increases this ratio from ~5 to 10, indicating a stronger effect on the pure POPC membrane. Most notably, \( 1/R \) for POPC:10FP is 13-fold larger than for chol:POPC:10FP, implying that the permeability to water is lower by a factor of 13 when cholesterol is present.

Theory

FP clustering: The inhomogeneous distribution of FPs on the surface of GUV at the time of poration is consistent with the hypothesis that, following FP surface binding, FP aggregation is an important driver of poration. The MD simulations at varying FP concentrations show a cooperative activity of multiple FPs: aggregates thin the membrane (Fig. 6) and exclude cis leaflet lipid tails and illustrate elastic deformation of the membrane at the FP/membrane interface for even a single FP. Thus, FPs incorporated into the lipid leaflet can be considered as generators of boundary conditions that induce membrane deformations. We calculated the elastic contribution to the FP cluster boundary energy in the framework of the theory of lipid membrane elasticity assuming symmetric FP, as described in the Methods section.

Bringing two FPs into close contact eliminates part of the peptide/membrane boundary, and consequently nullifies the deformations induced by that part of the boundary. Thus, nonzero boundary energy acts as a driving force for aggregation of FPs. Entropy-based forces lead to the lateral dispersion of the FPs at lower overall concentrations. The balance of these two forces results in some critical concentration of FPs, above which aggregation becomes possible. To ensure aggregation, the total free energy difference \( \Delta E \) between the ensemble of clusters containing \( n_{\text{clus}} \) FPs each and the system of separated FPs should be negative. We combine the elastic energy of the cluster \( E_{\text{clus}} \), the elastic energy induced by single FP \( E_{\text{fp}} \) and the free energy of water in each membrane.
energy arising from the entropy of mixing \( E_{\text{ent}} \) into \( \Delta E = E_{\text{cluster}} - E_{\text{FP}} + E_{\text{ent}} \) and require \( \Delta E \) to be negative as a condition for spontaneous clustering. FP aggregation is ensured when the normalized surface concentration \( x \) of FPs is above a critical concentration \( x_c \) where \( x_c \) is the value of \( x \) satisfying the equation \( \Delta E(x) = 0 \). For large clusters one can neglect the boundary energy of the cluster and entropy of the cluster ensemble (see Appendix in SI). The approximate expression \( x^0_c \) for the critical concentration reduces to a simple Boltzmann factor of the energy induced by insertion of a single FP (for details see SI):

\[
x^0_c = e^{\frac{E_{\text{FP}}}{k_B T}}
\] (1)

For the elastic parameters specified in Methods (Calculations of the energy of elastic deformations of lipid membranes.), \( x^0_c \approx 0.27 \). The dependence of the critical concentration \( x_c \) for aggregation on cluster size is weak and demonstrates that Eq. 1 is a reasonable estimate (see Supplementary Fig. 11). The optimal cluster size is calculated from the total cluster formation energy. There is a clear energy minimum at the cluster size \( x \approx 10 \) (Fig. 7). Note that this minimum is local, and at larger sizes the energy again decreases, predicting that larger systems should form macroscopic clusters. Thus, small FP clusters can form spontaneously, while the formation of larger clusters requires overcoming an energy barrier. Accounting for dipole-dipole attraction, one finds the formation of hydrogen bonds between contacting FPs will decrease the critical concentration \( x_c \) and enhance the formation of the cluster.

The pathway to pore formation: Since a cylindrical aqueous pore in a bilayer is the best pathway for lipid pore formation, the increased hydration of the FP domain bilayer (Fig. 8c, right) should lower the energy of formation of a critical intermediate state, the hydrophobic defect, without lipid reorientation\(^{40}\). Here, the side walls of the water filled defect are formed by hydrophobic lipid tails. The surface tension at the interface of lipid tails and water depends non-linearly (although monotonically) on cylinder radius, as water structure inside a small hydrophobic cavity differs from that in bulk water. This difference in water structure is accounted for in the framework of Marčelja theory\(^ {41,42}\), where the energy of a homogeneous hydrophobic cylinder filled with water is:

\[
E_H = \sigma_h 2\pi R \left( \frac{I_1(R) \Gamma_0(R)}{I_0(R) \Gamma_1(R)} \right)
\] (2)
where $\sigma_b$ is the surface tension at the macroscopic interface of lipid tails and water, $\xi_b \sim 1$ nm is the characteristic length of hydrophobic interactions, and $h_D$ are modified Bessel functions of the first and zeroth order, respectively. According to Mačelja’s theory, hydrophobic surface–water interactions are described by an order parameter that characterizes the hydrophobic surface-induced perturbation of the water structure. The associated free energy density is decomposed into a series with respect to the order parameter scalar field and its gradient. The final energy of the hydrophobic surface exposition is calculated by minimizing the total energy functional with respect to the order parameter distribution. The dependence of the cylindrical hydrophobic defect energy on its length $l$ is linear. The length of the cylinder, $l$, and the cylinder radius, $R$, completely describe the state of a membrane pore, and with these two parameters, one can build continuous trajectories—from intact bilayer ($R = 0, l = 2 h$, where $h$ is the leaflet thickness), to the hydrophilic pore ($l = 0, R > 0$). These trajectories have common features: 1) the transition of the hydrophobic defect to a subsequent hydrophilic pore requiring the surmounting of an energy barrier, and 2) the energy of the hydrophilic pore has a local minimum at some radius (approximately equal to the leaflet thickness $h$), i.e., the hydrophilic pore can be metastable. The metastability is provided by the fact that, at a pore radius around typical lipid leaflet thicknesses, meridional positive curvature cancels equatorial negative curvature, making this region less stressed. In POPC bilayers, the energy barrier to pore formation is high enough ($\sim 40 k_BT$, $k_BT \approx 4 \times 10^{−21}$ J) to essentially prohibit spontaneous pore formation. This agrees with the experimental observation (Fig. 1) that the dye leakage from the GUVs occurs only after FP application. We previously found that the maximum of this energy barrier occurs at a hydrophobic defect radius of $R = 0.7$ nm; the critical defect radius is weakly dependent on the elastic parameters of the membrane.

There are two main ways to induce membrane destabilization: (i) decreasing the energy of the hydrophobic defect by membrane thinning and (ii) making the pore edge energetically preferable by the addition of components with positive MSC, e.g., lysolipids. The membrane-disrupting amphiphilic peptide piscidin $^1$ acts in the first way. Once embedded in the cis lipid leaflet, it can tilt to form a funnel-like structure that locally decreases bilayer hydrophobic thickness, promoting hydrophobic defects. Our MD simulations showed that FPs decrease membrane thickness (Supplementary Fig. 3 and Fig. 5) which should facilitate the formation of hydrophobic defects (compare Figs. 7a and 7b). However, the average bilayer thinning induced by FPs and the thickening induced by cholesterol are comparable: a chol:POPC:FP membrane has approximately the same thickness as a pure POPC membrane, and thus both should have comparable energy barriers to pore formation. In contradiction, experimentally the former membrane is leaky, while the latter is almost non-leaky indicating an additional effect of FP apart from average membrane thinning occurs. This contradiction can be reconciled if FP clustering and tilting of the FPs towards each other in the cluster (Fig. 8c) leads to formation of a funnel-like structure. Now, the hydrophobic region of the cis leaflet almost vanishes, and the opening of the pore only requires a hydrophobic defect to span the trans leaflet.

We calculated the energy barrier towards pore formation for each system considered in the MD simulations. The barrier is defined by the energy of the water cylinder spanning the bilayer ($E_{\text{hydro}}$) and the deformation of the membrane near the hydrophobic defect ($E_{\text{def}}$). The energy is evaluated relative to the energy of the state of distantly separated (minimally interacting), n FPs ($nE_{\text{FP}}$).

$$E_{\text{barrier}} = E_{\text{hydro}} + E_{\text{def}} - nE_{\text{FP}}$$  \hspace{1cm} (3)

To calculate the membrane deformation energy, we utilized the Hamm & Kozlov$^{39}$ elastic model essentially in the same way used to calculate the deformation energy arising at the FP cluster boundary. We assume that the system is axially symmetric with respect to the axis passing through the center of the FP cluster or single FP and introduce a cylindrical coordinate system, $O-r\varphi$. As FPs are hypothesized to tilt, specific boundary conditions were imposed onto the projections of lipid directors (time-averaged lipid tail orientations) onto the $O-r\varphi$ axis ($n_\varphi(r=R, \varphi)$) and $O-\varphi$ axis ($n_\varphi(r=R, \varphi)$) of a cylindrical coordinate system. The boundary director depends on the FP tilt angle, FP insertion depth, and membrane thickness. Three reference points are considered for determining the dependence of the boundary director on these parameters. The first one is the single FP; the $n_\varphi$-projection of the boundary director ($n_{\text{mono}}$) is defined from geometrical considerations$^{14, 61}$:

$$n_{\text{mono}} = -\frac{h - h_{\text{FP}}}{\sqrt{(h - h_{\text{FP}})^2 + h_{\text{FP}}^2}}$$  \hspace{1cm} (4)

where $h_{\text{FP}}$ is FP hydrophobic thickness. By symmetry, the $n_\varphi$-projection of the boundary director is zero. The second reference point is the membrane, squeezed at the FP boundary to match its hydrophobic thickness. Now the boundary director $n_b$ is defined from the condition of the deformational energy minimum. The last reference point is the FP cluster, where boundary peptides are tilted inwards or outwards of the cluster, and the hydrophobic and leaflet thicknesses match. In that case, the boundary lipid director equals the FPs tilt $\theta$, describing the rotation of the FP’s long axis (see Supplementary Fig. 8). We interpolate these three reference points linearly:

$$n_b(h_{\text{fl}}, \theta) = n_{\text{mono}} + \left(n_b - n_{\text{mono}}\right)\frac{h - h_b}{h - h_{\text{FP}}} + \theta\frac{h_{\text{FP}}}{\pi}$$  \hspace{1cm} (5)

where $h_b$ is the thickness of the lipid leaflet at the point of contact with FP. The energy of the cylindrical hydrophobic defect is calculated in the framework of the Mačelja model$^{39, 42}$ according to Eq. 2. The calculation procedure was as follows. For a purely lipid membrane, we accounted for both the hydrophobic and elastic deformation energies arising in the hydrophobic defect vicinity. For FP-containing lipid membranes, we calculated the energy of membrane deformation around the FP cluster, in which FPs are tilted inwards by a $45^\circ$ angle. The hydrophobic defect in this case spans only the trans-leaflet. The lipid boundary director was set in accordance with Eq. 5; hydrophobic defect length was set equal to the hydrophobic thickness of the trans-leaflet.
The total energy was minimized with respect to the membrane deformations and the depth of the FP insertion $h_0$ (Fig. 5c). The hydrophobic defect radius, $r = 0.7$ nm, corresponding to the maximum of the energy barrier of pore formation, weakly depends on the elastic parameters and was taken equal for each system under consideration amended by the DOG-containing system. We assumed that the latter system has MSC equal to the cholesterol-containing one (as in the GUV experiments). The thickness and rigidity of this membrane are taken equal to the POPC membrane, as unsaturated DOG lipid tails should not affect lipid ordering. The results of the calculation are summarized in Table 1. There are two drivers of the poration inhibition - MSC and thickness of the membrane leaflet. A negative MSC restricts FP tilting, while increased thickness raises the energy of the hydrophobic defect. The chol:POPC system has the highest energy barrier for pore formation due to the highest bilayer hydrophobic thickness. The effect of FPs on the membrane poration is explained by the cooperative action of several FPs that can form a cluster capable of thinning the membrane during a fluctuation of mutual orientation and thus decreasing the energy barrier towards pore formation. DOG and cholesterol, due to their approximately equal negative MSC, inhibit pore formation in the presence of FPs by impeding FP mutual tilting. This suggests that it is not primarily the chemical structure of cholesterol per se that is responsible for cholesterol’s pore neutralization activity. However, cholesterol’s ability for leaflet ordering can inhibit membrane poration additionally by increasing membrane rigidity. An increase in FP concentration leads to an increased probability of cluster formation, and the larger cluster further lowers the barrier to pore formation; both factors facilitate pore formation.

**Discussion**

For viral pathogenesis, the essential topological event in cell entry occurs when viral and host cell membranes merge, an activity mediated by the FP. Here, synthetic HA FPs segregate on lipid bilayers to high density in occasional ‘hot spots’ at the time that pores form, replicating intact influenza virion and viral-like particles in porating POPC and cholesterol:POPC lipid bilayer membranes. This HA FP-induced poration was inhibited, and poration lifetime reduced by including cholesterol in the target membrane. To investigate mechanisms, in silico MD simulations were implemented on lipid bilayers with increasing FP densities. Surprisingly, a novel structure emerged at higher FP densities: FP aggregated in the cis leaflet such that lipids were depleted and replaced by peptides; even the formation of a peptide dimer is sufficient to exert this lipid-displacing effect (Fig. 5). The FP:lipid domain had increased water concentration in the bilayer midplane; such a thinner and more hydrated membrane domain is hypothesized to porate more easily than a peptide-free bilayer. Membrane elastic theory was extended to accommodate a new intermediate to water pore formation where a funnel-like defect, comprised of tilting FP, thins lipid further by $\alpha$ moving the hydrophilic edge of the FP towards the membrane midpoint and $\beta$ minimizing the length of the hydrophobic defect that acts as the barrier to poration. Increasing FP density increased hydration in the MD-simulated lipid tail region of the trans leaflet, in agreement with theory predictions for greater water entry under a FP aggregate due to a shorter hydrophobic defect. Thus, the synergy of these two effects on the membrane hydrophobic region under a FP aggregate (FP tilting to thin and increased lipid hydration) can explain how FP facilitate water pores in bilayers at the critical time when fluorescence evidence for FP aggregation is detected.

In the presence of FP, spectroscopic measurements demonstrated release of contents from large unilamellar vesicles (LUVs) composed of PC and PS/PC (1:1)27,46,47, consistent with subsequent electrophysiological recordings26, and electron and light microscopy22,25. While these findings indicated that the influenza FP and its analogs destabilize the membrane, they did not address whether these permeability changes are associated with stable pores. Here, in single FP-GLV experiments, fluorescent dye influx continued over minutes, suggesting that the FP-induced influx does not proceed via a short-lived transient flickering pore but rather via either a stable or constantly flickering pore48. The lipid dependence of FP-induced stable pore formation at neutral pH was the same as determined in the previous studies on intact virion and VLP9.

Molecular dynamics simulations have demonstrated the ability of isolated FPs to distort the bilayer47. These simulation results are consistent with several studies describing the interaction between the FPs and model membranes. For example, x-ray diffraction and differential scanning calorimetry indicate that the FPs destabilize DOPE membranes43,48. However, the effect of cholesterol on the estimated depth of penetration of FPs, based on tryptophan fluorescence in vesicles containing singly brominated lipids is less than reported here49.

MD simulations at higher density (6 and 10 FP) were motivated by the observation that hemagglutinin is densely packed on the virus surface. Specifically, the approximately 9.5 nm spacing50,51 implies that a centered hexagon of 7 HA can be contained in a square patch of 15.3 nm/side. Since there are 3 FP per HA, in principle, 21 FP could fit in such a patch; our 10 FP systems, while dense, are well below this upper limit. MD simulations of spontaneously emerging aggregates of FP on a single leaflet yielded qualitatively different results from those obtained at infinite dilution. Now, the FP aggregated and displaced many lipids in the cis (top) leaflet, thereby thinning that leaflet. Whereas a single FP coexists with a large ensemble of lipid configurations featuring tails that can spread under the FP, lipid tails adjacent to the FP aggregate were only partially able to shield the hydrophobic underside of that aggregate. Furthermore, at the highest FP densities (10 FPs) the trans leaflet thins and becomes negatively curved (concave) as the terminal methyl groups interact with the remaining exposed regions of the aggregate’s hydrophobic underside (Fig. 5, Supplementary Fig. 3, and Supplementary Fig. 5). This curved region of the membrane is more permeable to water, as indicated by the substantial changes to the water PMF in the region of the FP aggregate and the increase in the water permeability (Fig. 6). These structural changes to the bilayer are expected to modulate the spontaneous curvatures of the individual leaflets in ways not possible for non-interacting FPs.

Pores were not formed in any of the simulations, most likely due to an energy barrier incompatible with the microsecond atomistic simulation durations currently practical. Consequently, the analytic theory was developed to bridge the gap between simulation and experiments. Since the underlying ultrastructure of a FP membrane microdomain is radically different from that of a fluid mosaic FP-embedded bilayer, purely lipid leaflet elastic theory was modified to consider the work of bending the membrane into three possible intermediates of membrane poration. The tilting of FPs towards each other amid an aggregate, bringing their hydrophilic faces closer to the bilayer normal, was the most energetically favorable, with their hydrophobic faces shielding the largest fraction of the lipid tail from the full hydration of a water pore (see Fig. 5c). The use of membrane
physical parameters of cholesterol or DOG-containing membranes in the calculations made pore formation less energetically favorable due to their significant negative MSC. Analytical calculations supported the experimental observation that the DOG-containing vesicles with the same negative MSC as cholesterol-containing ones exhibited a similar inhibition of poration (Fig. 1c), suggesting that it is not the chemical structure of cholesterol per se that is responsible for cholesterol’s pore neutralization activity. Compared to previous estimates for the lowest energy pathway in lipid bilayers composed of longer chain lipids, the FP/lipid hybrid bilayer has an even lower energy pathway to membrane poration\(^{30,33-34}\). Illya and Deserno observed qualitatively similar aggregation in their simulations of highly coarse-grained peptide/bilayer systems\(^{25}\); coarse-grained methods should be useful for further studying aggregation of these FPs.

If an underlying evolving activity of the highly conserved FP is to aggregate, as demonstrated in this study and by the rosette formation described in the introduction, then aggregation may be due both to the hydrogen bond interactions of FP amino acid R groups seen in the MD simulations and membrane-mediated attraction shown in this work. Simulations of other amphipathic alpha helices\(^{45}\) do not show the aggregation described in this manuscript, potentially due to differential embedding of the two-component alpha helices unique to FP. The deep embedding of the N-terminal helix, consistent with previous measurements by the Lentz lab, is striking\(^{49}\). Is this aggregation of FP relevant to the membrane fusion of infection? A pore in a single bilayer (a target cell membrane) is not a fusion pore that links two bilayers (viral envelope and target cell membrane). Perhaps the work done by the FP microdomain comes later in the fusion process, after hemifusion.

The FP microdomain may be responsible for fusion pore formation at the lipid/protein boundary. In support of this idea, protein domain insertion at a distance (into a leaflet trans to the HD) may induce a barrier for fusion in one other membrane remodelling system: PH domain insertion in dynamin-mediated membrane fission\(^{47,49}\) and cell-cave in which myomaker acts after myomerger to form the fusion pore\(^{49}\). In both systems, the protein domain action is thought to occur near or at a three-way lipidic junction\(^{47,49}\). Alternatively, FP from intact enveloped virions might rupture endosomes and enter target cell cytoplasm for subsequent envelope disruption and intracellular trafficking of the viral genome to the nucleus. Regardless, the FP condensate revealed here, displacing lipids from one leaflet, is remarkably like the recent caveolin structure, also proposed to displace lipids from one leaflet to form a faceted surface of a caveola\(^{60-62}\); here, alpha-helices also stack and provide sufficient thickness to match the lipid leaflet thickness at the lipid/protein boundary.

### Methods

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), cholesterol, and 1,2-dioleoyl-sn-glycerol (DOG) were from Avanti Polar Lipids, Inc. (Alabaster, AL) and other chemicals were from Sigma-Aldrich (St. Louis, MO), unless specified. Stock solutions of POPC (13.15 mM), DOG (3.22 mM), and cholesterol (20 mM) were made in CHCl\(_3\) (Burdi & Jackson, Mexico City, Mexico, high purity solvent) and stored in brown glass vials at 20 °C. Structures of FP analogs (synthetic amphipathic peptide with additional hydrophilic amino acids to their C-terminus) in the presence of phospholipid bilayer membranes or micelles are alpha-helical in their secondary structure with either a closed coiled-coil tertiary structure\(^{20}\) or an open ‘boomerang’ shaped tertiary structure\(^{20}\). A 23 amino acid synthetic peptide corresponding to the X-31 FP domain\(^{20}\) was custom synthesized by GenScript, NJ labelled with tetra-methyl rhodamine (Sequence: GLFGAAGFIEGEGWMDGWG Y [LYS(TRITC)]). Stock solutions of FP in DMSO were stored at -20 °C. To compare the FP-induced membrane poration with intact X-31 influenza virus-induced poration\(^{20}\), this X-31 FP sequence was used\(^{20}\). To compare poration results to MD simulations, the H-serotype FP sequence was used\(^{20}\) (GLFGAAGFIEGEGWMDGWG Y). Poration results were similar for both FP sequences, irrespective of dye labelling.

### Experimental conditions

#### Preparation of giant unilamellar vesicles

GUVs were prepared by the gel swelling method as described previously\(^{20}\). Briefly, to prepare GUVs of a specific lipid composition the required amount of lipid drawn from individual stock solutions was diluted into 200 μl of CHCl\(_3\) to a concentration of 3.94 mM (3.35 mg/ml). The mixture was vortexed with -10 μl of MeOH for 2 min to avoid incomplete mixing and obtain a clear solution in CHCl\(_3\). 10 μl of DI water (Invitrogen; prepared in DMSO as a 5 μM stock solution) was added to this lipid mixture and vortexed for 2 min. The lipid mixture (in CHCl\(_3\)) was then deposited on a plasma cleaned (using a Harrick plasma cleaner, Ithaca, NY) microscope cover glass, coated with 5% (w/w in ddH\(_2\)O) polyvinyl alcohol (Merck Millipore). The organic solvent was evaporated by a gentle stream of nitrogen and then stored in high vacuum for 1h. The lipid film containing cover glass was then transferred to a 30-mm tissue culture dish. 500 μl of GUV formation buffer (1 mM EDTA, 1 mM HEDTA, 10 mM PIPES, 100 mM KCl, pH 7.4 with -200 mM sucrose) was added covering the entire surface of the cover glass and allowed to incubate for 30 min in the dark. After 30 min, the GUVs were harvested by gently tapping the sides of the dish, then gently removing the GUV suspension using a 1 ml pipette without touching the surface and transferring to a 1.5 ml micro-centrifuge tube. The GUV suspension was stored at 4 °C until further use. The total lipid concentration of the GUV suspension was 1.35 mg/ml (1.58 mM). Typically, GUVs were made the same day of the experiment. To vary the MSC systematically, POPC GUVs of varying concentrations of cholesterol, or DOG were prepared.

#### GUV poration assay

FP was added from the stock solution to a suspension of GUVs at a peptide-to-lipid ratio of 1:200 in a microcentrifuge tube. The total volume was diluted to 500 μl containing Alexa 488 or SRB. The mixture was then incubated at 37 °C for 15 min. While the FP-GUV mixture was incubating, 500 μl of a 5 mg/ml β-casein solution was added to a delta-TPG 0.17 mm dish (Bioteps, Butler, PA) allowed to sit for 10 min to passivate the glass surface, and then washed 10 times with ddH\(_2\)O. The FP-GUV suspension was then vortexed for 30 s and transferred to this imaging dish. For a given lipid composition, 50 - 100 GUV were observed and scored based on whether they have undergone influx of Alexa 488. Leakage was determined -15-18 min after FP addition to ensure equilibrium. The fluorescence intensity within GUV was monitored for an additional 30 min; no changes in the mean fluorescence intensity were detected after the additional waiting period. For kinetics measurements, the FP was directly added to the chamber contacting GUVs while mounted on the microscope objective.

#### Confocal microscopy

GUVs were imaged on a Zeiss LSM 880 microscope, controlled by the Zen Black software, using a 63× oil 1.4 NA Plan-Apochromat objective. The objective was heated (Bioteps, Butler, PA) to maintain 37 °C inside the chamber (verified with a microprobe thermometer). Alexa 488, SRB and DiD were excited with 488, 543 and 633 nm lasers respectively and detected using PMT detectors on separate imaging tracks.

#### Image, data, statistical and aggregation analyses

Microscopy images were analyzed using ImageJ (NIH). Data were analyzed using SigmaPlot (v12.5, Systat Software, Inc., Chicago, IL), Excel (v2016 and 2019, Microsoft, Inc., Redmond, WA), and MATLAB (v2018a – v2021a, The MathWorks, Inc., Natick, MA). The aggregation hypothesis predicts higher than expected concentrations of bound FP during poration. The polarization properties of a high NA objective produce a cosine-squared dependent modulation of an otherwise constant...
Fig. 9 | Kinetics of poration and FP accumulation on a POPC GUV. FP fluorescence signals were normalized by the FP mean intensity in solution (black data points). The time FP begins to accumulate on the vesicle, \( t_{\text{por}} \), was determined using piecewise linear fitting (solid black lines). The time, \( t_{\text{por}} \), poration begins (red arrow) was determined using piecewise linear and exponential fitting of the Alexa 488 influx (solid green line). In this example, the characteristic time between FP accumulation and poration is 197 sec with a mean FP intensity at poration 3.5 times the solution intensity. FP mean intensity in solution was constant over time (dotted black line). Inset) Confocal image of an FP labeled vesicle (red) and control ring (yellow), positioned in the solution, used to evaluate circumferential intensity of both the vesicle and solution as a function of time. Representative example from \( n=9 \) experiments. Source data are provided as a Source Data file.

To correct the intensity profiles recorded on GUVs, template matching alignment was employed (to minimize position jitter noise), using the Imagel plugins (Template Matching and Oval Profile Plot). However, neither the observed profiles at a single time point, nor averaged over a subset of time points (5, for example) nor the entire time series could be described using either a single uniform orientation or two component mixtures of orientations, unlike the profiles predicted for a chromophore with identical orientations uniformly bound to the surface. Statistical evidence of FP aggregation on GUV includes pixels with larger than expected intensity, requiring consideration of both the slowly varying modulation around the circumference and the magnitude of the intensity, since variance increases with intensity. The aggregation analysis first calculated the intensity surface at the time of poration followed by an analysis of the distributional properties of the residuals. Direct calibration issues were avoided by normalizing the GUV pixel intensities into units of solution intensity calculated from the distribution of intensities along a 1-pixel wide ring matching the size of a GUV in cross-section (Fig. 9, Inset). Since the distributions of FP intensity in solution, over time, often captured transient, FP-bound membrane structures, the distributions were modeled as a two component Gaussian mixture, with the lower intensity mean taken as the FP intensity in solution to be used in the normalization of membrane-bound FP. Possible changes to the chromophore properties following FP binding to the membrane were not observed because the initial normalized GUV intensity is approximately 1 (Fig. 9; solid horizontal line at 1 for normalized GUV intensity values less than \( t_{\text{por}} \), = 147 sec). Consequently, the intensity on the GUV surface is expressed in units of solution intensity that is directly related to the FP concentrations and designated as the normalized FP density.

Note, the solution values are invariant with time, whereas the intensity of the FP bound to the GUV begins to diverge and increase with time. The extent of Alexa 488 influx (\( R = F/I_{\text{norm}} \), green trace) is also plotted and the time of poration indicated by the red arrow.

A window +/- 2 timepoints around the estimated poration time, \( t_{\text{por}} \), was analyzed for the presence of pixels having FP intensity larger and smaller than expected. The data surface, defined by 3 parameters (radial angle, time, pixel intensity) were fit using locally weighted, smoothing quadratic regression (Lowess quadratic) with a data span of 20% to capture the complexity of the time, angle, and intensity of the peptide on the GUV. The same surface fits in solution were approximately planar. The spline-smoothed circumferential mean (over the five time points) removed any large scale (quadrant angle level) polarization-dependent intensity modulation of vesicle-bound FP. The resulting intensity deviations (residuals) were compared to the distributional behavior of FP in solution. The residuals represent both the positive and negative deviations around the circumferential mean; larger (smaller) than expected intensity deviations on the vesicle, when compared to the solution distribution are evidence for pixels with greater (lower) FP density. The spatial distribution of the FP intensities in solution represents the expected variation for orientationally random and spatially uniformly distributed data since the surface was \( a \) approximated by a plane at a constant value of 1 (the normalized intensity) and \( b \) the residuals were symmetric around 0.

Molecular dynamics simulations

System preparation. Two membrane compositions were compared: pure POPC and 1:1 chol:POPC. Large systems were constructed with 10, 6, or 1 copies of the 23-residue NMR structure of the FP (sequence: GLFGAAGFEFGWGMCMWOYG) determined by Loriet et al. \( ^6 \) placed in the cis (top) leaflet of ~150 & 150 Å membranes. Smaller symmetric systems were also constructed with a single copy of the FP in each leaflet of ~75 × 75 Å bilayers for the estimates of spontaneous curvature listed in Supplementary Table 1. All systems were constructed using the CHARMM-GUI Membrane Builder \( ^47,48 \) with a water thickness of 17.5 Å and 150 mM potassium chloride in the solvent. Simulations utilized the CHARMM36 force field \( ^49,50 \) and TIP3P water model \( ^51,52 \), and were all performed in the isothermal-isobaric (NPT) ensemble with a 2.0 fs time step.

Long-time scale simulations. All large simulations were simulated first in OpenMM version 7.4.1 \( ^15 \) using the Rick package version 0.7.0. OpenMM equilibrium times were 1000 ns for 10 FP systems and 100 ns each for 6 and 1 FP systems. Following this, because of the large fraction of FPs in the cis leaflet, the 10 FP systems were simulated for 30 ns in CHARMM \( ^6 \) using P2\( _1 \) boundary conditions \( ^53 \) to allow lipids to equilibrate between leaflets. The number of lipids per leaflet was averaged over the last 7.5 ns of these P2\( _1 \) simulations; representative frames with lipid distributions matching this average were selected as starting coordinates for simulation on Anton 2. \( ^7 \) All large systems were then simulated on Antion 2 for production runs using Antion software version 1.56.0.c7. The Antion 2 simulations times were 20 µs for 10 FP systems, and 2 µs each for 6 and 1 FP systems. The symmetric single FP systems were simulated for 500 ns in OpenMM. As controls for both system sizes, FP-free systems of comparable size (150 × 150 Å and 75 × 75 Å) were simulated for 500 ns in OpenMM.

All OpenMM simulations were performed at 310 K with the Nose-Hoover chain \( ^79,81 \) velocity Verlet integrator implemented in Openmmtools \( ^33 \) and the Monte-Carlo membrane barostat \( ^50 \). Bonds with hydrogen were constrained using the SETTLE and CCMA algorithm \( ^46,47 \). A 12.0 Å cutoff was used, with a force-switching function from 8–12 Å. Long-range electrostatics were treated using the particle mesh Ewald method \( ^48,50 \). Coordinates and velocities were saved every 50 ps. Simulations on Antion 2 utilized the Multigrator framework \( ^90 \) with the Nose-Hoover thermostat (310 K) and semi-isotropic MTK barostat. An 8 Å real space cutoff distance was used, and long-range electrostatics were evaluated with the u-series method \( ^92 \). Coordinates were saved every 200 ps.

Simulation analysis. Thickness maps and leaflet position maps were calculated using the MEMBPLUGIN v1.1 \( ^17 \) extension for VMD, and all system snapshots were rendering using VMD v1.9.4a51. Lipid density plots were calculated using LOOS \( ^67 \) v 3.3.0. FP clustering was assessed using the cluster module of the freud Python library \( ^22 \). All FP heavy atoms were considered in the analysis, and a cutoff of 3.5 Å.
consistent with a typical hydrogen bond donor-acceptor cutoff distance, was used to define points as belonging to the same cluster. Peptide tilt angles were calculated by defining a tilt vector between the alpha carbon of residue 13 (midpoint of the bend) and the geometric center of the alpha carbons of residues 3 and 22 (the helical residues furthest from the bend) and calculating the angle of this vector with respect to the xy-plane. Roll angles were calculated in a similar fashion, with the roll vector defined between the alpha carbons of residues 3 and 22 (see sketch in Supplementary Fig 8). Water, lipid, and peptide density distribution profiles were calculated using the density function in *cpptraj* [18](a) and a 0.5 Å bin width; only atoms within a cylinder of 30 Å centered about the origin (Supplementary Fig. 7) in the xy-plane were considered for this calculation (FP-containing systems were first imaged such that the FP aggregate was centered at the origin, in the plane of the membrane.) The PMF, *F*(z), was calculated from the normalized water density distribution, *p*(z):

\[ F(z) = -k_BT \ln(p(z)) \] (6)

**Permeability and partition coefficients.** The inhomogeneous solubility diffusion (ISD) model[38] provides a simple relation between the free energy profile and the resistance to permeability, *R*. The permeability *P* in the ISD model is written:

\[ \frac{1}{P} = e^{-\beta F_{ref}} \int_{-h/2}^{+h/2} \frac{d\phi(z)}{D_{z}(z)} dz \] (7)

where *D_(z)(z)* is the position dependent diffusion coefficient, \( \beta = 1/k_BT \), \( h \) is the thickness of the bilayer, and *F_(ref)* is the reference free energy in the water phase that is set to zero. The diffusion constant is not calculated here and will be assumed to be a constant (*D*). The resistance is then defined from Eq. 7 as:

\[ R = \int_{-h/2}^{+h/2} \frac{d\phi(z)}{D_{z}(z)} dz = \langle D(z) \rangle / P \] (8)

Note that the preceding definition of *R* differs slightly from the usual definition of *R*, where the diffusion constant remains in the integral.

**Calculations of the energy of elastic deformations of lipid membranes**

To calculate the energy of membrane deformations, we utilize the Hamm and Kozlov model[29] according to which the lipid monolayer is considered to be an elastic continuous 2D media. The state of the lipid leaflet in this model is defined by the field of unit vectors *n* corresponding to the average direction of the lipid molecules, and the shape of the monolayer-water interface. We assumed that the system has an elastic continuous 2D media. The state of the lipid leaflet is defined by the mean curvature (*f*), tilt (*t*), lateral compression/stretching (*α*) and Gaussian curvature (*K_g*) energy terms:

\[ E_{def} = \int_{\text{bilayer}} \left( \frac{B}{2} (1 - J_0)^2 + \frac{K_c}{2} t^2 + \frac{K_a}{2} \alpha^2 + \frac{K_g}{2} \right) dS \] (9)

where *B*, *K_c*, *K_a*, *K_g* are bending, tilt, compression/stretching and Gaussian elastic moduli; *J_0* is the monolayer spontaneous curvature. The integration is performed over both monolayer surfaces. The leaflet’s effective curvature is determined by the divergence of the director field: \( J = -\text{div}(n) \). The tilt vector is defined as a deviation of the lipid director from the normal *N* to the leaflet’s surface: \( t = n - N \). The Gaussian curvature in the axially symmetric system is defined using the following product: \( k_g = \frac{\nabla \cdot n}{n \cdot \nabla n} \), where *n* is the projection of the director on the Ox axis. The following parameters were used to calculate the energy of membrane deformations: Hydrophobic thickness and lipid MSC (Supplementary Table 1) were obtained from MD simulations; \( J_{\text{POPC}} = -0.043 \text{ nm}^{-1} \), \( J_{\text{LMM}} = -0.173 \text{ nm}^{-1} \) for pure POPC and mixed cho/POPC membranes/leaflets; bending rigidity for pure POPC membrane \( B_{\text{POPC}} = 11.2 \text{ kT} / \text{nm} \) and for mixed cho/POPC \( B_{\text{LMM}} = 13.7 \text{ kT} / \text{nm} \). The lateral area of FP was taken to be 4 nm². We used the following values for the remaining elastic constants: \( K_r = 10 \text{ kT} / \text{nm}^2 \), \( K_\alpha = 30 \text{ kT} / \text{nm}^2 \), \( K_g = -0.3B \text{ kT} \) equal for both pure POPC and mixed membranes.

To calculate the elastic energy due to insertion of a single FP, *E_F*, fusion peptides and mini-clusters were considered as rigid objects that impose boundary conditions on the directors \( n_r(r - r_0, \phi) \), \( n_\alpha(r - r_0, \phi) \), where *r_0* is the radius of single FP or their cluster and *n_0* is the projection of the director on the O_x axis; parameters were as above. Directors andleaflet surface were constrained to be continuous everywhere except in the regions occupied by the FPs. The membrane was required to be unperturbed far from the cluster boundary. The elastic energy was minimized with respect to the membrane deformations and FP’s insertion depth. The detailed description of the calculation procedure can be found in Kondrashov et al.[23].

**Reporting summary**

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

**Data availability**

All experimental data generated and analyzed in this article and its supplementary information files are available from the corresponding author upon request. The source data underlying Figs. 1, 2, 4, 6, 7, 9 and Supplementary Figures 1-3, 5, 6, 8-11 are provided as a Source Data file. Initial and final coordinates of all Molecular Dynamics simulations are provided as a Supplementary Dataset (Supplementary Data 1) in PDB format. All Molecular Dynamics trajectories generated on Anton 2 can be downloaded from the Pittsburgh Supercomputer Center (PSC) at https://antonweb.psc.edu/trajecories/ . The remaining Molecular Dynamics trajectories are available at the LoBoS cluster at NIH (contact R.W.P. at pastorr@nihbi.nih.gov). Source data are provided with this paper.

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