Supporting Information

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Membrane permeabilization can be crucially biased by a fusogenic lipid composition - leaky fusion caused by antimicrobial peptides in model membranes

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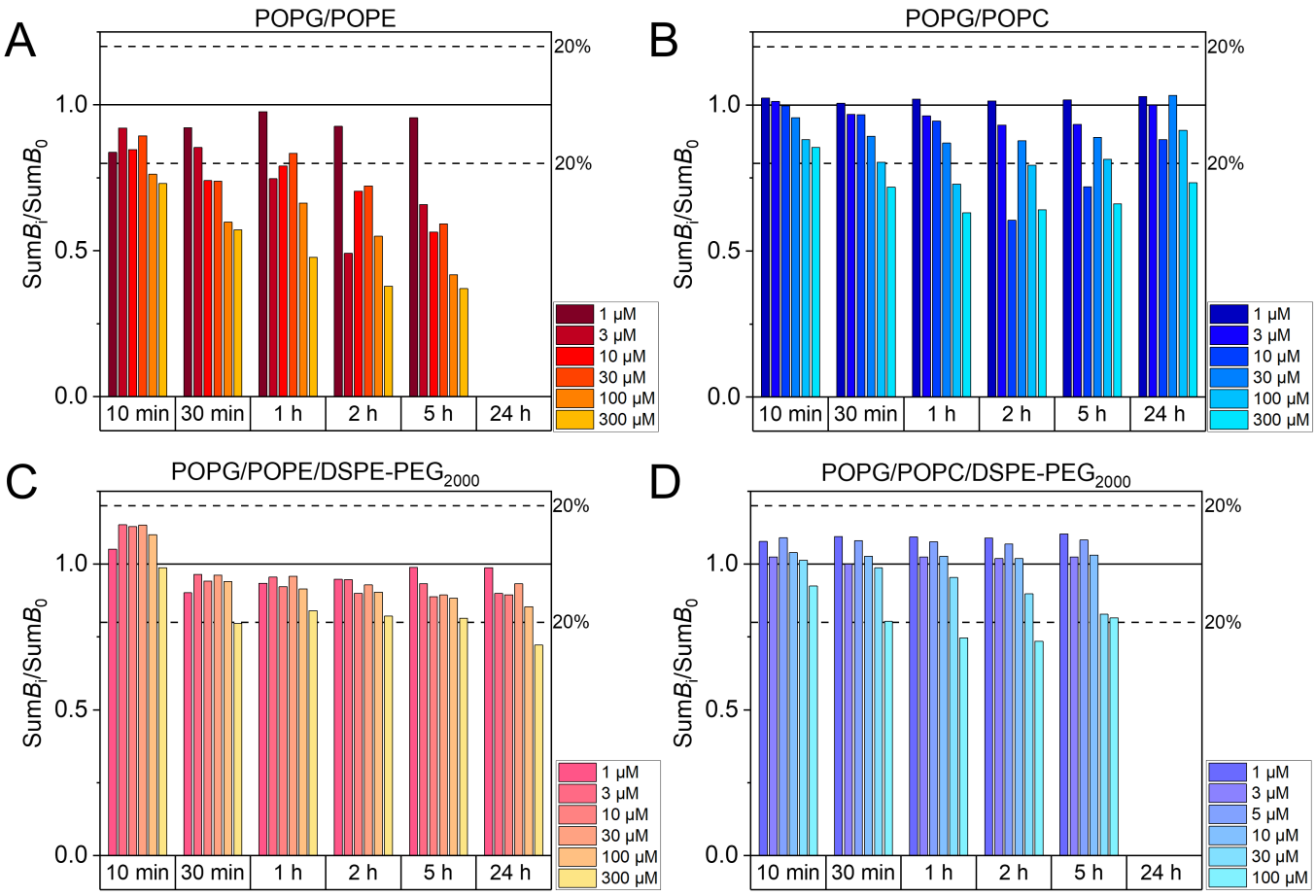

Fig. S 1 Changes in sum of B with respect to a sample without peptide for the data shown in figure 1. Calcein filled LUVs (all samples 30 µM lipid) were incubated with various concentrations of cR_3W_3 as stated. The criterion of 20% deviation is marked. The experiments were performed in standard TRIS buffer at 25 °C.

Table S 1 Estimation of the thermodynamic parameters characterizing the interaction between cR_3W_3 and model membranes. The one-set-of-sites model of the software provided with the instrument was fitted to the data.

Lipid mixture	n	K 10^4 [M^{-1}]	ΔH [kJ mol^{-1}]
POPG/POPC (50:50)	6.1	11	-4.3
POPG/POPE (50:50)	4.5	7.2	-4.9
POPG/POPE/DSPE-PEG_{2000} (50:50:4)	5.4	5.6	-4.3

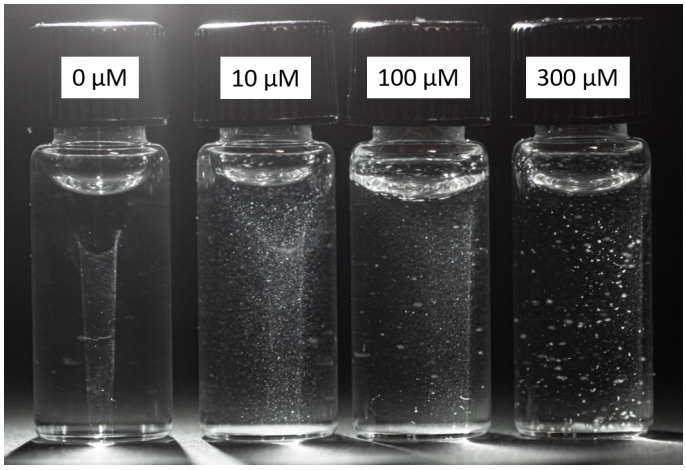

Fig. S 2 Visual particles in LUVS of POPG/POPE (30 µM lipids, initial diameter approximately 100 nm) incubated for 24h without or with cR_3W_3 as indicated. The particles in the right sample tend to sediment. For better visibility, light from the back was used. The experiments were performed in standard TRIS buffer at 25 °C.

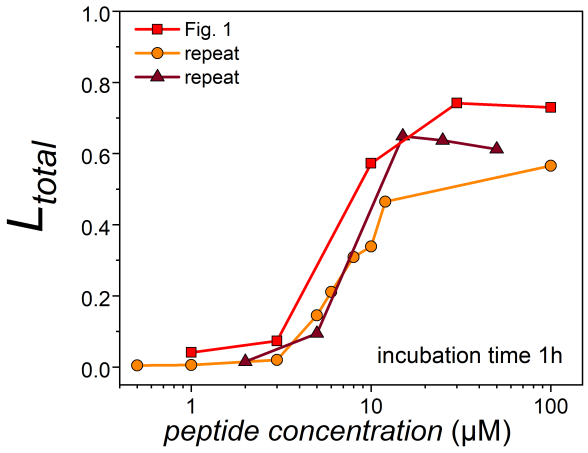

Fig. S 3 Calcein leakage induced by different concentrations of cR_3W_3 in 30 µM POPG/POPE (50:50) after one hour incubation. Repetition measurements of the data in figure 2A at different peptide concentrations. All experiments were performed under similar conditions in standard TRIS buffer at 25 °C.

Leakage when vesicle aggregation and fusion are promoted

Because of changes in light scattering and interference of tryptophan fluorescence with other fluorescence labels, the determination of fusion is only possible reliably in a narrow range of peptide concentration and in short incubation times (figures 4 and S 5). Even though PEG-lipids do not affect the binding of the peptide to the lipid membrane, there is a small chance that the PEG-lipid alter leakage by other effects than preventing leaky fusion. We therefore intended to increase vesicle fusion by increasing the probability of vesicle-vesicle contacts at increased vesicle density.[5]

After 1 or 2 hours of pre-incubation of the peptide with POPG/POPE, or POPG/POPC vesicles, respectively, the same amount of vesicles was added and incubated further while leakage was quantified. In the case of fusion-related leakage, leakage should increase more than in the control sample ($L_{excess} > 0$). If both binding and leakage are unaffected by the vesicle concentration and density, there should be no excess leakage ($L_{excess} = 0$). Leakage can indeed decrease because the peptides can redistribute over more membrane surface and the number of bound peptides might become too low for leakage ($L_{excess} < 0$). For more details see [Shi S., Fan H., Hoernke M., 2022 Nanoscale Adv.5109-5122, DOI: 10.1039/D2NA00464J].

A sample without additional LUVs is used as reference. When the same amount of calcein-filled vesicles is added after preincubation, the excess leakage (L_{excess}) is defined as:

L_{excess} = L_{total} ($2t$, after addition) - 1/2 L_{total} ($2t$, reference sample without addition) - 1/2 L_{total} (t, directly before addition)

With t being the time of pre-incubation chosen to yield intermediate extents of leakage before addition.

Unfortunately, we found the determination of excess leakage especially sensitive to fluctuations in fluorescence intensity (sum of B in lifetime-based fluorescence) because of vesicle aggregation (data not shown).

Lipid mixing

Lipid mixing is determined from the extent of FRET between NBD- and rhomdamine-labeled lipids. The spectra reveal unexpected behaviour as shown in figure S 5. At very low peptide concentration of 1 μM, there is a unusual reduction in the intensity at the maximum intensity of the donor NBD at approximately 520 nm (figure S 5 A). This is possibly caused by a participation of tryptophan side chains of the peptide in FRET. The addition of 3 μM peptides to the model vesicles (figure S 5 B) results in the expected reduction in FRET upon membrane fusion during the fist 21 minutes, *i.e.* slight increase in intensity at 520 nm (donor), and decrease in intensity at 580 nm (acceptor). At higher peptides concentrations (figure S 5 C), a marked decrease in intensity in the overall spectrum is intensified with increasing incubation times. This is perhaps caused by aggregation. This is also reflected in the increased error bars at higher peptide concentrations and long incubation times (figure S 4).

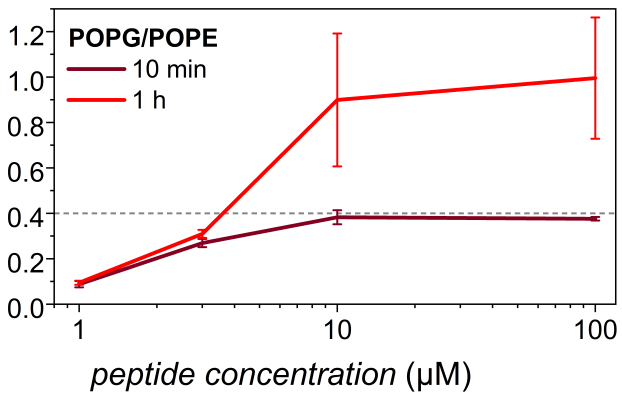

Fig. S 4 Selected data points of lipid mixing in POPE/POPG (50:50) vesicles (figure 4) with error bars (n = 3). The experiments were performed in standard TRIS buffer at 25 °C.

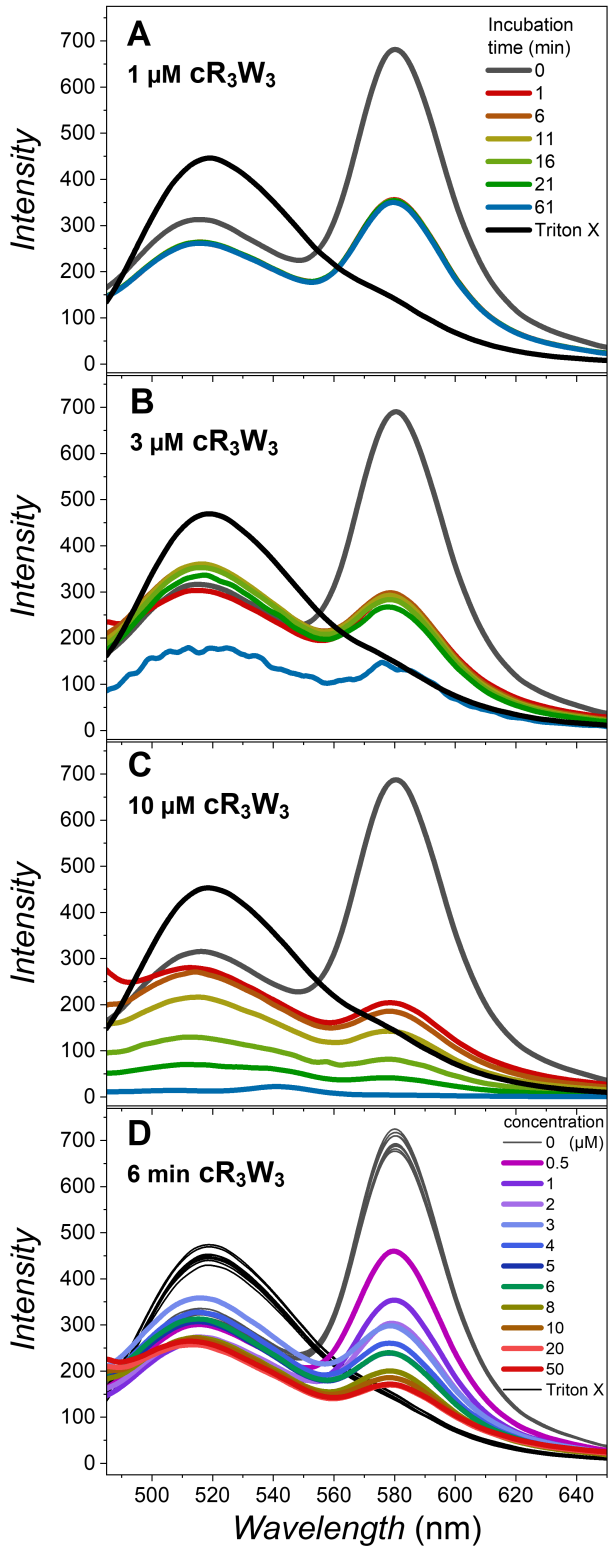

Fig. S 5 Fluorescence spectra used to evaluate lipid mixing depicted in figure 4. LUVs composed of POPG/POPE (1:1) and POPG/POPE (1:1) labelled with 0.5 mol% NBD-PE and 0.5 mol% rhodamine-PE were incubated at a ratio of 4:1 to yield a total lipid concentration of 30 μM. 1 μM (A), 3 μM (B), or 10 μM (C) cR_3W_3 were added and incubation times up to one hour were taken into account. (D) compares the fluorescence spectra after an incubation time of 6 minutes of various peptide concentrations. All experiments were performed in standard TRIS buffer at 25 °C with an excitation at 463 nm.

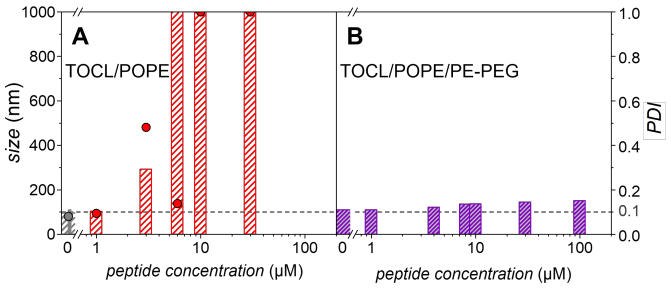

Fig. S 6 *Z*-average size and polydispersity index (PDI) obtained by dynamic light scattering for A: TOCL/POPC (50:50) (red), and B: TOCL/POPC/DSPE-PEG$_{2000}$ (50:50:4) (purple) vesicles (initial diameter approximately 100 nm) after 24 h of incubation with various concentrations of cR_3W_3. The experiments were performed in standard TRIS buffer at 25 °C.

PEG-lipids do not affect the binding of cR_3W_3 to lipid layer, but may lead to membrane instability

To investigate whether binding of cR_3W_3 changes when PEG-lipids are part of the lipid monolayers, the extend of peptide insertion (expressed as $\Delta\pi$) was measured in DMPG/DMPE and DMPG/DMPE/DSPE-PEG$_{2000}$ at different initial surface pressures (π_0). After injection of cR_3W_3 underneath preformed lipid monolayers, indistinguishable insertion and maximum insertion pressure occured (figure S 7). This agrees well with the ITC data (figure 6) and indicates that the binding of the peptide is not sterically prevented by the PEG-chains.

Since premature leakage of POPG/POPC/DSPE-PEG$_{2000}$ vesicles was observed, the behavior of DMPG/DMPE and DMPG/DMPE/DSPE-PEG$_{2000}$ as well as DMPG/DMPC and DMPG/DMPC/DSPE-PEG$_{2000}$ monolayers upon compression was examined (figure S 8). Notably, DMPG/DMPC/DSPE-PEG$_{2000}$ monolayers show a flatter compression isotherm compared to the monolayer without PEG-lipids. Furthermore, the collapse of the monolayer is shifted to lower areas per molecule (well below 40 Å^2 per molecule). This indicated a loss of lipids into the subphase in DMPG/DMPC/DSPE-PEG$_{2000}$ but not in DMPG/DMPE/DSPE-PEG$_{2000}$ and might provide an explanation for the tendency to premature leakage only in vesicles composed of POPG/POPC/DSPE-PEG$_{2000}$ but not of POPG/POPE/DSPE-PEG$_{2000}$.

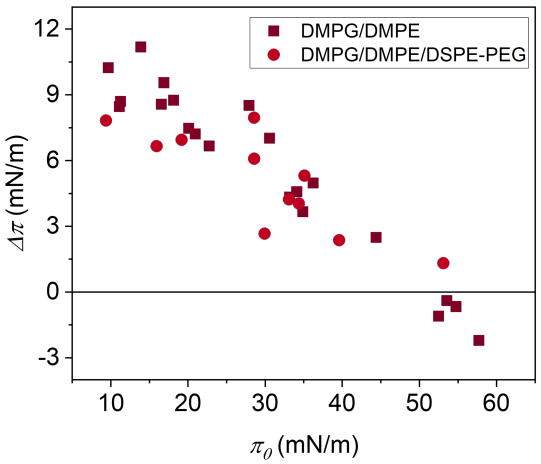

Fig. S 7 Adsorption of 900 nM cR_3W_3 to DMPG/DMPE (50:50) and DMPG/DMPE/DSPE-PEG$_{2000}$ (50:50:4) monolayers. The increase in surface pressure ($\Delta\pi$) upon addition of the peptide into the subphase is visualized as a function of the initial surface pressure (π_0). The experiments were performed in standard TRIS buffer at 25 °C.

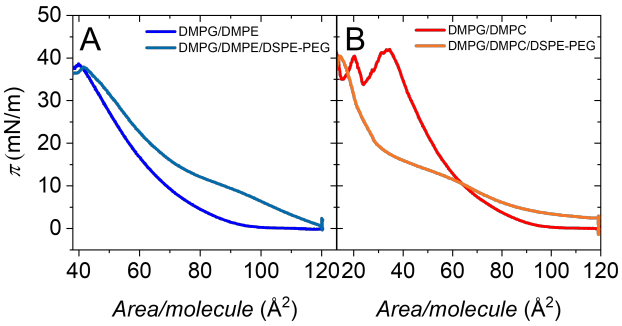

Fig. S 8 Compression isotherm of monolayers composed of A: DMPG/DMPE (50:50), DMPG/DMPE/DSPE-PEG$_{2000}$ (50:50:4) and B: DMPG/DMPC (50:50), DMPG/DMPC/DSPE-PEG$_{2000}$ (50:50:4). The experiments were performed in standard TRIS buffer at 25 °C.