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Coiled-coil mediated liposomal fusion: Asymmetric behaving peptide fusogens

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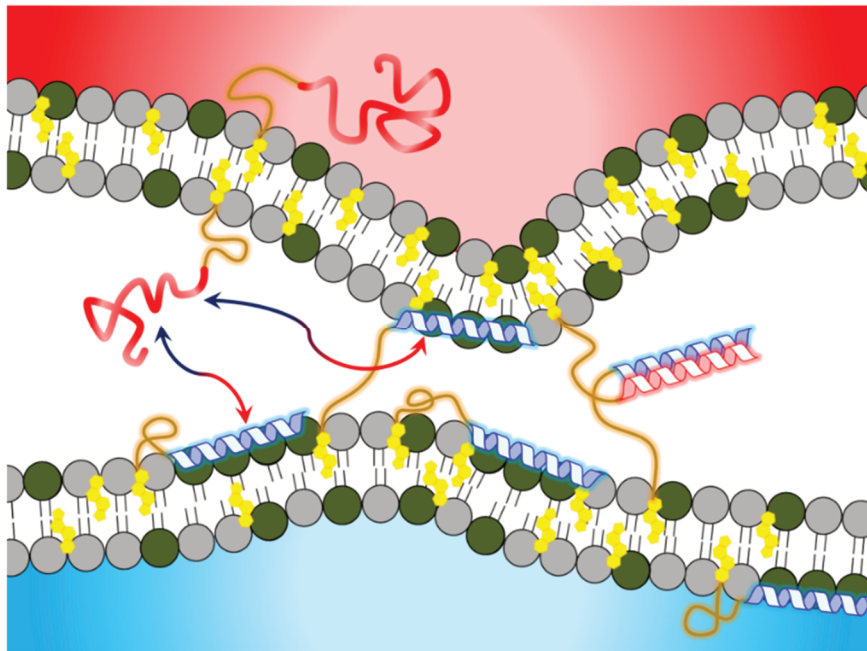
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CHAPTER II

MEMBRANE-FUSOGEN DISTANCE IS
CRITICAL FOR EFFICIENT COILED-COIL-PEPTIDE
MEDIATED LIPOSOME FUSION



ABSTRACT

We have developed a model system for membrane fusion that utilizes lipidated derivatives of a heterodimeric coiled-coil pair dubbed E (EIAALEK)₃ and K (KIAALKE)₃. In this system peptides are conjugated to a lipid anchor via a polyethyleneglycol (PEG) spacer, and this Chapter describes the influence of the PEG-spacer length, coupled with the type of lipid anchor, on liposome-liposome fusion. The effects of these modifications on peptide secondary structure, their interactions with liposomes, and their ability to mediate fusion were studied using a variety of different content mixing experiments and CD spectroscopy. Our results demonstrate the asymmetric role of the peptides in the fusion process, as alterations to the PEG-spacer length affects E and K differently. We conclude that negatively charged E acts as a 'handle' for positively-charged K and facilitates liposome docking, the first stage of the fusion process, through coiled-coil formation. The efficacy of this E handle is enhanced by longer spacer lengths. K directs the fusion process via peptide-membrane interactions, but the length of the PEG-spacer plays two competing roles: a PEG₄/PEG₈ spacer length is optimal for membrane destabilization, however a PEG₁₂ spacer increases fusion efficiency over time by improving the peptide accessibility for successive fusion events. Both anchor type and spacer length affect peptide structure; a cholesterol anchor appears to enhance K-membrane interactions and thus mediates fusion more efficiently.

INTRODUCTION

SNARE (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor) proteins are a well conserved class of fusion proteins that are primarily involved in vesicle trafficking and fusion.¹⁻³ Complementary SNARE protein subunits are located on opposing membranes and, in the first step of the fusion process, they form a tetrameric coiled-coil, which brings the opposing membranes into close proximity. The fusion cascade, initiated by this docking process, consists of 3 more steps⁴⁻⁵: (1) disruption of the membrane at the site of contact; (2) merging of outer membranes, i.e. lipid mixing or hemifusion; (3) formation of a fusion pore, facilitating content mixing between the two fused compartments.⁶ This SNARE fusion system has been studied extensively using the natural proteins,⁷⁻¹⁰ and has served as inspiration for the design of model systems. Such systems are primarily based on phospholipid vesicles functionalized with either DNA,¹¹⁻¹⁵ peptides,¹⁶⁻¹⁹ or small molecules,²⁰⁻²⁴ that act as recognition motifs, but molecular recognition between functionalized cyclodextrin vesicles has also been reported.²⁵⁻²⁸ In our lab, a model system for membrane fusion has been developed with the lipidated heterodimeric coiled-coil pair E (EIAALEK)₃ and K (KIAALKE)₃ as fusogens.²⁹⁻³⁰ These peptides are held in lipid membranes by a lipid anchor, either cholesterol (C) or 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-amine (DOPE), (L) and this is conjugated to the peptide via a PEG-spacer of variable length (*m*, *n*) to yield C/LP_{*m*}E and C/LP_{*n*}K.

The role of these lipopeptide constructs in the fusion process is revealed by studying the relationship between rational lipopeptide modifications and their resulting influence on fusion efficiency. The consequences of the anchor type were previously investigated,³¹ and it was shown that only DOPE and cholesterol anchors yielded significant fusion, with the latter being the most efficient. Increasing the length of the peptide (which increases coiled-coil binding strength and peptide-membrane interactions) also had a positive effect on fusion efficiency.³² Conversely, parallel or anti-parallel coiled-coil orientation,³³ and coiled-coil oligomer state,³⁴ did not influence the rate of fusion. A recent study has also proposed an asymmetric role for the peptides in the fusion process, as shown in Figure 4 (B).³⁵ A key function of K is its interaction with the liposome membrane, primarily via its hydrophobic face which comprises leucine and isoleucine

residues but also via the flanking lysine residues in a process known as snorkeling.³⁵⁻³⁶ This K-membrane interaction leads to destabilization of the membrane and initiation of the fusion process, whereas E facilitates coiled-coil formation, bringing the membranes into close proximity, allowing fusion to occur. These different roles lead to competing peptide interactions, which provides an equilibrium between K – E coiled-coil formation and K – membrane interactions.

In all these liposomal fusion studies a PEG₁₂ spacer was used to tether the fusogenic peptides to their membranes, but it is probable that spacer length is critical for efficient fusion. Indeed, it has been shown that the introduction of a (GGS)₁₋₃ spacer in the natural SNARE complex reduced the overall fusogenicity of the system with increasing spacer length,³⁷ likely due to an increase in distance between the proteins and their membranes. The same relationship between fusion efficiency and spacer length was also reported with DNA-based fusogens,¹² however faster docking kinetics were found with increasing spacer length, which was rationalized with a diffusion-driven collision model. This demonstrates that a spacer has opposing effects at different steps in the fusion cascade, and it indicates that, in our model system, the PEG-spacer length is likely to influence fusion efficiency.

In light of these previous studies, it was decided that the influence of PEG length on fusion efficiency should be studied using our model system. Both cholesterol and DOPE anchors were employed so that separate and cumulative effects of anchor and spacer could be studied as it is likely that the components of this model system (peptides, spacer and anchor) do not act in isolation but have a synergistic effect on fusion. (*Figure 4*) To demonstrate the asymmetry in function of the two complementary peptides the influence of PEG-spacer length for both E and K was measured individually. It was anticipated that this approach would provide further insights as to the fusion mechanism and the roles of the different components, in addition to giving a detailed picture of the functions of the lipopeptides at different stages of the fusion process.

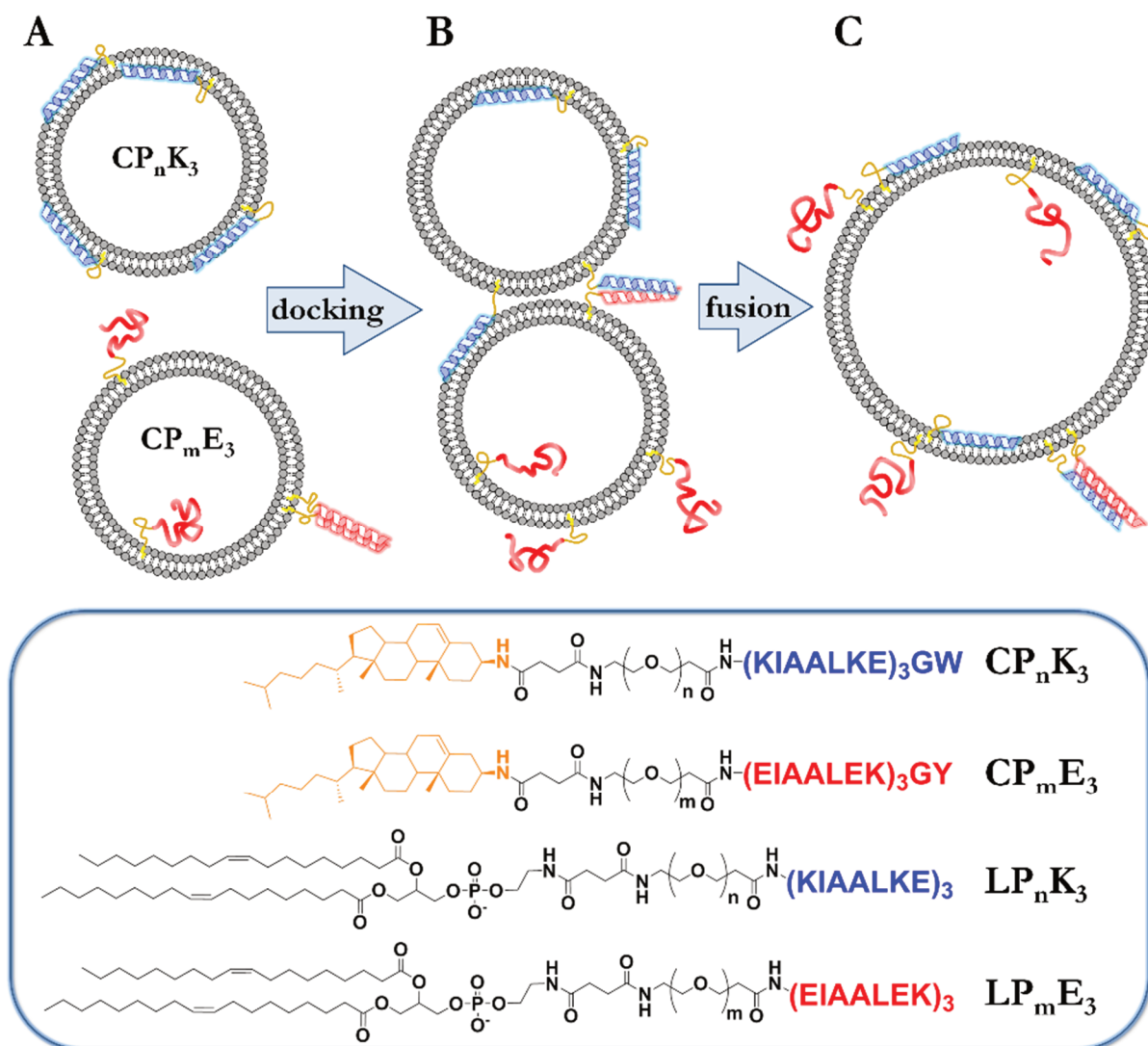


Figure 4. Schematic overview of the process of peptide-mediated liposomal fusion and lipopeptide structures. The peptides are drawn in their appropriate secondary structures at distinct points of the fusion cascade: (A) liposomes functionalized with complementary peptides are initially separated; (B) liposomes dock due to coiled-coil formation, and; (C), liposomes become fully fused. Peptide E (red) can adopt homo-coils, hetero-coils and random coils, while peptide K (blue) adopts only hetero-coils or membrane immersed α -helical structures.³⁸ The lower boxed region shows the structures of the lipopeptide constructs used in this study; succinic anhydride was used to facilitate conjugation of the PEG spacer to the DOPE anchor; the corresponding cholesterol derivative was also employed.

DESIGN AND SYNTHESIS

Both DOPE and cholesterol have been demonstrated to be effective anchor moieties and so using both anchors, a PEG series of 0, 4, 8, and 12 repeating units was synthesized initially. During the course of this study, the spacer series was extended to cover a significant range close to the maximum fusion efficiency, and LP_2E/K and $CP_{16}E/K$ derivatives were subsequently synthesized and evaluated. (Table 1)

To visualize the influence of the PEG-spacer length, its stretched end-to-end distance was calculated with the software AVOGADRO,³⁹ and from this value the distance between the peptide and the surface of the membrane was derived and termed L_{PEG} . It should be noted that, for longer linkers this effective distance may be reduced as it is unlikely the PEG will retain a stretched conformation. Since the membrane surface is defined by the polar headgroups, it was assumed that, in the case of a 75% DOPC/DOPE membrane and a lipopeptide DOPE anchor, the PEG length and the peptide-membrane distance are equal. For cholesterol containing DOPC/DOPE membranes, it is known that cholesterol is buried in the membrane,⁴⁰ and so to calculate L_{PEG} for cholesterol-anchored peptides, the average thickness of the membrane hydration layer (1.0 nm)⁴¹ was subtracted because cholesterol resides at the glycerol level in the phospholipid membrane.⁴²

Designing experiments to obtain insight as to the details of the fusion process is challenging due to complex short-lived intermediate stages and fast dynamics of fusion events. In addition, it is possible that the PEG-spacer will have opposing effects during the fusion process,³⁰ therefore it is necessary to identify the impact of these effects on the fusion efficiency. The cumulative effect of the combination of PEG-spacer length and anchor type on lipopeptide-mediated membrane fusion was studied using a content mixing assay. Further experiments were then conducted to determine the effects of the PEG-spacer length at different stages of the fusion process. Peptide ratio-dependent fusion provided insights as to the influence of the equilibria between different peptide conformations on the fusion mechanism. Furthermore, an experiment was designed to measure the ability of the lipopeptides to mediate multiple rounds of liposomal fusion.

The influence of membrane-tethering on the peptide structure was measured using circular dichroism (CD) spectroscopy. The results here, supported by previous studies,^{35, 38, 43} suggest that the process of membrane fusion is rate-limited by equilibria between different peptide conformations in the initial fusion stages and that PEG-length influences these equilibria and therefore the extent of fusion.

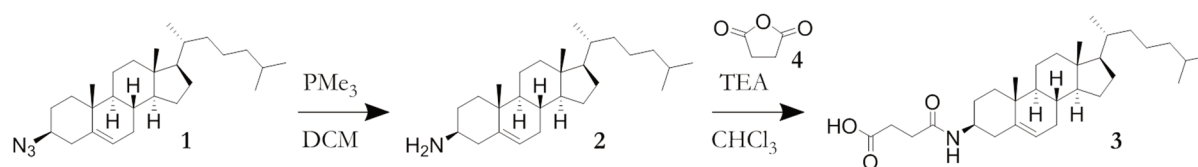
Table 1. Overview of the derivatives of peptides E and K used in this study.

Spacer	DOPE anchor	L_{PEG}^3	Cholesterol anchor	L_{PEG}^3
None ¹	LP ₀ E / LP ₀ K	0.5	CP ₀ E / CP ₀ K	-0.5
PEG ₂ ²	LP ₂ E / LP ₂ K	1.6	n.a.	n.a.
PEG ₄	LP ₄ E / LP ₄ K	2.3	CP ₄ E / CP ₄ K	1.3
PEG ₈	LP ₈ E / LP ₈ K	3.9	CP ₈ E / CP ₈ K	2.9
PEG ₁₂	LP ₁₂ E / LP ₁₂ K	5.4	CP ₁₂ E / CP ₁₂ K	4.4
PEG ₁₆ ²	n.a.	n.a.	CP ₁₆ E / CP ₁₆ K	5.8

Peptide purities of >95% were confirmed using LC-MS for all constructs. ¹The peptide is connected to the anchor via a succinic di-amide, with a length of 0.5 nm. ²A LP₂ spacer and a CP₁₆ spacer were added to the PEG-series to cover a significant range close to the maximum fusion efficiency. ³ L_{PEG} is the distance between the N-terminus of the peptide and the surface of the membrane in nm; for DOPE anchors this is the maximum theoretical chain length of the PEG spacer (nm), as estimated by AVOGADRO,³⁹ and for cholesterol anchors this is the maximum theoretical chain length - 1.0nm,⁴⁰ due to the intrinsic membrane insertion property of cholesterol in DOPC/DOPE membranes.

CHOLESTEROL-ANCHOR MODIFICATION

The yield of lipopeptide synthesis is significantly reduced by acidic hydrolysis of the ester bond present in a cholesterolhemisuccinate anchor during cleavage of the synthesized lipopeptides from the resin. Therefore, we designed and synthesized a TFA-stable derivative (**3**), as shown in Scheme 1. Cholesterolazide (**1**)⁴⁴ was converted to **2** via a Staudinger reduction, and a subsequent coupling of succinic anhydride yielded the cholesterol anchor **3** which was purified via recrystallization.⁴⁵

**Scheme 1.** Synthetic pathway towards cholesterol-amide-hemisuccinate **3**.

RESULTS AND DISCUSSION

CONTENT MIXING ASSAY

The final stage of a full fusion process between two membranes is content mixing. Although efficient and leakage-free content mixing remains a challenge for many fusion model systems, it has been successfully demonstrated using this system in previous studies. To facilitate this process, a quantitative content mixing model was developed by encapsulating the water-soluble dye Sulforhodamine B.⁴⁵⁻⁵⁰ This dye self-quenches at high

concentrations, but dilution results in relief of self-quenching and an increase in fluorescence. Liposomes incorporating E lipopeptides were loaded with 20 mM Sulforhodamine B in PBS and mixed with K-functionalized buffer-containing liposomes. The effect of anchor type (with a PEG₁₂ spacer length) on fusion efficiency was demonstrated previously, resulting in a variable degree of membrane fusion between LP₁₂E/K and CP₁₂E/K.³¹ Here, the influence of both spacer length and anchor type on content mixing (as a measure for full fusion), was determined, Figure 5, and Figure S1.

It is evident that liposomal fusion with CP_mE/CP_nK is most efficient if both lipopeptide constructs use a PEG₁₂ spacer, with fusion being less efficient if shorter, or longer, spacers are employed. For DOPE-anchored peptides, a PEG₄ spacer is necessary for the K construct, but the length of the spacer for E appears to be irrelevant. Furthermore, fusion mediated by cholesterol anchored peptides is twice as efficient as DOPE-peptide mediated fusion.

This spacer-dependent trend for DOPE-peptide mediated fusion shows that the L_{PEG} of peptide K highly influences the fusion efficiency, indicating that the K peptide is crucial for efficient fusion and that the linker length strongly influences the efficiency of this process. The observed differences in PEG spacer length preference between cholesterol- and DOPE-anchored peptides can't be explained by differences in L_{PEG} , since their various spacers all cover the same distance range (0 - 6 nm), although cholesterol is bound more tightly to the membrane than DOPE.³¹ Since liposomal membranes already contain 25 mol% cholesterol, significant influences in general membrane characteristics attributed to cholesterol were not expected.⁵⁰⁻⁵¹ However, recent studies on the influence of the lipid anchor on membrane-tethered PEG-spacers showed that a cholesterol anchor increased the flexibility of the spacers,⁵² and influenced their function.⁵³ This reveals that the anchor type does affect the behaviour of the other components (spacer and peptide) in this construct, and it is hypothesized that the cholesterol anchor enhances the membrane-interacting and disrupting role of K in the fusion process, which may explain why increased levels of content-mixing are observed with cholesterol-anchored peptides.

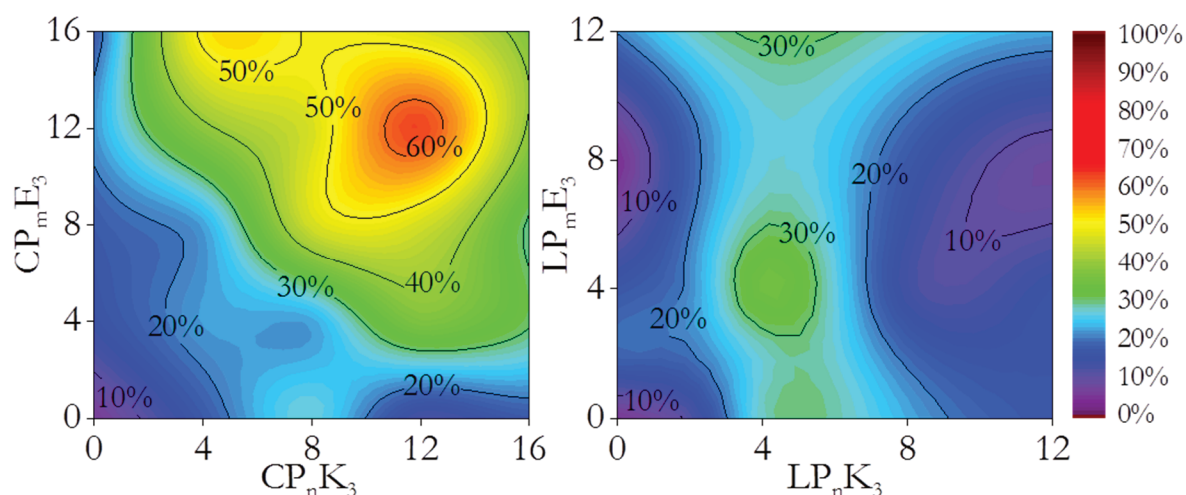


Figure 5. Content mixing between E and K-decorated liposomes as a function of PEG spacer length (m/n) as indicated by an increase in sulforhodamine B emission after 30 min. Left, cholesterol anchored Pm/n -series and, right, DOPE anchored Pm/n -peptides. [Total lipid] = 0.1 mM, with 1 mol% lipopeptide, PBS pH 7.4.

CIRCULAR DICHROISM SPECTROSCOPY

Since $LP_{12}E$ and $LP_{12}K$ are known to adopt distinct structures when tethered to lipid membranes,^{35, 38} the influence of linker length on peptide secondary structure was examined using circular dichroism (CD) spectroscopy. In addition to exploring these linker effects, both anchors were tested to determine whether these also influence peptide structure. The membrane-tethered peptide constructs were tested in isolation, and when mixed to determine whether heteromeric coiled-coil species were formed. The results are illustrated in Figure 6, and Figure S4. A $\theta_{222}/\theta_{208}$ ratio ≥ 1 is indicative for coiled-coil formation, however this ratio can be skewed due to inherent scattering properties of liposomes. While θ_{222} is a measure for helicity, both peptide-membrane interactions and coiled-coil formation contribute to the observed helicity, therefore identifying whether peptides are folded as α -helices or coiled-coils is non-trivial.

It has been demonstrated previously that the α -helical conformation of membrane tethered K is stabilized via spontaneous incorporation into the membrane,^{38, 43} and so a correlation between the observed helicity of the peptides and the relative strength of this membrane interaction may be inferred. For CP_nK derivatives, increasing helicity upon decreasing spacer length shows that peptides that are in closer proximity to the membrane experience more stabilization by, and therefore are likely to interact more strongly with, the membrane. The helicity of LP_n -anchored K peptides is lower compared

to the CP_nK peptides and does not change significantly with altering PEG-lengths. This indicates that the peptide membrane interaction is less pronounced for LP_n lipopeptides and that the peptide conformation is not significantly affected by varying the spacer length. The decreased fusion efficiency measured when liposomes are decorated with $LP_{m/n}$ anchored peptides is likely a result of this weaker membrane interaction of LP_nK , which provides further evidence for a correlation between the strength of the peptide-membrane interaction and the fusion efficiency of peptide decorated liposomes.

To explain this influence of anchor type on peptide-membrane interactions, it is necessary to examine the characteristics of the membrane and the tethered lipopeptides. When peptide K interacts with the membrane, it tends to be surrounded by lipids with smaller headgroups (i.e. DOPE, cholesterol).^{35, 54-55} The accumulation of DOPE in the proximity of peptide K destabilizes the membrane, promoting the formation of a protrusion centered at the peptide's position,³⁵ and supports the highly curved membranes of fusion intermediates.^{50, 56} The cholesterol anchor could enhance the membrane insertion of peptide CP_nK due to its relatively small headgroup, and because cholesterol promotes chain-flexibility of conjugated PEG-spacers,⁵² these factors could increase the tendency of the conjugated peptides to incorporate in the supporting membrane.

Peptide E adopts structures with relatively low amounts of helicity, supporting previous findings that peptide E does not significantly interact with DOPC/DOPE/cholesterol membranes, and adopts a mixture of weakly helical homodimers and random coils in solution.³⁸ Although α -helical peptide stabilization by PEG has been reported,⁵⁷ here these PEG-peptide interactions are not likely to be significant due to the short length of the used PEG-spacers.

The structural characteristics of the mixture of E and K decorated liposomes show the ability of the peptides to form a coiled-coil. The helicity measured in a mixture of LP_mE and LP_nK decorated liposomes is, for all m/n , higher than the helicity of the separated LP_mE and LP_nK functionalized liposomes and demonstrates coiled-coil formation between peptides tethered to different liposomes. The absence of coiled-coil formation for $CP_0E + CP_0K$ as measured by the $\theta_{222}/\theta_{208}$ ratio supports the previous observation that this lipopeptide-pair does not mediate fusion efficiently, most likely due to the

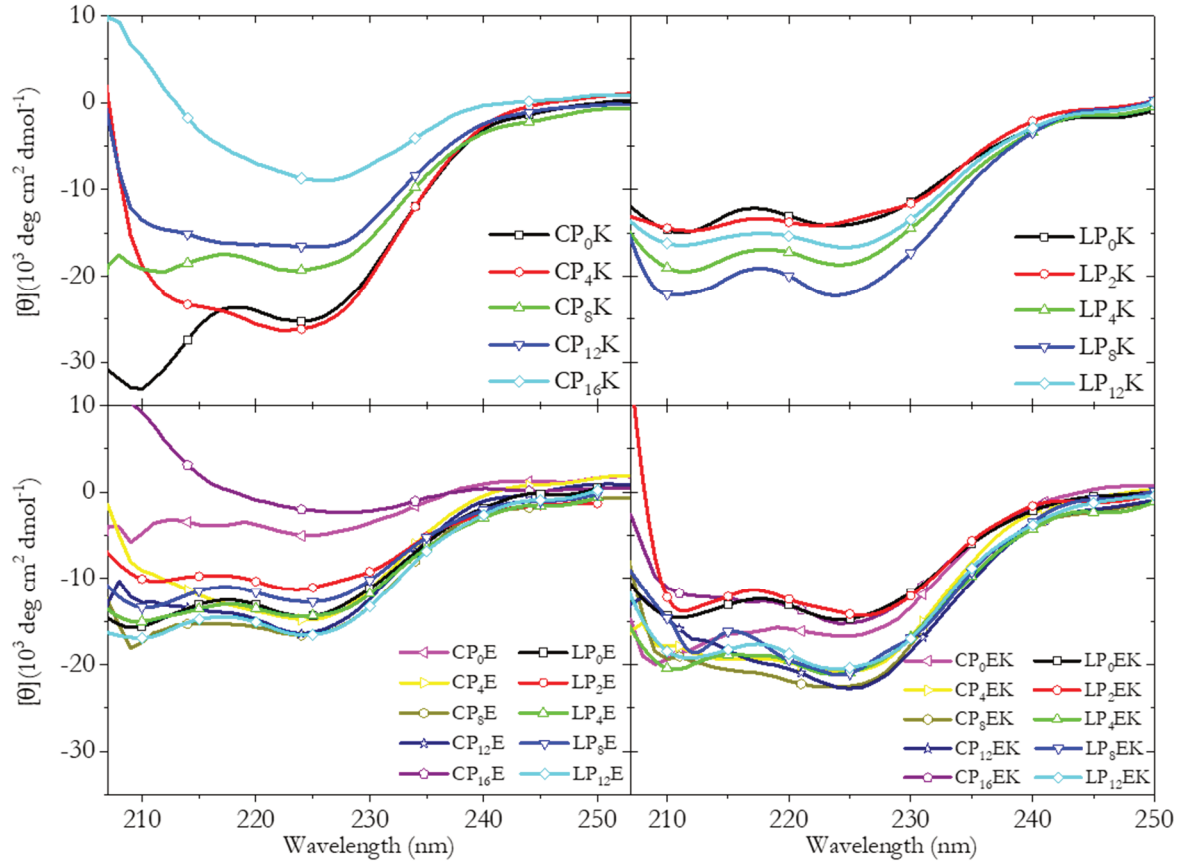


Figure 6. CD spectra of liposome-tethered lipopeptides. [Total lipid] = 0.5 mM, with 1 mol% lipopeptides, PBS pH 7.4.

Table 2. Degree of peptide helicity as determined by circular dichroism.

Anchor + spacer	E		K		E + K	
	$\theta_{222}/\theta_{208}$	%H ¹	$\theta_{222}/\theta_{208}$	%H ¹	$\theta_{222}/\theta_{208}$	%H ¹
Acyl ²	0.4	20	0.4	19	1.0	60
CP ₀	0.9	15	0.8	72	0.8	47
CP ₄	1.5	42	1.2	75	0.9	59
CP ₈	0.9	47	1.0	55	1.2	65
CP ₁₂	0.6	47	1.1	47	1.3	65
CP ₁₆	n.a. ³	6	n.a. ³	26	1.3	44
LP ₀	1.0	37	1.0	37	1.0	38
LP ₂	1.1	30	1.0	39	1.1	36
LP ₄	1.0	39	1.0	49	1.0	57
LP ₈	0.9	34	1.0	57	1.1	57
LP ₁₂	1.0	44	1.0	44	1.1	55

¹Percentage α -helicity was calculated using the formula $[\theta]_{222} = -39500 \cdot (1 - 2.57/n)$ to obtain a 100% helicity value for an α -helical peptide of n residues.⁵⁸ ²Measurements with acylated peptides, in PBS pH 7.4, without vesicles. ³Negative ratio due to positive θ_{208} value caused by a scattering artifact. [Total lipid] = 0.5 mM, with 1 mol% lipopeptides, PBS pH 7.4.

peptides being held too close to the membrane to be able to form a coiled-coil efficiently, thus preventing liposome docking. For cholesterol anchored peptides with elongated spacers, coiled-coils are formed, which correlates with the efficient fusion observed from the content-mixing experiments.

TIME RESOLVED RATE OF FUSION

Experiments using model systems incorporating DNA-based fusogens showed that while increasing spacer length decreased fusion efficiency, docking of liposomes (i.e. the initial stage of the fusion process) was accelerated with longer spacers.¹² To separate these influences of time and L_{PEG} on the fusion efficiency of this model system, it is necessary to identify and isolate distinct time periods in the fusion experiment. During the initial minutes of the content-mixing assays, the rate of fusion is linear, and provides a reliable correlation between L_{PEG} and docking/fusion efficiency. This linearity is largely due to the fact that fusion events occur with unfused liposomes randomly dispersed in a well-mixed experimental solution. After a few minutes the experimental mixture becomes increasingly more complex as larger fusion products appear and the available number of unfused liposomes decreases.³⁰

Since CP_mE/CP_nK mediated liposomal fusion is significantly more efficient than LP_mE/LP_nK mediated liposomal fusion, fusion efficiency with CP_mE/CP_nK was measured for all possible combinations of m/n , continuously for 30 minutes. To compare the different stages in the fusion experiment, the fusion rate was determined after 4 and 30 minutes. (*Figure 7*) Complete datasets, including control experiments which demonstrate a fluorescence increase is only obtained when both peptide-functionalized liposome populations are present, are available in Supporting Information, Figure S1 / S2.

After 4 minutes all fusion events involving the CP₁₂E lipopeptide showed the highest fusion efficiency, regardless of the length of the PEG-spacer in the CP_nK construct, although a CP₈K/CP₁₂E combination gives the highest initial fusion efficiency. Liposomal docking is dependent on the length of CP_mE because peptide K is fully incorporated in the membrane prior to fusion, as evidenced by Infrared Reflection Absorption Spectroscopy (IRRAS) measurements performed previously.^{35, 38} This indicates that the L_{PEG} of E is critical for liposome docking, with longer linkers being

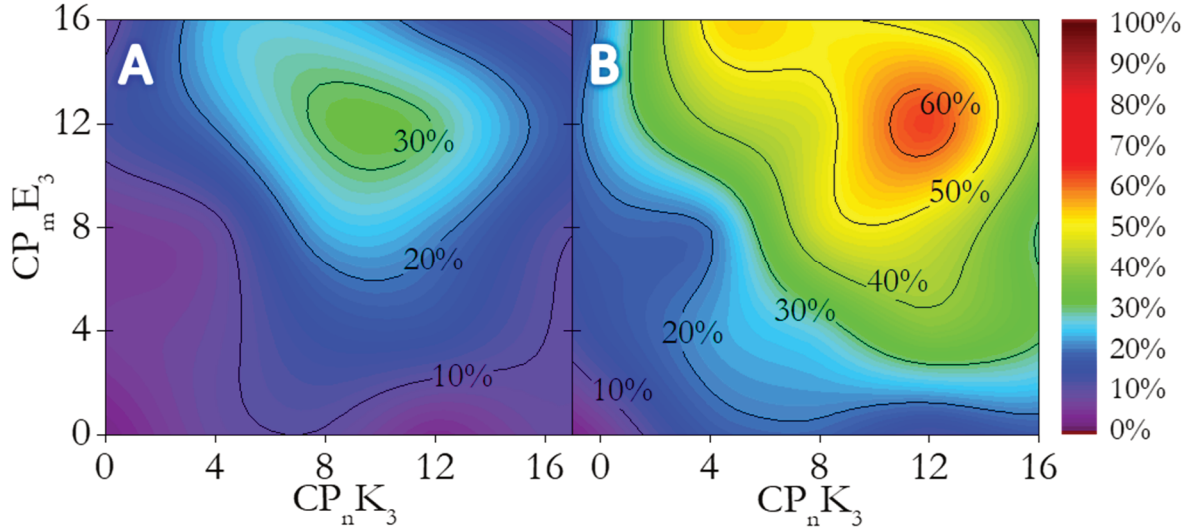


Figure 7. Time resolved rate of fusion (measured using content-mixing assays) with CP_mE and CP_nK decorated liposomes. Data is presented as a function of m/n , indicated by an increase in sulforhodamine B emission after (A) 4 mins, and (B) 30 mins. [Total lipid] = 0.1 mM, with 1 mol% lipopeptide, PBS pH 7.4.

more efficient. This supports the theory that E acts as a handle for K, searching for a K peptide to form a coiled-coil with, a process resulting in liposome docking. It has been shown that, during liposomal docking, peptide K is in dynamic equilibrium between a heterodimeric coiled-coil structure and a membrane-immersed α -helix; the length of the CP_nK spacer could highly influence this equilibrium. Membrane immersion of multiple K-peptides in both liposomes creates the necessary protrusions to initiate fusion and simultaneously dictates the maximum distance between connected liposomes.³⁵ Since optimal initial fusion efficiency, with an equimolar ratio of both peptides, is obtained with CP_8K a spacer length of 2.9 nm appears optimal to bring both membranes into close proximity and to overcome the energy barriers in the fusion process.

After 30 minutes, $CP_{12}K + CP_{12}E$ decorated liposomes show the highest overall fusion efficiency. These results oppose a diffusion driven collision model with docking of liposomes as the rate-limiting step,³⁷ because that implies a linear correlation between observed fusion efficiency and PEG-spacer length. Additionally, fusion with $CP_{16}E$ is less efficient compared to $CP_{12}E$, which also demonstrates that the rate limiting step in this fusion system is not liposomal docking. The shift in fusion efficiency to K peptides functionalized with elongated spacers can be caused by different phenomena: liposomal diffusion, better peptide accessibility and the occurrence of multiple rounds of fusion, all of which can be tested by changing the conditions of the content-mixing experiments (*vide infra*).

Although the fusion process is not driven by diffusion, liposomal diffusion could still have a minor effect on the fusion efficiency, although only over longer time scales when the number of liposomes decreases as a result of fusion. With decreased liposomal concentration, liposomes functionalized with elongated PEG spacers have a higher probability to find unfused liposomes with available coiled-coil binding partners. This is illustrated by data showing that initially, there is no difference between fusion of liposomes functionalized with CP₄K and liposomes bearing CP₈E or CP₁₆E, but over time fusion with CP₁₆E decorated liposomes is more efficient.

INFLUENCE OF CPE / CPK RATIO ON FUSION EFFICIENCY

Liposomal fusion efficiency mediated by LP₁₂E / LP₁₂K is highly dependent on peptide concentration;³⁰ with concentrations ≥ 0.5 mol%, efficient lipid mixing has been reported. To date, both liposome populations used in the fusion studies were functionalized with LP₁₂E or LP₁₂K in an equimolar ratio. A ratio $\neq 1$, should support the asymmetric function of the lipopeptides in the fusion process however, as the roles of both peptides will be affected differently by altering their concentrations.

To test this hypothesis, the concentration of CP_mE was lowered to 0.5 mol% while [CP_nK] was maintained at 1 mol%. Here, changes in the fusion efficiency will provide more-detailed insights as to the peptide's roles in the fusion process. Furthermore, lowering the concentration of CP_mE will affect the handle function of E and it is expected that the change in peptide ratio enhances the impact of L_{PEG} of CP_nK on the fusion process. Since the most striking differences in efficiency are found with elongated CP_mE spacers, only liposomal fusion with CP₁₂E and CP₁₆E decorated liposomes was studied. (*Figure 8, Figure S3*)

As is evident from Figure 8, the influence of CP_nK on the fusion process is enhanced by lowering the concentration of CP_mE, and the resulting fusion efficiencies reveal that CP₈K, with a calculated L_{PEG} of 2.9 nm, is optimal for fusion. Remarkably, initial fusion efficiencies were shifted to elongated spacers with decreasing CP_mE concentration, which supports the handle function of E for liposomal docking, and demonstrates the effect of

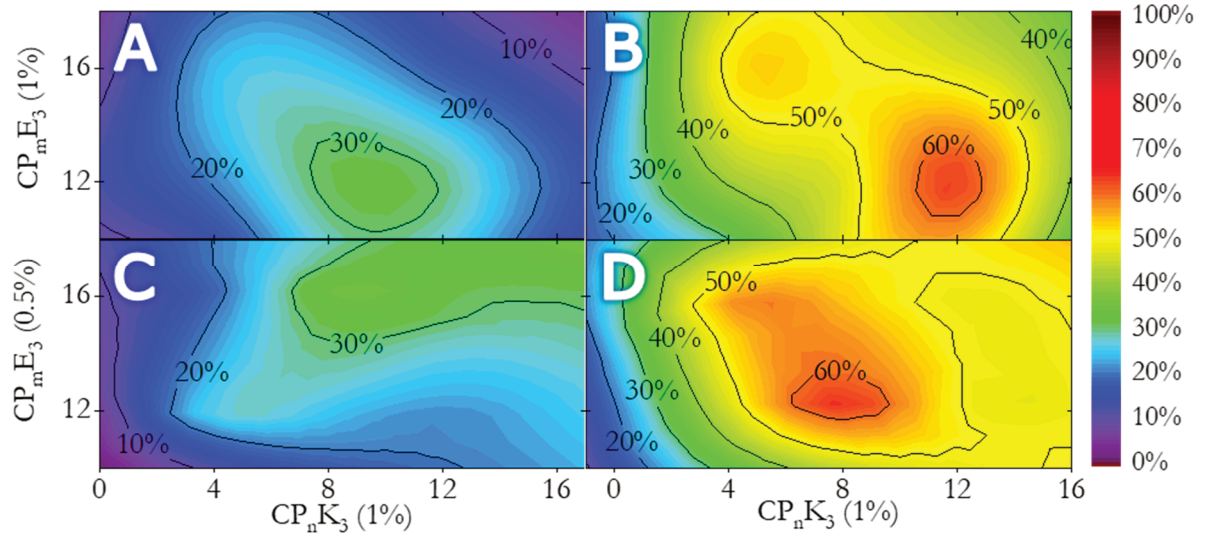


Figure 8. Peptide ratio dependent and time resolved rate of fusion (monitored using a content mixing assay) with CP_mE and CP_nK decorated liposomes as a function of m/n and $[peptide]$, as indicated by an increase in sulforhodamine B emission. Fusion of liposomes decorated with 1 mol% CP_mE and 1 mol% CP_nK is measured after 4 min (A) and 30 min (B) and taken as a reference plot from Figure 2; and fusion of liposomes decorated with 0.5 mol% CP_mE and 1 mol% CP_nK measured after 4 min (C) and 30 min (D). $[Total\ lipid] = 0.1\ mM$, PBS pH 7.4.

diffusion driven collision of liposomes. Over time however, no significant difference between $CP_{12}E$ and $CP_{16}E$ was observed indicating a minor role for L_{PEG} of E at these conditions. The impact of L_{PEG} of K is demonstrated by the shift in fusion efficiency to CP_8K decorated liposomes.

In the presence of membrane tethered E, K will either form a coiled-coil with E or insert in the lipid membrane. It has been demonstrated previously that insertion of unbound peptide K in a membrane is favored over coiled-coil formation, and the presence of membrane tethered E highly enhances the speed of this process.³⁵ This means that, when the concentration of CP_mE is lowered, initial docking of both liposomes via a transient coiled-coil interaction is still efficient, but the equilibrium between coiled-coil formation and membrane insertion of CP_nK (in the opposing liposomal membrane; if not, membrane fusion would be less efficient) shifts to membrane insertion, which in turn results in higher fusion efficiencies for shorter spacers. These results demonstrate that peptide equilibria between different conformations after initial docking highly influences fusion efficiency, and that the interaction between lipopeptide K and the phospholipid membrane is crucial for membrane fusion.

MULTIPLE ROUNDS OF FUSION ASSAY

Finally, a different content mixing experiment was conducted using CP_mE/CP_nK ($m/n = 8, 12, 16$) in order to visualise the influence of the PEG-spacer length on the efficiency of multiple rounds of fusion. To measure this parameter, a population of (self-quenched) fluorescent, CP_mE decorated liposomes with an excess of CP_nK decorated plain liposomes which should result in a higher loss of self-quenching, with respect to the standard content mixing assay using an equimolar mixture of fluorescent and non-fluorescent liposomes, upon every round of fusion. Therefore, CP_nK decorated plain liposomes were mixed with sulforhodamine B loaded CP_mE decorated liposomes in 2 ratios (1:1 and 10:1), and fusion efficiencies were determined by measuring the fluorescence increase after 30 minutes, Table 3. Remarkably, the efficiency of multiple-rounds of fusion appears to be dictated by the PEG-spacer length used for peptide K only, and is not affected significantly by L_{PEG} of CP_mE, confirming that K controls the fusion process, whilst E serves only to mediate coiled-coil formation which leads to liposome docking. The fluorescence increase measured with fusion of CP_mE decorated liposomes and a 10-fold excess of CP₈K decorated liposomes was as efficient as the fusion experiment with an equimolar mixture of the peptide functionalized liposomes. This shows that the abundant availability of CP₈K decorated liposomes doesn't yield a higher fusion efficiency. For CP₁₂K decorated liposomes, fusion experiments with a 10-fold excess of K decorated liposomes yielded a fluorescence increase of $115 \pm 18\%$, demonstrating the fusion of one CP_mE decorated liposome with multiple CP₁₂K decorated liposomes. For CP₁₆K, the multiple round fusion efficiency was $53 \pm 7\%$, indicating the presence of multiple rounds of fusion with this lipopeptide also.

Although it has been shown that CP₈K is most efficient in mediating the early rounds of fusion, it is not able to mediate multiple rounds of fusion in the setting described above. To address this, the behavior of the peptides just before the second fusion event, when both E and K are tethered to the same membrane and are approached by an unfused K decorated liposome have to be considered. E tethered to the membrane of the fluorescent liposome should be accessible to unfused K bearing liposomes, but efficient fusion only occurs when these liposomes are functionalized with CP₁₂K lipopeptides. It is possible that the L_{PEG} distance is again crucial, by shifting peptide equilibria between membrane-incorporated and coiled-coil conformations, and/or by bridging the gap

Table 3. *Multiple rounds of fusion assay.*

[CP _n K]/ [CP _m E] ^a	CP ₈ K +			CP ₁₂ K +			CP ₁₆ K +		
	CP ₈ E	CP ₁₂ E	CP ₁₆ E	CP ₈ E	CP ₁₂ E	CP ₁₆ E	CP ₈ E	CP ₁₂ E	CP ₁₆ E
1 ^b	47	47	51	46	64	48	29	40	38
10	51	50	55	95	150	97	47	61	55
$\Delta^0\%$ ³	10%	7%	8%	104%	135%	104%	61%	53%	46%

^a Fusion efficiency is indicated by an increase in sulforhodamine B emission after 30 mins, with K decorated liposomes in 1 fold or 10 fold excess with respect to dye loaded E decorated liposomes. ^b Fusion efficiency percentages are normalized to efficiency percentages reported for the equimolar mixture at [total lipid] = 0.1 mmol, shown in Figure 5. ³Differences in fluorescence increase between both experiments are calculated as a measure for multiple rounds of fusion and the value of the normalized equimolar mixing entry is used as 100%. [Total lipid of CP_mE decorated liposomes] = 0.05 mM, with 1 mol% lipopeptides, PBS pH 7.4.

between opposite membranes, thus enabling or disabling the next round of liposomal fusion. As a consequence, the mechanism of liposomal fusion is dictated by peptide equilibria affecting the function of peptide K, and a difference of 1 or 2 nm in the length of the spacers employed highly influences the efficiency of the peptides to mediate multiple rounds of fusion.

IMPLICATIONS FOR THE INITIAL STAGES OF LIPOSOMAL FUSION.

Based on these results the following mechanism for the initial stages of E-K mediated liposomal fusion is proposed: initially, lipopeptide K is fully immersed in the liposomal membrane, while lipopeptide E adopts a largely unfolded state. Interliposomal coiled-coil formation occurs when liposomes collide and lipopeptide E binds transiently with lipopeptide K. K-membrane interactions are favored over coiled-coil formation; therefore peptides are in an equilibrium, switching between the different states. Once folded as a coiled-coil, K is in close proximity to both membranes and hence can interact with either. Immersing itself in the opposite membrane will force both membranes into even closer proximity, destabilizing both membranes by forming protrusions, and will lead to the dehydration of both membrane solvation layers, thus providing the necessary initial conditions for the merging of the membrane bilayers.^{35, 55} (Figure 9)

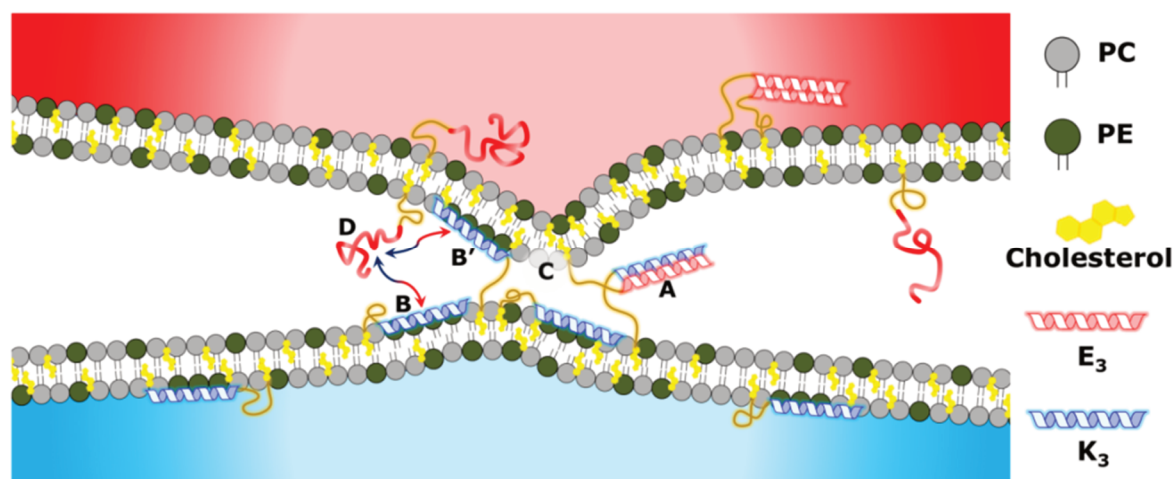


Figure 9. *Peptide equilibria prior to fusion. A transient coiled-coil complex (A) forms upon contact of liposomes. From the coiled-coil conformation, peptide K can immerse in both membranes (B – B’); membrane insertion in the opposing membrane will force both membranes in close proximity, destabilize both membranes with protrusions (C), providing the necessary conditions for membrane merging. Equilibria of peptide-interactions between (D) and (B – B’) are highly affected by the length of the PEG-spacer used for both E and K.*

CONCLUSIONS

This study has demonstrated that the membrane interaction propensity of K, a factor crucial for efficient membrane fusion, is dependent on both the anchor and PEG spacer-length employed. When conjugated to a cholesterol anchor, K shows increasing helicity with decreasing PEG-length, indicating a more profound peptide-membrane interaction for shorter spacers. In contrast, the LP_nK derivatives do not affect the peptide structure significantly, and the helical secondary structure of all LP_nK derivatives appears to be less membrane stabilized when compared to CP₁₂K lipopeptides. The influence of the anchor on peptide-membrane interaction is likely caused by altering local membrane characteristics, and is therefore also a factor in the fusion efficiency; cholesterol anchored lipopeptides are more efficient in mediating fusion than DOPE anchored lipopeptides, as evidenced by the content mixing assays.

The intrinsic stability of liposomes prevents coiled-coil formation when peptides are tethered directly to the membrane, (i.e. when a spacer is not used) and lessens the fusion efficiency when very long spacer lengths are applied. Changing the length of the spacer region between E and K independently demonstrated that the peptides clearly have different roles in the fusion process, as shown with the various content-mixing experiments that reflect the initial stages of fusion. It can be concluded that peptide E

acts as a handle for peptide K to enable initial liposomal docking via coiled-coil formation, and, a CP₁₂E or CP₁₆E construct is most efficient.

After coiled-coil formation, peptide K migrates to the lipid membrane,³⁵ and lowering the concentration of CP_mE at the liposomal surface from 1% to 0.5% revealed the effect of K equilibria between coiled-coil formation and K-membrane interaction on the rate and efficiency of liposomal fusion.³⁵ This change in CP_mE/CP_nK ratio increased the probability of peptide K immersing itself in the opposing membrane directly after coiled-coil formation, which in turn significantly increased the fusion efficiency for liposomes with shorter spacers, showing the strong influence of L_{PEG} on the rate of membrane fusion. Multiple rounds of liposomal fusion were only observed when liposomes were functionalized with CP₁₂K and CP₁₆K, while no apparent influence of CP_mE on the fusion efficiency was measured. Here, the fusion efficiency depends only on the spacer length of lipopeptide K. This pronounced influence of the PEG-spacer illustrates the extreme sensitivity of the fusion system to L_{PEG} and to the dynamics of the involved peptides in the initial stages of fusion.

The differences in the (initial) rate of fusion revealed that lipopeptides LP₄K and CP₈K are most efficient in mediating fusion compared to other LP_nK / CP_nK derivatives which means that a distance of ± 2 nm between peptide K and the membrane is most favorable, and this lipopeptide is an ideal candidate for further investigations in similar fusion systems. The length of the spacer highly influences the speed and efficiency of the fusion process and reveals the roles of the peptides in the fusion mechanism.

EXPERIMENTAL SECTION

MATERIALS

Fmoc-protected amino acids, rink amide resin, and O-(1H-6-Chloroben-zotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) were purchased from NovaBioChem. Diisopropylethylamine (DIPEA), piperidine, acetic anhydride, N-methylpyrrolidine (NMP), dimethylformamide (DMF), acetonitrile, and trifluoroacetic acid (TFA) were obtained from Biosolve. Dichloromethane (DCM), diethyl ether, triisopropylsilane (TIS), trimethylamine (TEA), cholesterol, trimethylphosphine (PMe₃, 1M in toluene) (1H-benzotriazol-1-yloxy)tripyrrolidinophosphoniumhexafluorophosphate (PyBOP), succinic anhydride, and sulforhodamine B were obtained from Sigma Aldrich. 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), and 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) were purchased from Avanti polar lipids. The Fmoc-NH-(PEG)_{2/8/12/16}-COOH spacers were purchased from Iris Biotech. The N₃-(PEG)₄-COOH spacer was synthesized according to a literature procedure, as reported previously.⁵⁹⁻⁶⁰ Cholesteryl-4-amino-4-oxobutanoic acid (**3**) was synthesized according to a modified literature procedure,⁴⁵ synthesis of the starting compound 3-Azido-5-cholestene (**1**) is described elsewhere.⁴⁴ PBS buffer contains 5 mM KH₂PO₄, 15 mM K₂HPO₄, 150 mM NaCl, pH 7.4. Data analysis and visualization was performed using OriginPro 9.1.

SYNTHESIS OF CHOLESTERYL-AMINO-HEMISUCCINATE

3-Amino-5-cholestene (2): To a solution of 3-Azido-5-cholestene (**1**) (15.8 g, 38.4 mmol) in dichloromethane, 4 equivalents of trimethylphosphine (1M solution in THF or toluene) was added and the reaction was stirred overnight. When 3-Azido-5-cholestene was consumed as evidenced by TLC (eluent: hexane, *rf* = 0.7), the reaction was concentrated *in vacuo* to yield **2** (14.8 g, 100%) as a pure compound. ¹H NMR (300 MHz, CDCl₃) δ = 5.27 (s, 1H), 2.60 (m, 1H), 2.17-0.85 (m, 40H), 0.64 (s, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 141.8, 120.7, 56.8, 56.2, 52.0, 50.3, 43.4, 42.3, 39.8, 39.5, 38.2, 36.6, 36.2, 35.8, 32.7, 31.9 ($\times$ 2), 28.3, 28.0, 24.3, 23.9, 22.8, 22.6, 21.0, 19.5, 18.7, 11.9; IR (film) ν 3354, 3260, 3154, 2936, 2896, 2849, 1464, 1437, 1381 cm⁻¹; LRMS (ESI⁺) *m/z* 386.4 ([M+H]⁺, C₂₇H₄₈N calcd. 386.4).

Cholesteryl- 4-amino-4- oxobutanoic acid (3): To a solution of **2** (10 g, 26 mmol) in chloroform (150 mL) succinic anhydride (7.8 g, 78 mmol) was added. Triethylamine (8mL, 57 mmol) was added drop-wise to the solution and the reaction mixture was stirred overnight under nitrogen atmosphere at room temperature, until the starting material was completely consumed as evidenced by TLC (eluent: hexane: ethylacetate 1:1, $r_f = 0.4$). The reaction mixture was transferred to a separatory funnel, and the organic layer was washed with water and brine, was dried with $MgSO_4$ and was concentrated in *vacuo* to afford cholesterol amide acid (10 g) as a yellow solid. The crude cholesterol amide acid was purified by recrystallization in Acetonitrile: MeOH (1:1) at 4°C and was vacuum filtered to afford **3** as an off-white solid (8.7 g, 76%) 1H NMR (400MHz, $CDCl_3$) $\delta = 5.36$ (s, 1H), 3.69 (s, 1H), 3.60 (m, 1H) 2.62 (m, 2H), 2.44 (m, 2H), 2.20-1.72 (m, 7H), 1.67-1.03 (m, 20H) 1.01 (s, 3H) 0.92 (d, $J = 6.5$ Hz, 3H), 0.87 (dd, $J = 6.6, 1.7$ Hz, 6H), 0.69 (s, 3H). TLC-MS (ESI⁺) m/z 486.4 ($[M+H]^+$, $C_{31}H_{51}NO_3$ calcd. 486.4 g/mol).

LIPOPEPTIDE SYNTHESIS

PEG_n-peptide synthesis: Peptide synthesis was performed on a CEM Liberty I on a 100 μ mol scale using Rink amide resin of 0.55-0.73 mmol/g substitution. Amino acid activation was achieved using HCTU/DIPEA in DMF. Fmoc deprotection was carried out using 2 cycles of 20% piperidine in DMF. All automated reactions were carried out at 70-80 °C with the use of a microwave. The N-terminal free amine was used to couple the PEG-spacer through standard solid phase chemistry using Py-BOP/DIPEA activation. With the exception of N3-PEG4-COOH containing lipopeptides, Fmoc-deprotection of the PEG component was achieved using 20% piperidine for 20 mins to obtain a free amine at the N-terminus. N3-PEG4-COOH containing lipopeptides were reduced using 2 cycles of PME in dioxane/water (4:1).

DOPE anchor attachment: Succinic anhydride was coupled to the N-terminal free amine using a succinic anhydride/TEA (10 eq./10 eq.) mixture in NMP. The reaction was left overnight on a shaker at room temperature. The resin was washed thoroughly with NMP, DCM, and NMP to remove excess reactants. Next, the resin (1 eq.) was activated using PyBOP/DIPEA (3 eq./5 eq.) in DCM:DMF (1:1) for 1hr. DOPE (2 eq.) was dissolved in a minimal volume (2 ml) of DCM: DMF (1:1) and a small amount of TEA (2 eq.) was added to improve solubility. After 1 hr this DOPE solution was added

to the resin, warmed to 50 °C and the reaction was left overnight on a shaker. The resin was washed thoroughly with DMF and DCM to remove excess reactants.

CHOL anchor attachment: Cholesteryl- 4-amino-4-oxobutanoic acid (**3**) (1.2 eq.) was activated with PyBOP/DIPEA (3eq/5eq) in DCM:DMF 1:1, added to the peptide-bound resin and the reaction was left for 2 days on a shaker. The resin was washed thoroughly with DMF and DCM to remove excess reactants.

Cleavage: The resin bound peptide was cleaved from the resin and deprotected for 2 hours in 4ml of TFA: TIS: Water (95%: 2.5%:2.5%), The cholesterol-anchored peptides were precipitated in cold diethyl ether followed by centrifugation and dried under vacuum, DOPE-anchored peptides were concentrated in vacuo, dissolved in a minimal amount of acetic acid/H₂O and subsequently purified.

Purification: RP-HPLC was performed with a Shimadzu HPLC system with two LC-8A pumps, and a SPD-10AVP UV-VIS detector. Sample elution was monitored by UV detection at 214 nm and 256 nm. Samples were eluted with a linear gradient from A to B, A being 10 % (v/v) H₂O, 0.1 % (v/v) TFA, and B being 90 % (v/v), 0.1 % (v/v) TFA in acetonitrile. Purification of DOPE-lipopeptides was performed on a Vydac C4 reversed phase column (214TP1022, 22 mm diameter, 250 mm length, 10.00 μM particle size) with a flow rate of 20 mL min⁻¹. Purification of CHOL-lipopeptides was performed on a Phenomenex C18 reversed phase column (21.2 mm diameter, 150 mm length, 5.00 μM particle size) with a flow rate of 15 mL min⁻¹. Collected fractions were tested for >95% purity using LC-MS with Gemini C18 column and further freeze dried to store at -20°C.

GENERAL METHODS

Liposome preparation. A 1 mM stock solution containing DOPC : DOPE : cholesterol (50 : 25 : 25 mol%) lipids in 1 : 1 (v/ v) methanol : chloroform was prepared for all fusion experiments. Lipidated peptides were dissolved in a 1 : 1 (v/v) chloroform : methanol mixture, to a concentration of 50 μM. 1 mol% of these lipidated peptides were mixed with the appropriate liposome solution and the solvent was removed under a stream of air. For content mixing experiments, PBS buffer containing 20 mM sulforhodamine B was added to the dried lipid/peptide mixtures containing lipopeptide E. These solutions were subsequently sonicated for 5-10 minutes at 55 °C to yield 100 nm diameter liposomes²⁹. The solutions were used without further purification, except

for the sulforhodamine B containing liposomes, which were purified using a Sephadex column to remove any non-encapsulated sulforhodamine B.

Fluorescence spectroscopy. Lipid- and content-mixing experiments were performed on a TECAN Infinite M1000 PRO fluorimeter using a 96-well plate at 25 °C. The percentage of fluorescence increase $\%F$ was calculated as:

$$\%F = \frac{F(t) - F_0}{F_{max} - F_0} * 100$$

Content mixing experiments: Sulphorhodamine B fluorescence intensity $F(t)$ at 580 nm was monitored in a continuous fashion for 30 min after mixing non-fluorescent K-liposomes with sulphorhodamine B loaded E-liposomes. F_0 was obtained by measuring emission of sulphorhodamine B-loaded E-liposomes to which an equal amount of PBS was added, and F_{max} was obtained by measuring the emission of plain liposomes loaded with 10 mM sulphorhodamine B. [Total lipid] = 0.1 mM, in PBS pH 7.4.

Multiple round content mixing experiments: Experimental settings of regular content mixing experiments were used as described above. E decorated, dye loaded, liposomes were prepared with a total lipid concentration of 0.05 mM, in PBS. K decorated liposomes were prepared with a total lipid concentration of 0.5 mM, in PBS. Total experimental mixtures for equimolar fusion experiments contained 100 uL E liposomes, 10 uL K liposomes and 90 uL PBS. Total experimental mixtures for fusion experiments at 10:1 ratio contained 100 uL E liposomes and 100 uL K liposomes.

Data visualization: 2D contour graphs were constructed in Origin 9.1, and subjected to a 100pnt smoothing algorithm. Major and minor levels were set at 10% and 1% fluorescence increase respectively. As color scheme, the multicolored and intuitive gradient ‘Rainbow’ was chosen.

Dynamic Light Scattering. Particle size distributions were measured by dynamic light scattering using a Malvern Zetasizer Nano ZS ZEN3500 equipped with a peltier thermostatic cell holder. The laser wavelength was 633 nm and the scattering angle was 173°. The Stokes Einstein relationship

$$D = \frac{k_B T}{3\pi\eta D_h}$$

was used to estimate the hydrodynamic diameter D_h . Here, k_B is the Boltzmann constant, η is the solvent viscosity, and T was set to 25 °C.

Circular dichroism spectroscopy. CD spectra were obtained using a Jasco J-815 spectropolarimeter equipped with a peltier temperature controller. The ellipticity, given as mean residue molar ellipticity, $[\theta]$ (deg cm² dmol⁻¹), is calculated using the following equation:

$$[\theta] = \frac{100 * \theta_{obs}}{nlc}$$

where θ_{obs} is the observed ellipticity (mdeg), n is the number of peptide residues, l is the path length of the cuvette (cm) and c is the peptide concentration (mM). Spectra were recorded from 260 nm to 200 nm at 20 °C. Data points were collected with a 1 nm bandwidth at 1 nm intervals, using a scan speed of 1 nm s⁻¹. Each spectrum was an average of 5 scans. For analysis, each spectrum had the background spectrum (plain liposomes in PBS) subtracted. Percentage α -helicity was calculated using the predicted value $[\theta]_{222} = -40000 * (1 - 4.6/n)$ as 100% value for an α -helical peptide of n residues.⁵⁸ [Total lipid] = 0.5 mM, with 1 mol% lipopeptide, in PBS pH 7.4.

LIPOSOMAL CALCULATIONS

To visualize the peptide density at the surface of a liposome, we calculated the amount of lipid molecules and peptides (N) in an ideal liposome with the following formula⁶¹:

$$N = 4\pi \frac{r^2 + (r - d_m)^2}{A_{Lipid}}$$

where r = liposomal radius, d_m = membrane thickness and A = surface area of respective lipid headgroups.⁶² When $A_{lipid} = \frac{2A_{DOPC} + A_{DOPE} + A_{Chol}}{4} = \frac{2*0.67 + 0.75 + 0.34}{4} = 0.61$ nm², r = 50 nm, and d_m = 4.6 nm, N is approximately 94400. The number of peptides (1% M/M) exposed at the outer surface of the liposomes is approximately 520 upon assumption of equal preference for inner and outer membrane, with a surface area per peptide of 61 nm² (square of 7.8nm). At [total lipid] = 0.1 mM, the liposomal concentration is calculated to be $7.5*10^8$ liposomes μ L⁻¹.

LIPOPEPTIDE ANALYSIS

Purity was confirmed using LC-MS analysis equipped with Gemini 3 μ C18 column coupled with Finnigan LCQ advantage max (Thermo) ESI-MS analyzer and the results are summarized in Table S1. LCMS eluents were A) H₂O + 0.1% v/v TFA, B) MeCN + 0.1 v/v TFA, with a flow rate of 1 mL min⁻¹. Gradient was applied between 2 and 12 min, from 10% B in A to 90% B in A. Retention time RT is rounded to 2 significant numbers.

Table S1. Characteristics of synthesized lipopeptides.

Peptide	formula	Calc. mass	Found mass	RT (min)
CP ₀ E	C ₁₄₄ H ₂₃₆ N ₂₆ O ₄₀	2971.57	2972.14, [M+H] ⁺	9.0
CP ₀ K	C ₁₄₉ H ₂₅₂ N ₃₀ O ₃₃	2991.78	2992.60, [M+H] ⁺	7.7
CP ₄ E	C ₁₅₄ H ₂₅₅ N ₂₇ O ₄₅	3204.83	3206.63, [M+H] ⁺	8.3
CP ₄ K	C ₁₅₉ H ₂₇₁ N ₃₁ O ₃₈	3225.04	1611.48, [M+2H] ⁺²	6.7
CP ₈ E	C ₁₆₃ H ₂₇₃ N ₂₇ O ₄₉	3395.06	1696.40, [M+2H] ⁺²	8.4
CP ₈ K	C ₁₆₈ H ₂₈₉ N ₃₁ O ₄₂	3415.28	1706.40, [M+2H] ⁺²	6.8
CP ₁₂ E	C ₁₇₁ H ₂₈₉ N ₂₇ O ₅₃	3571.27	1190.53, [M+3H] ⁺³	8.5
CP ₁₂ K	C ₁₇₆ H ₃₀₅ N ₃₁ O ₄₆	3591.49	1197.07, [M+3H] ⁺³	7.3
CP ₁₆ E	C ₁₇₉ H ₃₀₅ N ₂₇ O ₅₇	3747.48	1249.27, [M+3H] ⁺³	8.5
CP ₁₆ K	C ₁₈₄ H ₃₂₁ N ₃₁ O ₅₀	3767.70	1255.93, [M+3H] ⁺³	7.4
LP ₀ E	C ₁₄₇ H ₂₅₆ N ₂₆ O ₄₃ P ⁻	3106.78	1563.44, [M+H+NH ₄] ⁺²	12
LP ₀ K	C ₁₅₀ H ₂₇₁ N ₂₉ O ₃₇ P ⁻	3103.96	1553.01, [M+2H] ⁺²	10
LP ₂ E	C ₁₅₃ H ₂₆₇ N ₂₇ O ₄₆ P ⁻	3251.94	1084.98, [M+3H] ⁺³	12
LP ₂ K	C ₁₅₆ H ₂₈₂ N ₃₀ O ₄₀ P ⁻	3249.12	1084.03, [M+3H] ⁺³	10
LP ₄ E	C ₁₅₇ H ₂₇₅ N ₂₇ O ₄₈ P ⁻	3340.04	1114.33, [M+3H] ⁺³	12
LP ₄ K	C ₁₆₀ H ₂₉₀ N ₃₀ O ₄₂ P ⁻	3337.22	1113.72, [M+3H] ⁺³	10
LP ₈ E	C ₁₆₆ H ₂₉₃ N ₂₇ O ₅₂ P ⁻	3530.28	1178.04, [M+3H] ⁺³	12
LP ₈ K	C ₁₆₉ H ₃₀₈ N ₃₀ O ₄₆ P ⁻	3527.46	1177.09, [M+3H] ⁺³	10
LP ₁₂ E	C ₁₇₄ H ₃₀₉ N ₂₇ O ₅₆ P ⁻	3706.49	1236.74, [M+3H] ⁺³	12
LP ₁₂ K	C ₁₇₇ H ₃₂₄ N ₃₀ O ₅₀ P ⁻	3703.67	1235.79, [M+3H] ⁺³	10

CD SPECTROSCOPY

Characteristics ($\theta_{222}/\theta_{208}$ and percentage helicity at θ_{222}) of the spectra as summarized in Table 2, are supported with the recorded spectra between 207nm and 250 nm (*Figure S1*). Below 207nm, spectra were too noisy due to scattering of the signal by liposomes.

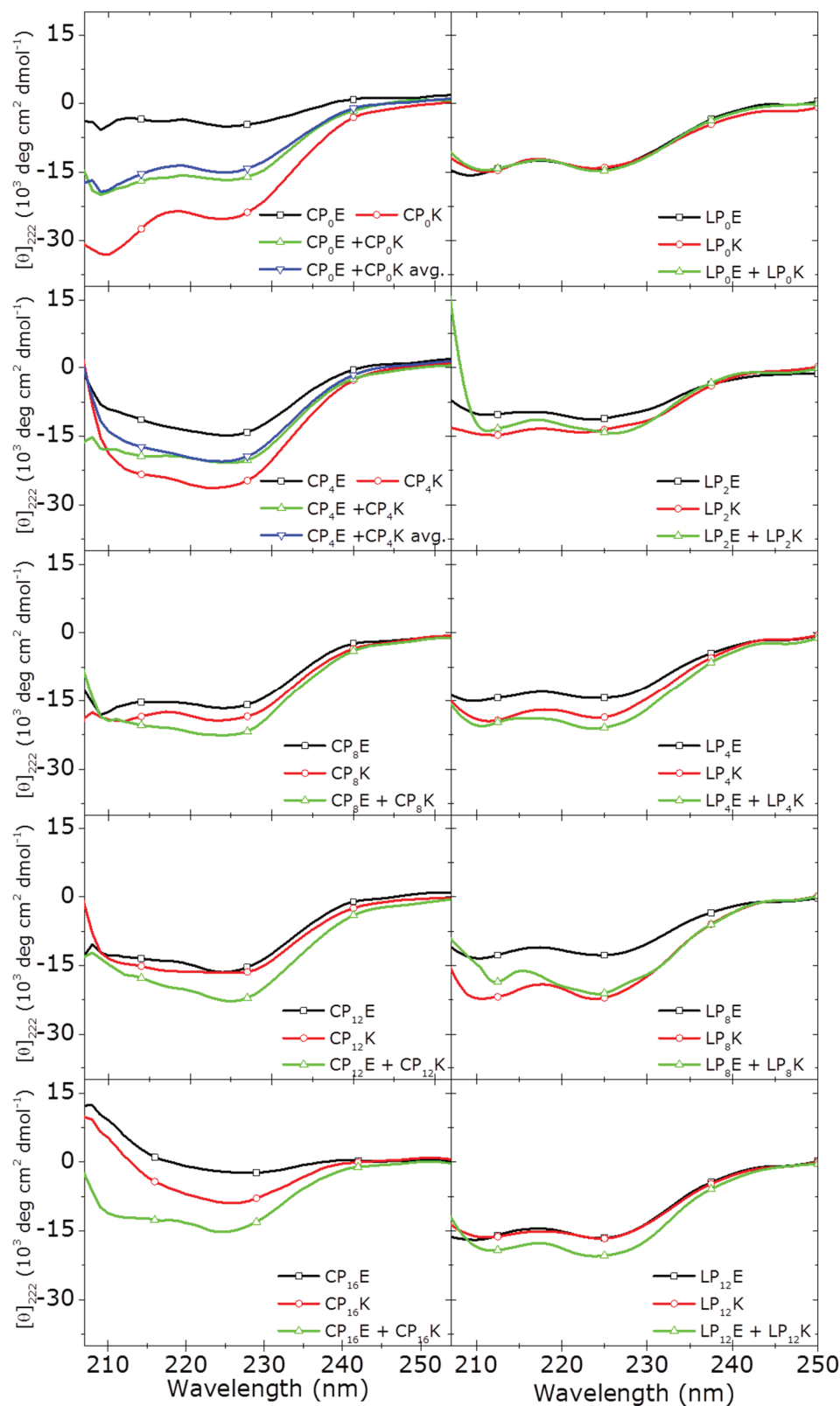


Figure S1. Peptide secondary structure of E (black) and K (red)- decorated liposomes before and after mixing (green), measured by Circular Dichroism. Left series is with cholesterol anchor, right series contain a DOPE anchor. Only symmetric pairs ($m = n$) are used in this study. With CP_0 and CP_4 anchor coiled-coil formation is not evident, so the calculated average of the individual E and K spectra is added (blue). $[Total\ lipid] = 0.5\ mM$ with $1\ mol\%$ lipopeptide, PBS pH 7.4.

FULL DATA OF 30MIN CONTENT MIXING ASSAY

Content mixing experiments were followed for 30 minutes, error bars and absolute values after 4 and 30 minutes were obtained by at least 3 individual experiments and are shown in Figure S2. Fluorescence increase was measured as a function of m , n , in a continuous fashion and showed characteristic 1st order kinetics in the initial minutes (Figure S3). Therefore, fluorescence increase after 4 minutes is used to estimate the relationship of L_{PEG} and the fusion efficiency. In negative control experiments, peptide bearing liposomes (m , n = 4 or 8) were mixed with plain liposomes and the fluorescence increase was recorded for 30 minutes. Left out peptides E or K , (X), are denoted with X , n or m , X .

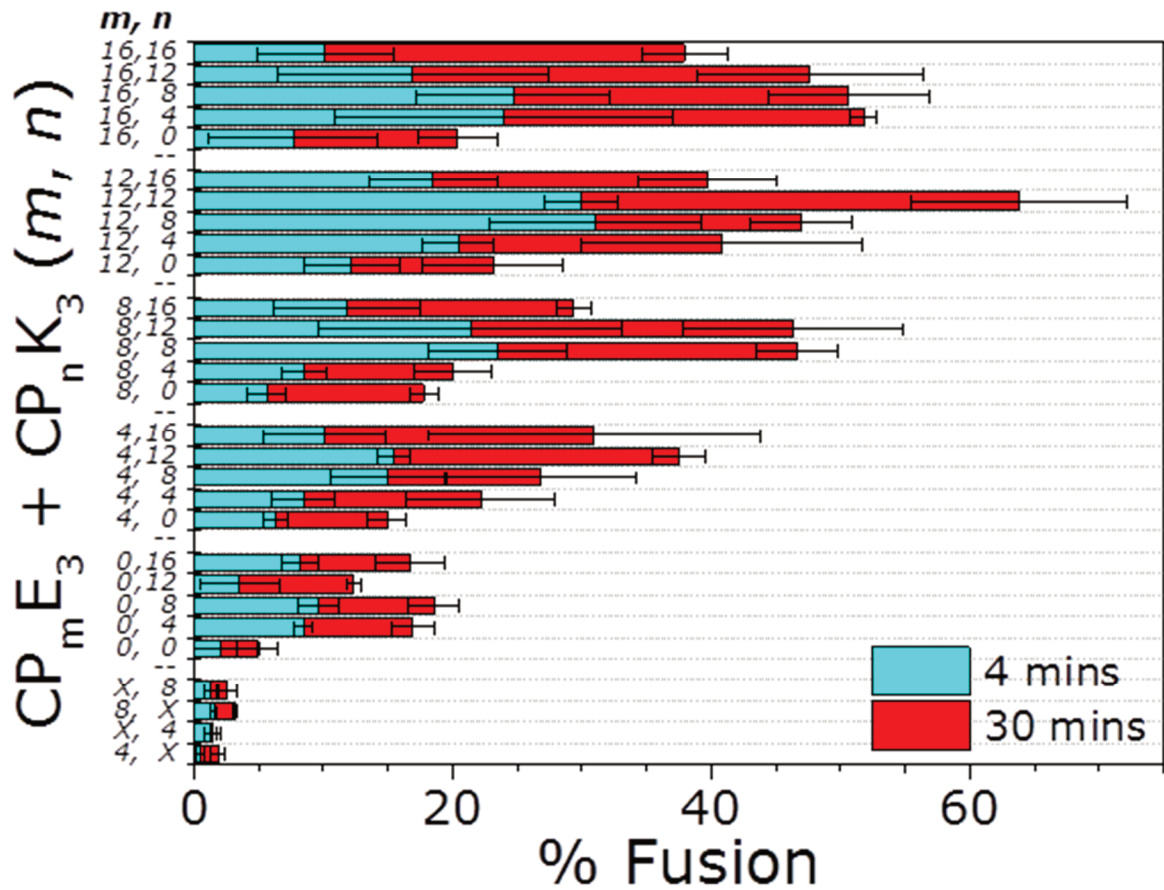


Figure S2. Time resolved rate of fusion (Content mixing) with lipopeptide CPmE or CPnK decorated liposomes as a function of m , n , indicated by an increase in sulforhodamine B emission after 4 and 30 minutes. Left out peptides E or K , (X), in negative control experiments are denoted with X , n or m , X . [Total lipid] = 0.1 mM with 1 mol% lipopeptide, in PBS pH 7.4.

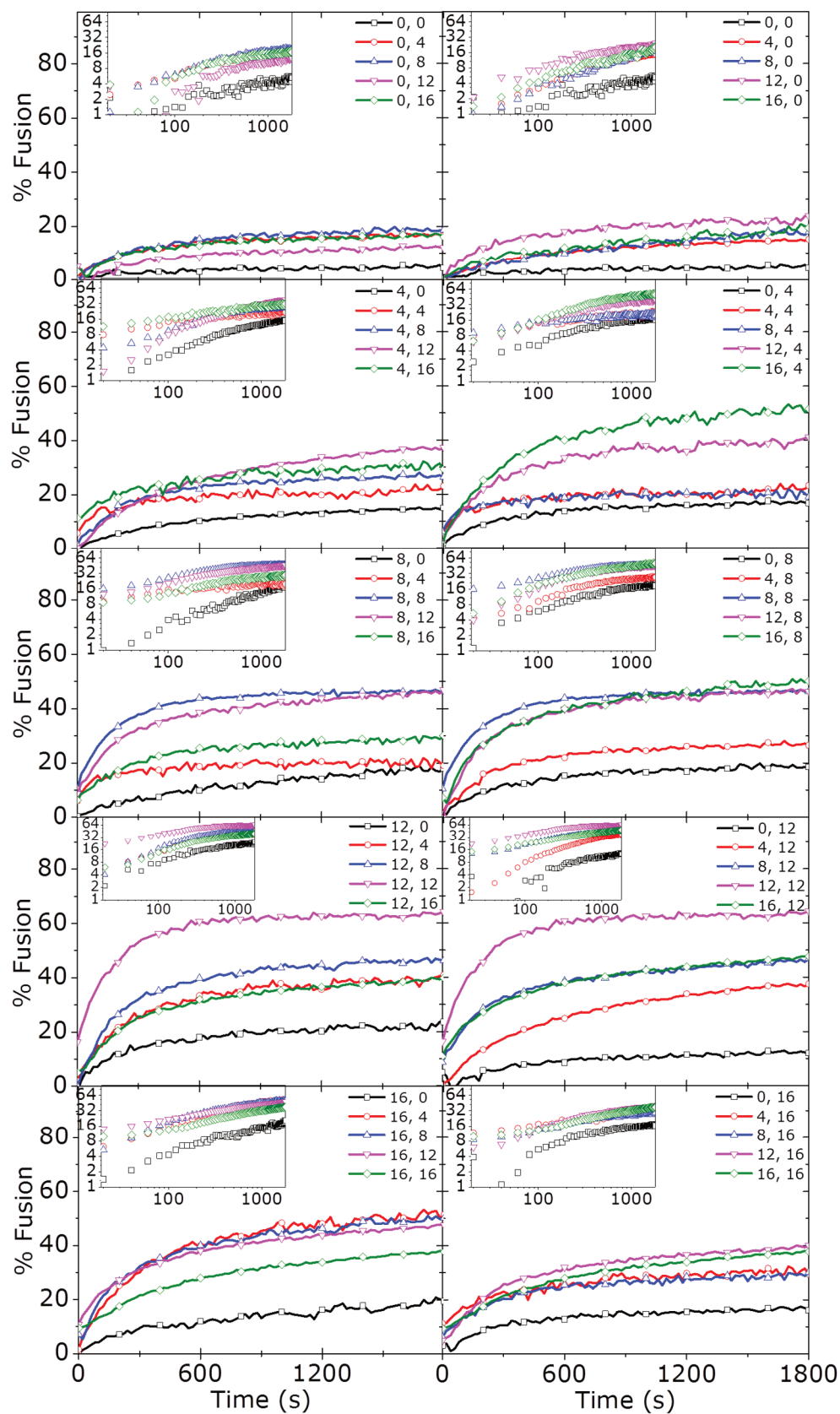


Figure S3. Content mixing of CP_mE and CP_nK decorated liposomes as a function of m , n . Insets with log scale illustrate 1st order kinetics until at least the 4th minute. [Total lipid] = 0.1 mM with 1 mol% lipopeptide, PBS pH 7.4.

INFLUENCE OF CPE / CPK RATIO ON FUSION EFFICIENCY

A change in peptide functionalization ratio revealed the influence of peptide equilibria on the fusion process. Again, results are shown as a function of m, n . (Figure S4)

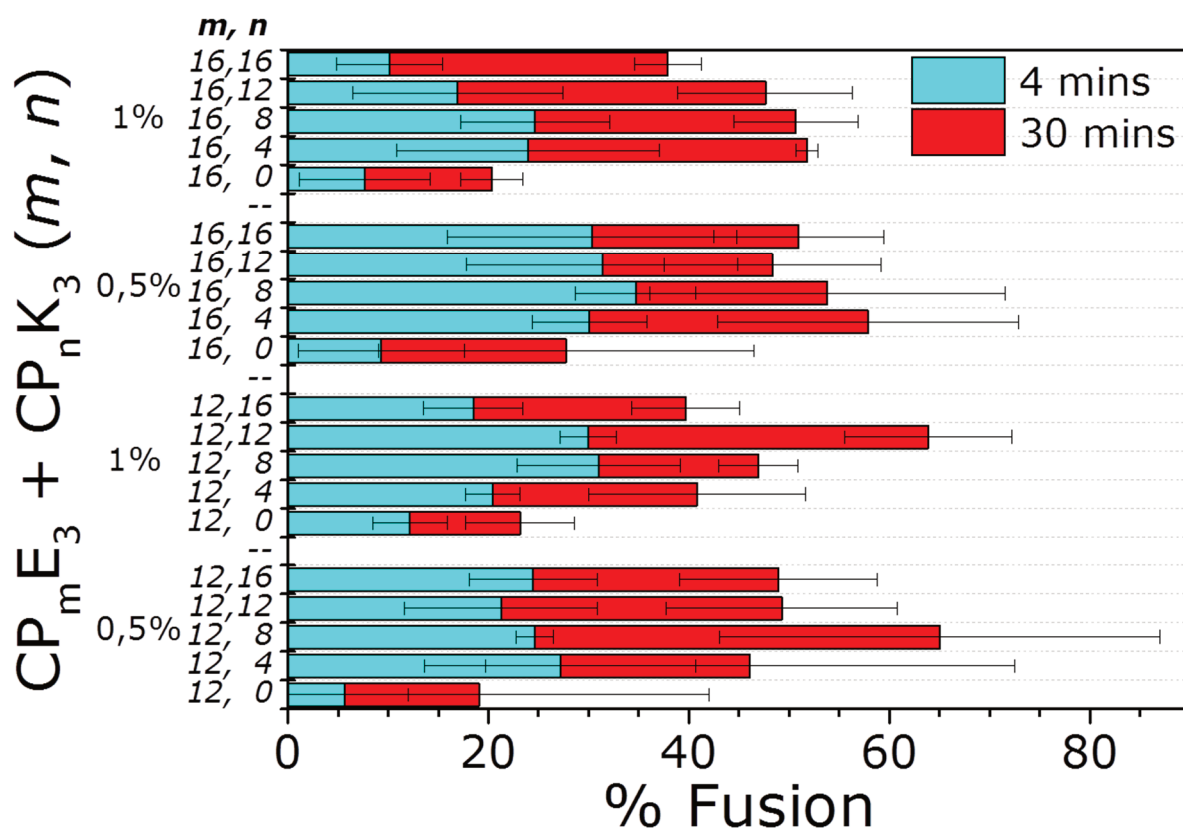


Figure S4. Time resolved rate of fusion (Content mixing) with peptide CP_mE and CP_nK decorated liposomes as a function of m, n , and $[CP_mE]$, as indicated by an increase in sulforhodamine B emission after 4 and 30 minutes. $[Total\ lipid] = 0.1\ mM$ with 1 mol% CP_nK and 1 mol% or 0.5 mol% CP_mE , PBS pH 7.4.

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