# Zipping into fusion

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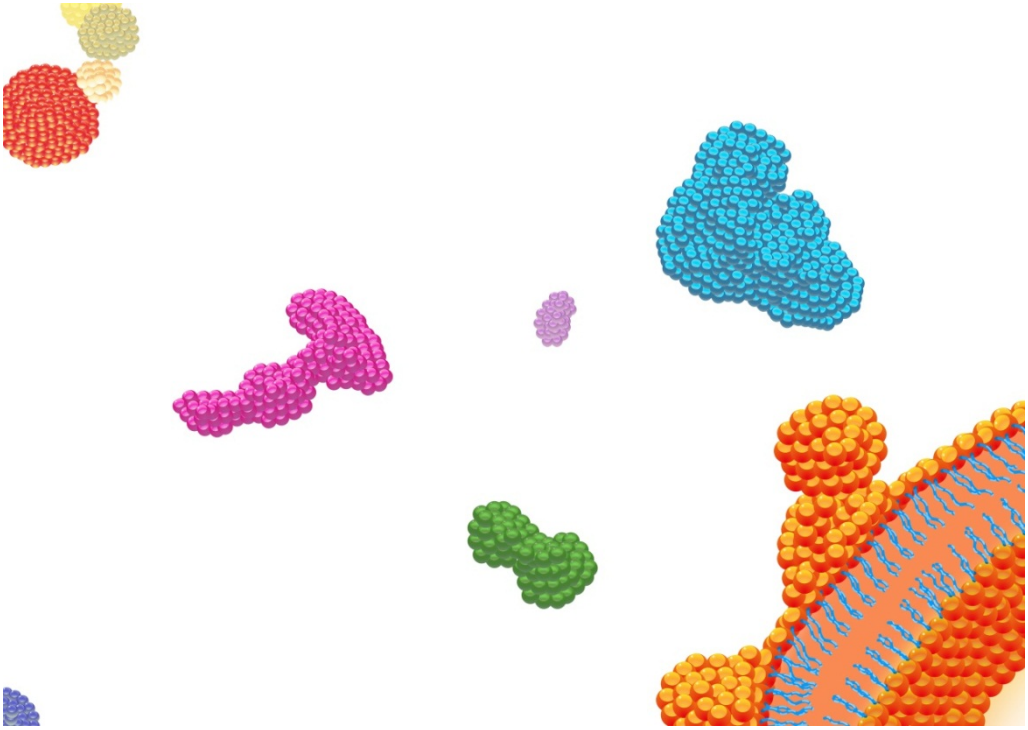
# Chapter 4

## Controlled liposome fusion mediated by SNARE protein mimics



Marsden, H. R.; Zheng, T. T.; Voskuhl, J.; Kros, A., Controlled liposome fusion mediated by SNARE protein mimics. Biomaterials Science 2013, 1 (10), 1046-1054.

## Abstract

The fusion of lipid membranes is essential for the delivery of chemicals across biological barriers to specific cellular locations. Intracellular membrane fusion is particularly precise, and is critically mediated by SNARE proteins. To allow membrane fusion to be better understood and harnessed, a simple bottom-up model in which synthetic fusogens replicate the essential features of SNARE proteins has been used to mimic this important process. In our fusogens, the coiled-coil molecular recognition motif of SNARE proteins is replaced by the coiled-coil E/K peptide complex, which is one ninth the size. The peptides are anchored in liposome membranes via pegylated lipids. Here, how the liposome fusion process is controlled by different parameters within the minimal model has been discussed. The lipopeptide fusogens form specific coiled coils that dock liposomes together, resulting in the merging of membranes via the stalk intermediate. Unusually for model systems, the lipopeptides can rapidly lead to fusion of entire liposome populations and the liposomes can undergo many rounds of fusion. The rate and extent of fusion and the number of fusion rounds can be manipulated by adjusting the fusogen and liposome concentrations. For example, these parameters can be tuned such that tens of thousands of ~100 nm liposomes fuse into a single giant liposome ~10 μm in diameter, alternatively, conditions can be selected such that only two liposomes fuse. The improved understanding of membrane fusion shows how application-specific fusion attributes can be achieved, and paves the way for controlled nanoreactor mixing and the controlled delivery of cargo to cells.

## Introduction

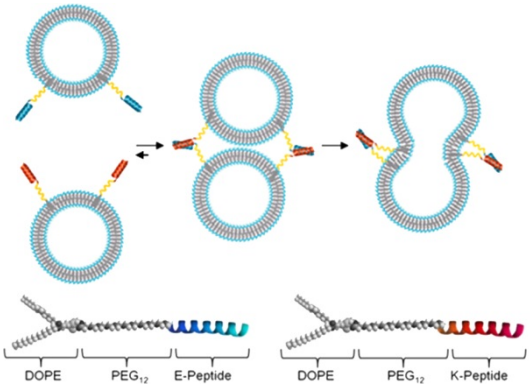
The fusion of biological membranes is a very significant process as it allows the delivery of molecules across lipid bilayers, barriers that are usually impervious to the molecules. Intracellular membrane fusion, which is mediated by SNARE-proteins, is of particular interest as it is highly controlled in terms of which membranes will fuse and the location of fusion. The mechanism relies on the specific coiled-coil interaction between complementary proteins that are spatially organized.[1] The most widely studied SNARE-proteins are involved with cell-to-cell communication in the nervous system. Within neurons there are small liposomes called synaptic vesicles that are packed with neurotransmitters and whose outer lipid surface is extensively covered by proteins.[2] SNARE-proteins make up ~0.35 mol% of the molecules of the synaptic vesicle.[2] The

fusion process can be dissected into three stages: collision, docking, fusion. Firstly synaptic vesicles approach the neuronal membrane, they then dock to the target membrane by way of a coiled-coil bundle, which forms between the SNARE protein on the vesicle and two other complementary SNARE proteins, one anchored in the neuronal membrane, and one in the cytoplasm. Finally, lipid mixing results in the transfer of the neurotransmitters out of the neuron. In vitro experiments using reconstituted SNARE proteins have shown that the formation of the coiled-coil bundle docking the vesicles to the target membrane is sufficient to locally disrupt the lipids and cause the membranes to merge.[3-5]

The ability to controllably fuse specific lipid membranes has much potential, and an improved understanding of membrane fusion will allow the development of more sophisticated applications. The most highly anticipated application of controlled membrane fusion is the targeted transport of cargo such as drugs or gene therapies into cells. This would reduce side effects for patients and increase the efficacy of treatments.

For the application of controlled membrane fusion it is advantageous to use small synthetic fusogens rather than the groups of large proteins that have evolved in competitive cellular environments, which are cumbersome to manage. For the same reasons of ease of use and interpretation it is preferable to use liposomes or supported lipid bilayers as model membranes rather than whole cells. There have been previous membrane fusion models proposed using metal ions[6, 7] or synthetic fusogens, most of which are based on the concept of the fusion of viruses with cells, in which case a conformational change in a peptide causes it to bury a hydrophobic domain into the 'target' membrane.[8-13] In this case the binding is non-specific, with binding occurring randomly on the surface, and it is often a leaky process.[11] In other models the fusion is more SNARE-like in that it relies on the specific interaction between molecules.[14-20] In a previous contribution, our group presented the first model system in which the fusogens are simplified versions of SNARE proteins.[21] As with native SNARE-based fusion, the mechanism is based on the formation of coiled-coil complexes which dock and fuse the target membranes (Scheme 1). Two coiled-coil forming peptides, denoted E and K, were lipidated via a short PEG spacer, and are denoted LPE and LPK.[22] The lipid tail (DOPE) anchors the peptides into lipid membranes. One set of liposomes is decorated with LPE and another with LPK. It was shown that when the liposome populations are mixed, the interaction between the coiled-coil forming peptides E and K leads to mixing of the liposome membranes and contents mixing without leakage, ie. clean liposome fusion.[21] The model displays the same key characteristics as native

membrane fusion and the reduced SNARE proteins are also simple enough to enable ready synthesis and use in a range of settings.




**Scheme. 1.** Schematic illustration of liposome fusion mediated by simple SNARE protein mimics. Liposomes are modified with the lipopeptides LPE or LPK and upon mixing the peptide interactions trigger liposome fusion. E-peptide is $(EIAALEK)_3$ and K-peptide is $(KIAALKE)_3$ from N- to C- terminus.

Having established a model that achieves membrane fusion showing the desired characteristics, the boundaries within which it functions and it how the fusion process differs within this scope need to be thoroughly investigated to maximize its future use. Here the determinants for liposome fusion using this minimal model has been assessed. The influence of lipopeptide concentration, lipid concentration, and lipids of positive curvature on liposome fusion have been characterized. This chapter describes how the juxtaposition of liposome collision, docking and lipid mixing rates shapes the varied outcomes of liposome fusion within the reduced SNARE model.

## Results and Discussion

## 1. Targeted fusion

When anchored in liposome membranes the fusogens LPK and LPE can cause rapid and complete fusion of all initial liposomes, as determined by a fluorescence-based lipid mixing assay (Figure 2).[23] Control experiments did not result in significant lipid mixing, confirming that the specific molecular recognition between peptides E and K is necessary for fusion, and that this is the only route to fusion in this model (Figure 2).

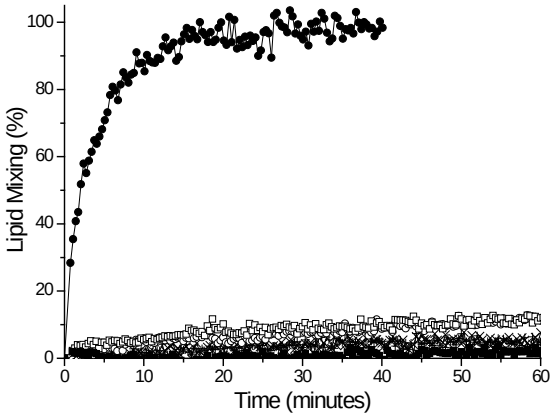



**Figure. 2.** Lipid mixing as monitored by fluorescence spectroscopy between LPK-modified and LPE-modified liposomes (●), LPK-modified and plain liposomes (×), LPE-modified and plain liposomes (+), LPK-modified liposomes (○), LPE-modified liposomes (□), and plain liposomes (■). Fusogen proportion 1 mol%, [lipids] = 0.25 mM.

Models for membrane fusion can be divided into two classes: non-targeted, in which the fusion is caused by interactions between the fusogen and lipid bilayers, and targeted, i.e. in which the fusion is caused by molecular recognition between membrane-bound fusogens.[24] Many peptide fusogens, both synthetic and native (i.e. those involved with virus-cell membrane fusion), are non-targeted. They adopt an amphipathic helical conformation in the presence of lipid bilayers and penetrate into the membranes, destabilizing them and causing membrane fusion,[24] which is often accompanied by leakage.[11] When anchored in liposomes, the peptide components of LPE and LPK are in the α-helical conformation, with isoleucine and leucine residues making up a hydrophobic face running the length of the α-helix. However, when either LPK- or LPE-modified liposomes are added to non-modified liposomes there is no significant lipid mixing observed by fluorescence spectroscopy (Figure 2). These results demonstrate that the penetration of E or K peptides into the membrane of an opposing liposome does not cause membrane fusion, i.e. the fusion mechanism is not 'virus-like', but rather is 'SNARE-like', with membrane fusion triggered by molecular recognition between fusogens. As with SNARE-mediated fusion, this molecular recognition is specific to the hetero coiled-coil complex formation, with no significant lipid mixing occurring between LPK-modified liposomes or between LPE-modified liposomes due to homocoiling (Figure 2). The possibility of the electrostatically neutral E/K peptide complex inserting into the bilayer was investigated with tryptophan fluorescence, which is highly sensitive to the polarity of its local environment (see

Appendix). For liposomes decorated with tryptophan-labeled LPK the tryptophan fluorescence indicates that the C-terminus of LPK is in close proximity to the liposome membrane. Upon mixing LPK- and LPE-modified liposomes the tryptophan emission maximum becomes typical of a completely water-exposed environment.[25] These results indicate that after the molecular recognition between E and K the peptide complex remains outside the liposomes rather than penetrating into the liposome membrane.

## 2. Effect of fusogen concentration on liposome fusion

The specific molecular recognition between the peptides E and K can rapidly lead to complete membrane fusion of all liposomes (Figure 2). This efficiency is important for potential applications. To evaluate the effect of fusogen concentration on the fusion process, the lipid mixing assay was conducted with samples containing a fixed concentration of total lipids (1 mM), but with decreasing proportions of the lipopeptide fusogens (Figure 3A).[26] Incorporating as little as 0.5 mol% lipopeptide results in all of the initial liposomes going through at least one round of fusion (which is the limit of lipid mixing that can be monitored with this assay) within 30 minutes. Upon the incorporation of lower proportions of fusogen the rate of lipid mixing decreases, with the lower limit under these conditions being ~0.05 mol% lipopeptides, corresponding to an average of only 40 lipopeptides decorating the outer surface of a 120 nm liposome. In comparison, synaptic vesicles are ~40 nm in diameter and are decorated with ~70 copies of the SNARE proteins that dock to the target area of the neuronal membrane.[2]

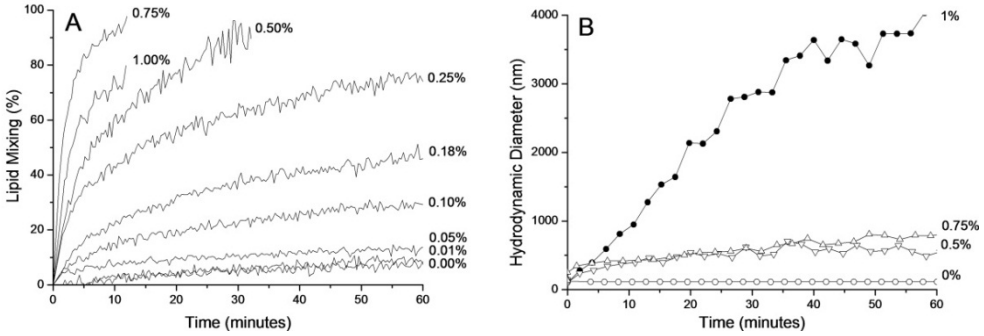



**Figure. 3**. A) Total lipid mixing recorded by fluorescence spectroscopy for liposomes modified with between 0 and 1 mol% LPE and LPK. B) Average particle sizes determined by DLS for liposomes modified with between 0 and 1 mol% LPE and LPK. [Lipids] = 1 mM.

The general trend of increasing membrane fusion rates with increasing proportions of fusogens arises because two liposomes that diffuse into close proximity are more likely to be displaying complementary peptides in the correct orientation for binding in the approaching area, and hence are more likely to dock and undergo fusion. However, the most rapid lipid mixing occurs for liposomes with a surface modification of 0.75 mol% lipopeptides. Circular dichroism (CD) data demonstrates that when 0.75 mol% LPE or LPK are incorporated into the liposome membranes the peptides E and K have typical α-helical conformations, with minima at 208 and 222 nm and an ellipticity ratio <<1 (Figure 4A), implying that the lipopeptides are molecularly dispersed across the surface.[27, 28] Upon mixing, E and K bind together forming coiled coils (ellipticity ratio >1, Figure 4A), which dock liposomes together and lead to their fusion. However, when the amount of fusogen decorating the liposomes is increased to 1 mol%, peptide K is more constrained on the surface of the decorated liposomes, with CD spectra indicative of LPK homo-coiled coils (Figure 4B). No change in liposome size, lipid mixing, or peptide structure is detected with time for LPK-modified liposomes, thus peptide K forms homo-coiled coils on the surface of individual liposomes rather than between liposomes. Upon mixing LPE and LPK liposomes with 1 mol% surface modification E/K coiled coils still form (Figure 4B), docking liposomes together, however the rate of lipid mixing is decreased in comparison to 0.75 mol% fusogen due to the reduced accessibility of peptide K and the reduced energy gain of E/K binding.

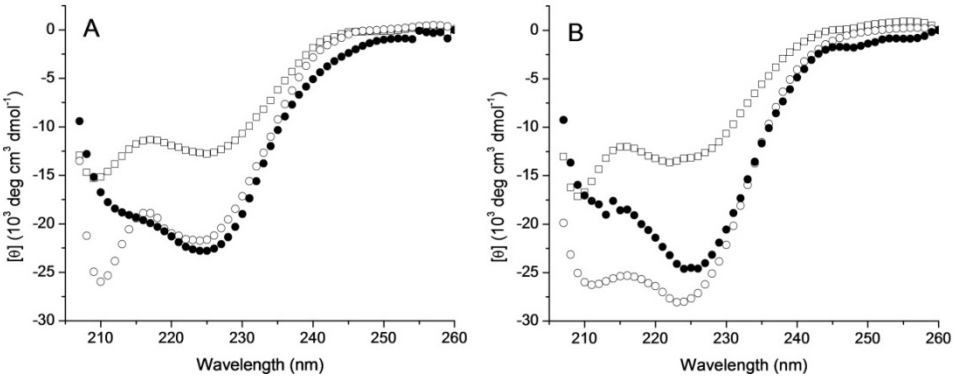



**Figure 4.** Circular dichroism data of A) liposomes modified with 0.75 mol% LPE (□), LPK (○) and an equimolar mixture thereof (●). B) liposomes modified with 1 mol% LPE (□), LPK (○) and an equimolar mixture thereof (●). [Lipids] = 0.5 mM.

## 2.1 Rapid Fusion of multiple liposomes

At lipopeptide proportions above ~0.5 mol%, when complete lipid mixing is rapidly achieved (Figure 3A), the particle sizes increased upon mixing LPE- and LPK-modified liposomes, as monitored by DLS (Figure 3B) and the liposomes sedimented to the bottom of the flask within hours (note that this size increase is the reason for the truncated fluorescence data in Figure 3A).[29] While the initial liposomes have an average diameter of ~120 nm, upon mixing the LPE- and LPK-modified liposomes particles with a larger hydrodynamic diameter are formed which increase in diameter and intensity with time, while the distribution containing the original LPE and LPK modified liposomes decreases in intensity.

Optical microscopy revealed that the macroscopic particles detected by DLS are clusters of docked and fused liposomes. An example of the morphological development is shown in Figure 5 for liposomes bearing 0.75 mol% fusogens. Initially the LPK- and LPE-modified liposomes are ~120 nm in diameter and are not visible by optical microscopy (Figure 5A). One hour after mixing the liposomes, particles ~1 µm in diameter are visible (Figure 5B), consistent with the sizes measured by DLS. Five hours after mixing, the liposomes are predominantly docked into large clusters and giant liposomes 5 µm in diameter can be discerned within the clusters (Figure 5C). These clusters of giant liposomes sediment out of solution. The liposomes continue to increase in size, with images taken three days after mixing showing the membranes of giant liposomes up to 10 µm in diameter (Figure 5D). These giant liposomes sometimes displayed elongated morphologies (Figure 5E), which may be a mechanism to conserve membrane area and internal volume.

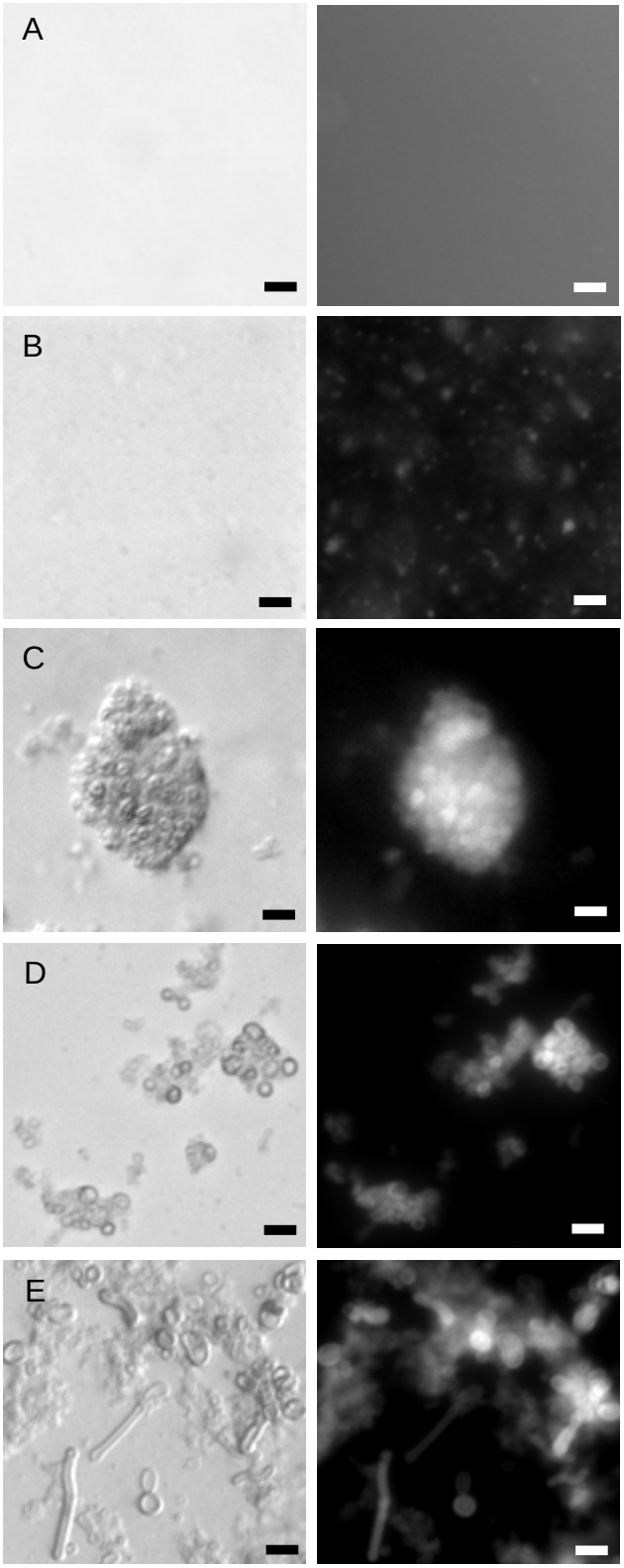

A
B
C
D
E

**Figure 5.** Optical and fluorescent microscopy images (left and right columns respectively) showing docking and fusion of liposomes modified with 0.75 mol% LPE and LPK. A) 5 minutes after mixing. B) 1 hour after mixing. C) 5 hours after mixing. D, E) 3 days after mixing. [Lipids] = 1mM lipids. Scale bars 10 μm.

For conditions under which liposome clustering occurs, the complete lipid mixing coupled with the large liposome size increases have molecular scale implications. The observation of liposome clusters means that a single liposome can be docked to multiple other liposomes, with the inference that not all of the lipopeptides that decorate a liposome are involved in a single fusion event. The accumulation of docked liposomes rather than their immediate transition through the fusion process is in line with expectations because each stage of liposome fusion – docking, hemifusion, complete fusion – requires further energy input.[30] It is presumed that after docking the energy input required to complete the fusion process arises from the more gradual attainment of a critical number and/or arrangement of lipopeptides. This finding is in line with the enhancement of membrane fusion through the physical arrangement of multiple complexes as observed for SNARE-protein mediated membrane fusion by experimental and computational studies.[29-335]

The increase in liposome diameter over two orders of magnitude, from ~100 nm to ~10 μm (meaning that over $5x10^5$ of the original liposomes have fused to grow into a single giant liposome), demonstrates that the liposomes can go through many rounds of fusion. This means that some fusogens on liposomes that have already fused are able to participate in subsequent fusion events. It is presumed that unless physically or kinetically impeded, the lipopeptides are able to laterally diffuse through the lipid leaflet in which they are anchored, such that following a fusion event the peptides E and K that were not involved with the fusion event would diffuse into close proximity with one another and form a coiled coil on the surface of the liposome. Due to the low dissociation constant for an unmodified and isolated E/K coiled coil complex in solution (~ $3.3x10^{-8}$ M at 25 °C, see Appendix), lipopeptides that have formed a complex on the surface of a single liposome are presumed to be effectively blocked from further fusion events. It is therefore assumed that some of the lipopeptides are physically prevented from forming a complex on the surface of a pre-fused liposome, most likely because the rate of docking is higher than the rate of lateral diffusion of the lipopeptides. Further study is required to elucidate how lipopeptides on pre-fused liposomes are available for subsequent fusion rounds.

## 2.2 Fusion of two liposomes

When the total lipid concentration is maintained at 1 mM but the liposomes are decorated with lower proportions of lipopeptide, the fusion process results in distinctly different physical changes to the liposome populations. For mixtures of liposomes modified with 0.25 mol% of each lipopeptide or less, the lipid mixing (e.g. Figure 6A) is accompanied by an initial small size increase of the particles as detected by DLS followed by a gradual decrease (e.g. Figure 6B). After mixing LPE- and LPK-modified liposomes the hydrodynamic diameter of the particles increases close to that expected if two liposomes have docked. The hydrodynamic diameter then decreases with time, reaching a plateau at the size corresponding to the fusion of two liposomes in which the volume is conserved rather than the outer surface area. These results are in accordance with our previous experiments in which no leakage was detected during fusion, and cryo-TEM images, in which internalized bilayers were observed.[21]

The two liposome fusion regimes, i.e., the fusion of tens of thousands of liposomes and the fusion of two liposomes, have the same total lipid concentration, therefore the liposome collision rate is the same. The dramatically different outcome arises because with fewer fusogens decorating the surface of the liposomes there is a lower probability that colliding liposomes are properly oriented to allow docking. The observation that with 0.25 mol% fusogen or less present an average of two liposomes fuse is presumably because after the first fusion event the lipopeptides that were not involved in the fusion event diffuse on the surface and form non-active E/K complexes before there is another opportunity for docking.

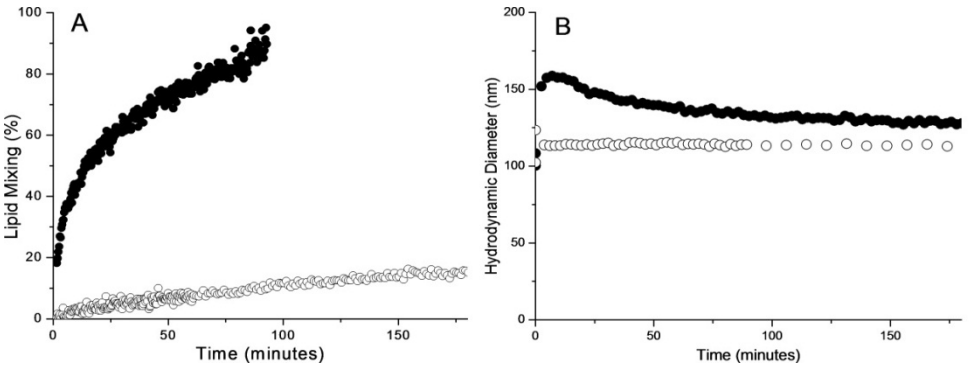



**Figure 6.** A) Total lipid mixing recorded by fluorescence spectroscopy for liposomes modified with 0.25 mol% (●) and 0 mol% (○) LPE and LPK. B) Average particle sizes determined by DLS for liposomes modified with 0.25 mol% (●) and 0 mol% (○) LPE and LPK. [Lipids] = 1 mM.

## 3. Effect of lipid concentration on membrane fusion

The fusion process is less sensitive to dilution of the liposomes, i.e. changing the liposome collision rate, than it is to the lipopeptide concentration, i.e. changing the probability that peptides are at the correct position and orientation for docking. Fluorescence time series were recorded to follow the total lipid mixing of mixtures containing 1 mol% fusogen and different concentrations of lipid (Figure 7A). Complete lipid mixing is observed within 30 minutes for lipid concentrations from 1 mM to 0.25 mM. In more dilute samples the rate of lipid mixing decreases, but remains significant at 0.01mM lipids. The lipid mixing is more efficient if there are more fusogens on fewer liposomes rather than fewer fusogens on more liposomes (Figure 7A vs. Figure 3A). DLS time series (Figure 7B) showed large particle size increases for lipid concentrations down to 0.1mM, while at 0.025 mM lipids (i.e. 0.25 μM lipopeptide) and lower there is no sustained size increase with time, rather a slight increase is followed by a decrease to the hydrodynamic diameter expected for volume-conserved two-liposome fusion. For mixtures with 1 mM lipids and different lipopeptides concentrations this transition between the multiple liposome fusion regime and the two liposome fusion regime occurred at 0.25 mol% fusogen (i.e. 2.5 μM lipopeptide). This means that the multiple liposome fusion regime is maintained with ~10x less fusogen if the liposomes are diluted in buffer rather than the number of fusogens modifying the surface of the liposomes is reduced. The docking efficiency is determined more by the number of fusogens (so that two approaching liposomes are more likely to be in the correct orientation

for the complementary lipopeptides to form a coiled-coil complex bridging the liposomes), than by the number of liposomes (the time between liposomes diffusing into close proximity).

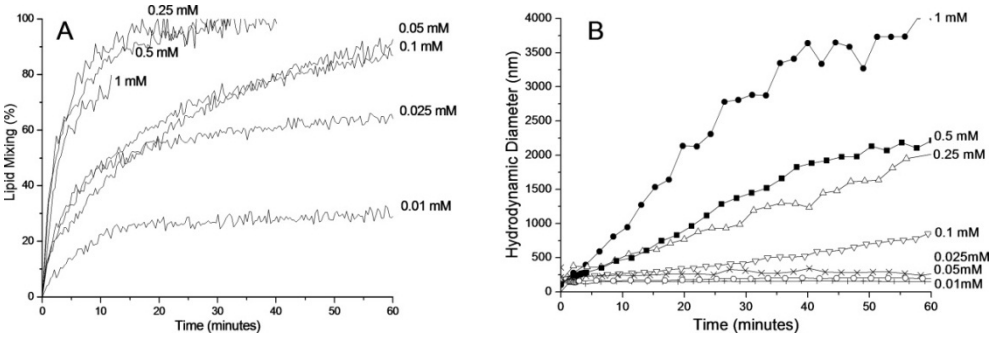



**Figure 7.** A) Total lipid mixing recorded by fluorescence spectroscopy for liposomes modified with 1 mol% LPE and LPK. Total [lipid] is varied between 0.01 and 1 mM. B) Average particle sizes determined by DLS for liposomes modified with 1 mol% LPE and LPK. Total [lipid] is varied between 0.01 and 1 mM.

## 4. Effect of positive curvature lipids on fusion process

In a previous contribution introducing this minimal model it was shown that the fusion mechanism proceeds through the stalk intermediate that is thought to be part of native fusion.[21] In the stalk intermediate the outer leaflets of two approaching liposomes have merged at the contact point, forming a 'stalk' that connects the liposomes. The stalk intermediate requires a negative curvature, and while lipids of positive curvature are able to form part of a bilayer they are physically unsuited to the structure of the stalk intermediate, therefore their incorporation into liposomes reduces lipid mixing. The role of the positive curvature lipid lysophosphatidylcholine (LPC) in preventing fusion was further investigated.

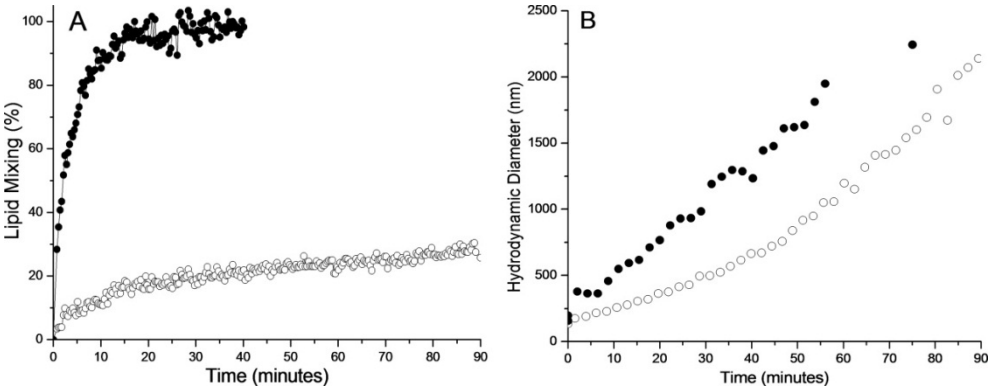



**Figure 8.** A) Total lipid mixing recorded by fluorescence spectroscopy for liposomes with the standard composition (●) and with 15 mol% LPC (○). B) Average particle sizes determined by DLS for liposomes with the standard composition (●) and with 15 mol% LPC (○). 1 mol% lipopeptides, [lipid] = 0.25 mM.

As expected, the inclusion of 15 mol% LPC to liposomes dramatically decreases lipid mixing (Figure 8A). However, a large size increase was observed with DLS for liposomes with and without LPC (Figure 8B). With optical microscopy the cause of this size increase is apparent (Figure 9). It can be seen that for liposomes of the standard composition there are many giant liposomes ~5 μm in diameter one day after mixing, with ~10 giant liposomes docked together in clusters. In contrast, for liposomes containing 15 mol% LPC there are hundreds of liposomes of less than 1 μm docked into clusters. These results show that LPC does not prevent the first step of fusion, docking, which is insensitive to the liposome composition, but it does inhibit the following two stages, hemifusion and full fusion.

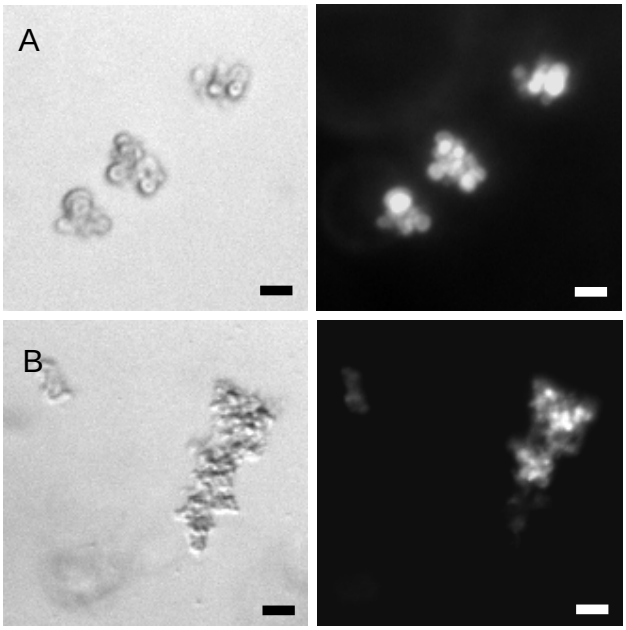



**Figure 9.** Optical and fluorescent microscopy images (left and right columns respectively) showing docking and fusion of liposomes one day after mixing. A) Standard liposome composition, i.e. DOPE/DOPC/CH 50/25/25 mol%. B) With 15 mol% LPC, i.e. DOPC/DOPE/CH/LPC 43/21/21/15 mol%. 1 mol% lipopeptide, [lipid] = 0.25 mM. Scale bars 10 μm.

## Conclusions

The functional determinants of a reduced SNARE model for membrane fusion were investigated. Complementary lipopeptides are incorporated into liposomes and lead to their docking, then via the stalk intermediate to complete membrane fusion. As with SNARE protein mediated fusion, molecular recognition occurs through coiled-coil binding, without fusion caused by viral-like aspecific peptide-lipid interactions. The coiled-coil formation can lead to the rapid membrane mixing of all initial liposomes, and the effects of lipopeptide and lipid concentrations on this process were studied. The optimal surface modification for rapid lipid mixing occurs when 0.75 mol% lipopeptide is incorporated into the liposome membrane, while complete lipid mixing still occurs with lower proportions of fusogens than there are SNARE proteins in synaptic vesicles. DLS and optical microscopy revealed that there are two fusion regimes – the fusion of thousands of

liposomes through multiple fusion rounds into giant liposomes up to 10 μm in diameter, and the fusion of two liposomes. This mapping of the rate and route of liposome fusion under different conditions gives a detailed understanding of the capacity of the reduced SNARE model to fuse liposome membranes. This understanding paves the way for future applications of the minimal model such as controlled nanoreactor mixing and the directed delivery of drugs to cells.

# Experimental section

## Materials

Lipopeptides were synthesized and purified as described previously.[21] The amino acid sequence of peptide K was (KIAALKE)3-NH2, and that of peptide E was (EIAALEK)3-NH2. The molecular weights of LPK and LPE were 3703.7 $gmol^{-1}$ and 3706.6 $gmol^{-1}$ respectively. DOPE was purchased from Lipoid AG, and cholesterol was obtained from Fluka. DOPE-NBD and DOPE-LR were obtained from Avanti Polar Lipids. All other reagents and solvents were obtained at the highest purity available from Sigma-Aldrich or BioSolve Ltd. and used without further purification. Milli-Q water with a resistance of more than 18.2 MΩ/cm was provided by a Millipore Milli-Q filtering system with filtration through a 0.22 μm Millipak filter.

## Liposome Preparation

Liposomes were composed of DOPC/DOPE/CH (50:25:25 mol%). Fluorescently labeled liposomes also contained 0.5 mol% LR-DOPE and NBD-DOPE. Lipid stock solutions (1 mM) were prepared in chloroform. Lipopeptide stock solutions (10 μM) were prepared in 1:1 (v/v) chloroform:methanol. Liposomes were prepared by drying appropriate volumes of the lipid and lipopeptide stock solutions in a 20 mL bottle under reduced pressure, addition of TES buffer (*N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid sodium salt 10 mM, NaCl 100 mM, adjusted to pH 7.4) and sonication for ~5 minutes in a bath sonicator with the water bath at ~60°C.

## Experimental Methods

Differential interference contrast (DIC) optical micrographs were recorded with a Zeiss axiovert-200 inverted microscope equipped with a 63 x objective long-range working lens. The images were recorded with a black and white CCD camera (AxioCam MRm) connected to an image-recording and -processing system (Axiovision 4.4).

Experimental diffusion coefficients, D, were measured at 25 °C by dynamic light scattering using a Malvern Zetasizer Nano ZS ZEN3500 equipped with a peltier-controlled thermostatic cell holder. The laser wavelength was 633 nm and the scattering angle was 173˚. The Stokes-Einstein relationship $D = k_BT/3\pi\eta D_h$ was used to estimate the hydrodynamic radius, $D_h$. Here $k_B$ is the Boltzman constant, and η is the solvent viscosity.

The results are expressed as the hydrodynamic diameter with units of nm. For individual liposome batches the sample was allowed to equilibrate for 2 minutes. For DLS time series the solutions were mixed in the cuvette (1000 rpm for 30 seconds). Measurements were started immediately after mixing.

FRET-based lipid mixing experiments were conducted on a Tecan X fluorometer using a 96 well plate. The z-position was 12500 µm, and the gain was optimized according to the amount of fluorophore in the sample. Excitation and emission slits were set at 10 nm. The excitation wavelength was 460 nm, and NBD emission was monitored 535 nm. The temperature was set at 24 °C and the measured temperature was 28 – 29 °C**.** 100 µL of fluorescent and non-fluorescent liposomes were combined, and for consistent mixing the plate was shaken inside the fluorometer for 30 seconds (2mm linearly, 70 x per minute). Data was collected every 20 seconds for at least 1 hour. Using 0.5 mol% of each fluorophore in the fluorescent liposomes and mixing fluorescent and non-fluorescent liposomes in a 1:1 molar ratio the increase in NBD fluorescence is proportional to lipid mixing (see Appendix). The data was calibrated to show the percentage of liposomes that have undergone lipid mixing by LM (%) = $(I_t - I_0)/(I_{100}-I_0)$ x 100, where $I_0$ is the NBD intensity of 1:1 (v/v) fluorescent liposomes:TES, and $I_{100}$ is the NBD intensity of liposomes of the same concentration prepared using an equimolar mixture of fluorescent and non-fluorescent stock solutions. $I_0$ and $I_{100}$ were monitored with time as they are temperature sensitive. This assay only detects fusion between the original liposomes. e.g. if two pre-fused liposomes fuse the distance between the fluorophores does not change so the event is not detected.

Circular dichroism spectra were obtained using a Jasco J-815 spectropolarimeter equipped with a peltier-controlled thermostatic cell holder (Jasco PTC-423S). Spectra were recorded from 260 nm to 200 nm in a quartz cuvette with 5.0 mm pathlength at 25 °C. Data were collected at 1.0 nm intervals with a 1 nm bandwidth and 1 s readings. Each spectrum was the average of 5 scans. The spectra had a baseline of plain liposomes in TES buffer subtracted. The ellipticity is given as the mean residue molar ellipticity, [θ] ($10^3$ deg $cm^2$ $dmol^{-1}$), calculated from $[\theta] = (\theta_{obs} \times MRW)/(10 \times l \times c)$, where $\theta_{obs}$ is the observed ellipticity in millidegrees, MRW is the mean residue molecular weight (i.e. the molecular weight of the peptide divided by the number of amino acid residues), l is the path length of the cuvette in cm and c is the peptide concentration in mg $mL^{-1}$. The mean residue molecular weights are 107.8 g $mol^{-1}$ and 107.7 g $mol^{-1}$, and the peptide concentrations of 1

mol% in 0.5 mM lipid suspensions correspond to 11.3 mg $L^{-1}$ and 11.3 mg $L^{-1}$ for peptides LPE and LPK, respectively.

# Appendix

## Part 1. CD of acetylated peptides in TES

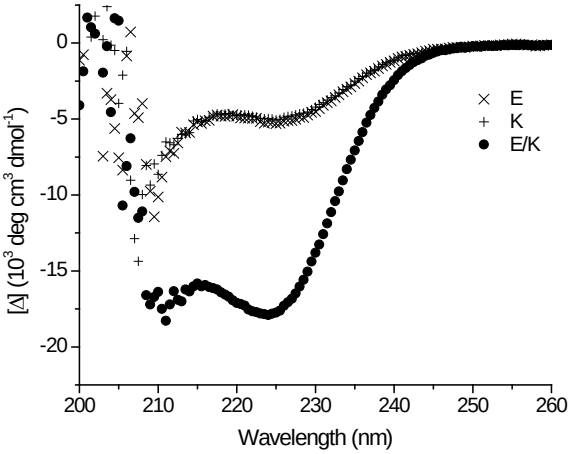



**Figure A1.** CD Spectrospcopic Data of E (Δ), K (+), and an equimolar mixture of E and K (●) in TES, pH 7.4, 25 °C. [Total Peptide] = 40 μM.

## Part 2. Temperature dependent CD data and analysis

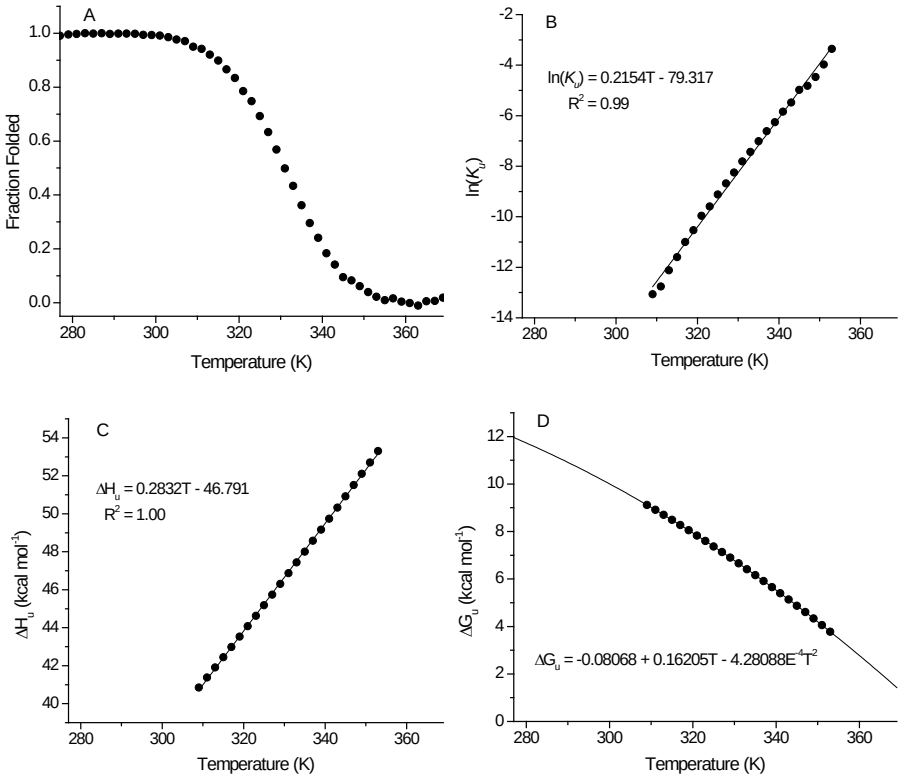



**Figure A2**. A) Thermal unfolding curve of unmodified E/K in TES buffer, pH 7.4, as followed by CD. [Total Peptide] = 40 μM. B) Van't Hoff plot of the thermal denaturation of E/K. C) Dependence of the enthalpy of unfolding of E/K on temperature. ΔHu values were obtained from the derivative of the van't Hoff plot. D) Free energy associated with the unfolding of E/K as a function of temperature. The least-squares fit gives a ΔGu value at 25 °C of 10.2 kcal $mol^{-1}$.

Temperature dependent CD spectra were obtained using an external temperature sensor immersed in the sample. The temperature was controlled with the internal sensor and measured with the external sensor. A 10 mm quartz cuvette was used, and the solutions were stirred at 900 rpm. Spectra were recorded from 260 nm to 200 nm, with data collected at 0.5 nm intervals with a 1 nm bandwidth and 1 s readings. Each spectrum was one scan. The temperature range was 4 °C to 96 °C with a temperature gradient of 2.0 °C/minute and a 60 s delay after reaching the set temperature. The spectrum of TES at 4 ° C (average of 5 scans) was subtracted from each spectrum.

The data was analyzed using a two-state unfolding model to determine the fraction folded using Eqn. (1),

$$F_f = (\theta - \theta_u) / (\theta_f - \theta_u) \quad (1)$$

Where θ is the observed ellipticity at 222 nm, θu is the ellipticity at 222 nm of the denatured state, as determined from the plateau of the ellipticity vs. temperature curve, and θf is the ellipticity at 222 nm of the folded state at that temperature as determined from a linear fit of the initial stages of the ellipticity vs. temperature curve.

The fraction unfolded, Fu, was calculated by Eqn. (2),

$$F_u = 1 - F_f \quad (2)$$

The dimer dissociation constant in the transition zone was calculated using Eqn. (3),

$$K_u = 2 P_t F_u^2 / F_f \quad (3)$$

where Pt is the total peptide concentration. By taking the derivative of the ln(Ku) vs. temperature and using this in the van't Hoff equation, Eqn. (4), the change in enthalpy associated with unfolding with temperature can be plotted:

$$d\ln(K_u) / dT = \Delta H_u / RT^2 \quad (4)$$

The gradient of this plot, ΔCp, is the difference in heat capacity between the folded and unfolded forms, and can be used in the Gibbs-Helmholtz equation adapted to monomer-dimer equilibrium, Eqn. (5), to obtain the Gibbs free energy of unfolding as a function of temperature:

$$\Delta G_u = \Delta H_m (1 - T / T_m) + \Delta C_p [T - T_m - T\ln(T/T_m)] - RT\ln[P_t] \quad (5)$$

where Tm and Hm are the temperature and enthalpy at the midpoint of the transition, as determined by the maximum of the derivative of the ellipticity vs. temperature graph.

## Part 3. Calibration of fluorescence based lipid mixing assay

The lipid mixing assay monitors NBD fluorescence, which depends on the distance between the FRET pair NBD and LR. One liposome population contains 0.5 mol% of DOPE-NBD and DOPE-LR (headgroup labeled), while the other population is non-fluorescent. As non-fluorescent and fluorescent liposomes fuse the distance between the fluorophores increases, resulting in decreased FRET efficiency and an increase in NBD fluorescence. Using 0.5 mol% of each fluorophore the increase in NBD fluorescence is proportional to lipid mixing (Figure A3).

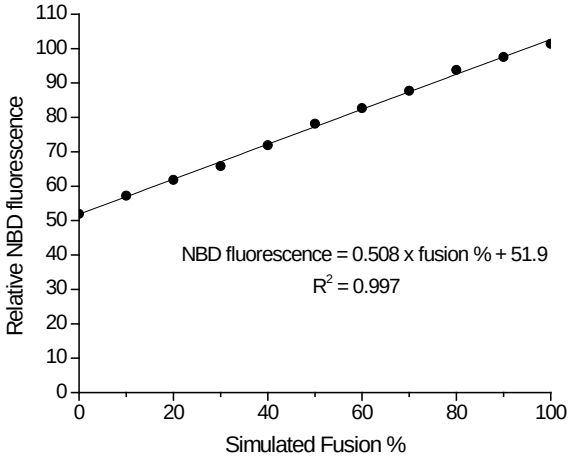



**Figure A3.** Samples which simulated 0-100% lipid mixing were prepared by combining the appropriate ratios of fluorescent and plain lipid stock solutions. The NBD fluorescence of these samples was monitored for 1 hr. The relative NBD fluorescence increases linearly with increasing fusion percent.

For the lipid mixing experiments each sample was calibrated by monitoring its fluorescence over time relative to an I0 sample (the fluorescent liposomes were diluted 1:1 with TES) and an I100 sample (liposomes prepared from an equimolar mixture of fluorescent and plain lipid stock solutions). The NBD fluorescence of mixed liposomes is not affected by the lipopeptide, thus the I100 samples did not contain lipopeptide.

Using this assay the lipid mixing is calibrated using an "I100" sample that simulates the distance between the fluorophores (and hence NBD intensity) if all of the original liposomes have fused. The most commonly used method for calibrating the fluorescence data is to add Triton-X to the sample after monitoring. This method results in a very large distance between the fluoropohores, much larger than is possible using 1:1 fluorescent and non-fluorescent liposomes. In that case the monitored fluorescence increase is not proportional to lipid mixing. Using Triton-X 100% lipid mixing corresponds to ~20-30% fluorescence increase.

## Part 4. Tryptophan fluorescence

The manner in which the coiled-coil formation between SNARE proteins leads to membrane distortion and fusion is unknown. A possible mechanism for both SNARE-mediated and LPK/LPE-mediated membrane fusion is molecular recognition followed by 'virus-like' membrane penetration and destabilization. This is of particular consideration for the minimal model as the E/K coiled-coil dimer is electrostatically neutral and hence may have a greater propensity for membrane insertion. In order to probe this possibility a tryptophan residue was included in the first generation LPK. Tryptophan fluorescence is highly sensitive to the polarity of its local environment, and to monitor environmental changes during the fusion process the LPK fusogen was synthesized containing tryptophan at the C-terminus (with the PEG spacer and lipid anchor at the N-terminus). Before fusion the C-terminus of LPK is in a relatively polar environment (the tryptophan emission maximum of LPK-decorated liposomes is at 342.5 nm, Figure A4).[25] Within one minute of mixing LPK- and LPE-modified liposomes the tryptophan emission maximum had red-shifted to 350 nm, indicating that upon E/K complex formation the C-terminus of the peptide moves from a relatively polar to a completely water-exposed environment.[25, 34] These results indicate that after the molecular recognition between E and K initiates membrane fusion the peptide complex is situated outside the liposomes, rather than buried in the liposome membrane. Thus, the membrane distortion required for membrane fusion does not appear to arise via penetration of the peptide complex into the bilayer. Having established that the E/K peptide complex is unlikely to cause liposome fusion by 'viral-like' membrane burial, the second generation LPK, as employed for all other experiments in this paper, does not contain the tryptophan residue.

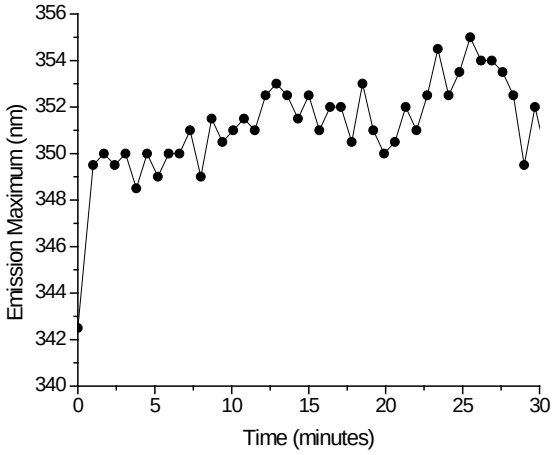



**Figure A4.** The wavelength of the tryptophan fluorescence maximum before (t = 0 minutes) and during lipopeptide induced fusion of liposomes. Lipid concentration 1mM, fusogen proportion 3%.

Tryptophan emission spectra were measured with a FS920 fluorometer from Edinburgh Instruments with a DTMS-300X excitation monochrometer and a peltier-controlled thermostatic cell. Spectra were obtained at 25 °C using a quartz cuvette with a 1 cm path length. The step size was 0.5 nm, with a sampling time of 0.5 s at each wavelength, and 1 scan was measured for each spectrum. The excitation and emission slits were 5 nm. Emission spectra were measured from 330 nm to 360 nm at a fixed excitation wavelength of 280 nm. Measurements were started immediately after mixing the solutions in the cuvette (30 seconds stirring at 1000 rpm).

## Part 5. Optical microscopy control experiment

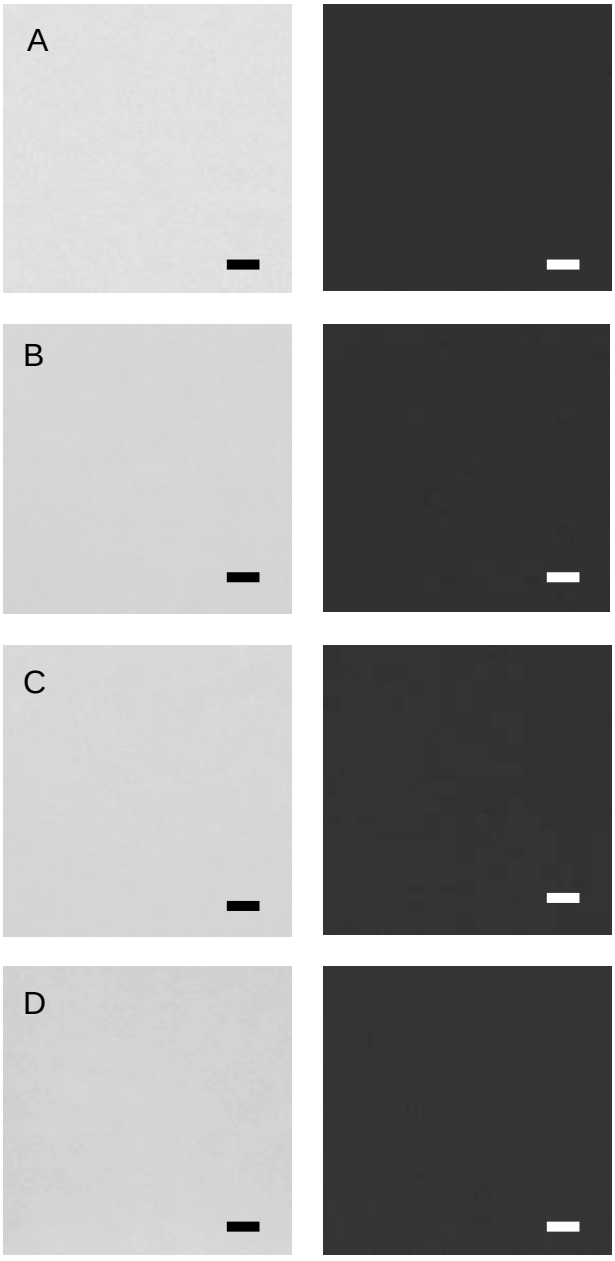



**Figure A5.** Optical and fluorescent microscopy images (left and right columns respectively) of liposomes without lipopdetide modification. A) 5 minutes after mixing plain and fluorescently labeled liposomes. B) 1 hour after mixing. C) 5 hours after mixing. D) 3 days after mixing. [Lipids] = 1mM lipids. Scale bars 10 μm.

## Part 6. Content mixing

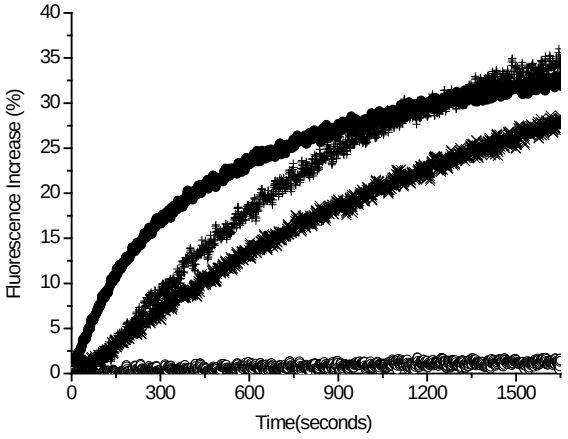



**Figure A6.** Content mixing recorded by fluorescence spectroscopy for liposomes modified with 1 mol% LPE and LPK. [lipid] 0.25 mM (●), 0.1 mM (+), 0.025 mM (×). Control with liposomes modified with 1 mol% LPE and 0 mol% LPK, [lipid] 0.1 mM (○).

To measure content mixing, LPE-modified liposomes containing sulforhodamine B at a self-quenching concentration were added to LPK-modified liposomes. Upon fusion the contents of the liposomes mix, resulting in an increased sulforhodamine B fluorescence signal due to the relief of self-quenching.3,4 The fluorescence signal is calibrated by adding Triton-X to lyse the liposomes and reach the maximum dilution. The sulforhodamine B dilution is much greater than the two-fold dilution expected if all LPE- and LPK-modified liposomes fuse, therefore the extent of the content mixing cannot be compared with the extent of the lipid mixing. In previous experiments (3 mol% fusogen, [lipid] 0.4 mM, PBS buffer), it was observed that content mixing proceeded without leakage.5

For content mixing experiments a dried film containing DOPC/DOPE/CH 50:25:25 mol% and 1 mol % LPE was hydrated and sonicated (5 min at 50 °C) in TES buffer solution (N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid sodium salt 10 mM, NaCl 100 mM, adjusted to pH 7.4) containing sulforhodamine B (20 mM). The final lipid concentration was 1 mM. To remove the non-encapsulated dye, the liposome suspension was subjected to Sephadex (G50, Superfine) using TES buffer as the eluent. The fraction containing liposomes was collected and diluted to the final lipid concentration. Sulforhodamine fluorescence was measured with a Perkin Elmer Luminescence

Spectrometer LS 50B at room temperature. The excitation and emission slits were 2.5 nm. The excitation wavelength was 520 nm and the emission wavelength was 580 nm. 800 µL of the LPE-decorated liposomes with encapsulated sulforhodamine B were added to a small volume disposable cuvette. The fluorescence signal of the sulforhodamine was detected and 800 µL of unmodified or LPK-modified liposomes (1:1 molar ratio with the LPE-modified liposomes) were added and the increase of sulforhodamine B fluorescence was detected due to a relief of self quenching. After 30 minutes 160 µL of 10% (v/v) solution of Triton X was added. To calculate the percentage of fluorescence increase the following equation was used:

$F\% = (F_{(t)} - F_{(0)}) / (F_{(max)} - F_{(0)}) \times 100$

where $F_{(t)}$ is the fluorescence at a certain time, $F_{(max)}$ is the fluorescence after lyses of the liposomes with Triton X and $F_{(0)}$ is the starting fluorescence after addition of the LPK-modified liposomes.

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