Peptide Amphiphiles and their use in Supramolecular Chemistry
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Citation
Versluis, F. (2013, December 9). *Peptide Amphiphiles and their use in Supramolecular Chemistry*. Retrieved from https://hdl.handle.net/1887/22801

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Title: Peptide amphiphiles and their use in supramolecular chemistry
Issue Date: 2013-12-09

Chapter 5

Coiled coil driven membrane fusion: zipper-like vs. non-zipper-like peptide orientation

Membrane fusion plays a central role in biological processes such as neurotransmission and exocytosis. An important class of proteins that induce membrane fusion are SNARE proteins. To induce membrane fusion, two SNARE proteins that are embedded in opposing membranes form a four-helix coiled-coil motif together with a third, cytoplasmic, SNARE protein. Coiled coil formation brings the two membranes into close proximity allowing fusion to occur. Importantly, structural investigations have demonstrated that native membrane fusion only occurs when the orientation of the coiled coil motif resembles that of a zipper. The zipper orientation arises when parallel coiled coil formation takes place between peptides that are anchored into opposing membranes at identical termini. Recently, a synthetic model for membrane fusion was designed, which is based on a set of lipidated coiled coil forming peptide pair which are denoted E/K. When incorporated into liposomal membranes, coiled coil formation between these lipidated peptides induces targeted and efficient membrane fusion of liposomes. The model system studied here here mimics SNARE driven membrane fusion, as it contains a coiled coil motif which has a zipper-like orientation. Here it was investigated whether the zipper-like orientation of the coiled coil motifs is a prerequisite for membrane fusion in our model system. Our strategy is based on conjugation of the transmembrane anchor to either the N- or the C-terminus of peptides E and K. Surprisingly, it was observed that efficient and targeted membrane fusion was induced even when the coiled coil motif did not form the zipper-like orientation. This demonstrates that for our model system, the zipper model for membrane fusion does not apply.

This work is published: F. Versluis, J. Dominguez, J. Voskuhl and A. Kros. Coiled Coil driven membrane fusion: zipper-like vs. non-zipper-like peptide orientation. *Faraday Discuss.,* 2013. DOI: 10.1039/C3FD00061C

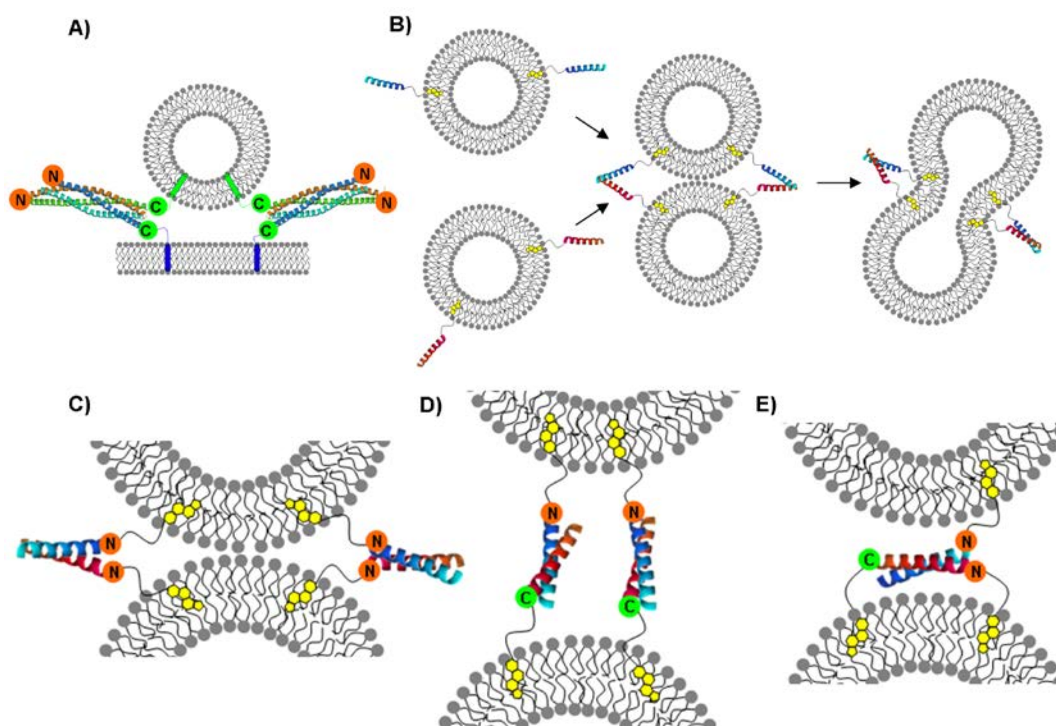
Introduction

Membrane fusion is a vital transport mechanism in all living systems. Trafficking usually involves generation of a vesicle from a precursor membrane, the transport of the vesicle to its destination and, last, the fusion of the vesicle with the target compartment.[1-3] A well conserved family of proteins denoted SNAREs induce membrane fusion and the bulk of experimental data on membrane fusion is based on these proteins. Although the exact mechanism of membrane fusion is not yet entirely clear, a widely recognized model has emerged that is supported by a steadily increasing body of evidence. This model consists of several different steps: 1) opposite membranes come into close contact, 2) local disruption of the membrane structure, 3) formation of a stalk intermediate and 4) pore formation.[4] In the first step, SNARE proteins that are located on opposite membranes come together to form a coiled coil motif, which forces them into close proximity. Importantly, structural investigations of purified neuronal SNARE complexes using deep-etch electron microscopy,[5] electron spin resonance[6] and x-ray crystallography[7, 8] have revealed that SNARE proteins typically bind in a parallel fashion. Binding starts at the N-termini of the proteins and subsequently "zippers up" in the direction of the C-termini. This exerts a force on the membranes towards each other which induces membrane fusion **(Scheme 1A)**.[9] Furthermore, it has been shown that anti-parallel binding of SNARE proteins does not lead to vesicle membrane fusion.[10] This finding indicates that upon coiled coil formation of the SNARE proteins, the transmembrane domains located at the C-termini of the coiled coil forming membrane proteins have to be in close proximity. Both the parallel arrangement and the identical location of the transmembrane domain are crucial elements for coiled coil formation in membrane fusion. All together these results can be used to construct a cumulative case for a model of vesicle-membrane fusion that is now commonly referred to as the zipper model, wherein directional folding of parallel SNAREs from the N to the C terminus drives membrane fusion **(Scheme 1A)**.[11]

Due to the complexity of the native fusion machinery, model systems for targeted membrane fusion have been designed in order to study its mechanisms and develop possible applications.[12] Fusogenic constructs that are able to induce targeted membrane fusion are typically based on recognition motifs such as DNA, peptides or

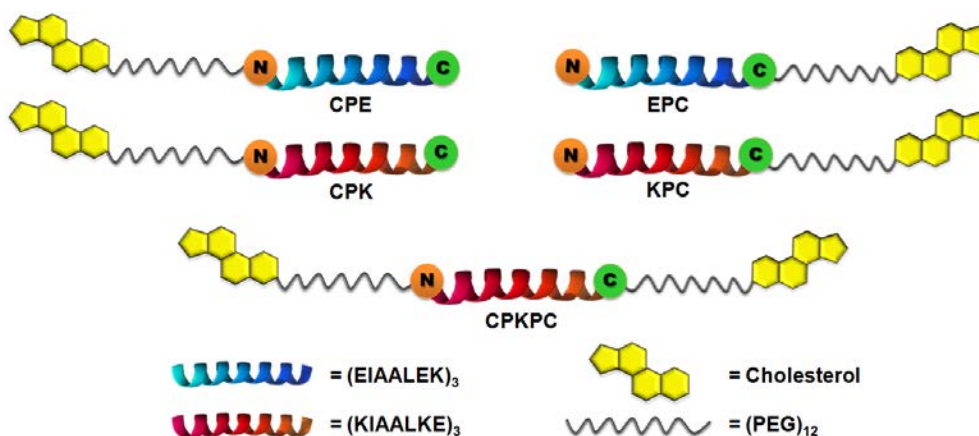
small molecules.[13-21] The model system for membrane fusion developed in our lab in recent years is based on parallel coiled coil formation between two complementary, membrane anchored peptides "E" $(EIAALEK)_3$ and "K" $(KIAALKE)_3$.[22-24] The structure of these fusogens is cholesterol-PEG_{12}-E and cholesterol-PEG_{12}-K (CPE and CPK). The cholesterol anchor ensures the confinement of the lipidated peptides to the membrane, whereas the PEG_{12} spacer facilitates coiled coil formation between E and K through the addition of flexibility to these constructs. When incorporated into the membranes of two separate batches of liposomes, parallel coiled coil formation between peptides E and K forces the liposomal membranes into close proximity, enabling full membrane fusion (i.e. lipid and content mixing) occurs **(Scheme 1B)**.[22, 24, 25] The investigation described here is an effort to better understand the mechanism through which fusion takes place in our model system. More precisely, it was examined whether the orientation of the coiled coil motif, as described in the zipper model, is a prerequisite for full membrane fusion in our model system. In this study the zipper-like orientation of the coiled coil motif was compared with the non-zipper-like motif **(Scheme 1C-E)**. In this regard two strategies can be envisaged: 1) the design of anti-parallel coiled coil forming peptides and 2) the design of coiled coil forming peptides in which the membrane anchor is located at opposite peptide termini. Both these strategies yield coiled coil motifs that do not force the membrane anchors into close proximity and are therefore expected not to induce membrane fusion as efficiently. The first approach was recently attempted in the lab of Diederichsen, who used fusogens which were also based on peptides E and K. At the C-terminus, peptide K contained the sequence GWGGGC, whereas the E peptide contained GGGGC at the C-terminus. The glycine residues acted as a spacer and the thiol group of the cysteine was used to couple the peptides to DOPE lipids which contained a maleimide group. They observed that reversing the amino acid sequence of one of the peptides (either E or K) yielded lower fusion rates.[26] Reversing the amino acid sequence of for example peptide K, was expected to give rise to anti-parallel coiled coil formation between reversed peptide K and E. The decreased fusion rates were explained through the hypothesis that the anti-parallel binding motif has a non-zipper-like orientation and therefore does not force the liposomal membranes into close proximity. However, a limitation of this study was that no data was shown to support the hypothesis that anti-parallel coiled coils were indeed formed between one reversed and one normal E and K peptide. Also non-peptide recognition motifs, such

as DNA like polymers, have been employed to investigate the influence of parallel vs. anti-parallel binding orientations. In another contribution of the Diederichsen group, the rate of fusion between liposomes that were forced into close contact through the formation of either parallel or anti-parallel peptide nucleic acid (PNA) duplexes, was found not to differ significantly.[27] However, as both binding orientations showed negligible content mixing the influence of PNA duplex orientation on full membrane fusion (i.e. lipid *and* content mixing) could not be tested effectively. The second strategy, which entails the conjugation of the lipid anchor to opposite locations of the recognition motif, was explored by Boxer et al.[15] They observed elevated levels of lipid and content mixing upon combining vesicles which carried complementary 5'- and 3'-lipidated DNA sequences, as compared to only 5'-lipidated DNA sequences. The zipper-like orientation of DNA duplexes thus induced fusion more efficiently than the non-zipper-like orientation. However, also here the levels of content mixing were found to be negligible. In conclusion, only a few studies have been performed regarding the orientation of the recognition motif in membrane fusion and mixed results were obtained. Furthermore, full fusion as indicated by significant content mixing was not reported in any of the mentioned studies.[28] As the lipidated peptides that constitute our model system are able to induce efficient content mixing,[22, 24, 29, 30] the aim was to investigate the influence of coiled coil orientation on full membrane fusion. In this study, the membrane anchor was conjugated to alternate peptide termini (N or C) as an attractive strategy to study the importance of the zipper-like binding motifs on fusion rates. Here, the peptide pair E/K was used that is known to form parallel coiled coils.[23] However, the location of the membrane anchor determines the binding orientation with respect to the liposomal membranes **(Scheme 1C-E)**.



Scheme 1. *Schematic representation of A) SNARE induced membrane fusion, B) membrane fusion induced by coiled coil formation between lipidated peptides that mimic SNAREs,*[22, 24, 25] *C) the zipper-like coiled coil orientation, D) the non-zipper-like coiled coil orientation and E) the coiled orientation that arises upon coiled coil formation between a peptide that is anchored in the membrane by a single anchor with a peptide that is anchored in the membrane with two anchors. The peptide C-termini are indicated by* C, *whereas the N-termini are indicated by* N.

To study the influence of zipper-like binding in our model system, peptides E and K were conjugated to cholesterol-PEG_{12} at the N-terminus, yielding CPE and CPK, or at the C-terminus, yielding EPC and KPC. When peptide pairs were used which contained the cholesterol-PEG_{12} unit at identical termini (i.e. CPE + CPK and EPC + KPC), zipper-like binding ensued **(Scheme 1C)**. In contrast, when peptide pairs with the cholesterol-PEG_{12} segment on opposite termini were used (i.e. CPE + KPC and EPC + CPK) events following non-zipper-like binding of peptides could be studied **(Scheme 1D)**. Furthermore, peptide K was conjugated with cholesterol-PEG_{12} anchors at both N- and C-termini, to study the influence of a double membrane anchor on membrane fusion (**Scheme 1E**).



Scheme 2. *Overview of the molecules used in this study. Combinations of peptide pairs which are anchored into liposomal membranes at identical termini (i.e. CPE + CPK and EPC + KPC) generate zipper-like coiled coil orientations with respect to the liposomal membranes. Combinations of peptide pairs which are anchored into liposomal membranes at opposite termini (i.e. CPE + KPC and EPC + CPK) form coiled coil motifs with a non-zipper-like orientation. Furthermore, a K peptide which was anchored into the liposomal membrane via two cholesterol anchors was tested against both E lipidated peptides (i.e. CPE and EPC).*

Results and discussion

First the pre-fusion state of the separate batches of E and K decorated liposomes was studied with the aid of circular dichroism (CD) spectroscopy. This allowed us to determine whether the position of cholesterol conjugation affected the secondary structure of the peptides E and K **(Figure 1A and B)**. As is evident from the graphs presented in **Figure 1**, all lipidated peptides display significant α-helical character at the liposomal surface. Furthermore, CD data reveal that the conjugation of the anchor to either N- or C-termini of peptides E or K does not significantly alter the secondary structure of the peptides. Finally, even the conjugation of membrane anchors to both N- and C-termini of peptide K did not alter the structure that the K peptide adopts. These findings are important as the aim is to study whether the zipper-like binding of coiled coil forming peptides leads to more efficient membrane fusion then non-zipper-like binding. As no notable differences in the initial secondary structures of the various lipidated peptides was observed, the fusion efficiencies observed in lipid and content mixing experiments can be solely attributed to the orientation of the coiled coil binding motif.

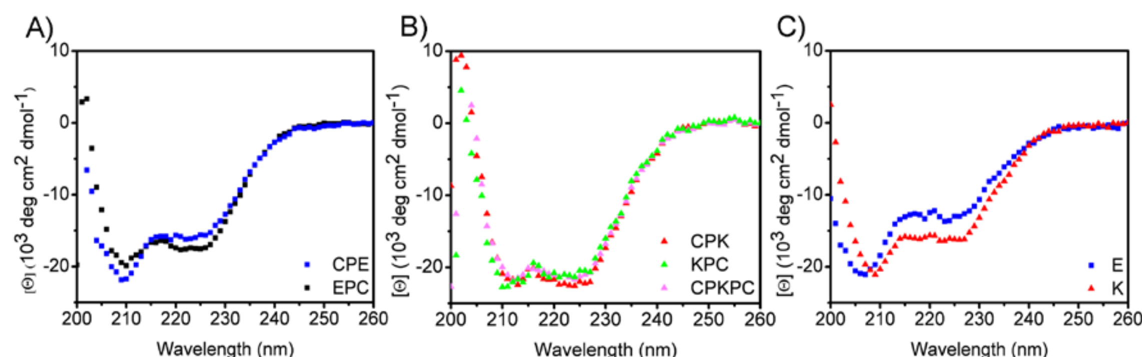


Figure 1. *CD spectra of lipidated E (left) and K (middle) peptides at the surface of liposomes and acetylated peptides (right). For A) and B) the total lipid concentrations were 0.25 mM with 3 mol% of lipidated peptide in PBS. For C, the peptide concentration was 7.5 µM in PBS.*

Next, a comparison of the CD data of the membrane anchored lipidated peptides to acetylated peptides **(Figure 1C)** revealed a number of interesting differences. First, the lipidated peptides adopt a more helical structure than the acetylated counterparts **(Table 1)**. This is in line with the well known observation that attaching peptides to a scaffold leads to peptide structures that are more ordered.[31, 32] Interestingly, the ellipticity ratio of the minima around 222/208 nm changes dramatically upon lipid conjugation and membrane anchoring of peptides E and K. The lipidated K peptides show values of ~1, which is an indication of interacting helices. It is possible that these ellipticity ratios reflect a process of aggregating K peptides. Lipid conjugation of peptide E leads to larger ellipticity ratios, albeit to a lesser extend. It can be hypothesized that peptide aggregation in the prefusion state aids membrane fusion, as it is very likely that multiple coiled coils have to be formed between complementary peptides E and K to induce fusion, as also multiple SNAREs need to bind to spark membrane fusion.[33-35] Aggregation of the lipidated peptides would result in a high local concentration of peptides, which might be necessary to initiate the local formation of multiple coiled coils, resulting in sufficient disturbance of bilayer lipid packing which is most likely needed to induce fusion.

Table 1. *Helical content and ellipticity ratios of the lipidated peptides at the surface of liposomes, compared to the acetylated peptides. The helical content and ellipticity ratios were calculated*[36] *using CD data (0.25 mM total lipid concentration and 3 mol% peptide).*

Compound	Helicity (%)	Ellipticity ratio ~222/208 nm
Ac-E	38	0,61
CPE	45	0,72
EPC	51	0,83
Ac-K	47	0,79
CPK	66	1,05
KPC	62	1,01
CPKPC	63	1,06

Upon combining separate batches of E and K decorated liposomes, coiled coil formation of peptides E and K at the surface of these liposomes forces the liposomes into close contact. This will result in an increase of the average particle size, which was evaluated by measuring the optical density of the liposomal dispersions at a wavelength of 400 nm (**Figure 2**). Interestingly, a similar rate of optical density increase was observed for samples where peptides bound in a zipper-like as well as in a non-zipper-like manner. This indicates that coiled coil formation is taking place in both binding orientations, causing similar increases in optical density. Furthermore, when the K peptide was embedded into liposomal membranes via two cholesterol anchors, addition of E decorated liposomes resulted in less aggregation. Most likely, the increased steric hindrance caused by the additional membrane anchor reduces the flexibility of the K peptide and thereby its ability to reorient in the appropriate manner to form a coiled coil complex with peptide E. To investigate this further, CD spectra of mixtures of E and K decorated liposomes were recorded **(Appendix, Figure S1)**. Efficient coiled coil formation was observed for all the combinations of E and K decorated liposomes, as the ratio of the ellipticity minima at 222/208 nm was >1 for all combinations. These data reveal the formation of highly stable coiled coil complexes, which is likely due to aggregation of coiled coils. However, these CD measurements do not necessarily indicate that coiled coil formation between E and K peptides at opposing liposomes occurred in comparable rates. The process of recognition and complex formation between complementary peptides resulting in

fusion tends to be fast. Therefore, the CD data is a reflection of an ensemble of liposomes that are in pre and postfusion states.

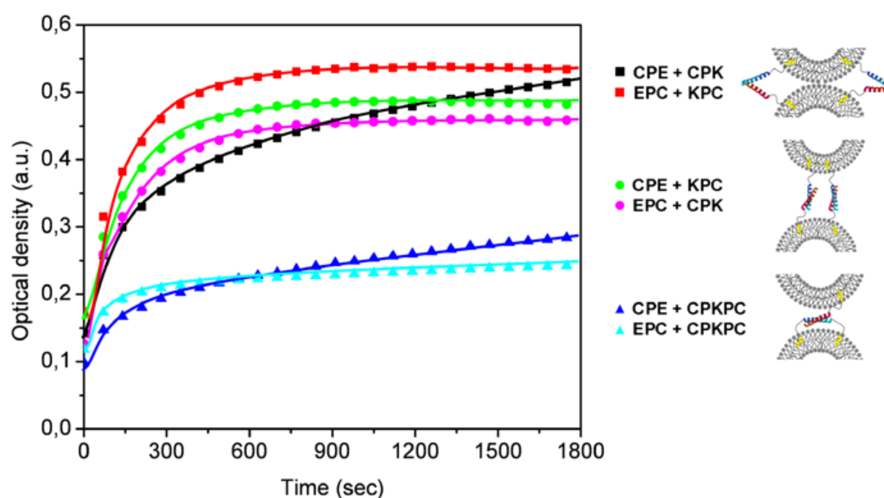


Figure 2. *Optical density measurements at λ= 400 nm. Samples consisted of mixtures of 0.25 mM E decorated (1 mol%) liposomes with 0.25 mM K decorated (1 mol%) liposomes. The medium was PBS.*

Next, the fusion events were characterized by the extend of lipid mixing that was caused by zipper-like and non-zipper-like coiled coil formation. The merger of the lipid membranes is the first step in the fusion process and typically this is assessed by using a standard FRET assay.[12, 37] A FRET pair (DOPE-NBD (donor) and DOPE-LR (acceptor)) was incorporated in the E decorated liposomes, whereas the K decorated liposomes were non-fluorescent. Prior to mixing E and K decorated liposomes, excitation of the donor leads to efficient energy transfer from the donor to the acceptor due to their close proximity. As a result of lipid mixing the average distance between the two probes increases, resulting in an increase of NBD emission. Surprisingly, the zipper-like and non-zipper-like coiled coil configurations were both able to induce efficient membrane fusion in similar rates **(Figure 3)**. This suggests that the orientation of the coiled coil motif does not influence the rates with which lipid mixing occurs. Based on these results it seems likely that the mechanism through which coiled coil formation between E and K is not dependent on the zipper-like binding orientation. Control experiments were performed in which one of the peptides was omitted. In these experiments, plain liposomes were added to E or K decorated fluorescent liposomes. The results show that the fluorescence increase for E

liposomes (i.e. CPE and EPC) is negligible **(Appendix, Figure S2)**, whereas the K liposomes (i.e. CPK, KPC and CPKPC) show fluorescence increase of only ~10%. These results demonstrate that the lipid mixing observed upon mixing E and K decorated liposomes is targeted and that both peptides have to be present at opposing membranes. Surprisingly, the double anchored peptide CPKPC showed lipid mixing results similar to the other single anchored peptides, even though the aggregation was found to be significantly lower. This phenomenon can be explained by taking two factors into account. First, liposome fusion does not result in a dramatic increase of the average particle diameter. Fusion of two liposomes with a radius of 50 nm will generate a particle with a radius of ~62 nm.[38] Therefore, an increase in optical density can only be caused by aggregation or multiple rounds of fusion. Second, it is possible that even though coiled coil formation is more sterically hindered, membrane fusion is more efficient once they have been formed due to the double cholesterol anchor.[13]

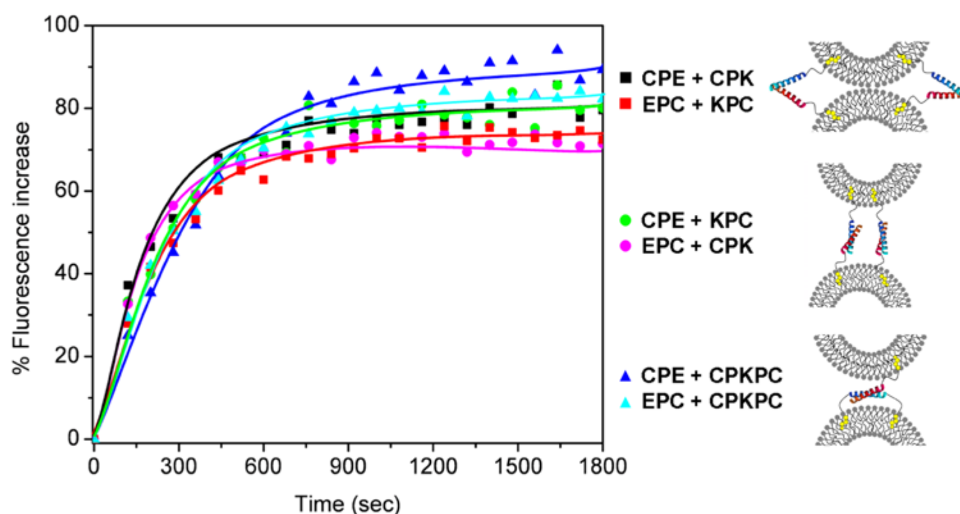


Figure 3. *Fluorescence traces showing lipid mixing between E and K decorated liposomes, as measured through an increase in NBD fluorescence. Total lipid concentrations were 0.1 mM with 1 mol% of lipidated peptide, in PBS.*

Although no significant difference in lipid mixing rates were observed between complementary coiled coil forming peptides that bind in a zipper-like manner versus a non-zipper-like manner, lipid mixing is only the first step of membrane fusion. Therefore, performing a content mixing assay is vital, also as fusion induced by synthetic model systems often does not proceed beyond hemifusion in which lipid

mixing is observed, but no content mixing.[15, 26, 27] In this assay, E decorated liposomes were loaded with sulphorhodamine B at a self-quenching concentration of 20 mM. Upon full membrane fusion of these E decorated liposomes with non-fluorescent K decorated liposomes, the dilution of sulphorhodamine leads to relief of the selfquenching and a concomitant increase in fluorescence intensity. As shown in the data presented in **Figure 4**, both zipper and non zipper orientations of the coiled coil motif induce efficient content mixing. Control experiments in which one of the peptides was omitted, revealed that content mixing only takes place when both E and K are present at opposite liposomal surfaces **(Appendix, Figure S3)**. This confirms that coiled coil formation between E and K is the driving force for full fusion. Consistent with the lipid mixing experiments this shows that the orientation of the coiled coil motif does not influence the fusion efficiency. It is therefore very likely that the mechanism through which membrane fusion occurs in our model system is independent of coiled coil orientation.

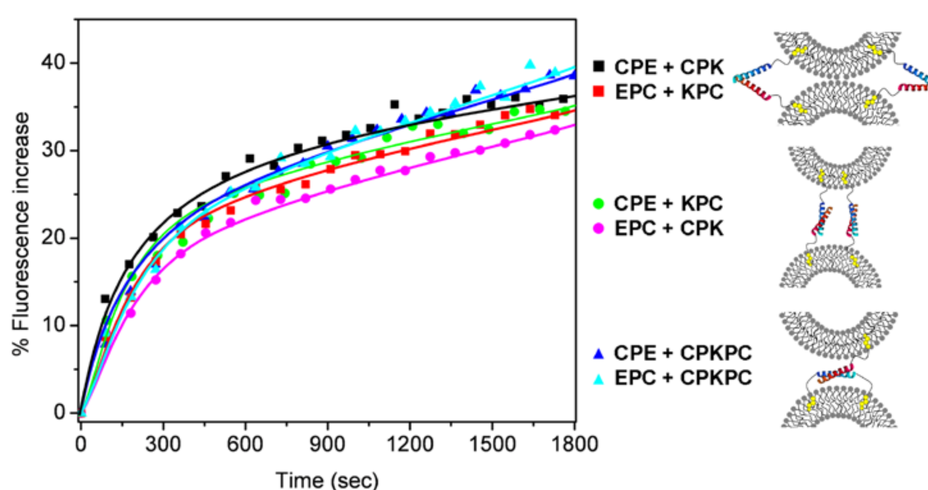


Figure 4. *Fluorescence graphs indicating content mixing between sulphorhodamine loaded (20 mM), peptide E decorated liposomes and non-fluorescent, K decorated liposomes. Total lipid concentrations were 0.1 mM with 1 mol% lipidated peptide in PBS.*

Although coiled coil orientation does not influence the rates with which membrane fusion occurs, the data obtained in this investigation does hint at a possible mechanistic model. In fusogenic liposomes in the prefusion state, higher ellipticity ratios for especially peptide K was observed, as compared to the acetylated counterpart. This is a good indication that peptide K forms homo interactions at the

surface of liposomes. This might lead to aggregation of the peptides at the surface of liposomes. This aggregation of helices results in locally high concentrations of peptide strands. As multiple coiled coils are necessary to spark fusion, as is also observed in SNARE induced membrane fusion, these locally elevated peptide concentrations might be vital for inducing efficient membrane fusion. Furthermore, postfusion measurements on coiled coil formation revealed highly stable coiled coil complexes, which is likely due to aggregation of coiled coil motifs. It is possible that the aggregation of coiled coils disturbs the membrane after which membrane fusion can proceed.

Conclusions

The influence of the orientation of the coiled coil motif with respect to the liposomal membranes on the rate of fusion was investigated. The strategy that was chosen to examine this phenomenon was to conjugate the membrane anchor to either the C- or the N-terminus of peptides E and K. When a set of complementary coiled coil forming peptides is anchored at identical termini this yields the zipper-like coiled coil orientation as displayed by native SNARE proteins. When the peptides are anchored on opposing peptide termini, a non-zipper-like coiled coil orientation arises. The data clearly demonstrates that both coiled coil orientations are able to induce targeted and efficient membrane fusion. Therefore, it is likely that the mechanism through which the lipidated peptides induce membrane fusion is not dependent on the specific orientation of the coiled coil motif with respect to the liposomal membranes. As our coiled coil forming peptides are much smaller than the native SNARE proteins, it is likely that the spatial dimensions of our coiled coil motif is not sufficiently large for its orientation to play a significant role. However, the data also hints at a mechanistic model of membrane fusion which is based on aggregation of helices in the prefusion state and aggregation of coiled coils which disturbs the membrane enabling fusion.

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28. As the various model systems use different assays for evaluating content mixing, caution should be taken in making these type of statements. References 20 and 21 used a content mixing assay based on relieve of self-

quenching of sulphorhodamine, reference 15 used an assay which is based on the formation of the fluorescent Tb3+-DPA complex. However, the comment is justified as we have used both assays and have observed a fluoresence increase of ~15% after 1 hour in the Tb3+-DPA assay, as compared to ~3% in ref 15 and an increase of ~20% after 10 minutes in the sulforhodamine assay as compared to ~1% in ref 21 and ~3% in ref 20.

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38. Assuming that the volume of a fused liposome is the sum of two liposomes before fusion, the radius of the fused liposome is r x (2^1/3), where r is the radius of a starting liposome.

Experimental Section

Peptide synthesis

Peptides E and K were synthesized at 100 μmol scale on a fully automated peptide synthesizer using standard solid phase peptide chemistry. In each coupling reaction, 4 eq. of amino acid, 4 eq. of HCTU and 8 eq. of DIPEA were dissolved in 2 mL of DMF and added to the resin for 45 minutes. For N-terminally anchored peptides, conjugation of PEG_{12} and cholesterol followed the synthesis of the amino acid sequence. 1.25 eq. of Fmoc-PEG_{12}-COOH, 3 eq. of HOBT and 3 eq. of DIC were dissolved in 2 mL NMP and added to the resin overnight. Fmoc deprotection was performed by the addition of 2 mL of a solution of pipiridine/NMP (40/60 v/v) to the resin for 2 min., 3 times. Next, 3 eq. of cholesterol hemisuccinate was dissolved in 2 mL NMP, together with 3 eq. of HOBT and 3 eq. of DIC and added to the resin for 3 hours. For the C-terminally anchored peptides, the synthesis was initiated by coupling an Fmoc protected lysine residue (4 eq. of amino acid, together with 4 eq. of HOBT and 4 eq. of DIC in 2 mL of NMP) directly to the resin. Boc deprotection of the lysine side chain amine was ensued by treating the resin two times with 4 mL of a 1:1 mixture of trifluoroacetic acid and dichloromethane. Cholesterol hemisuccinate was subsequently coupled to the side chain amine of the lysine residue as mentioned above. Next, Fmoc deprotection of the lysine was performed as mentioned earlier, after which Fmoc-PEG_{12}-COOH was coupled. Subsequently, deprotection of the Fmoc group of the PEG moiety was performed and the amino acid sequences that peptide E and K consist of were coupled to the pegylated resin using the solid phase peptide strategy. Cleavage of the crude products from the resin was performed by the addition of a 95/2.5/2.5 (v/v) mixture of TFA/H_2O/TIS for 1.5 hours. Precipitation in cold ether gave a white solid. Purification was performed by reversed phase HPLC. The purity of the products was confirmed with LCMS and was found always to be >95%.

Characterization

The conditions for DLS, UV, CD, lipid and content mixing experiments are detailed in chapter 4.

Appendix: Supplementary information

CD experiments on the combination of E and K decorated liposomes

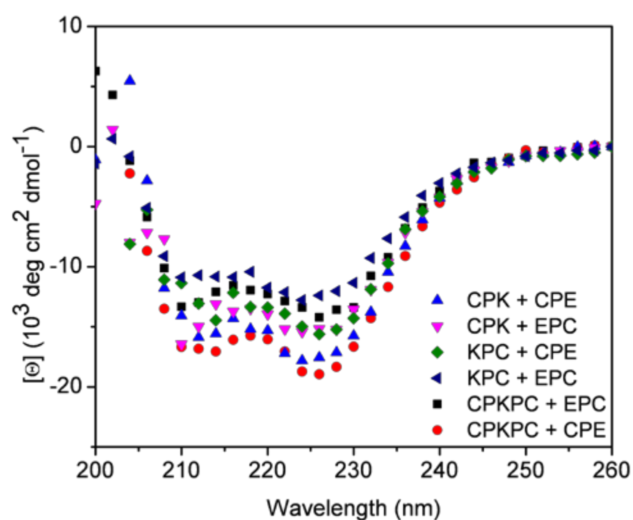

Figure S2. *CD data for a 1:1 mixture of E and K decorated liposomes. Total lipid concentration 0.25 mM and 3 mol% lipidated peptide, in PBS.*

Control experiments for lipid mixing

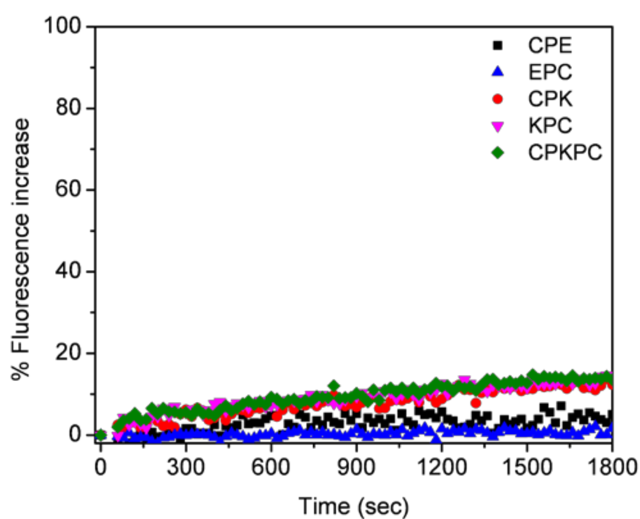

Figure S3. *Lipid mixing between E or K decorated liposomes and plain liposomes, as indicated by an increase in NBD fluorescence. Non-fluorescent liposomes (0.1 mM) were added to fluorescent K liposomes (0.1 mM, 1% peptide E or K).*

Control experiments for content mixing

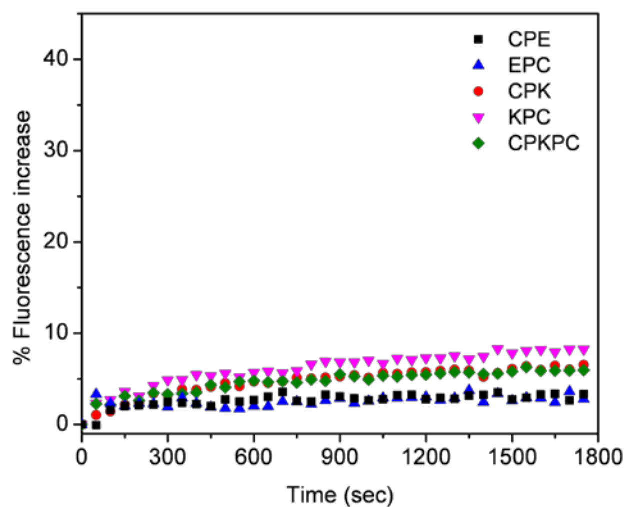

Figure S3. *Content mixing between E or K decorated liposomes and sulphorhodamine loaded (20 mM) plain liposomes, as indicated by an increase in sulphorhodamine emission. Non-fluorescent liposomes (0.1 mM) were added to fluorescent K liposomes (0.1 mM, 1% peptide E or K).*