Coiled-coil mediated liposomal fusion: Asymmetric behaving peptide fusogens

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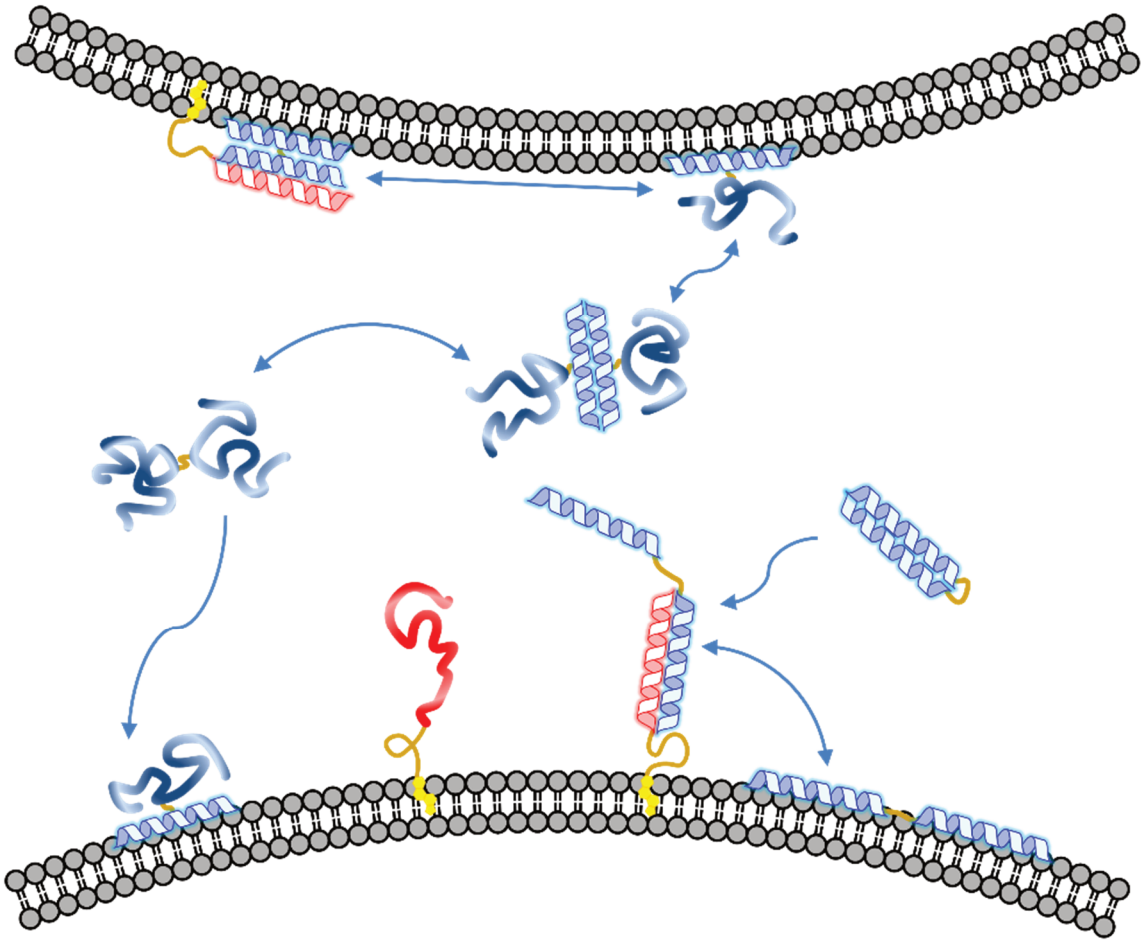
CHAPTER IV

DIMERS OF PEPTIDE K: (SUPER)SECONDARY STRUCTURES AND FUSOGENIC ACTIVITY



ABSTRACT

In Chapter III, we demonstrated the asymmetric roleplay of membrane anchored coiled-coil lipopeptides CPE and CPK in the process of liposome fusion. The asymmetric behavior of these lipopeptides originates from the different affinities of the E and K peptides to phospholipid membranes. To further study the membrane affinity of peptide K, covalently linked K dimers were synthesized with disulfide bridges in the second heptad of the peptide backbone (f-K*c*K) or between the N-terminal ends (n-K*c*K). The effect of the linker region was investigated with N-terminal K dimers by using a rigid *p*-fluorobenzene (n-K*b*K) or a flexible PEG_{600} (n-K*p*K) moiety as a spacer. We found that N-terminal modifications promoted intramolecular coiled-coil interactions accompanied with a Tm around 70 °C, but n-K*b*K showed both intra- and intermolecular coiled-coil interactions. Conversely, f-K*c*K showed only intermolecular interactions, and unfolded at 35 °C. Furthermore, f-K*c*K and n-K*c*K were found to trigger fusion of CPE functionalized liposomes, with N-terminally linked n-K*c*K being more efficient compared to Cys(14) linked f-K*c*K.

INTRODUCTION

In the previous chapters, structure and fusogenic activity relationships of the heterodimeric coiled-coil peptides E $(EIAALEK)_3$ and K $(KIAALKE)_3$ were investigated. The secondary structure of the peptides changed significant upon membrane immobilization, and the resulting (super)-secondary structures were found to be highly important for the fusogenic efficiency of these peptides. Membrane tethering of these peptides is achieved via lipidation, but the position of the membrane anchor at the peptide sequence was found to influence the peptide structure and the related fusogenic efficiency.[1-2] Besides the designed E-K heterodimeric coiled-coil formation, it has been demonstrated that peptide K interacts strongly with phospholipid membranes, while membrane tethered peptide E was mainly found as homomeric coiled-coils, thereby both enhancing the fusion efficiency.[3-4]

Recently, an intramolecular homomeric coiled-coil formation was found for polymers with two covalent peptide K strands.[5] This non-favorable coiled-coil interaction depends on the distance between the 2 peptide K strands and was stronger at a shorter distance, but only minor effort was put in the understanding of this forced homodimer formation. Furthermore, by connecting two or more peptides using short linkers, fairly complex assembled structures can be formed as a result of the well-defined folding pattern of each single peptide folding motif.[6-10] Peptides have also been conjugated to larger synthetic polymeric backbones, such as poly(ethylene glycol) (PEG),[11-12] in order to create a wider range of supramolecular hybrid materials and nanostructures, including peptide-polymer hybrid hydrogels,[13-15] fibrils,[16] and micelles.[17]

Therefore, conjugated homodimers of peptide K can be used to further probe the peptide behavior. A high local peptide concentration will influence the secondary structures and equilibria between various interactions of these peptide dimers. Secondly, the type and position of the linker could have an additional influence on the peptide structure by favoring or disabling intramolecular coiled-coil formation of the covalent peptide dimer. Also, the various peptide derivatives will exhibit differences in binding affinities and peptide-membrane interactions, which would allow for an in-depth study of peptide homodimer formation. Furthermore, strong K-K homodimer formation could

interfere with the formation of the E-K heterodimer, which can be used to influence equilibria between various peptide-peptide interactions.

Since the coiled-coil peptide pair E and K is highly fusogenic, it is envisaged that these K dimers could trigger fusion of liposomes bearing peptide E. Usually, fusion of liposomes is initiated via heterodimeric coiled-coil formation between liposomes functionalized with lipopeptide derivatives of E and K.[18] However, here two liposomes functionalized with lipopeptide E will be brought into close proximity and concomitant fusion via the sequential binding to one K dimer present in solution. The influence of the used K dimer derivative on the fusion efficiency will reveal the structure-activity relationships of these peptide fusogens. This approach will allow the study of peptide fusogens with both membrane-bound and solvated peptides.

DESIGN OF THE STUDY

A versatile peptide modification is the incorporation of a cysteine residue into the primary sequence, which is frequently used for intramolecular peptide stapling.[19] To control intramolecular peptide-peptide interactions, two different linking positions were introduced in the peptidic backbone; a N-terminal Cys functionalization, or a Cys incorporation at the **f** position in the second heptad, which yield peptides n-K and f-K respectively. Intramolecular homodimer formation is disabled by the latter modification via steric hindrance, while the N-terminal modification could enhance homodimer formation. To investigate the influence of the length and the nature of the linker region on homodimer formation, we introduced various linkers: a short disulfide bridge, a rigid *p*-fluorobenzene spacer,[20] and a PEG-based linker with an average mass of 600 Da (±13 repeating ethylene glycol units based on NMR measurements). (*Figure 17*)

Binding affinities of the different homomers are quantified via concentration- and temperature-dependent CD measurements, and analyzed using *FitDis!*.[21] The peptide secondary/tertiary structure was also evaluated in the presence of plain liposomes and CPE functionalized liposomes, to investigate whether K-dimers interact with lipid membranes in the absence or presence of membrane anchored CPE. Furthermore, lipid mixing assays were used to determine the fusion efficiency of f-K*c*K and n-K*c*K with CPE functionalized liposomes.

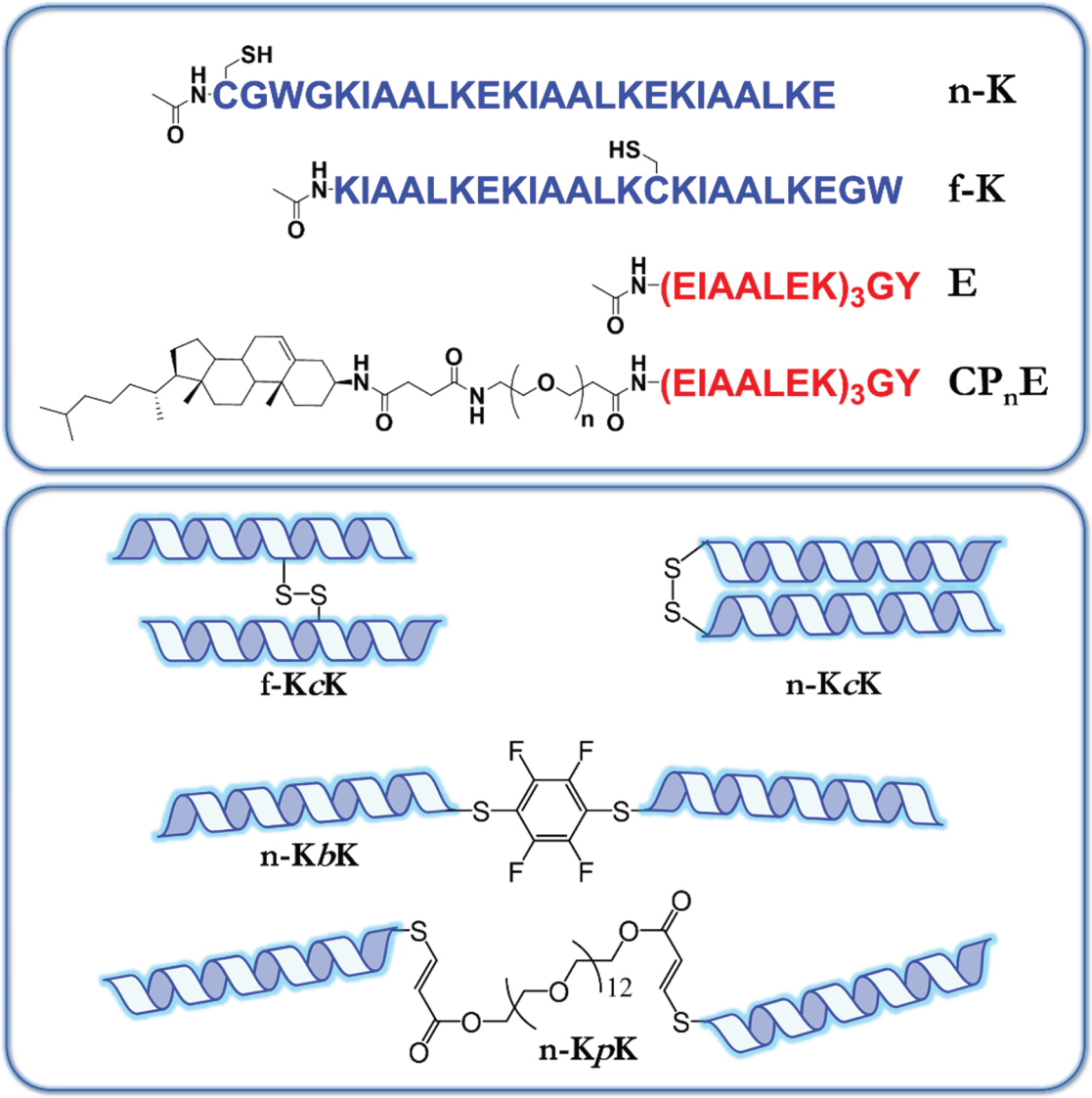


Figure 17. *Overview of peptide sequences of K and its cognate partner E, and structure of cholesterol-anchored E peptides (n = 8, 12). The lower box depicts the four K dimers used in this study.*

RESULTS AND DISCUSSION

DISULFIDE DIMERS

The (super)secondary structure of K*c*K dimers is affected by the position of the linker at the peptide backbone, as illustrated in Figure 18. For f-K*c*K, the extend of helicity is highly dependent on peptide concentration and shows a T_m of 35 °C at 150 mM. The peptide structure of the N-terminal linked n-K*c*K is independent of peptide concentration and shows a relatively high T_m of 70 °C. These observations suggest an intermolecular interaction for the sterically hindered f-K*c*K dimer, and a more stable intramolecular interaction for the N-terminal linked n-K*c*K dimer.

N-TERMINAL K DIMERS WITH LINKER

The type of the linker highly affected the peptide structure of N-terminally dimerized K peptides, as shown in Figure 19. The rigid *p*-fluorobenzene linker of n-K*b*K induced structural characteristics of both intra- and intermolecular interactions, as shown by a high melting temperature and a concentration dependent peptide helicity. The flexible PEG_{600} linker of n-K*p*K showed only a slight dependency on peptide concentration and dimerized predominantly via intramolecular interactions.

ANALYSIS OF K-HOMODIMER MELTING CURVES

Analysis of concentration dependent thermal unfolding curves by FitDis![21] revealed the oligomeric state of the peptide K dimers and provided the binding characteristics of the coiled-coil peptide interactions. (*Table 7, Figure 20*) The exclusive intramolecular interaction of n-K*c*K is supported by a best fit with an oligomer state (*n*) of 1. On the other hand, similar values for K_F and Tm of f-K*c*K and monomeric K demonstrate the intermolecular coiled-coil interaction of f-K*c*K, which is further supported by a best fit with $n = 2$. The high RMSE values obtained for n-K*b*K show that these peptide interactions are less defined, and do not form a solely intra- or intermolecular coiled-coil structure.

Table 7. *FitDis analysis of CD melting curves.*

Peptide	K monomer [a]	f-K*c*K	n-K*c*K	n-K*b*K	n-K*p*K
$P_{Tmin} - P_{Tmax}$ / µM [b]	22 – 178	73 - 300	40 - 360	50 - 400	40 – 300
Found $\upsilon_1, \upsilon_2, \upsilon_3$, [c]	2	2	1	2	2
Tm / °C (50uM)	23	23	70	65	70
ΔH° / kJ mol^{-1}	163	189	121	212	333
T° / °C	92	93	78	131	103
ΔC_P / kJ mol^{-1} K^{-1}	1.5	1.9	1.8	1.9	4.2
θ_{F0} / 10^3	-27.8	-17.9	-17.2	-19.4	-14.5
m_{F0}	(0)	65.4	64.5	80.0	78.2
θ_{U0} / 10^3	-3.9	-3.9	-4.0	-3.8	-4.0
m_{U0}	-10	(0)	(0)	(0)	(0)
ΔS_{25} / J mol^{-1} K^{-1}	150	119	44	-46	-101
ΔG_{25} / kJ mol^{-1}	20.2	22.0	10.6	26.9	32.2
ΔH_{25} / kJ mol^{-1}	65.0	57.6	23.6	13.1	1.97
$K_{F\,25}$ / M^{-1}	3.4×10^3	7.2×10^3	7.1×10^1	5.1×10^4	4.4×10^5
$K_{U\,25}$ / M^{-1}	2.9×10^{-4}	1.4×10^{-4}	1.4×10^{-2}	1.9×10^{-5}	2.3×10^{-6}

[a] Data taken from Rabe et al.[22] [b] Range of total peptide complex concentration used in experiment. [c] Stoichiometric factors of best fitting model. Values in parenthesis were fixed during all fitting procedures.

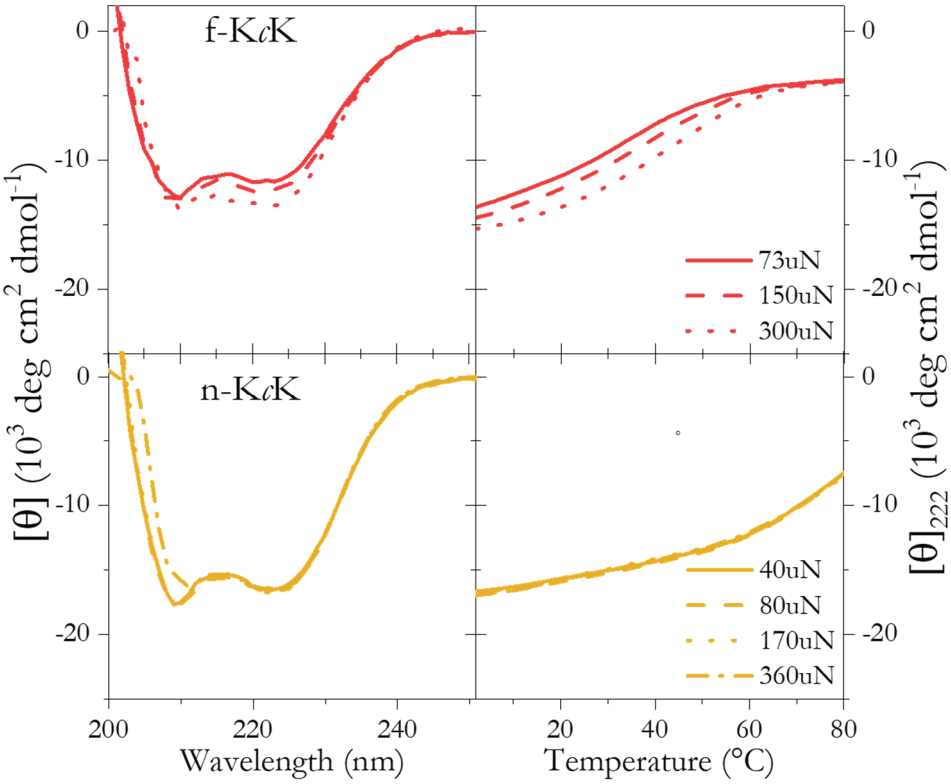


Figure 18. *Left: CD spectra of f-KcK and n-KcK at various concentrations, recorded at 20 °C. Right: Concentration dependent thermal unfolding curves of f-KcK and n-KcK. PBS, pH 7.4.*

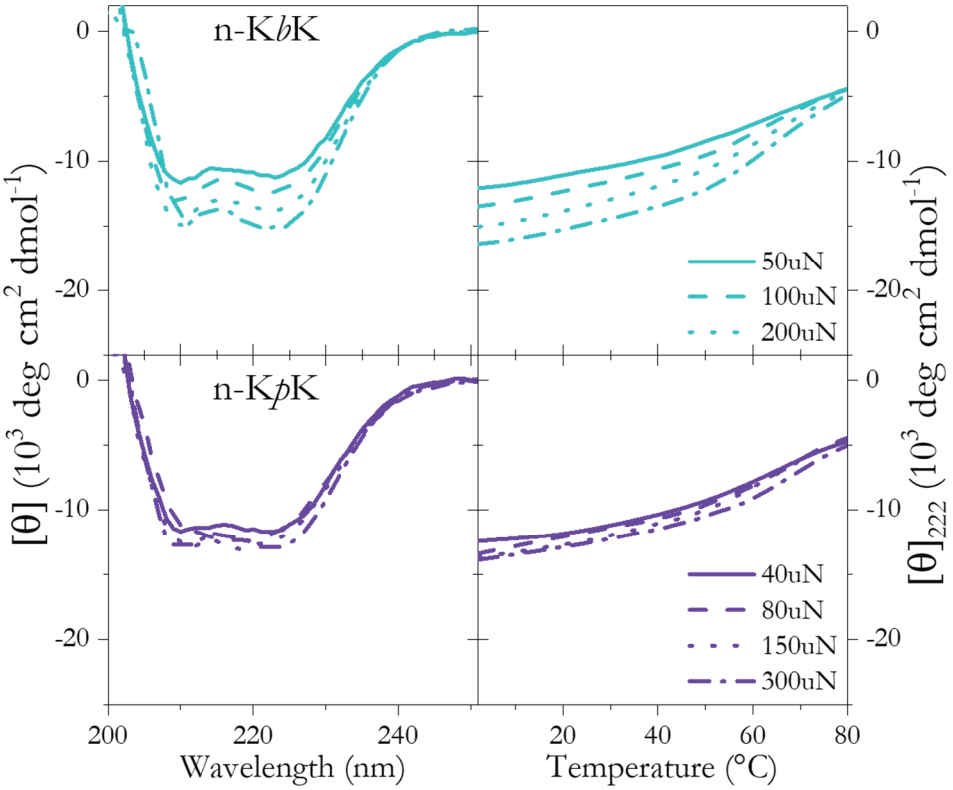


Figure 19. *Left: CD spectra of n-KbK and n-KpK at various concentrations, recorded at 20 °C. Right: Concentration dependent thermal unfolding curves of n-K dimers. PBS, pH 7.4.*

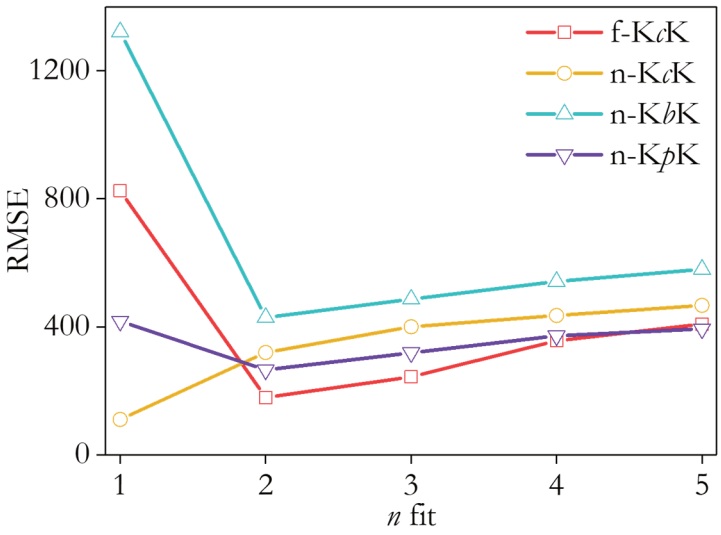


Figure 20. *Root mean square error (RMSE) of the fitting of concentration dependent thermal unfolding curves, indicative for coiled-coil oligomer state (n).*

The small differences in RMSE values obtained for n-K*p*K with different oligomer state *n* shows that the data can be described by various parameters, which indicates that both intra- and intermolecular interactions contribute to the coiled-coil formation.

E + K COILED-COIL INTERACTION

Next, we probed the interaction of the K-dimers with its cognate partner E at room temperature (*Figure 21*). The K-peptides were analyzed with CD-spectroscopy before and after mixing with equimolar amounts of peptide E, and the results are shown in Figure 22 and Table 8.

The N-terminal disulfide dimer n-K*c*K showed an increased helicity and stability over the K monomer and the other dimeric derivatives, which supports the intramolecular stabilization of the homomeric peptide interaction. The slight decrease in helicity with respect to the K monomer observed for all other K-dimers is accompanied by a slight increase in $\theta_{222}/\theta_{208}$ ratio, which points towards slightly distorted helices with a higher degree of coiled-coil formation for dimeric peptides.

Upon mixing dimeric K and peptide E in equal normality, a heterodimeric coiled-coil formation is evidenced in all cases by an increase in helicity. However, coiled-coil formation between E and K dimers is less pronounced compared to coiled-coils involving monomeric K. The covalent linker could very well interfere with heteromeric

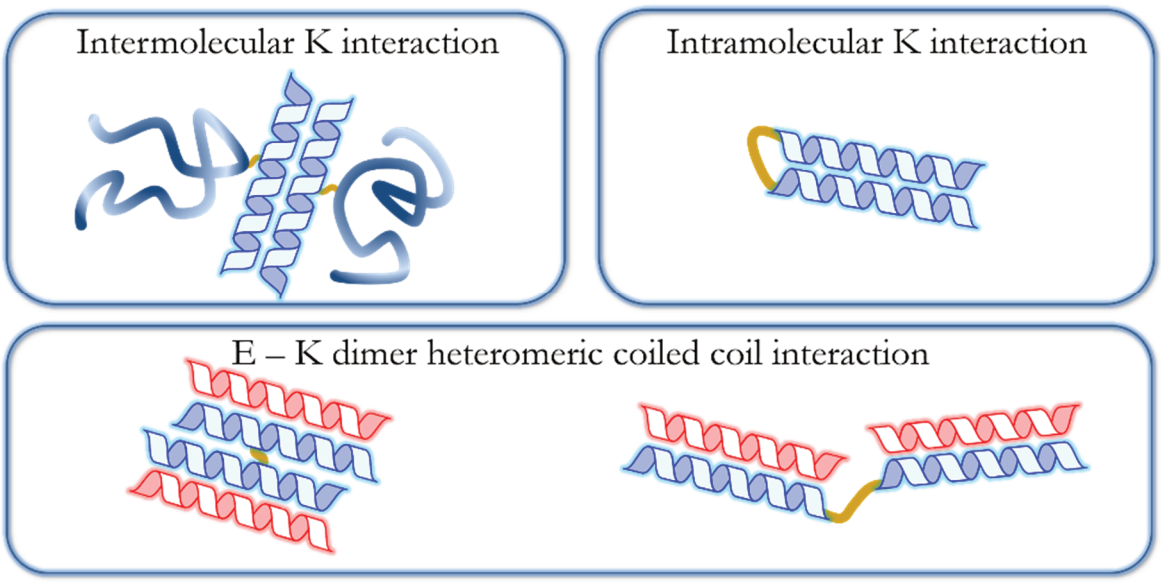


Figure 21. *Overview of inter- and intramolecular homodimeric interactions in K dimers, and the heterodimeric coiled-coil formation between E and K dimers.*

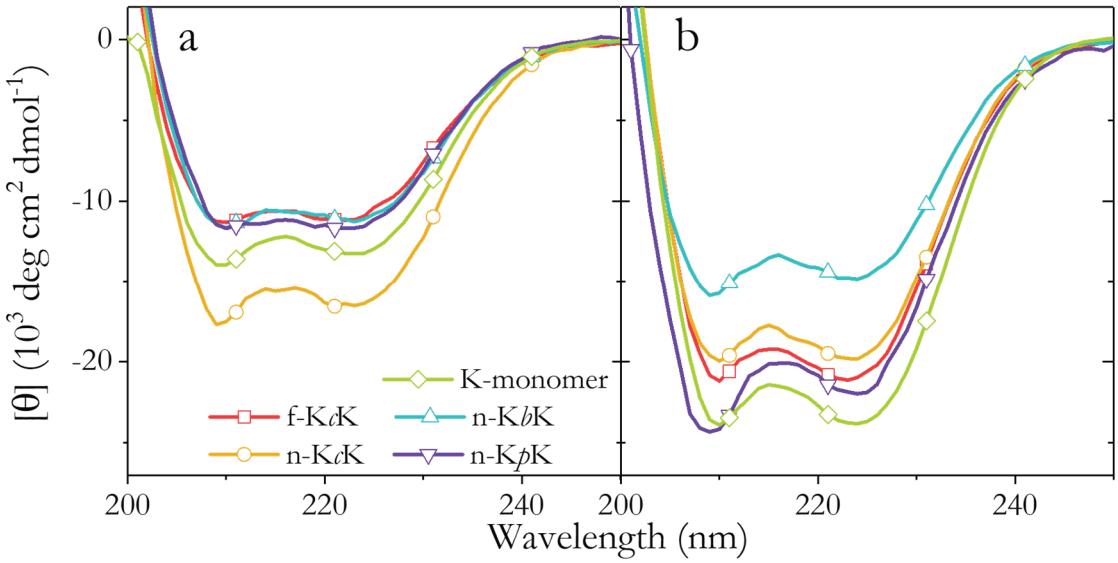


Figure 22. *CD spectra of K dimers (a), and an equimolar mixture of K dimers with peptide E (b). [Total peptide] = 40uN, in PBS pH 7.4, 20 °C.*

Table 8. *Degree of peptide helicity of K – E heterodimeric coiled-coil formation.*

		K-monomer	f-K*c*K	n-K*c*K	n-K*b*K	n-K*p*K
PBS	%H	41	35	52	35	36
	$\theta_{222}/\theta_{208}$	0.9	1	0.9	1	1
K + E	%H	74	66	62	46	69
	$\theta_{222}/\theta_{208}$	1	1	1	0.9	0.9
Helicity increase (%)		100	79	45	29	85

coiled-coil formation via steric effects, causing an equilibrium between K-K homodimers and E-K heterodimers.The influence of the linker on this equilibrium can be estimated via the relative helicity increase, compared to native E and K. To calculate the relative increase in helicity for the K dimers upon mixing with E, the helicity of the coiled-coil formation of monomeric K and E was taken as 100%. (*Table 8*) The data shows that heterodimeric coiled-coil formation of E with f-K*c*K and n-K*p*K closely match with the hetero coiled-coil structure of E and monomeric K, with 80% relative helicities at θ_{222}. Minor differences in absorbance at θ_{208} could indicate small linker-induced structural differences for these two K-dimers. Conversely, for n-K*c*K a relative helicity around 45% was found. Combined with the relatively high association constant of this homodimer formation, this data suggests a competition between intramolecular homodimeric and intermolecular heterodimeric coiled-coil formation. For n-K*b*K, the relative helicity of only 30% indicates that heteromeric coiled-coil formation is inhibited. Since the homodimer interaction and the coiled-coil melting temperature are similar to n-K*c*K, an additional factor should be accounted for, next to homodimer stability. This is most likely an electrostatic π – peptide or π – π interaction of the rigid tetrafluoro-biscysteine spacer moiety which could be further investigated by UV spectroscopy but is not performed in the scope of this study.

PEPTIDE MEMBRANE INTERACTION

As demonstrated in Chapters II and III, peptide K interacts spontaneously with lipid membranes, and this behavior becomes more pronounced with elongated peptides sequences resulting in more efficient membrane fusion[23] or when peptides are tethered to a membrane.[3] It was anticipated that the K dimers also interact with membranes, but differences between the various derivatives are clearly visible, as shown in Figure 23 and Table 9.

For n-K*c*K and n-K*p*K, peptide helicities increase only slightly in the presence of plain vesicles, supporting the very stable intramolecular interaction shown for these peptide dimers; these interactions are not disrupted in the presence of vesicles. When the membrane surface is functionalized with $CP_{12}E$ however, the helicity of the resulting peptide structures is increased, via disruption of the homodimeric intramolecular K – K coiled-coil formation and formation of the heterodimeric $CP_{12}E$ – K coiled-coil

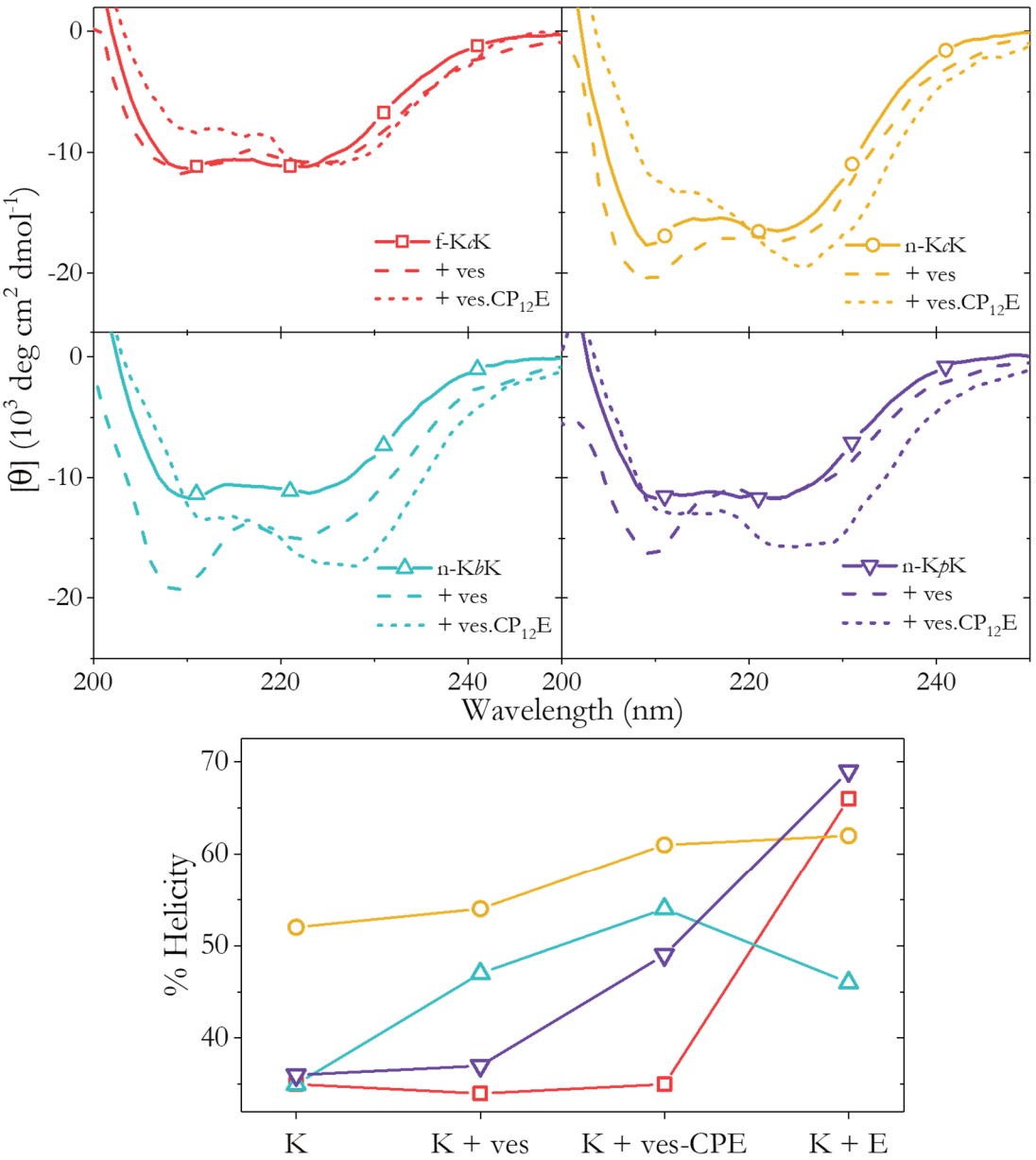


Figure 23. *CD spectra of solvated K dimers, a mixture of K dimers and vesicles and a mixture of K dimers and $CP_{12}E$ decorated vesicles. Lower graph shows degree of peptide helicity as determined from CD. [Total lipid] = 0.5mM, [total peptide] = 10 uN, in PBS pH 7.4. Functionalized vesicles contained 2% $CP_{12}E$.*

Table 9. *Degree of peptide helicity of K – vesicle interactions.*

K-dimer +		f-K*c*K	n-K*c*K	n-K*b*K	n-K*p*K
PBS	%H	35	52	35	36
	$\Theta_{222}/\theta_{208}$	1.0	0.9	1.0	1.0
Vesicles (0.5 mM)	%H	34	54	47	37
	$\Theta_{222}/\theta_{208}$	0.9	0.8	0.8	0.7
Vesicles + $CP_{12}E$ [a]	%H	35	61	54	49
	$\Theta_{222}/\theta_{208}$	1.3	1.5	1.3	1.2

[a] *2 mol% lipopeptide functionalization, [K] / [E] = 1.*

formation. It has been shown that this structural motif is an intermediate stage for the K – membrane interaction, since the membrane interaction occurs concomitant to heteromeric coiled-coil formation of lipopeptides.[24] Therefore, the K-dimer is expected to fully immerse in the membrane, but an equilibrium could be established between heteromeric coiled-coil formation and K-dimer – membrane interaction, as is reported for similar peptide systems in Chapter III.

In the presence of plain vesicles, an increase in helicity is only observed for the n-K*b*K dimer. This shows that the membrane interaction is favored over the coiled-coil interaction, and that the transition between these two conformations occurs spontaneously. Therefore, the coiled-coil interaction of this derivative is mostly intermolecular, since intramolecular coiled-coil forming dimers (n-K*c*K, n-K*p*K) did not show this spontaneous incorporation in plain membranes.

Remarkably, f-K*c*K shows similar helicities in both the absence and presence of vesicles. Since this derivative is sterically constrained by the linking site at the backbone, peptide – membrane interactions are accompanied by an energy penalty because one peptide of the peptide dimer is forced into the aqueous phase. This also reduces the number of peptides available for membrane incorporation by at least 50%. Consequently, most of the peptide interactions are intermolecular peptide – peptide interactions, and the presence of vesicles does not influence the overall peptide structures significantly.

LIPOSOME FUSION

One of the important stages in the fusion cascade, besides full fusion, is hemifusion, when the outer membranes of opposing liposomes are merged. This mixing of phospholipids is measured via the incorporation of a lipidated FRET pair (DOPE-NBD and DOPE-LR) in the membrane of one population of liposomes. Fusion events with non-functionalized liposomes increases the average distance between the FRET components, which increases the donor emission. The standard liposomal fusion model system, as discussed in Chapter II, uses $CP_{12}E$ and $CP_{12}K$ functionalized liposomes to mediate the fusion process. Here, K-dimers are used as solvated fusogens (without a cholesterol anchor) bringing two CP_nE functionalized liposomes in close proximity and concomitant fusion. Therefore, the standard fusion assay was slightly altered: one batch of liposomes bearing peptide CP_nE was decorated with the FRET pair and was mixed

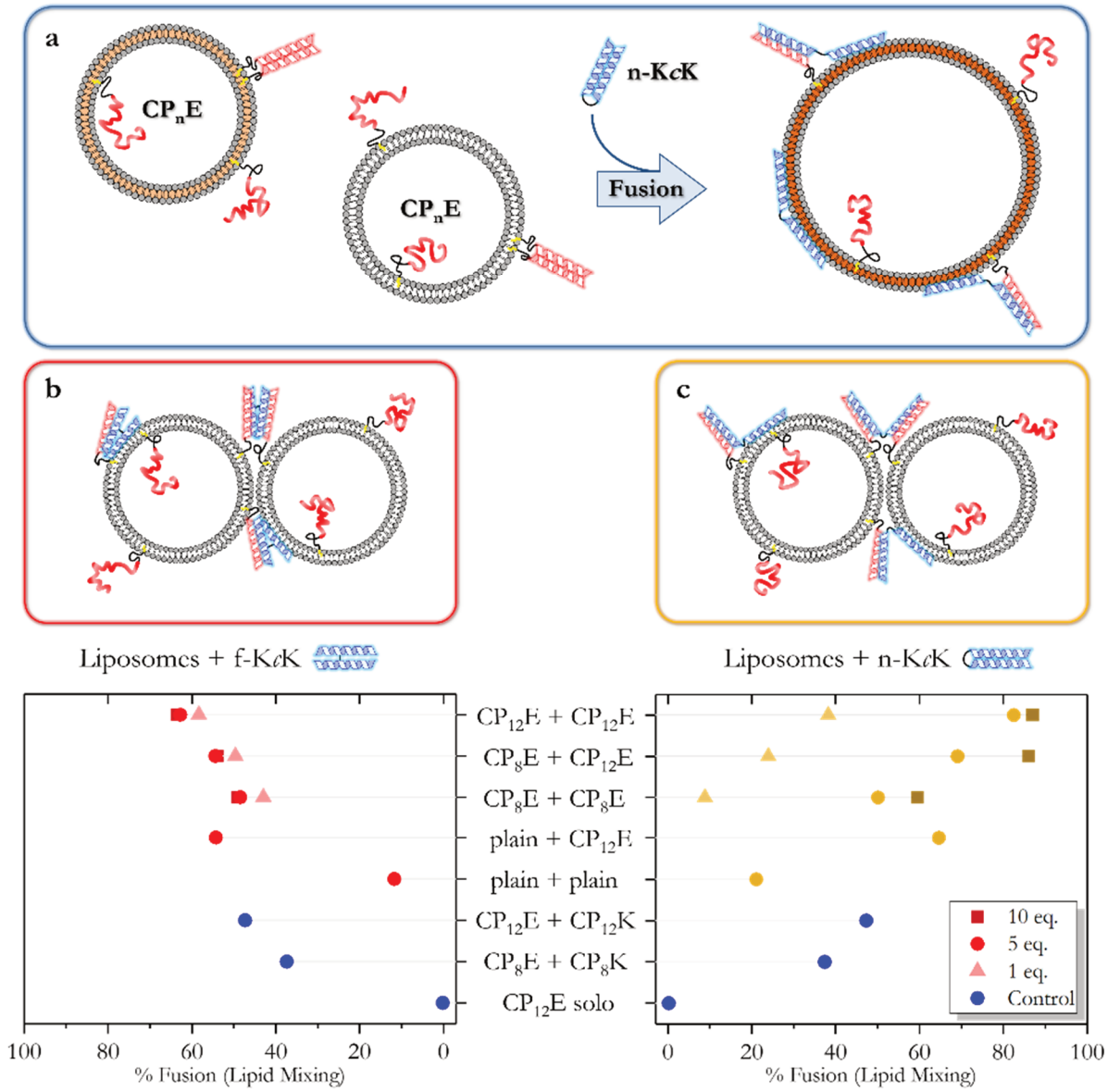


Figure 24. *(a) For a successful fusion event, a KcK dimer should form a (simultaneous or sequential) coiled-coil interaction with 2 CPnE peptides, tethered to opposing liposomes. This will force both liposomes in close proximity and concomitant fusion. Relative emission of DOPE-NBD before and after fusion is shown with pale and bright orange colored membranes. Lipid mixing fusion efficiency of CPnE decorated vesicles (n = 8, 12), in the presence of f-KcK (b, red) or n-KcK (c, yellow) at various concentrations (square, 5uN; circle, 2.5 uN; triangle, 0.5 uN; with appropriate color gradient). Control experiments of liposomes lacking E-lipopeptides in the presence of KcK dimer are denoted 'plain'. The fusion efficiency of liposomes functionalized with CPnE and CPnK is added for comparison (blue). [Total lipid] = 0.1 mM, with 1 mol% CPnE, in PBS pH 7.4 20 °C. [KcK] = 5 μN, 2.5 μN, 0.5 μN.*

with non-fluorescent, peptide CP$_n$E bearing liposomes. The K dimer (aq) was added and the NBD emission was measured for 20 minutes. (*Figure 24, Table S3*) The structurally very different dimers f-K*c*K and n-K*c*K were used in this fusion assay as proof of principle. Also, these K-dimers have a high affinity for membrane incorporation, and highly dynamic intermediate stages are expected prior to fusion with several peptides

involved, both K dimers as multiple E peptides, tethered to opposing liposomes. Since it was also shown that the fusion efficiency is influenced by the PEG spacer length of both CP_nE and CP_nK,[24] here CP_8E and $CP_{12}E$ are used to determine this effect on the fusion process. The results show that fusion efficiency depends on the K-dimer and on the spacer length of the employed CP_nE derivative.

First of all, with the intermolecular interaction promoting f-K*c*K, fusion between $CP_{12}E$ functionalized liposomes is even more efficient as fusion between $CP_{12}E$ and $CP_{12}K$ decorated liposomes (Chapter II),[24] and did not depend significantly on the peptide dimer concentration in the range of 0.5 μN to 5 μN ([K]:[E] = 1:1 to 10:1). For n-K*c*K however, fusion does depend on peptide dimer concentration. At [n-K*c*K] = 0.5uN, fusion is less efficient compared to f-K*c*K, while at higher concentrations fusion becomes more efficient, reaching fusion efficiencies around 90% at 10-fold peptide excess.

The increasing fusion efficiency for liposomes functionalized with E lipopeptides with increasing spacer length, is illustrated here by a linear correlation between cumulative spacer lengths ($n + n$) and fusion efficiency, and this trend was observed with both K*c*K-dimers at all measured concentrations. Furthermore, control experiments showed that efficient fusion already occurred between non-functionalized liposomes and $CP_{12}E$ functionalized liposomes (plain + $CP_{12}E$), for both K*c*K-dimers. This shows that peptide K first interacts with $CP_{12}E$ decorated liposomes, which then proceed to interact and fuse with non-functionalized liposomes. This supports the findings of Chapters II and III that the fusion mechanism involves both E and K peptides and the K – membrane interaction. It further demonstrates the main role of peptide E in the fusion mechanism: trigger the dissociation of the stable homodimer of n-K*c*K and facilitate docking of the liposomes.

CONCLUSIONS

Conjugation of two K peptides increases the stability of the homodimer formation, but the site and nature of the linker moiety highly influences the equilibrium between intra- and intermolecular interactions. A linking site at the second heptad and opposed to the hydrophobic core prohibited the intramolecular peptide interaction via steric hindrance, and yielded a T_m similar to single K, around 35 °C. Furthermore, we found no significant peptide stabilization by lipid membranes. Since massive peptide induced liposomal aggregation was found at high lipid concentrations (indicative for significant peptide – membrane interactions), we strongly think that the peptide structure is similar in peptide – peptide interactions and peptide – membrane interactions.

With a N-terminal linking site and a flexible spacer, n-K*p*K showed a $T_m \approx 70$ °C, while a spontaneous peptide – membrane interaction was not observed. Therefore, n-K*p*K forms highly stable intramolecular coiled-coil homodimers. In the presence of membrane tethered $CP_{12}E$ however, peptide helicities increased sharply, indicative for disruption of the homodimer structure, and the formation of heteromeric coiled-coil structures and/or membrane immersion.

A rigid *p*-fluorobenzene linker affected the heteromeric coiled-coil formation between E and n-K*b*K, as only 30% helicity increase was observed in the presence of E. Remarkably, a spontaneous peptide-membrane interaction was only measured with this derivative.

Solvated K dimers are very efficient in mediating fusion between $CP_{12}E$ functionalized liposomes, as measured by lipid mixing, with efficiencies reaching 90%, even higher than the reported values of fusion mediated by membrane-tethered $CP_{12}E$ and $CP_{12}K$. No significant influence of [f-K*c*K] was found in the range of 1 eq. to 10 eq. with respect to the peptide decoration at the liposomes, while [n-K*c*K] highly enhanced the fusion efficiency with increasing concentration. Fusion efficiencies depended linearly on the cumulative spacer lengths of the E lipopeptides. Also, the fusion mechanisms involve both E and K lipopeptides and the K – membrane interaction as $CP_{12}E$ decorated liposomes were found to fuse with non-functionalized liposomes in the presence of K*c*K dimers. Finally, the main role of peptide E is to cause the dissociation of the stable homodimer formation of n-K*c*K, and to facilitate docking of the liposomes.

FUTURE WORK

In this work, the fundamental characteristics of K dimers are explored, and compared to the native K structure and behavior. The understanding of structure and function relationships opens ways to the rational design of coiled-coil peptide dimers and multimers, with tunable binding strengths and defined equilibria between intra- and intermolecular interactions. Investigations could start with the evaluation of f-K dimers with a rigid hexafluorobenzene linker or a flexible PEG_{600} linker. Also, a C-terminal Cys handle can be introduced in the peptides, and the effect of this modification can be correlated to the behavior of N-terminal linked dimers. The linker series can be extended with a well-defined tetraethyleneglycol spacer, in combination with Cysteine containing peptides n-K and f-K. Furthermore, a PEG_{2000}-dialkyne, and PEG_{5000}-tetraalkyne are available and azide-functionalized peptides can be conjugated to these linkers via the CuAAC click reaction. These multimeric peptide-dimers with elongated spacers can also be useful to trigger aggregation of peptide functionalized particles.

Next, K*c*K and K*b*K dimers can be used to determine the effect of the linker type and linker position on their fusogenic activity via lipid and content mixing assays. The understanding of the fusion mechanism with non-anchored peptides will allow the development of fusion systems with an external and site-specific trigger, which could have broad implications for drug delivery systems, and would increase the understanding of naturally occurring multicomponent coiled-coil interactions. Furthermore, the f-K*c*K dimer can be functionalized with a single or double N-terminal Chol-PEG_4 anchor. The resulting constructs can be tethered to liposomes, which could yield very high fusion efficiencies with $CP_{12}E$ decorated liposomes. Furthermore, surprising peptide conformations and membrane interactions could be obtained, due to the intrinsic steric hindrance of these peptide dimers and the tight membrane immobilization via the double cholesterol anchor. Finally, various E dimers can be synthesized like the different K dimers. Since membrane tethered E shows a tendency to form homodimers, and even homotetramers *in silico*,[25] surprisingly stable binding affinities could be found. Mixing of E dimers with K dimers could result in many different interlocked coiled-coil structures, depending on concentration and peptide ratio, and would allow the study of multivalent coiled-coil peptide aggregates.

EXPERIMENTAL SECTION

MATERIALS

See Chapter II. Fmoc-Cys was purchased from NovaBioChem. PEG(600)-OH and propiolic acid were obtained from Sigma Aldrich. PBS buffer contains 20 mM PO_4^{3-}, 150 mM NaCl, pH 7.4. Peptide concentrations are calculated in Normality, to compensate for dimerization (2 uN = 1 uM).

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. Lipopeptides were dissolved in 1 : 1 (v/v) chloroform : methanol at 50 μM concentration.

Lipid mixing experiments: liposomes bearing 1 mol% of a FRET pair are required besides plain liposomes, and an additional 1 mM stock solution of DOPC : DOPE : cholesterol : DOPE-LR : DOPE-NBD (49.5 : 24.75 : 24.75 : 0.5 : 0.5mol%) in 1 : 1 (v:v) methanol : chloroform was made. 1 mol% of the lipopeptide solution was mixed with the appropriate liposome solution and the solvent was removed under a stream of air. The dry lipid/peptide layer was rehydrated with PBS. These solutions were subsequently sonicated for 5-10 minutes at 55 °C to yield ~100 nm diameter liposomes,[27] and used without further purification.

Fluorescence spectroscopy. Lipid-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:

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

For lipid-mixing experiments the fluorescence intensity F(t) was monitored, by measuring NBD emission at 530 nm in a continuous fashion for 20 min upon addition of K*d*K dimer solution to a equimolar mixture of fluorescent liposomes and non-fluorescent

liposomes. F_0 was determined by measuring NBD emission of fluorescent liposomes to which an equal amount of PBS was added. F_{max} was determined by using plain liposomes which contained half the concentration of fluorescent lipids. [Total lipid] = 0.1mM, in PBS pH 7.4.

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, [θ] (deg cm^2 $dmol^{-1}$), 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 proper background spectrum (PBS, or plain liposomes in PBS) subtracted. Percentage α-helicity was calculated using the predicted value $[\theta]_{222}$=-39500*(1-2.57/n) as 100% value for an α-helical peptide of *n* residues.[26] For temperature dependent measurements $[\theta]_{222}$ was recorded as a function of temperature, with a range of 2 - 80°C and ΔT = 40°C/h. For experiments involving liposomes, [Total lipid] = 0.5mM, with 1mol% lipopeptide, in PBS pH 7.4.

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 633nm 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 measurements were carried out at room temperature.

SYNTHESIS

PEG(600)-dialkyne: PEG(600)-OH (6 grams, 10 mmol) was dissolved in toluene (50 mL) and conc. H_2SO_4 (0.1 mL, cat.) was added. The obtained solution was stirred and heated to 80 °C to obtain a clear homogenous solution. Propiolic acid (2,8 grams, 40 mmol, 4 eq.) was added, and the solution was heated to reflux under Dean Stark conditions (± 140 °C). After no more water was collected (± 20 hours), the solution was allowed to cool to rt. Toluene was evaporated *in vacuo* and the residue was dissolved in 20 mL DCM. The obtained solution was washed with saturated $NaHCO_3$ (10 mL) and Brine (10 mL). The organic phase was dried with Na_2SO_4, and 0,1 g of activated carbon was added. The activated carbon was removed through vacuum filtration over Celite®545. Vacuum filtration was performed twice for effective removal of the activated coal. The solvent was removed *in vacuo* and the product (5.9g, 96%) was obtained as a clear oil, and was stored in a glass vial in the dark at -20 °C. ^{1}H NMR (400MHz, $CDCl_3$) δ = 4.37 (m, 4H), 3.76 (m, 4H), 3.68 (m, 44H) 2.98 (s, 2H). IR (film) ν 3213, 2870, 2112, 1713, 1452, 1349, 1225, 1095 cm^{-1}; MALDI/TOF m/z 761.211 ($[M+Na]^+$ $C_{34}H_{58}O_{17}Na$ cald. 761.36).

PEPTIDE SYNTHESIS

SPPS: Peptide synthesis was performed on a CEM Liberty I on a 100 μmol scale using Rink amide resin of 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 acylated using a mixture of Ac_2O (50 mM) and DIPEA (12.5 mM) in NMP.

Cleavage: The peptides were cleaved from the resin and side-chain deprotected using a mixture of TFA/Thioanisole/EDT/Anisole (90:5:3:2 v/v) for 2h. The peptides were precipitated in cold diethyl ether followed by centrifugation and dried under vacuum.

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 278 nm. Samples were eluted with a linear gradient from 10% to 50% (v/v) B in A, A being H_2O, 0.1% (v/v) TFA, and B being MeCN, 0.1% (v/v) TFA. A Phenomenex C18 reversed phase column (21.2 mm diameter, 150 mm length, 5.00 μM

particle size) was used with a flow rate of 15 mL min^{-1}. Collected fractions were tested for >95% purity using LC-MS with Gemini C18 column, pooled and freeze dried.

K*c*K dimer formation: Purified K peptides were dissolved in PBS (pH 8.5) and cysteine oxidation was achieved with bubbling of air through the solution for 30 min. The reaction mixture was subjected to RP-HPLC with a linear gradient from 10% to 50% (v/v) B in A, A being H_2O, 0.1% (v/v) TFA, and B being MeCN, 0.1% (v/v) TFA. Collected fractions were tested for >95% purity using MALDI/TOF, pooled and freeze dried.

K*b*K dimer formation: A stock solution was prepared of hexafluorobenzene in DMSO:H_2O (1:1 v/v). Of this solution, 0.48 eq. were added to a solution of peptide n-K (1 mM) in PBS (0.5 mL, pH 8.5) and the mixture was shaken for 2h at room temperature. The reaction mixture was subjected to RP-HPLC with a linear gradient from 10% to 50% (v/v) B in A, A being H_2O, 0.1% (v/v) TFA, and B being MeCN, 0.1% (v/v) TFA. Collected fractions were tested for >95% purity using MALDI/TOF, pooled and freeze dried.

K*p*K dimer formation: A stock solution was prepared of PEG(600)-dialkyne in DMSO:H_2O (1:1 v/v). Of this solution, 0.48 eq. were added to a solution of peptide n-K (1 mM) in PBS (0.5 mL, pH 8.5) and the mixture was shaken for 2h at room temperature. The reaction mixture was subjected to RP-HPLC with a linear gradient from 10% to 50% (v/v) B in A, A being H_2O, 0.1% (v/v) TFA, and B being MeCN, 0.1% (v/v) TFA. Collected fractions were tested for >95% purity using LC-MS with Gemini C18 column, pooled and freeze dried.

PEPTIDE ANALYSIS

Table S2. *Analysis of synthesized peptides.*

Peptide	Formula	Calc mass	Observed mass	RT (min)	isoelectric point [a]	Charge [a]
f-K	$C_{118}H_{205}N_{31}O_{28}S$	2538.16	1269.53 $[M+2H]^{2+}$	5.1	8.94	3.4
n-K	$C_{125}H_{215}N_{33}O_{32}S$	2724.33	908.87 $[M+3H]^{3+}$	5.1	8.68	2.4
f-K*c*K	$C_{236}H_{408}N_{62}O_{56}S_2$	5074.28	MALDI/TOF	n.d.		
n-K*c*K	$C_{250}H_{428}N_{66}O_{64}S_2$	5446.61	MALDI/TOF	n.d.		
n-K*b*K	$C_{256}H_{428}N_{66}O_{64}S_2F_4$	5594.66	MALDI/TOF	n.d.		
n-K*p*K	$C_{282}H_{484}N_{66}O_{80}S_2$	6143.38	1536.60 $[M+4H]^{4+}$	7.7		

[a] *Calculated with Isoelectric Point Calculator and peptide charge is calculated at pH 7.4.*[28]

LIPID MIXING FUSION EFFICIENCY

Table S3. *Fusion efficiency of CP_nE decorated vesicles in the presence of f-KcK and n-KcK*

functionalized liposomes 0.1 mM	f-K*c*K 10 uN	f-K*c*K 5 uN	f-K*c*K 1 uN	n-K*c*K 10 uN	n-K*c*K 5 uN	n-K*c*K 1 uN
CP_8E + CP_8E	49	48	43	60	61	9
CP_8E + $CP_{12}E$	54	54	50	86	69	24
$CP_{12}E$ + $CP_{12}E$	64	63	58	87	82	38
Plain + $CP_{12}E$		54			65	
Plain + Plain		12			21	

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