

Coiled-coil biomaterials for biological applications Shen, M.

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Chapter 4

Coiled-coil Forming Peptide Dimers: Structure, Self-Assembly and Fusogenicity

ABSTRACT

Efficient delivery of extracellular molecules into mammalian cells is essential yet challenging for cell therapeutics. Current drug delivery strategies adopt vesicles or nanoparticles as drug carriers capable of transporting drugs into cells via endocytosis to enhance drug delivery efficiency with minimal toxicity. However, this drug delivery strategy suffers from low drug release due to poor endolysosomal escape. Thus the majority of the drug is eliminated from the cell before it reaches the cytoplasm. Here, we developed a drug delivery system using a membrane fusion strategy, triggered by heterodimeric coiled-coil formation between peptides K₄ and E₄. This drug delivery system is independent of endolysosomal escape pathways thereby enhancing cytosolic drug delivery. In this chapter, we investigate how the secondary structure, self-assembly and lipid membrane affinity of three novel K₄ dimers affects fusion and concomitant drug delivery. Previous studies revealed that K₄ has a dual role in membrane fusion: coiled-coil formation with peptide E bringing opposing membranes into close proximity, but also interacting with the membrane which facilitates membrane fusion. Therefore we hypothesized that dimeric K₄ variants should be more efficient at promoting fusion as they can fulfil both these roles simultaneously. Three K₄ dimers were designed by linking K₄ monomer at either their N- or their C-terminus, to form linear dimers (NLK₄ or CLK₄), or in the center of the peptide to form a parallel dimer (PK₄). Self-assembly studies revealed that linear K₄ dimers tend to dimerize to a possible tetramer-like homodimer, while PK4 tends to form temperature-dependent aggregates. Fusion assays revealed that PK4 has the highest capacity to trigger both liposome-liposome fusion and cell-liposome fusion using peptide modified liposomes. This study, therefore, describes a novel fusogenic drug delivery system but also yields insights as to the influence of conformation, charge, and steric effects of the K/E coiled-coil peptides in triggering efficient membrane fusion.

INTRODUCTION

Efficient intracellular delivery of drugs is pivotal to treating diseases efficiently.¹⁻³ However, many drugs are unable to enter cells due to their charge, size, or solubility and are therefore unable to cross the plasma membrane.⁴⁻⁶ Furthermore, drugs that have a therapeutic effect in one organ/tissue may be toxic in other tissues.⁷⁻⁸ Although significantly advanced drug discoveries have been made,⁹⁻¹¹ target specificity and control over drug delivery into cells remain challenging.

Liposome-assisted drug delivery systems have progressed rapidly in many biological areas because of their advantages in stabilizing therapeutic compounds, overcoming the barrier for cellular and tissue uptake, and reducing cytotoxicity.¹²⁻¹⁴ Liposomes can be used to deliver not only traditional lipophilic and hydrophilic drugs into cells, but also biomacromolecular proteins and nucleic acids.¹⁵⁻¹⁷ However, a major challenge for many applications of liposome-assisted drug delivery systems remains the inefficiency of cytosolic drug release due to inefficient endo-lysosomal escape, resulting in drug degradation or exocytosis.¹⁸⁻²¹

Membrane fusion is a fundamental process in life, which occurs between separate lipid bilayers that merge into one membrane resulting ultimately in content transport.²²⁻²⁴ Studies show that the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) family of proteins play a key role in non-viral membrane fusion during various intracellular exocytosis processes, such as autophagy and neuronal endocytosis.²⁵⁻²⁷ Inspired by this biological process, artificial membrane fusion models systems have been constructed and these systems are able to induce liposome-liposome fusion resulting in lipid mixing and content mixing.²⁸⁻³¹ Unfortunately, only a few of these systems are able to induce liposome-cell fusion, limiting their utility in drug delivery applications.

In previous studies, a minimal membrane fusion model was designed based on the complementary coiled-coil forming peptides "K" (KIAALKE)₃ and "E" (EIAALEK)₃.³²⁻³³ Conjugating these peptides via short poly(ethene glycol) (PEG) linker to cholesterol resulted in the lipopeptides CPK₃ and CPE₃. These molecules readily insert into lipid bilayers and have the ability to induce liposome-liposome membrane fusion. Mechanistic studies showed that peptide K plays an essential role in membrane fusion.³⁴⁻³⁶ Not only does it form a coiled-coil with the complimentary peptide E, but it also has a high affinity to phospholipid membranes because of its structural specificity and positive charge.³⁴⁻³⁶ Peptide K membrane

interactions induce positive membrane curvature and destabilize the membrane, facilitating membrane fusion. Extension of the peptides from 3 to 4 heptads (*i.e.* CPK₄ and CPE₄) increases membrane interactions, enabling cell-liposome fusion while only liposome docking onto the membrane of cells was observed for CPK₃/CPE₃.³⁷⁻³⁸ A study investigating the effect of the PEG spacer shows that the distance between peptide and membrane is another key factor which influences membrane fusion efficiency.³⁹⁻⁴⁰ A short spacer prevents efficient coiled-coil formation due to steric effects, while too long a spacer decreases membrane fusion efficiency.

Considering that the membrane affinity of peptide K and coiled-coil formation influences membrane fusion, three dimeric K_4 peptides with different structures were designed (Scheme 1A). Ideally, K₄ dimers are able to form coiled coils with two complimentary E₄ peptides attached to two lipid membranes, thus connecting two opposing membranes and inducing efficient membrane fusion. Furthermore, a K_4 dimer is able to interact with two lipid membranes, which also enhances membrane fusion efficiency. These K₄ dimers may have different fusion efficiencies due to different membrane affinities and different interaction manners when forming a coiled-coil with peptide E_4 (Scheme 1B). In this chapter, we characterized the peptide folding, self-assembly and fusogenicity of these dimeric K₄ peptides. The secondary structure and thermal stability of the K₄ dimers and their coiled-coil formation were studied by circular dichroism (CD) spectroscopy. Homodimer formation of linear K₄ dimers was evaluated employing a CD titration assay. Using dynamic light scattering (DLS), particle formation of K₄ dimers was studied. Next, we determined the affinity of the K₄ dimers for lipid membranes using a tryptophan fluorescence assay. The ability to induce fusion was evaluated by performing lipid-mixing and content-mixing assays. Cell studies show the potential application of K₄ dimers to deliver liposomes as a potential drug delivery tool. Moreover, this research could help to better understand the influence of peptide conformation, charge, and steric effects on the E/K coiled-coil induced membrane fusion.



Scheme 1. Schematic representation of (A) three K_4 dimers, (B) the coiled-coil structure of three K_4 dimers and peptide E_4 , (C) cell-liposome fusion induced by different coiled-coils.

RESULTS AND DISCUSSION

1. Dimeric K₄ peptide design and synthesis

To synthesize K_4 dimers, a cysteine was introduced into the original amino acid sequence either at the terminus or at the *f*-position in the second heptad of peptide K. Through disulfide bond formation, the various K_4 dimers were obtained (Figure S1). Parallel PK₄ was obtained by conjugating two K_4 peptides with glutamic acid at the *f*-position in the second heptad mutated to cysteine (Scheme 1A). One peptide was extended with a glycine-tryptophan (-GW) for peptide concentration determination and tryptophan fluorescence measurements. Two linear K₄ dimers were also synthesized. A cysteine was added either to the N-terminus or the Cterminus of K₄. Disulfide bond formation between two K₄ monomers at the Nterminus resulted in a linear dimeric structure, denoted NLK₄. In CLK₄, a disulfide bond between cysteines at the C-terminus of the K₄ monomer peptides was formed.

The positive charge of the lysine side chains, and the hydrophobic face of the helix, are two factors influencing the membrane affinity of peptide K_{4} .³⁶ The synthesized

 K_4 dimers have double the amount of positive charge and a well-defined hydrophobic interface, which is expected to result in a higher membrane affinity and destabilization. Since the two K_4 monomers are close to each other in PK₄, this dimer will have a high charge density in solution. However, the positive charge may be distributed more evenly on linear K_4 dimers, therefore the charge density on NLK₄ and CLK₄ is likely to be lower than for PK₄. Such a charge density difference may influence the affinity of the different K₄ dimers for the membrane.

Apart from charge density, the three-dimensional structure was another factor we considered for K₄ dimer design. Peptides K and E form a parallel heterodimeric coiled-coil structure,⁴¹ so one K₄ dimer is expected to interact with two E₄ peptides. Scheme 1B shows the possible binding modes of each K₄ dimer with free peptide E₄ in solution and CPE₄ incorporated into the cell membrane. For PK₄, one K₄ dimer interacts with two E₄ peptides to form a 'sandwich' double dimeric coiledcoil structure. On a cell membrane, such a 'sandwich' structure can be also formed between one K_4 dimer and two CPE₄ modified membranes (Scheme 1C). In the case of the linear K₄ dimers, one K₄ dimer can also bind to two E₄ peptides to form a linear coiled-coil structure. Due to the parallel coiled-coil interaction between peptide K and peptide E, NLK₄ and CLK₄ have different orientations of coiled-coil formation (Scheme 1B). CPE₄ modified cell membranes and liposomes binding by NLK₄ will be very close to each other while liposomes tend to be further away from the cell membrane when using CLK₄ (Scheme 1C). These differences between the three K₄ dimers can yield a better understanding of how charge properties and steric effects in K peptide dimers influence membrane fusion.

2. Secondary Structure Analysis

The secondary structure of the K₄ dimers in solution was studied using circular dichroism (CD) spectroscopy (Figure 1A). Both CLK₄ and NLK₄ adopted a highly helical conformation which was comparable to monomeric K₄ (Table 1). Previous studies have shown that both K₄ and E₄ tend to form homodimers.^{34, 42} We suspect that linear K₄ dimers may form a 'tetramer-like' dimeric structure or a hairpin structure, stabilizing the α -helix structure. Surprisingly, PK₄ shows a highly distorted non- α -helix signal. This might be because the high charge density of PK₄ hinders α -helix formation. Alternatively, due to the specific structure, PK₄ may not be able to dimerize, but rather interacts with other peptides resulting in aggregation.

Peptide	$[\theta] / \text{deg cm}^2 \text{dmol}^{-1}$	Helicity (%) ^a
K4	-29894.2	89
\mathbf{E}_4	-25063.8	80
PK ₄	-8550.3	25
NLK ₄	-34322.4	92
CLK ₄	-39613.2	105
PK ₄ -E ₄	-34330.3	90
NLK4-E4	-31438.4	83
CLK ₄ -E ₄	-35300.8	92

Table 1. Normalized mean residue molar ellipticity and percentage helicity of peptides

^aThe percentage of α-helicity was calculated using Equation 2 (see experimental section).

Next, the interaction of the K_4 dimers with peptide E_4 was investigated. Equimolar mixing of PK₄ and E_4 resulted in a strong increase of the CD signal with minima at 208 and 222 nm, indicative of coiled-coil formation (Figure 1B). In contrast, peptide E_4 and CLK₄ didn't interact as the resulting CD spectrum was the average of the two individual spectra (Figure 1C). This suggests that the 'tetramer-like' homodimer or helical hairpin of CLK₄ is too stable, preventing interaction with E_4 . NLK₄ did interact with E_4 as an increase in CD intensity was obtained (Figure 1D). This result suggests that NLK₄ may also form a tetramer-like homodimer or a hairpin, albeit with a lower stability such that it still interacts with peptide E_4 to form heteromeric coiled coils.



Figure 1. CD specta of (A) K₄ dimers and monomer. (B) $PK_4 \pm E_4$, (C) $CLK_4 \pm E_4$, D) $NLK_4 \pm E_4$. The blue line is the measured spectrum, the dotted line is the calculated average spectrum from the K₄ dimer and peptide E₄. Spectra were recorded in PBS (pH 7.2) at 20 °C. [K₄ dimer] = 5 μ M, [K₄ monomer] = 10 μ M and [E₄] = 10 μ M.

Temperature-dependent CD spectroscopy was applied to determine the thermostability of the peptides. The K₄ monomer shows a typical "S" shaped melting curve with the melting temperature (T_m) at 50 °C. In contrast, the linear K₄ dimers CLK₄ and NLK₄ remained highly helical over the entire temperature range. Even at 95 °C, a decrease of only 44% and 46% in ellipticity was observed (Figure 2A), revealing the very high thermal stability of these two K₄ dimers. PK₄ formed a non- α -helical secondary structure (Figure 1A), and the temperature-dependent CD measurement was also unusual, showing a "V" shape melting curve with the highest ellipticity at ~ 60 °C. To better understand this unusual behaviour, full CD spectra were obtained at different temperatures (Figure 2B). A signature α -helical CD signal was gradually obtained when the temperature was raised from 5 °C to 60 °C. Upon further heating to 90 °C, the helical secondary structure disappeared again. Thus, PK₄ changes from a distorted non- α -helical state to an α -helix and then eventually to a different non-helical state, revealing the temperaturedependent nature of PK4.

Next, the thermal stability of the helical peptides was determined in the presence of peptide E_4 (Figure 2C). The high thermal stability of NLK₄ and CLK₄ was further enhanced in the presence of E_4 , with only a 30% and 32% decrease in ellipticity upon heating to 90 °C. This suggests that both linear dimers indeed interact with E_4 . It is also possible that the homodimeric CLK₄ and NLK₄ species gradually dissociate to monomeric species and thus form more stable CLK₄/ E_4 heterodimers when the temperature is increased. In contrast, the coiled coil formed by PK₄ and E_4 shows poor thermal stability as an 89% ellipticity decline was observed. Indeed, a typical sigmoidal melting curve was obtained with a T_m of 55 °C. The overall thermo-stability study shows that the linear K₄ dimers have high thermo-stability, which increases further upon the addition of E_4 . In summary, PK₄ shows complex (self) assembly behaviour at different temperatures and the interaction with peptide E_4 is rather weak.



Figure 2. (A) Melting curves of K_4 dimers and monomer. (B) CD spectra of PK_4 at different temperatures. (C) Melting curves of $PK_4 + E_4$, $NLK_4 + E_4$, $CLK_4 + E_4$ and $K_4 + E_4$. Spectra were recorded in PBS (pH 7.2). [K₄ dimers] = 5 μ M, [K₄] = 10 μ M, [E₄] = 10 μ M.

3. Homodimer formation of linear K4 dimers

The CD study revealed that a higher-order structure may exist for the linear K_4 dimers as evidenced by the increased helicity and T_m. Based on the structure of the linear K₄ dimeric peptides, we suspect that either a hairpin structure or a 'tetramerlike' homodimer structure may form, resulting in homomeric coiled-coil formation. If intramolecular-homodimerization occurs, its (dis)-assembly should be concentration-independent. However, if intermolecular dimerization occurs, the CD spectrum is expected to be concentration-dependent. Therefore, a concentration-dependent CD titration assay was performed (Figure 3).⁴³ A significant change in CD signal as a function of linear K₄ dimer concentration was observed, suggesting that these linear K₄ dimers tend to form tetramer-like homodimeric structures instead of hairpin structures. The dimerization affinity constant, K_a was determined for NLK₄ and CLK₄ (Table 2). CLK₄ has a higher K_a, which is consistent with the slightly increased stability of CLK₄ in comparison to NLK₄ as observed in the previous CD experiments. Figure 4 shows a representation of the possible tetramer-like homodimers of NLK4 and CLK4. Homodimer formation may influence membrane fusion induced by these peptides as homodimerization blocks the hydrophobic face of the helical peptide, hindering the coiled-coil interaction with E₄.



Figure 3. CD titration to determine homodimer formation of linear K_4 dimers and nonlinear fitting obtained based on equation 3 (see experimental section). The mean residue molar ellipticity at 222 nm was plotted against the concentration. Data were recorded in PBS (pH 7.2) at 20 °C.



Figure 4. Proposed tetramer-like homodimer formation of linear K₄ dimers, A) NLK₄ and B) CLK₄.

Table 2. Non-linear fitting results from CD titration using equation 3 (see experimental section).

K ₄ dimer	$\left[\Theta\right]_{m}^{a}$ deg cm ² dmol ⁻¹	$\left[\Theta\right]_{d}^{b}$ deg cm ² dmol ⁻¹	$\mathrm{K_a}(\mathrm{10}^4)^{\mathrm{c}}$	R ^{2 d}
NLK ₄	-18241.3	-44648.1	4.30	0.965
CLK ₄	-30822.1	-49993.5	13.0	0.974

Determined using equation 3, ${}^{a}[\Theta]_{m}$ and ${}^{b}[\Theta]_{d}$ are the fitted constant which represents the normalized mean residue molar ellipticity when the K₄ dimer forms no tetramer-like homodimer or a fully tetramer-like homodimer respectively. c K_a is the fitted peptide affinity constant of homodimer tetramer-like formation. d R-squared represents the coefficient of determination, which has been used to determine the "goodness of fit".

4. Self-assembly of K4 dimers

 PK_4 consists of two K_4 monomers connected by a disulfide bond between cysteines at *f* positions.⁴⁴ Since K_4 tends to form a helical structure in solution, PK_4 may have two hydrophobic faces exposed to the solution, which makes the structure susceptible to high order self-assembly in order to shield the hydrophobic face. Indeed, PK_4 shows a completely different CD spectrum compared to the other peptides (Figure 1A), indicative of self-assembly. In contrast, the linear K_4 dimer variants form an intermolecular homodimer and this discrete assembly not only enhances the helicity but also shields the hydrophobic face. To study the selfassembly behaviour of all dimeric K_4 peptides, dynamic light scattering (DLS) studies were performed. We used the derived count rate (DCR) as an indicator of particle formation, as it is dependent on particle concentration and size. The solution behavior of all K_4 dimers and K_4 monomer were studied in H₂O and PBS. In H₂O, no particles formed as evidenced by the low DCR (Figure 5A). In PBS, a high DCR was obtained for PK₄ while the other peptides show a very low DCR, revealing the tendency of PK₄ to aggregate while the other peptides were fully soluble (Figure 5A). This finding is in line with the CD data of PK₄, which can also be explained by aggregates to shield the exposed hydrophobic faces of the helical peptides from the solvent.



Figure 5. (A) DCR of K_4 dimers (10 μ M) and K_4 monomer (20 μ M) in H₂O or PBS (pH 7.2) at 20 °C. (B) DLS titration assay: peptide E_4 was titrated into the K_4 dimers (10 μ M) in PBS (pH 7.2). (C) Temperature-dependent DCR for PK₄ in PBS (pH 7.2), [PK₄] =10 μ M.

To verify this hypothesis, a DLS titration assay was performed (Figure 5B), where peptide E_4 was titrated into a solution of PK₄. Coiled-coil formation between peptide E/K is stronger than homodimerization.³⁴ Thus the addition of peptide E_4 should result in the disappearance of large assemblies. At a low E/K ratio, the DCR

gradually increased. This might be because E_4 initially interacts with PK_4 exposed on the surface of the assembled particles, resulting in the size increase. With additional amounts of E_4 , the DCR decreased dramatically. Quite low DCR was obtained when two equivalents of E_4 was added, which indicates that large assemblies were no longer present (Figure 5B). Thus, the DLS titration assay revealed that PK_4 forms coiled coils with peptide E_4 at a 1:2 stoichiometric ratio, as anticipated. The scheme illustrating the process of particle formation of PK_4 as determined by the DLS titration assay is shown in Figure 6.

The CD study reveals that the secondary structure of PK₄ depends on temperature. Therefore, a temperature-dependent DLS assay was performed (Figure 5C). The highest DCR was observed at 25 °C, suggesting the presence of many large particles. Increasing the temperature further resulted in a decrease of the DCR, revealing that the PK₄ assemblies gradually dissociate at higher temperatures, confirming the temperature-dependent CD spectra of PK₄. Upon heating, the particles gradually dissociate to fully dissociated isolated dimers or small assemblies resulting in a CD spectrum typical for α -helices (Figure 2B). Further heating resulted in unfolding of the peptide (Figure 2B, figure 6D & 6E).

Surprisingly, no particles were reformed upon slowly cooling from 90 °C to 20 °C as the DCR remained low (Figure 5C). This suggests that upon cooling, the PK₄ monomers form more thermodynamically stable, aligned, homomeric coiled coils which further self-assemble into more homogeneous small particles, Figure 6F. This process seems similar to DNA annealing, in which slow cooling of two single-stranded oligonucleotides with complementary sequences results in perfectly matched double-stranded DNA. To study whether the speed of cooling influences PK₄ self-assembly, the experiment was repeated with fast cooling to 20 °C from 90 °C within 2 min. A high DCR close to the initial value was obtained, revealing that large particles were re-formed after fast cooling (Figure 5C & Figure 6G). This shows that these large particles are kinetically-trapped assemblies while the smaller particles are the more stable thermodynamically favoured state.



Figure 6. PK_4 (dis) assembly (A-C) in the presence of peptide E_4 and as a function of temperature (D-G).

Transmission Electron Microscopy (TEM) imaging was applied to visualize the PK_4 assemblies. Unstructured aggregates were observed for PK_4 in PBS (Figure 7A). In the presence of two equivalents of peptide E_4 , the aggregates were no longer observed (Figure 7B), consistent with the DLS study. This further supports the hypothesis that the addition of peptide E_4 results in the formation of soluble PK_4/E_4 coiled-coil complexes. The temperature-dependent DLS study showed that the DCR change depends on the rate of cooling of the PK₄ solution. As expected, TEM imaging reveals quite different structures of PK₄. In the fast cooled sample (~45 °C/min), large aggregates were observed; which is similar to the PK₄ aggregates obtained without heating and cooling (Figure 7C). In contrast, only small particles were observed upon slow cooling samples (~1 °C/min, see Figure 7D). In summary, these results reveal that PK₄ aggregates in PBS are able to interact with E_4 to form more stable and soluble coiled-coil complexes. Furthermore, PK₄ self-assembly is a kinetic process and the size of the self-assembled PK₄ aggregates depends on the rate of cooling.



Figure 7. TEM images of PK₄ based structures. A) PK₄ peptide aggregates, B) PK₄ + E₄, C) PK₄ subjected to a fast cooling process and D) PK₄ after a slow cooling process. [PK₄] = 10 μ M, [E₄] = 20 μ M, all samples were prepared in PBS (pH 7.2) at 20 °C. Scale bar: 500 μ m.

5. Membrane affinity of K₄ dimers

As described previously, peptide K₄ plays an essential role in the E/K coiled-coil membrane fusion system because it induces membrane curvature, and destabilizes the lipid membrane facilitating membrane fusion.³⁶ The lysine snorkelling mechanism was proposed to explain the interaction between the lysine-rich K peptides and the lipid membrane,⁴⁵ which suggests that structural specificity plays a crucial role in peptide-membrane affinity. Peptide K₄ adopts a helical structure in which the hydrophobic amino acids (at the "*a*" and "*d*" positions) form one face of the peptide while the lysines (at "*e*" and "*g*" positions) lie on both sides of this hydrophobic face. When interacting with a lipid membrane, the hydrophobic face of the peptide inserts into the hydrophobic centre of the membrane and the positive amines on the side chain of lysine interacts with the negative phosphate groups of the lipids through electrostatic interactions. Therefore, the K₄ dimers in the current





Figure 8. (A) Tryptophan fluorescence titration assay and nonlinear fitting based on equation 4 (see experimental section). The shadow following each fitting curve represents the 95% confidence interval of the fit. (B) The membrane partition coefficient (K_p) of three K_4 dimers and K_4 monomer was calculated during fitting.

Tryptophan is intrinsically fluorescent and its fluorescence is sensitive to its environment; insertion into a hydrophobic membrane leads to a fluorescence increase. To compare the membrane partition coefficient (K_p) of the K_4 peptide variants, a tryptophan fluorescence titration assay was performed (Figure 8 and Table 3). The tryptophan fluorescence of PK₄ increased nearly fivefold in the presence of a lipid membrane. In contrast, the fluorescence of the linear K₄ dimers hardly increased, revealing an inability to interact with a lipid membrane. The K_p of each peptide was determined by a non-linear fitting procedure (Figure 8B). The highest partition coefficient was calculated for PK4, revealing that this dimer has the highest lipid membrane affinity, confirming our hypothesis that as PK₄ has a higher charge density it interacts more strongly with lipid membranes. Although the linear K₄ dimers are identical in composition but differ in structure, they show a low affinity for lipid membranes. This result can be explained by the fact that linear K₄ dimers tend to form a 'tetramer-like' dimeric structure thereby shielding the hydrophobic face of the peptides. As a result, these linear dimers are unable to interact with a membrane.

peptide	$K_{p}^{a}(10^{3})$	$\mathbf{R}^{2 b}$	
K ₄	91.1	0.99	
PK_4	140.4	0.99	
NLK_4	34.5	0.94	
CLK_4	19.4	0.88	

Table 3. Lipid membrane affinity determined by tryptophan fluorescence titrations.

^a The membrane partition coefficient (K_p) was obtained by a nonlinear fitting curve (Figure 8) based on equation 4 (see experimental section). ^b The R-squared represent the coefficient of determination.

6. Liposome fusion

The fusogenic properties of the different coiled-coil peptides were studied using lipid-mixing and content-mixing assays. Previous studies have shown that the fusogenic properties of coiled-coil peptides are correlated to the stability of the peptides and the affinity of the K peptide for the lipid membrane.^{32, 35} PK₄ forms a strong coiled-coil with peptide E_4 and has a high affinity for lipid membranes, suggesting that the PK₄-E₄ coiled-coil combination may be highly fusogenic.

The lipid mixing assay was based on the Förster resonance energy transfer (FRET) pair nitrobenzoxadiazole (NBD, donor fluorophore) and lissamine rhodamine (LR, acceptor fluorophore).^{33, 43} These dyes were attached to lipids and incorporated into the same lipid membrane. When membrane fusion occurs between fluorescent lipid-modified liposomes and non-fluorescent liposomes, the average distance between the NBD and LR fluorophores increases resulting in enhanced NBD emission. In this assay, both sets of liposomes contained 1% CPE4 and liposome fusion was initiated by K_4 dimer addition. For comparison, lipid mixing facilitated by CPK_4 and CPE_4 was studied as well. PK_4 addition triggered efficient lipid mixing in contrast to the other two linear K₄ dimers (Figure 9A). The final lipid mixing efficiency of PK₄/CPE₄ is even higher than measured for CPK₄/CPE₄. The linear K₄ dimers cannot trigger membrane docking between CPE₄ modified liposomes due to their low lipid membrane affinity as a result of their tendency to form homodimers. In control experiments (Figure S2), a low fluorescence increase was observed when PK4 was added to plain liposomes (i.e. liposomes without CPE₄).

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Figure 9. Lipid mixing (A) and content mixing (B) assays. The y-axis represents the percentage fluorescence increase due to liposome docking or membrane fusion.

Next, a content mixing assay was performed. Liposomes were either loaded with sulphorhodamine-B (SRb) at a self-quenching concentration or contained only buffer. Both liposomes were modified with 1% CPE₄. Upon membrane fusion, content mixing results in SRb dilution and fluorescence dequenching. Efficient content mixing was obtained using PK4 while no fluorescence increase was observed for NLK₄ and CLK₄ (Figure 9B). Surprisingly, content mixing was significantly higher for PK₄/CPE₄ compared to CPK₄/CPE₄. As a control experiment (Figure S2), a leakage test was performed by mixing SRb liposomes with the different K₄ dimers. PK₄ induced liposome leakage but at a low level compared to the PK₄/CPE₄ induced content mixing. This is not surprising because PK₄ has a very high affinity for lipid membranes. When PK₄ was added to a liposome mixture containing both non-CPE4 modified liposomes (no dye) and SRb liposomes, the content mixing curve was slightly higher than the leakage control, revealing that even in the absence of CPE₄, PK₄ weakly induces liposome fusion, along with leakage. In contrast, no linear K₄ dimer was able to induce non-CPE₄ modified liposome fusion or leakage which might be due to the low membrane affinity or homodimer formation. This high membrane affinity of fusogenic PK_4 makes the PK₄/CPE₄ pair suitable for liposome-cell fusion.

7. Cell membrane labelling

Decorating cell membranes with lipopeptides was demonstrated in previous studies.^{37-38, 46} In order to confirm coiled-coil formation between K_4 dimers and lipopeptide- E_4 , and also to determine whether K_4 dimers interact with another E_4 peptide after combining with lipopeptide CPE₄, a cell membrane labelling assay

was performed (Figure 10A). K_4 dimers were added to CPE₄ modified Hela cells resulting in coiled-coil formation. Next, carboxyfluorescein labeled peptide E₄ (fluo-E₄) was added to probe binding to the K4 dimer at the cell surface.

In a control experiment, CPE_4 decorated cells were incubated with fluo-E₄ and no fluorescence was detected at the cell membrane (Figure S3A). Fluorescence was observed when the membrane labelling assay was performed using CPK₄ decorated cells + fluo-E₄ (Figure 10B), which is in line with the previous study.³⁸ The cell membrane with the highest amount of fluorescence was observed by performing the cell membrane labeling assay using PK_4 (Figure 10C), indicative of efficient coiled-coil interaction between PK₄ and E₄ on the membrane. Figure S3B shows that even for cells without CPE_4 decoration, the addition of PK_4 and fluo- E_4 resulted in some fluorescence at the cell membrane. However, the fluorescence intensity is significantly lower and the distribution is less homogeneous. The PK₄ peptides likely form particles in DMEM (Figure S4) which interact with the negatively charged cell membrane through electrostatic interactions. These particles also mediate the interaction between the CPE₄ labelled cell membrane and the fluorescent E_4 peptides, as in these images there also appears to be 'clusters' of fluorescence. In contrast, cell membrane labelling with NLK_4 resulted in only treated cells with very weak fluorescently labelled membranes (Figure 10D) while CLK₄ showed almost no fluorescent labeling (Figure 10E). In the control experiment, no cell membrane labelling was observed when the cell was not decorated with CPE₄ (Figure S3C & S3D). These results suggest that the linear dimeric K₄ peptides are unable to interact with CPE₄ at the cell membrane or fluo-E4. The tetramer-like homodimer formation of the linear K4 dimers could be responsible for the low membrane labelling. The hydrophobic face of the linear dimeric K_4 peptides is buried, which makes the interaction with peptide E_4 less favourable. Because CLK₄ forms more stable homodimers than NLK₄, the cell membrane labelling efficiency with CLK₄ was very low.

The cell membrane labelling assay reveals that PK_4 efficiently forms coiled coils with CPE_4 and free fluo- E_4 on the cell membrane. Due to homodimer formation, the linear K_4 dimer variants show very low cell membrane labelling affinity. Combined this study suggests that PK_4 is the best choice for future drug delivery applications.



Figure 10. (A) Scheme of cell membrane labelling using PK_4 as an example. (B) CPK_4 decorated Hela cell membrane labelling by adding fluo-E₄. CPE_4 decorated Hela cell incubated with PK_4 (C), NLK_4 (D) or CLK_4 (E), then fluorescent membrane labelling is obtained by adding fluo-E₄. Green channel: fluorescein. Scale bar: 30 µm.

8. Cell-liposome fusion - NBD/propidium iodide (PI) delivery

To investigate whether it is possible to use K_4 dimers to induce efficient cellliposome membrane fusion, a membrane fusion assay was performed (Figure 11A). Cells were decorated with CPE₄ and the K₄ dimers were added. Next, CPE₄ modified liposomes containing PI and labelled with NBD were added to induce cell-liposome membrane fusion. PI is a membrane-impermeable fluorescent dye, therefore delivery of liposomal propidium iodide (PI) into cells is a convincing indicator for the membrane fusion pathway.

Confocal imaging was applied to visualize cell-liposome fusion induced by the different coiled-coil peptides. Figure 11A shows the result of cell-liposome membrane fusion between CPK₄ decorated cells and CPE₄ modified liposomes. NBD fluorescence was observed at the membranes while PI stained the cytoplasm red (Figure 11B), which is consistent with previous results.³⁷ In this study, the highest PI delivery efficiency was obtained when CPE4 decorated cells were fused with CPE₄ modified liposomes induced by coiled-coil formation with the addition of peptide PK₄ (Figure 11C). In contrast, cell-liposome fusion using the linear K₄ dimers resulted in very low efficiency. With the NLK₄ peptide, only weak fluorescence was obtained both on the cell membrane and in the cytoplasm (Figure 11D), suggesting a low efficiency of cell-liposome fusion. Almost no membraneand cytoplasm-fluorescence was observed when using CLK_4 (Figure 11E). These results are consistent with the cell membrane labeling results which are because CLK₄ forms homodimers more readily and has a lower affinity for lipid membranes than NLK₄. In contrast, the PK_4 dimer forms coiled coils with the E_4 peptide efficiently and it has a high affinity for the cell membrane, resulting in highly efficient cell-liposome membrane fusion.

In control experiments, no fluorescence was observed in cells when plain liposomes (liposome contains no CPE₄) were used (Figure S5), revealing that coiled-coil formation plays an essential role in membrane fusion. In the absence of a K₄ dimer, fusion between cells and CPE₄ modified liposomes was not achieved (Figure S6A). However, fluorescent cells were observed when PK₄ was used in combination with CPE₄ modified liposomes, (Figure S6B), even without CPE₄ on the cell membrane. This can be explained by the fact that PK₄ assembles into positively charged particles (Figure S4) and is capable of interacting with negatively charged cell membranes. This makes it possible for CPE₄ modified liposomes to interact with PK₄ clusters through coiled-coil formation, resulting in

liposome uptake. There is also weak NBD staining on the cell membrane with high PI staining inside the cells, (Figure S6B). We suspect that, in this case, most liposomes enter the cells via endocytosis, thus NBD does stain the cell membrane homogeneously or strongly, but PI is released into the cytoplasm. Due to the low affinity of linear K₄ dimers for the lipid membrane, membrane fusion between non-CPE₄ decorated cells and CPE₄ modified liposomes was not observed (Figure S6C & 6D).



Figure 11. (A) Scheme of the cell-liposome fusion assay. (B) Cell-liposome fusion between CPK₄ decorated Hela cells and CPE₄ modified liposomes containing NBD and PI. Cell-liposome fusion between CPE₄ decorated Hela cells and CPE₄ modified liposomes triggered by different K₄ dimers, (C) PK₄, (D) NLK₄ and (E) NLK₄. Green channel: NBD, red channel: PI. Scale bar: 30 μ m.

CONCLUSION

In this study, three different K_4 dimers were designed, synthesized and characterized. CD measurements show that these K_4 dimers adopt different secondary structures in buffered solutions. The CD spectrum of PK4 suggests aggregation of the peptides while the linear K₄ dimers appeared to fold into typical helical structures. The three K₄ dimers show different affinities for the complementary peptide E_4 resulting in coiled coils with different thermostabilities. The DLS study revealed that PK₄ indeed forms particles in solution, confirming the non-helical CD spectra. In the DLS titration assay, peptide E_4 induces the PK₄ particles to dissociate by forming soluble heterodimeric coiled coils evidenced by the strong α-helical signal observed by CD spectroscopy. The linear K₄ dimers were found to self-associate by CD titration assays and this self-assembly behaviour influences heterodimeric coiled-coil formation with peptide E₄. Furthermore, these linear K₄ dimers have a low affinity for the lipid membrane and are also incapable of triggering efficient membrane fusion. Temperature-dependent CD studies reveal that the secondary structure of PK₄ changes with temperature and it adopts maximum helicity at 60 °C. The membrane affinity study of the K_4 dimers reveals that PK_4 has the highest lipid membrane affinity while linear K_4 dimers hardly interact with a lipid membrane, most likely due to homodimer formation. These results are consistent with the liposome membrane fusion results that show that the PK_4 - E_4 coiled-coil results in the highest efficiency in both lipid and content mixing, while almost no liposome membrane fusion was obtained using linear K_4 dimers. Because of the high affinity of PK_4 to lipid membranes, PK₄ induces some membrane fusion by itself, along with liposome leakage. These results confirm our hypothesis that the affinity properties of peptide K for cell membranes, which resulting in destabilization of the lipid membrane is pivotal for inducing efficient membrane fusion.

In cell assays, cell membrane labelling and cell-liposome fusion were achieved using the K₄-dimer/E₄ system. The highest level of NBD and PI delivery was obtained using the PK₄/CPE₄ combination, consistent with the liposome fusion assays. In contrast, weak membrane labelling and NBD-PI delivery were obtained using the NLK₄/E₄ coiled-coil pair, while no membrane labelling and NBD-PI delivery were achieved with CLK₄/E₄ coiled-coil. This can be explained by the peptide study showing that NLK₄ has a lower tendency to dimerize and a higher affinity for the lipid membrane than CLK₄. In summary, this study of K₄ dimer based self-assembly and coiled-coil formation, together with their properties for triggering membrane fusion could not only contribute to the design and development of coiled-coil peptide-based membrane fusion systems but also provides a more efficient system for potential drug delivery applications.

EXPERIMENTAL SECTION

Chemicals and materials

All chemicals were purchased from Sigma and used directly without further purification unless otherwise stated. All amino acids and HCTU were obtained from Novabiochem. All solvents, in addition to piperidine, trifluoroacetic acid (TFA) and acetic anhydride were purchased from Biosolve. Tentagel HL RAM resin was purchased from Iris Biotech GmbH. Oxyma pure was obtained from Carl Roth GmbH. All lipids were purchased from Avanti Polar Lipids. μ -Slide 8 Well confocal chambered coverslips were purchased from Ibidi. All cell culture supplies were purchased from Starstedt. Ultrapure water was obtained from Milli-QTM purification system from Millipore (Amsterdam, The Netherlands).

Peptides synthesis

All peptides were synthesized using Fmoc chemistry on a CEM Liberty Blue microwave-assisted peptide synthesizer. A Tentagel HL RAM resin (0.22 mmol/g) was used as the solid phase for peptide synthesis. The Fmoc group was removed with 20% piperidine in DMF by heating to 90 °C for 1 min. In the reaction, 5 eqv. of DIC and 5 eqv. Oxyma and 5 eqv. of amino acid were added to the reaction vessel and heated to 90 °C for 4 minutes. DMF was used as the solvent. All the peptides sequences are listed in Table S1. Except for the ones for synthesising lipidated and fluorescent peptides, all peptides were acetylated at N-terminus.

Synthesis of PK_4 : K₄GW-Cys14 and K₄-Cys14 were synthesized and purified by HPLC (see below). K₄GW-Cys14 (66 mg, 20 µmol) was dissolved in 15 mL of water and activated with 2,2'-Dithiobis(5-nitropyridine) (62 mg, 200 µmol) dissolved in 5 mL of acetone. For this, the K₄GW-Cys14 solution was added to the flask containing 2,2'-Dithiobis(5-nitropyridine) dropwise while stirring. The reaction was stirred overnight and turned yellow gradually. After filtration, the reaction mixture was dried under a N₂ flow. The crude peptide was dissolved in 20 mL water and purified by HPLC (see below) and lyophilized yielding a white K₄GW-Cys14-S-nitropyridine solid powder (50 mg, 14.5 µmol, yield: 72.5%).

Peptide K₄GW-Cys14-S-nitropyridine (20 mg, 5.8 μ mol) was mixed with peptide K₄-Cys14 (20 mg, 6.6 μ mol) and dissolved in 10 mL HEPES buffer (pH 8.1). The solution turned yellow gradually and after 30 minutes, the peptide was purified by direct injecting of the reaction mixture in HEPES into the HPLC (see below) and lyophilized to yield a white powder (22mg, 3.5 μ mol, yield: 59.8%).

Synthesis of NLK₄: CG-K₄GW and CG-K₄ were synthesized and purified. CG-K₄GW (70 mg, 20 μ mol) was dissolved in 15 mL of water in a flask and 5 mL 2,2'-Dithiobis(5-nitropyridine) (62mg, 200 μ mol) solution in acetone was added dropwise while stirring. After filtration, the reaction mixture was dried under an N₂ flow. The crude peptide was dissolved in 20 mL water and purified by HPLC (see below) and lyophilized yielding a white CG-K₄GW-S-nitropyridine solid powder (60 mg, 16.5 μ mol, yield: 82%). Peptide CG-K₄GW-S-nitropyridine (20 mg, 5.5 μ mol) was mixed with peptide CG-K₄ (20 mg, 6.2 μ mol) and dissolved in 10 mL HEPES buffer (pH 8.1). The solution turned yellow gradually and after 30 minutes, the peptide was purified by directly injecting the reaction mixture into the HPLC (see below), followed by lyophilization to yield a white powder (20 mg, 3 μ mol, yield: 54.2%).

Synthesis of CLK₄: K₄GW-GC and K₄-GC were synthesized and purified. CG-K₄GW (70 mg, 20 μ mol) was dissolved in 15 mL of water in a flask and 5 mL 2,2'-Dithiobis(5-nitropyridine) (62 mg, 200 μ mol) solution in acetone was added dropwise while stirring. After filtration, the reaction mixture was dried under an N₂ flow. The crude peptide was dissolved in 20 mL water and purified by HPLC (see below) and lyophilized yielding a white CG-K₄GW-S-nitropyridine solid powder (55 mg, 15,7 μ mol, yield: 78%). Peptide CG-K₄GW-S-nitropyridine (20 mg, 5.5 μ mol) was mixed with peptide CG-K₄ (20 mg, 6.2 μ mol) and dissolved in 10 mL HEPES buffer (pH 8.1). The solution turned yellow gradually and after 30 minutes, the peptide was purified by directly injecting the reaction mixture into the HPLC (see below) and lyophilized to yield a white powder (18 mg, 2.7 μ mol, yield: 48.8%).

Lipopeptide Synthesis

The synthesis of the lipopeptides (CPK₄ and CPE₄) is described in **Chapter 3**.

Fluo-K₄/Fluo-E₄ Synthesis

The synthesis of the fluorescent peptides (fluo-K₄ and fluo-E4) is described in

Chapter 3.

Peptide purification

All peptides were purified with reversed-phase HPLC on a Shimadzu system with two LC-8A pumps and an SPD-20A or MPD-20A UV-Vis detector. A Vydac C4 column (22 mm diameter, 250 mm length, 10 μ m particle size) was used for lipopeptides CPK₄ and CPE₄. A linear gradient from 20% to 80% acetonitrile (with 0.1% TFA) in water (with 0.1% TFA) was performed at a 12 mL/min flow rate over 36 mins.

All other peptides were purified using a Phenomenex Kinetix Evo C18 column (21.2 mm diameter, 150 mm length, 5 μ m particle size). The 'K' peptides, which include K₄, K₄-Cys14, K₄GW-Cys14, CG-K₄, CG-K₄-GW, K₄-GC, WG-K₄-GC, fluo-K₄ and all the three K₄ dimers were purified using a linear gradient from 20% to 45% acetonitrile (with 0.1% TFA) in water (with 0.1% TFA) with a 12 mL/min flow rate over 28 mins. The E4 and fluo-E₄ peptides were purified using a linear method gradient from 20% to 55% acetonitrile (with 0.1% TFA) in water (with 0.1% TFA) with a 12 mL/min flow rate over 28 mins. All peptides were characterized by LC-MS, see Figure S9-11 and Table S2.

CD Spectroscopy

CD measurements were performed on a JASCO J-815 CD spectrometer equipped with a Peltier temperature controller. All room temperature CD was carried out at 20 °C with a 2 mm path length quartz cuvette. Data points were recorded every 1 nm from 190 nm to 260 nm, with a 1 second response time, 2 nm bandwidth and 5 sequentially recorded spectra averaged at a scanning speed of 200 nm/min.

After the data were collected, the mean residue molar ellipticity was calculated using equation 1:

$$[\theta] = (1000 \times [\theta]_{obs})/(cnl) \quad (1)$$

Where $[\theta]$ is the mean residue molar ellipticity in deg·cm²·dmol⁻¹, $[\theta]_{obs}$ represents the observed ellipticity in mdeg, c is the concentration of peptide in mM, n represents the number of amino acids in the peptide and 1 is the path length of the cuvette in mm.

The percentage of helicity of the peptides (F_{helix}) can be calculated by equation 2:

$$F_{\text{helix}} = 100\% ([\theta]_{222} - [\theta]_0) / ([\theta]_{\text{max}} - [\theta]_0)$$
 (2)

In equation 2, $[\theta]_{222}$ represents the mean residue molar ellipticity of peptide at 222 nm, $[\theta]_0$ is the mean residue ellipticity of the peptide when the peptide is in an entirely random coil conformation, $[\theta]_{max}$ is the maximum theoretical mean residue ellipticity. The details for calculating $[\theta]_0$ and $[\theta]_{max}$ can be found in chapter 3.

Temperature-dependent CD measurements were performed with the same instrument, using the same cuvette. The melting curves of all peptides were obtained by recording the ellipticity at 222 nm from 5 °C to 95 °C with a 1 °C data pitch and with a 60 °C/h temperature ramp. The delay time for each data point was 2 seconds.

CD titration and nonlinear fitting for homodimer formation

The CD titration study was performed on the same JASCO J-815 CD spectrometer. The measurements were carried out at 20 °C with a 1 mm path length quartz cuvette. All the parameters are the same as described above except the low sensitivity detection mode was used in this study. The concentration of each linear K₄ dimer varied from 2.5 μ M to 150 μ M. The normalized mean residue molar ellipticity was calculated based on equation 1.

The non-linear fitting curve for the concentration-dependent CD titration used to determine homodimer formation was based on equation 3:

$$[\theta] = \frac{K_d \left(-1 + \sqrt{1 + \frac{8[P_t]}{K_d}}\right) \left\{2[\theta_m] + \left(-1 + \sqrt{1 + \frac{8[P_t]}{K_d}}\right) [\theta_d]\right\}}{8[P_t]} \qquad (3)$$

where $[\theta]$ represents the mean residue ellipticity in deg·cm²·dmol⁻¹, $[\theta_m]$ and $[\theta_d]$ represents the normalized mean residue molar ellipticity contributed by the peptide monomer or homodimer, K_d represents the dissociation constant and $[p_t]$ represents the total concentration of peptide. The K_a ($K_a = 1 / K_d$) of each K_4 dimer was calculated and summarized in Table 2.

Dynamic light scattering (DLS)

DLS measurements were performed on a Zetasizer Nano S (Malvern Instruments, Malvern, UK) equipped with a green laser (532 nm). All the derived count rate (DCR) measurements were performed in a low volume cuvette (Brand, Wertheim, Germany) by non-invasive backscatter mode (automatic mode), detecting the

scattered light at 175°. Except for the temperature-dependent DLS assay, data was collected in triplicate at 20 °C. All K₄ dimers were measured at a concentration of 10 μ M and K₄ at 20 μ M.

DLS titration

For the DLS titration assay (PK₄ as the example), a guest solution that contained 200 μ M of E₄ and 10 μ M of PK₄ was gradually added into the host solution containing 10 μ M of PK₄. During the titration assay, the concentration of PK₄ was kept at 10 μ M while the concentration of E₄ was varied from 0 to 20 μ M. In every titration, the DCR was measured after 3 minutes of incubation to stabilize the particles. The process for all the other titration assays is similar.

Temperature-dependent DLS

For temperature-dependent DLS measurements, data were collected in duplicate every 5 °C from 20 °C to 90 °C and then the temperature was decreased from 90 °C to 20 °C at a speed of 1 °C/min. In the fast temperature drop temperature-dependent DLS assay, the sample was slowly heated up from 20 °C to 90 °C (1 °C/min), then a DLS measurement was performed at 90 °C. Next, the sample was cooled from 90 °C to 20 °C over 2 min (~ 45 °C/min), the DCR was measured after 3 min of incubation.

Tryptophan fluorescence titration assay

Tryptophan fluorescence titration assays were performed in 96-well plates using a TECAN Infinite M1000 Pro microplate reader. Details of liposome preparation are described in the next section. For each titration assay, 2.5 μ M of the peptide was mixed with liposomes with a series of lipid concentrations ranging from 25 μ M to 4500 μ M. After mixing, the 96-well plate was incubated at room temperature for 60 min before fluorescence measurements were recorded. The fluorescence spectrum was obtained by recording the emission from 450 to 310 nm using an excitation wavelength of 275 nm.

The fluorescence of each measurement was normalized to the free peptide fluorescence intensity, *i.e.* in the absence of liposomes. Maximum tryptophan fluorescence was observed around 340 nm and used to obtain the titration curve. The nonlinear fitting and partition constant were based on equation 4^{43} .

$$F = 1 + (F_{max} - 1)(K_pX)/(55.3 + K_pX)$$
(4)

F is the normalized fluorescence and F_{max} is the maximum fluorescence when all peptide interacts with the lipid membrane. K_p is the molar partition coefficient. X represents the concentration of peptide in M and 55.3M is the assumed constant of water concentration at room temperature. The partition coefficient and the standard error of fitting was achieved using the least-squares method by fitting the experimental result to equation 4.

Lipopeptide Micelles and Liposome Preparation

Lipopeptide (CPK₄ or CPE₄) was dissolved in chloroform/methanol (1:1) at a concentration of 200 μ M. 50 μ L CPK₄ or CPE₄ stock solution was dried to a film in a 20 ml glass vial under N₂ flow. DMEM (1 mL) was added to the glass vials to rehydrate the lipopeptide film. The vials were sonicated in a sonication bath for 5 min at 55 °C to obtain lipopeptide micelle solutions (10 μ M).

The liposomes used for tryptophan fluorescence titration studies were prepared by the extrusion method. DOPC/DOPE/cholesterol (50/25/25%) was dissolved in chloroform/methanol (1:1) at a total concentration of 10 mM. 1 mL of this solution was dried to form a lipid film in a 20 ml glass vial under N₂ flow for 2 h, followed by incubation in a vacuum desiccator for 30 min. Lipid film rehydration in 1 mL of PBS was followed by vortexing at maximum speed for 30 s. Liposomes were obtained by extrusion using an Avanti mini extruder with 100 nm polycarbonate membranes at 55 °C. The liposomes were characterized by DLS and the average size was found to be 100 nm with a polydispersity index (PDI) below 0.2.

All liposomes used in liposome membrane fusion and cell-liposome fusion studies were prepared similarly. The list of liposomes and their lipid composition is shown in Table S3. The preparation of lipo-NBD-PI- E_4 liposomes is shown as an example. mixture containing DOPC/DOPE/cholesterol/DOPE-NBD The lipid (49.5/24.75/24.75/1%) was dissolved in chloroform/methanol (1:1) at a total lipid concentration of 10 mM. The lipid film was prepared in 20 mL glass vials by drying 100 μ L of lipid stock solution mixed with 100 μ L lipopeptide solution (1% mole ratio of total lipid) under N_2 flow for 2h. The glass vials were transferred to a vacuum desiccator for 30 min to remove the remaining solvent. A propidium iodide (PI) solution was prepared by dissolving 10 mg of PI in 1 mL hot PBS (60 °C). The lipid film was rehydrated by adding 1 mL of PI solution and vortexing at high speed for 30s. Liposomes were prepared using the sonication method by sonicating the lipid solution using a Branson 2510 bath sonicator for 3 min at 55 °C. G25 size-exclusion PD-10 Columns (GE-Healthcare, USA) were used to separate liposomes from the non-encapsulated PI. The quality of the liposome was determined by DLS and the average size was found to be around 100 nm with a PDI below 0.25. A 500 μ M liposome solution contains 1% DOPE-NBD, 1% CPE₄ and 10 mg/mL of encapsulated PI. The PI loaded and sulphorhodamine-B (SRb) loaded liposomes were purified using a G25 size-exclusion PD-10 column, all other liposomes were used without additional purification.

Liposome membrane fusion—lipid mixing and content mixing

For the lipid mixing assays, lipo-NBD/LR-E₄ and lipo-E₄ were mixed (1:1) at a total lipid concentration of 200 μ M. For each experiment, 200 μ L of the liposome mixture was transferred to a black 96 well-plate followed by the addition of K₄ dimer (50 μ l, 8 μ M). For the non-CPE₄ modified liposome control, lipo-NBD/LR and lipo-free were mixed (1:1) at a total lipid concentration of 200 μ M. 200 μ L of this liposome mixture was used and K₄ dimer (50 μ l, 8 μ M) was added before the measurement. For the positive control, 200 μ L of 200 μ M lipo-NBD/LR-positive was mixed with 50 μ L of PBS. For the negative control, 100 μ L of 200 μ M lipo-NBD/LR-E₄ was mixed with 150 μ L PBS. For the CPK₄-CPE₄ control, 100 μ L of 200 μ M lipo-NBD/LR-E₄ mixed 50 μ L of PBS, following by adding 100 μ L of 200 μ M Lipo-K₄. The NBD emission at 530 nm was measured every 20 s and followed over 1 h. The standard deviation was calculated by two independent samples, and the experiment was repeated two times.

For content mixing assays, the process and concentrations of all components were identical to the lipid mixing assays except that the membrane dye (NBD/LR) was replaced by the soluble dye (SRb). An additional liposome leakage control was performed, which 100 μ L of lipo-SRb was mixed with 100 μ L PBS followed by the addition of 50 μ L of 8 μ M K₄ dimers. SRb emission at 585 nm was measured every 20 s for 1 h. The standard deviation was calculated from two independent samples, and the experiment was repeated twice.

All fluorescence measurements were performed using a TECAN Infinite M1000 Pro microplate reader. The fusion efficiency of both lipid mixing and content mixing was calculated based on equation 5:

$$\%F_t = 100\% \times (F_t - F_0) / (F_{max} - F_0)$$
 (5)

Where $\%F_t$ is the percentage of fluorescence increase, F_t is the fluorescence

intensity at a specific time t, F_{max} is the fluorescence intensity from the positive control at the same time t and F_0 is the fluorescence intensity from negative control at the same time.

Cell labelling

 5×10^4 Hela cells were seeded in ibidi µ-Slide 8 well plates with 200 µL of DMEM for 24h at 37 °C in a 7% CO₂ atmosphere. DMEM was removed from the cell culture and then 200 µL of CPK₄ or CPE₄ (10 µM) solution was added to each well containing cells and incubated for 1.5 h. After the lipopeptide was washed away, 200 µL of different K₄ dimers (10 µM in DMEM) was added and incubated for 10 min. The K₄ dimers solution was removed and the cells were washed three times with DMEM. Then fluo-E₄ (200 µL, 20 µM in DMEM) was added to the wells. After 15 min incubation, all wells were thoroughly washed using DMEM before imaging using confocal microscopy.

Cell-liposome fusion assay—PI delivery

 5×10^4 Hela cells were seeded in ibidi μ -Slide 8 well plates with 200 μ L of DMEM at 37 °C in a 7 % CO₂ atmosphere. After 24h, the medium was removed and cells were washed with fresh DMEM, following by the addition of 200 μ L CPK₄ (10 μ M) or CPE₄ (10 μ M) and incubated for 1.5 min. After excess lipopeptide was washed away, 200 μ L of the K₄ dimer (10 μ M in DMEM) was added and incubated for 10 min. Liposomes in PBS were diluted with DMEM to reduce the toxicity of cells by buffer (PBS < 50 %). After the cells were washed three times with DMEM, CPE₄decorated liposomes (200 μ L, [lipid] = 250 μ M) containing PI and labelled with NBD were added and incubated for 15 minutes at 37 °C. Next, the liposomes were removed and the cells were washed three times with DMEM. Confocal microscopy imaging was performed after the cells were incubated for another 30 min.

Confocal Microscopy

Confocal imaging was performed using a Leica TCS SP8 confocal laser scanning microscope with the Leica application suite advanced fluorescence software (LAS AF, Leica Microsystems B.V., Rijswijk, The Netherlands). The fluo-K₄, fluo-E₄ and NBD fluorescence was excited by a laser at 488 nm and the emission was detected from 495 nm to 550 nm. The PI fluorescence was excited by a laser at 535 nm and the emission was detected from 600 nm to 700 nm. For each set of experiments, all settings were kept the same.

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APPENDIX 4



Figure S1. Synthetic route of K4 dimers, (A) PK4, (B) NLK4 and (C) CLK4.

Table S1. Sequences of monomer peptides used in this work

Dontidosª	Sequence					
reputes	ef	gabcdef	gabcdef	gabcdef	gabcdef	ga
K4		KIAALKE	KIAALKE	KIAALKE	KIAALKE	${\tt GW-NH}_2$
\mathbf{E}_4		EIAALEK	EIAALEK	EIAALEK	EIAALEK	$GW-NH_2$
K ₄ -Cys14		KIAALKE	KIAALK <mark>C</mark>	KIAALKE	KIAALKE	$-NH_2$
K ₄ GW-Cys14		KIAALKE	KIAALK <mark>C</mark>	KIAALKE	KIAALKE	$GW-NH_2$
CG-K ₄	<mark>C</mark> G	KIAALKE	KIAALKE	KIAALKE	KIAALKE	$-NH_2$
CG-K ₄ GW	<mark>C</mark> G	KIAALKE	KIAALKE	KIAALKE	KIAALKE	$GW-NH_2$
K ₄ -GC		KIAALKE	KIAALKE	KIAALKE	KIAALKE	G <mark>C</mark> -NH ₂
WG-K ₄ GC	WG	KIAALKE	KIAALKE	KIAALKE	KIAALKE	G <mark>C</mark> -NH ₂
Fluo-E4	<mark>Fluo</mark> -GG	KIAALKE	KIAALKE	KIAALKE	KIAALKE	$GW-NH_2$

^aAll peptides except the ones for synthesising lipodated and fluorescent peptides were acetylated at N-terminus.

Peptide	Mass (calcd.) / Da	Mass (found) / Da
	$[M + 5H]^{5+}$ 1268.2	1267.5
PK_4	$[M + 4H]^{4+}$ 1585.0	1584.3
	$[M + 3H]^{3+} 2113.0$	2113.0
	$[M + 5H]^{5+}$ 1342.4	1341.8
NLK_4	$[M + 4H]^{4+}$ 1677.8	1677.2
	$[M + 3H]^{3+} 2236.7$	2237.5
	$[M + 5H]^{5+}$ 1342.4	1341.8
CLK_4	$[M + 4H]^{4+}$ 1677.8	1677.9
	$[M + 3H]^{3+} 2236.7$	2237.5

Table S2. Calculated mass and found mass via LC-MS of all K4 dimers.



Figure S2. Control experiments from lipid mixing (A) and content mixing (B) assays.



Figure S3. Control experiments from cell membrane labeling. Scale bar: 30 µM.



Figure S4. DCR results of K4 monomer and K4 dimers in DMEM.



Figure S5. Control experiments from cell-liposome fusion. CPE_4 decorated cells with non-CPE₄ liposomes. Scale bar: 30 μ M.



Figure S6. Control experiments from cell-liposome fusion. Cells were not decorated with CPE_4 and liposomes were modified with CPE_4 . Scale bar: 30 μ M.

Studies	liposome	Lipid composition	Content	Purification
	lipo- NBD/LR- positive	DOPC/DOPE/cholesterol/NBD/LR (49.75/24.87/24.87/0.25/0.25%)		No purification
	Lipo- NBD/LR- E ₄	DOPC/DOPE/cholesterol/NBD/LR/CPE4 (49/24.5/24.5/0.5/0.5/1%)	-	No purification
Lipid mixing	Lipo- NBD/LR	DOPC/DOPE/cholesterol /NBD/LR (49.5/24.75/24.75/0.5/0.5%)	-	No purification
	Lipo-E4	DOPC/DOPE/cholesterol/CPE ₄ (49.5/24.75/24.75/1%)	-	No purification
	Lipo-K ₄	DOPC/DOPE/cholesterol/CPK4 (49.5/24.75/24.75/1%)	-	No purification
	Lipo-free	DOPC/DOPE/cholesterol (50/25/25%)	-	No purification
	Lipo-SRb-	DOPC/DOPE/cholesterol	10mM	G25 size-
	positive	(50/25/25%)	SRb	exclusion
	Lipo-SRb-	DOPC/DOPE/cholesterol/CPE4	20mM	G25 size-
	E4	(49.5/24.75/24.75/1%)	SRb	exclusion
Content mixing	Lipo-SRb	DOPC/DOPE/cholesterol (50/25/25%)	20mM SRb	G25 size- exclusion
	Lipo-E ₄	DOPC/DOPE/cholesterol/CPE4 (49.5/24.75/24.75/1%)	-	No purification
	Lipo-K4	DOPC/DOPE/cholesterol/CPK4 (49.5/24.75/24.75/1%)	-	No purification
	Lipo-free	DOPC/DOPE/cholesterol/CPE ₄ (49.5/24.75/24.75/1%)	-	No purification
Cell- liposome fusion assay	Lipo- NBD-PI- E4	DOPC/DOPE/cholesterol/CPE ₄ (49.5/24.75/24.75/1%)	10 mg/mL PI	G25 size- exclusion
	Lipo- NBD-PI	DOPC/DOPE/cholesterol/CPE4 (49.5/24.75/24.75/1%)	10 mg/mL PI	G25 size- exclusion

Table S3. Liposomes used for liposome membrane fusion and cell-liposome membrane fusion.













Figure S11. LC-MS spectrum of CLK₄.