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CHAPTER 2

Effect of the linker region on the fusogenicity of a membrane fusion model system

Keywords: Peptides • membrane fusion • liposomes • coiled-coils

Abstract

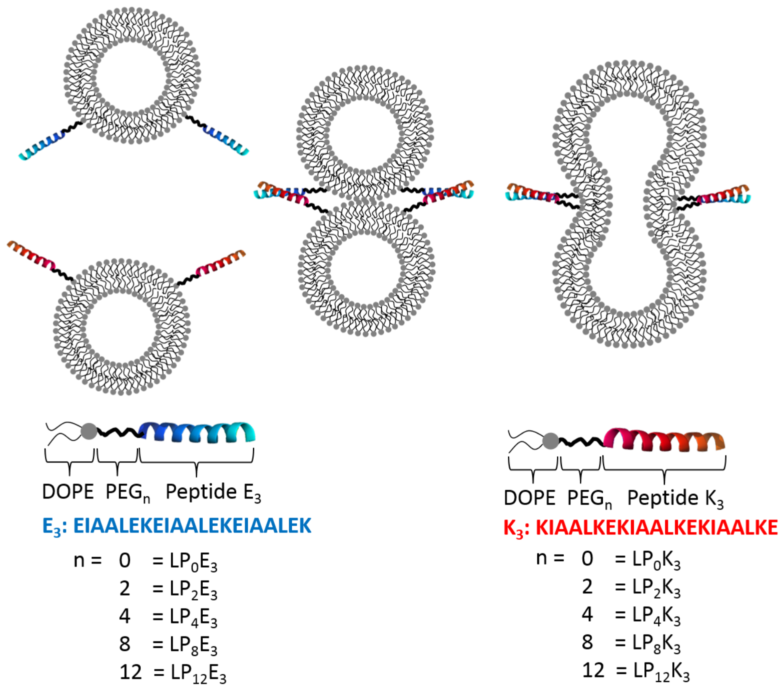
Liposome-Liposome fusion is an effective tool to study natural SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) mediated membrane fusion. To mimic this naturally occurring phenomenon, a model system has been developed consisting of a heterodimeric coiled-coil pair conjugated to a phospho-lipid anchor via a flexible poly(ethylene glycol) (PEG) linker. These peptide amphiphiles are tethered into the lipid bilayers of liposomes. When two batches of liposomes, modified with one of the complementary peptides are mixed, fusion occurs. In this chapter, the influence of the flexible PEG linker length on the Liposome-Liposome fusion efficiency is studied. Lipopeptides in which the PEG linker length is varied (0, 2, 4, 8 and 12 ethylene glycol units) were synthesized. The efficiency of liposome-liposome fusion was determined using dynamic light scattering (DLS), lipid mixing and content mixing assays. Our studies show that the linker has critical role in membrane fusion and PEG_4 provides optimum rate of fusion for the mentioned model system.

Introduction

Membrane fusion is a vital physiological process as it controls the transport of chemicals in cells and is a source of inspiration for supramolecular chemists. Fusion proceeds with molecular recognition between complementary elements present on the membranes. This interaction brings the two opposing membranes in close proximity resulting in the merging of the membranes.[1] Fusion of membranes has been studied intensively using either naturally occurring proteins or supramolecular model systems. For example, in order to achieve membrane fusion in model systems, metal ion co-ordination,[2] carbohydrates,[3] zwitterion dimerization,[4] peptides,[5] [6] cyanuric acid–melamine[7] and diol-boronic acid [8] as molecular recognition units as well light responsive systems[9] have been used.

In nature, the highly conserved class of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins play a crucial role in many non-viral membrane fusion events.[10] SNARES are membrane-bound proteins comprised of three functional domains; a tetrameric coiled coil motif is connected via a flexible linker region to a transmembrane domain. Even though SNARE mediated fusion has been studied intensively, the detailed mechanism of membrane fusion at the molecular level is still under debate.[11] Using the bottom-up approach, several groups have attempted to design SNARE protein mimics[12] using either pH as a stimulus,[13] DNA zipper recognition,[14] a PNA binding motif [15] or a fusogenic peptide based motif. [16] These SNARE mimicking motifs were anchored on the membrane either through lipids like stearic

acid[13a] cholesterol,[14a, 14b] phospholipids[14c, 14d, 16-17] or transmembrane domain peptides.[15, 18] All these studies predominantly focused on mimicking the binding motif or anchoring these motifs on lipid membranes, however there are only a few reports studying the role of the linker region.[14d, 19]



Scheme 1: *Liposome-liposome fusion mediated by membrane anchored lipopeptides, along with an overview of the lipopeptides used in this study. Upon mixing of* LP_nK_3 *(red) or* LP_nE_3 *(blue) decorated liposomes, coiled-coil formation between peptide* K_3 *and peptide* E_3 *brings the opposite membranes in close proximity leading to a membrane fusion. The amino acid sequence of* E_3 *and* K_3 *is shown, n denotes the number of ethylene glycol units in the linker region and L denotes DOPE lipid.*

Design and Synthesis

Our group has developed a reduced SNARE model composed of three functional domains. It is based on a small heterodimeric coiled-coil motif anchored to a phospholipid on the membrane of a liposome via a flexible poly(ethylene glycol), i.e. PEG linker (**Scheme 1**).[17] this modular approach enables a systematic study of the contributions of the individual functional domains on the process of membrane fusion. Varying the number of heptad repeats of the peptides forming the coiled coil binding motif[20] as well as changing hydrophobic anchors has a significant influence on the fusion efficiency.[21] In this chapter, the influence of the PEG linker length on fusion efficiency is studied. Other studies using DNA

and natural SNAREs fragments acting as the linker region have been studied for their effect on the extent of fusion.[14d, 19] Both studies showed that the addition of a linker region between the recognition unit and the membrane anchor resulted in a decreased fusion efficiency. Here we will show that the length of the linker region has a strong influence on the fusion efficiency in our model system as well.

The coiled-coil forming peptide pair consisting of $E_{3,}$ with the amino acid sequence $(EIAALEK)_{3,}$ and peptide K_3 with the sequence $(KIAALKE)_3$ was chosen as the recognition unit. Coiled-coil formation between these complementary peptides results in the assembly of a heterodimeric complex is driven by hydrophobic as well as electrostatic interactions between the charged side chains of the peptides.

To anchor the coiled coil binding motif on the membrane, the phospholipid 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE) was used as a trans-membrane domain. To study the influence of the length of a flexible poly(ethylene glycol) linker (PEG) on the efficiency of liposome-liposome fusion, lipopeptides LP_nK_3 and LP_nE_3 in which the number of ethylene glycol repeat units was varied between n= 0 and 12, were synthesized.

Results and Discussion

The peptides K_3 and E_3 were synthesized with automated standard solid phase peptide synthesis protocols using Fmoc chemistry. After coupling of the final amino acid, the resin was removed from the reaction vessel and Fmoc-NH-PEG_n-COOH was coupled manually to the immobilized peptides on the resin and subsequently the Fmoc group was removed. The PEG component was comprised of 2, 8 or 12 repeating units. For the synthesis of the tetra(ethylene glycol) linker, N_3-PEG_4-COOH was coupled to the resin and the azide was subsequently reduced using trimethylphosphine to obtain the free amine. Next, succinic anhydride was coupled to the n-terminus of the PEG-peptides in the presence of a base. The DOPE anchor was finally coupled to the succinate group by activation of the free acid group using HOBt/DIC in DMF:DCM (1:1) and an overnight reaction with DOPE. The lipopeptides were cleaved from the resin and subsequently purified using reversed phase chromatography yielding purities >95%. For the membrane fusion assays, lipopeptides were incorporated in liposomes composed of DOPC, DOPE and cholesterol (CH) with a molar ratio of 2:1:1. Liposomes were obtained by hydrating a lipid film containing mentioned lipid composition and 1 mol % of either LP_nK_3 or LP_nE_3 in a buffered solution followed by sonication at 50 °C for 5 min.[17] Dynamic light scattering (DLS) measurements revealed that the liposomes were typically 100 nm in diameter and stable for at least 24h.

Next, the effect of the PEG linker length on lipopeptide mediated membrane fusion was studied using lipid and content mixing assays as well as DLS

measurements. Mixing of LP_nK_3 and LP_nE_3 functionalized liposomes will led to an immediate molecular recognition between the complementary peptides K_3 and E_3 and thereby forcing the opposing membranes in close proximity resulting in docking and subsequent fusion. As a result, the size of the resulting assemblies increases and this process was quantified using DLS measurements. When only one of the lipopeptides was present on the liposomal membranes no increase in the hydrodynamic diameter was observed, showing that coiled coil formation is critical to drive fusion. In contrast, when liposomes functionalized with LP_nK_3 and LP_nE_3 were mixed, a strong increase in size was observed for linkers PEG_8 and PEG_{12} due to liposome docking and fusion events (**Figure 1**). Interestingly, the linker PEG_2 and linker PEG_4 showed a size increase up to ~ 160 nm and remained stable throughout the experiment suggesting that no significant aggregation occurred. Surprisingly, with lipopeptides lacking a linker (PEG_0), no increase in hydrodynamic diameter was observed indicating that fusion did not occur.

To confirm that the observed size increase is a result of membrane fusion and not just a result of docking events and without merging two opposing membranes, a lipid mixing assay was conducted. For this, LP_nK_3 liposomes with an average size of 100 nm were decorated with the Förster resonance energy transfer (FRET) pair composed of the donor dye N-(7-nitrobenz-2-oxa-1,3-diazol-4-YL)-dioleoyl-phosphatidyl-ethanolamine (DOPE-NBD) and the acceptor dye lissamine rhodamine-phosphatidyl-ethanolamine (DOPE-LR).[22] Upon mixing with plain liposomes negligible fluorescence increase was observed (**Figure A2**). However, upon mixing with LP_nE liposomes, a significant increase in the NBD fluorescence was observed due to an increase in the average distance of the FRET pair indicative of lipid mixing between the LP_nK_3 liposomes and the LP_nE_3 liposomes. The lipid mixing assay shows that the PEG_4 linker displays the highest efficiency in lipid mixing whereas all other PEG linkers with a longer length gave slightly weaker lipid mixing efficiency. Surprisingly, when lipopeptides were used that did not have a linker (i.e. LP_0K_3 and LP_nE_3), hardly any fusion could be detected (**Figure 2**). The observed low efficiency for the linker PEG_0 could be due to reduced molecular recognition between K_3 and E_3 which indicates that the linker region has a significant role in achieving efficient coiled coil formation in this model system (**Table 1**).

To determine that the observed lipid mixing is not due to hemi fusion which is an intermediate step in the fusion process, we performed a content mixing assay (**Figure 3**). The dye sulphorhodamine B was encapsulated at a self-quenching concentration (20 mM) in liposomes decorated with LP_nE_3. Non-encapsulated dye was separated from the liposomes using a sephadex G50 column. Upon fusion of sulphorhodamine B loaded LP_nE_3 liposomes with LP_nK_3 liposomes an increased fluorescence is observed due to the dilution of the fluorescent dye and concomitant relief of self-quenching. [18, 23] In the content mixing assay, the

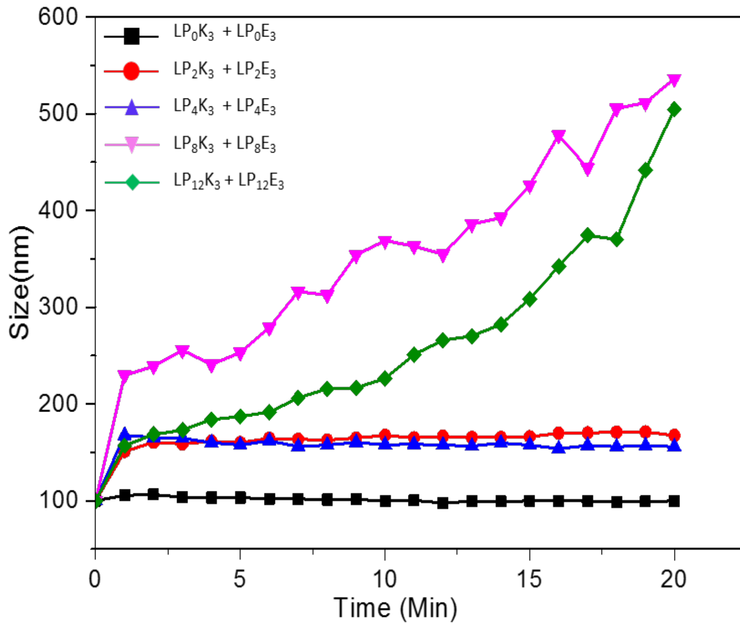


Figure 1: *The hydrodynamic diameter determined by DLS as a function of time and PEG-linker length. At t=0 minutes, E_3-decorated liposomes were mixed with K_3-decorated liposomes in equimolar amounts. The total lipid concentration was 0.5 mM, with 1 mol% of lipopeptide, in PBS, pH 7.4.*

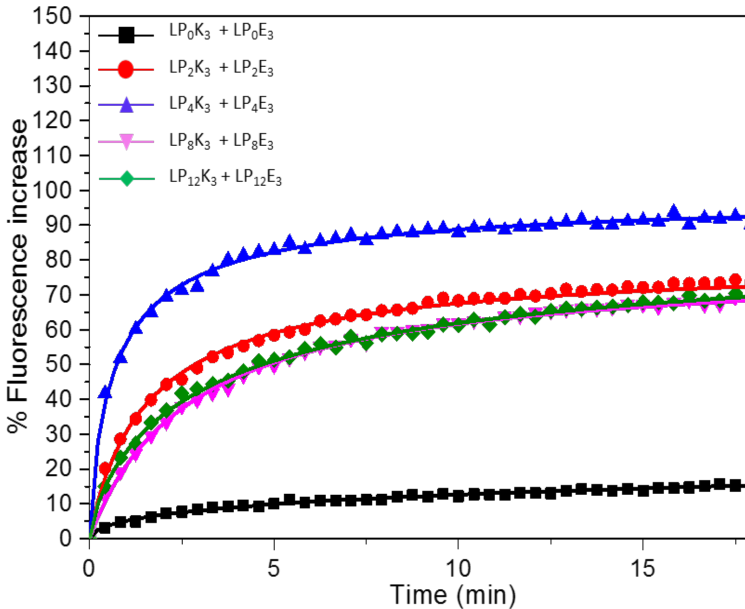


Figure 2: *Lipid mixing between E_3 and K_3 decorated liposomes as indicated by the NBD emission increase. Total lipid concentrations were 0.1 mM, with 1 mol% lipopeptide in PBS, pH 7.4.*

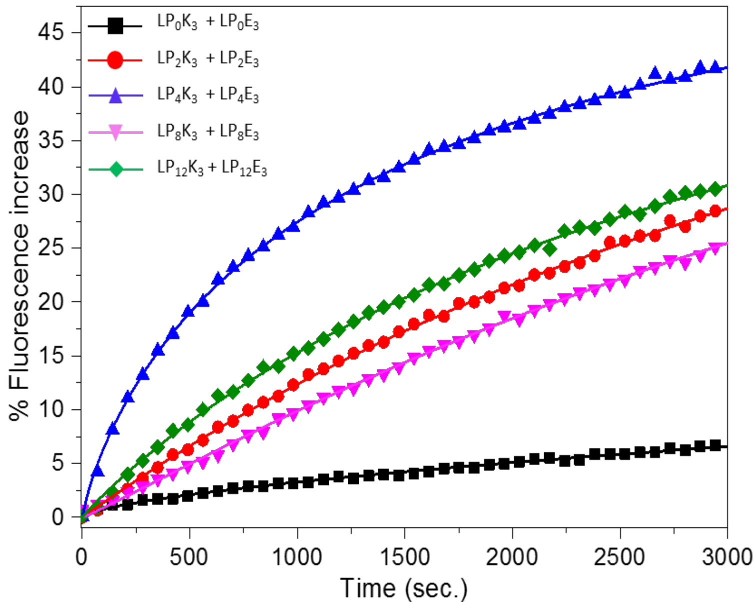


Figure 3: *Content mixing between E_3 and K_3 decorated liposomes as indicated by an increase in sulforhodamine emission. Total lipid concentrations were 0.1 mM, with 1 mol% lipopeptide in HEPES.*

same trend as for the lipid mixing assay was observed confirming that full fusion *i.e.* the mixing of the liposomal content has occurred. To ensure, that the obtained results are due to the heterodimerisation of the peptide "K_3" and "E_3"; control experiments were conducted. Plain liposomes were mixed with sulphorhodamine B containing LP_nE_3 liposomes and little or no fluorescence increase was observed (**Figure A3**) revealing that the fusion occurs only when coiled coil interaction between E_3 and K peptides is occurs.

Next, the effect of PEG linker length on the secondary structure of the membrane anchored lipopeptides was investigated to better understand the observed differences in the lipid- and content mixing assays. Circular Dichroism (CD) studies of the lipidated peptides in liposomes are shown in **Figure A4** and the main results are summarized in **Table 1**. We observed that all the lipopeptide pairs used in this study are able to form a coiled-coil complex. However, there are significant differences observed between the percentages of α-helicity at post fusion state. For PEG linkers with n = 4, 8 and 12, similar α-helicities in postfusion state (i.e. after mixing LP_nK_3 liposomes with LP_nE_3 liposomes) were observed, but the extent of fusion was different. This effect is presumably due to the fact that the distance between two membranes upon coiled coil formation is crucial in order to have efficient lipid and content mixing which is in good agreement with other previous studies performed in other model systems.[19, 24]

Table 1: Circular dichroism (CD) data for LP_nK_3 and LP_nE_3 lipopeptides bearing liposomes

PEG Linker	LP_nK_3		LP_nE_3		LP_nK_3+ LP_nE_3	
(n)	$[\theta]_{224}/[\theta]_{211}$	%H	$[\theta]_{224}/[\theta]_{211}$	%H	$[\theta]_{224}/[\theta]_{211}$	%H
0	0.95	31.5	0.91	32	1.02	32.9
2	0.96	31.4	1.08	25	1.07	31.9
4	0.96	41.4	0.95	31.9	1.02	46.8
8	1.01	49.4	0.94	28.2	1.1	47.2
12	1.01	37.01	0.97	36.9	1.07	45.5
Only Peptide	0.44	20.9	0.43	22.4	0.97	65.5

Ellipticity ratios using minima (~222/208 nm) for E_3-decorated liposomes and K_3-decorated liposomes in 1:1 mixtures, as measured with circular dichroism. The total lipid concentration was 0.5 mM, with 1 mol% lipopeptide, in PBS.

Boxer and co-workers used short DNA binding motifs as the recognition unit in a membrane fusion model system to study the role of the linker region on the fusion process.[14d] It was shown that the addition of a poly-T linker to the membrane proximal region reduces the efficiency of lipid and content mixing. This study has shown that a specific length of the linker (i.e. PEG_4) yields the highest efficiency in lipid and content mixing in our coiled-coil based model. It is difficult to proof what the exact role of the linker might be. It could be that the linker provides conformational flexibility for the lipidated peptides in order to bind to its complementary peptide at an opposing membrane. With longer linkers (i.e. PEG_8 or PEG_{12}) the distance between the two liposome membranes increases and/or the steric hindrance increases due to favorable peptide-PEG interactions results in a lower membrane fusion efficiency.[25] Future efforts will be dedicated to investigating and formulating a detailed understanding of the linker region by comparing with natural SNARE linkers.

Conclusion

In our membrane fusion model system, the length of the flexible PEG-linker is critical and significantly influences the kinetics and the final yield of liposomal fusion as shown by the differences in the DLS measurements, lipid mixing and content mixing assays. From this study it is clear that the tetra(ethylene glycol) linker is the optimal length in combination with the DOPE anchor to obtain efficient fusion between liposomes. A longer linker reduces the fusion efficiency,

while when the linker is too short or absent the ability of coiled coil formation is lowered resulting in low rates of membrane fusion. PEG_4 linker aids an optimal membrane fusion between liposomes due to an efficient coiled coil formation and providing a most favorable distance between two opposing membranes, which give rise to less aggregation and better rate of membrane fusion.

Appendix

Abbreviations

CD, circular dichroism; CH, cholesterol; DIPEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; NMP, N-Methyl-2-Pyrrolidone; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphatidyl-ethanol-amine; DOPE-LR, 1,2-dioleoyl-sn-glycero-3-hosphatidylethanolamine-lissamine-rhodamine B; DOPE-NBD, 1,2-dioleoyl-sn-glycero-3-phospoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt); Fmoc, fluorenylmethoxycarbonyl; DIC, N,N'-Diisopropylcarbodiimide; HCTU, 1H-Benzotriazolium 1-[bis(dimethylamino)methylene]-5chloro-hexafluorophosphate (1-),3-oxide; MALDI-TOF, matrix-assisted laser desorption-ionization time-of-flight; RP-HPLC, reversed-phase high-pressure liquid chromatography; TEA, triethyl amine; TFA, trifluoro-acetic acid; TIS, triisopropylsilane; PEG, polyethylene glycol.

Materials

Amino acids Fmoc-Lys(Boc)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH and Fmoc-Ala-OH as well as DMF, DCM and NMP (peptide synthesis grade) were purchased from Biosolve. trimethylphosphine and the lipids DOPE, DOPC and Cholesterol were obtained from Sigma-Aldrich while the fluorescent lipids, DOPE-NBD and DOPE-LR were purchased from Avanti Polar Lipids. Fmoc-NH-$(PEG)_2$–COOH (mw 385.42 g/mol), Fmoc-NH-d$(PEG)_8$–COOH (mw 663.75 g/mol) and Fmoc-NH-$(PEG)_{12}$–COOH (mw 839.98 g/mol) linkers were purchased from Iris biotech. N_3-PEG_4-COOH (mw 279 g/mol) was synthesized as described previously.[26] Milli-Q water with a resistance of more than 18.2 mΩ/cm was provided by a Millipore Milli-Q filter system with filtration through 0.22 µM millipak filter. Phosphated buffered saline, PBS: 5 mM KH_2PO_4, 15 mM K_2HPO_4, 150 mM NaCl, pH 7.4

Lipopeptide synthesis

Peptide synthesis was performed with a Biotage Syro I on a 100 µM scale using Rink amide resin with a degree of loading of 0.62 mmol/g. In each coupling reaction, 4 eq. of amino acid, 4 eq. of HCTU and 8 eq. of DIPEA were dissolved in 2 mL of DMF and added to the resin for 45 min. Fmoc deprotection was carried out using 2 cycles consisting 40 vol% piperidine and 20 vol% piperidine in DMF for 2 and 15 minutes respectively. The N-terminal free amine was used to couple the PEG linker through standard solid phase chemistry using 1.25 eq of Fmoc-PEG_n-COOH, 3 eq. of DIC and 3 eq. of HOBt in 3 ml DMF.

N_3-PEG_4-COOH was synthesized and utilized using procedure described earlier.[26c] The N-terminal free amine of resin was used to couple N_3-PEG_4-COOH,

by adding 2 eq. of N_3-PEG_4-COOH, 3 eq. of HOBT and 3 eq. of DIC in 3 ml DMF. Reaction was left over the shaker for 18 h. After several resin washes with DMF and DCM, the azide functionality was reduced to an amine with the aid of 10 eq of trimethylphosphine in 7 ml dioxane/water (6:1 v/v). After 2 h the reactants were removed from the reaction vessel and the procedure was repeated one more time to obtain N-terminal free amine. Afterwards, several washes with dioxane/ water and DMF were carried out.

Except for N_3-PEG_4-COOH containing lipopeptides, Fmoc deprotection of the PEG component was achieved using 20 vol% piperidine in DMF for 20 min to obtain an N-terminal free amine. Further, succinic anhydride was coupled to the N-terminal free amine using 10 eq. of succinic anhydride and 10 eq. TEA in 7 ml NMP. The reaction was left overnight on a shaker. The resin was washed thoroughly with NMP, DCM, and NMP to remove excess of reagents. Next, the resin was activated using 4 eq. of HOBt and 4 eq. of DIC in 2 ml of DCM:DMF (1:1, v/v) for 1hr. In separate vial, 2 eq. of DOPE was dissolved in 2 ml of DCM:DMF (1:1, v/v) containing 2 eq. of TEA. After 1 hr, the DOPE solution was added to the resin and gently heated to ~55 ^{0}C for 10 min to achieve efficient coupling and then the reaction was left shaking overnight at room temperature. Next, the resin was washed thoroughly with DMF and DCM to remove excess reactants. The lipopeptide was cleaved from the resin and concomitantly deprotected for 3 hours in 7 ml of a TFA:TIS:Water (95%:2.5%:2.5%) mixture and resulting mixture was co-evaporated with toluene on the rotary evaporator to ensure complete removal of TFA to obtain crude lipopeptides. Amount of crude lipopeptide was determined and dissolved in water: acetonitrile: tert-butanol (1:1:1, v/v/v) to obtain 20 mg/ml lipopeptide solution which was further injected in HPLC for purification.

RP-HPLC was performed with a Shimadzu HPLC system with two LC-8A pumps, and an SPD-10AVP UV-VIS detector. Sample elution was monitored by UV detection at 214 nm and 256 nm. Samples were eluted with a linear gradient from A to B, A being 10 % (v/v) acetonitrile, 0.1 % (v/v) TFA in water, and B being 90 % (v/v) acetonitrile, 0.1 % (v/v) TFA in water. Purification of the lipopeptides was performed on a Vydac C4 reversed phase column (214TP1022, 22 mm diameter, 250 mm length, 10.00 μm particle size) with a flow rate of 20 mL/min. Collected fractions were tested for >95% purity using LC-MS with a Gemini C18 column, lyophilized and to store at -20 ^{0}C.

LC-MS analysis

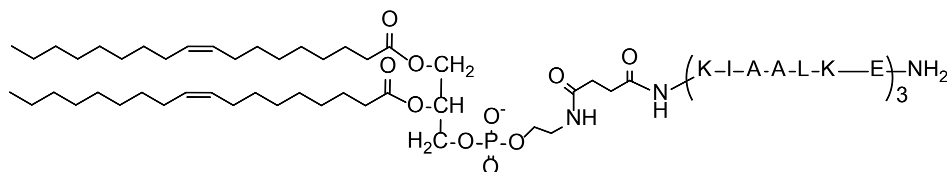
Purity was confirmed using LC-MS analysis equipped with Gemini 3μ C18 column coupled with Finningan LCQ advantage max (Thermo) ESI-MS analyzer. 0.1%TFA

containing acetonitrile/water was used as a mobile phase. The following gradient (Table A1) was used with a flow rate of 1ml/min.

Table A1: *Gradient used in LCMS analysis of peptides and lipopeptides.*

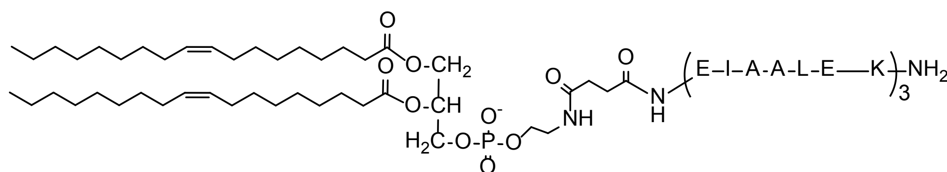
Time (min)	Gradient
0-2	10% (v/v) acetonitrile in water (0.1% TFA)
2-12	10%-90%(v/v) acetonitrile in water (0.1% TFA)
12-13	90%(v/v) acetonitrile in water (0.1% TFA)
13-15	10%(v/v) acetonitrile in water (0.1% TFA)

1. LP_0K



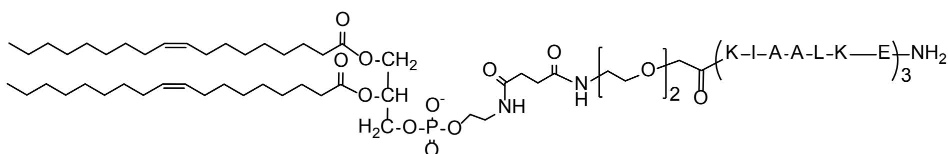
Chemical Formula: $C_{150}H_{271}N_{29}O_{37}P^-$ [3103.96]
Retention time: 10 min
LC-MS: Calcd. [1552.9, $M+2H]^{+2}$, Found 1553.01

2. LP_0E



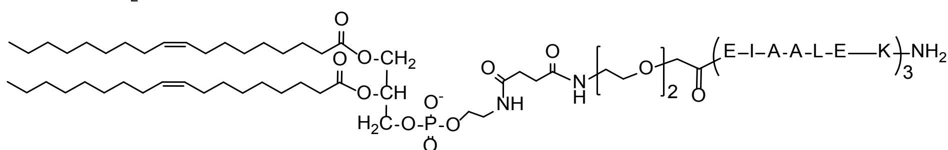
Chemical Formula: $C_{147}H_{256}N_{26}O_{43}P^-$ [3106.78]
Retention time: 12 min
LC-MS: Calcd. [1554.39, $M+2H]^{+2}$, Found 1563.44, $[H+NH_4]^{+2}$

3. LP_2K



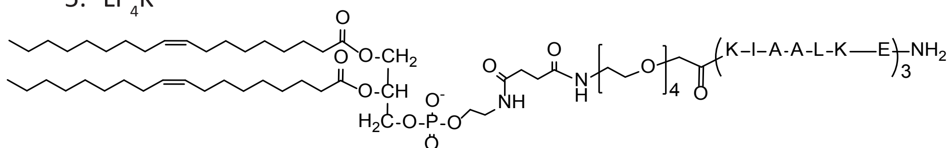
Chemical Formula: $C_{156}H_{282}N_{30}O_{40}P^{-}$ [3249.12]
Retention time: 10min
LC-MS: Calcd. [1084.04, $M+2H]^{+2}$, Found 1084.03

4. LP_2E



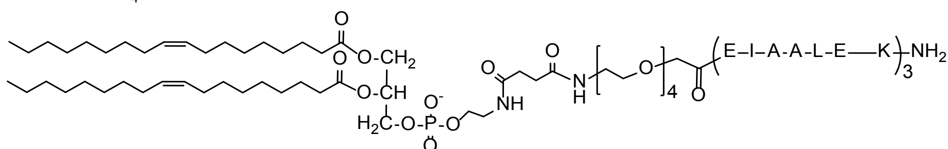
Chemical Formula: $C_{153}H_{267}N_{27}O_{46}P^{-}$ [3251.94]
Retention time: 12 min
LC-MS: Calcd. [1084.98, $M+2H]^{+2}$, Found 1084.98

5. LP_4K



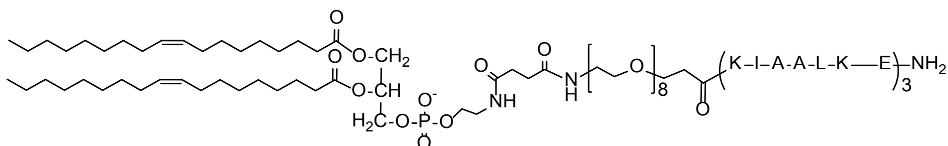
Chemical Formula: $C_{160}H_{290}N_{30}O_{42}P^{-}$ [3337.22]
Retention time: 10 min
LC-MS: Calcd. [1113.41, $M+3H]^{+3}$, Found 1113.72

6. LP_4E



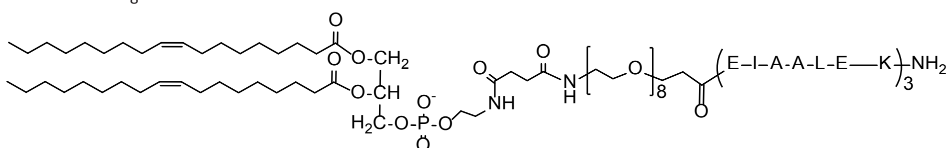
Chemical Formula: $C_{157}H_{275}N_{27}O_{48}P^{-}$ [3340.04]
Retention time: 12 min
LC-MS: Calcd. [1114.34, $M+3H]^{+3}$, Found 1114.33

7. LP_8K



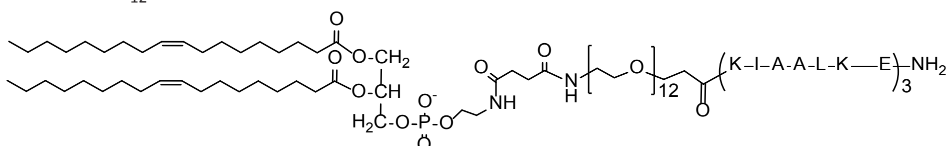
Chemical Formula: $C_{169}H_{308}N_{30}O_{46}P^{-}$ [3527.46]
Retention time: 10 min
LC-MS: Calcd. [1176.82, $M+3H]^{+3}$, Found 1177.09

8. LP_8E



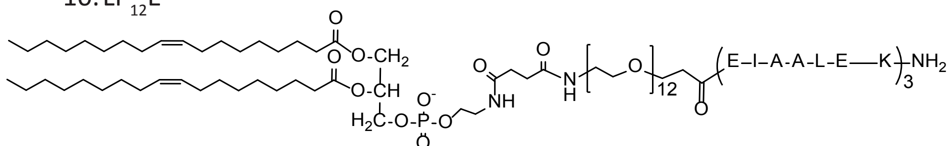
Chemical Formula: $C_{166}H_{293}N_{27}O_{52}P^{-}$ [3530.28]
Retention time: 12min
LC-MS: Calcd. [1177.76, $M+3H]^{+3}$, Found 1178.04

9. $LP_{12}K$



Chemical Formula: $C_{177}H_{324}N_{30}O_{50}P^{-}$ [3703.67]
Retention time: 10 min
LC-MS: Calcd. [1235.55, $M+3H]^{+3}$, Found 1235.79

10. $LP_{12}E$



Chemical Formula: $C_{174}H_{309}N_{27}O_{56}P^{-}$ [3706.49]
Retention time: 12 min
LC-MS: Calcd. [1236.49, $M+3H]^{+3}$, Found 1236.74

Liposome Preparation

A 1 mM stock solution of DOPC: DOPE: CH (50:25:25 mol%) was prepared in dry chloroform and stored at -20 °C. Lipopeptide stock solutions (10 μM) were prepared in chloroform:methanol (1:1) and also stored at -20 °C. Liposomes (0.1 mM) in PBS buffer were prepared containing 1 mol% lipopeptide as reported before.[17] In 20 ml glass vial, lipids (100 μl from stock solution) and lipopeptide (100 μl from stock solution) were mixed and the mixture was dried under continuous air flow to obtain lipid/lipopeptide film. This film was hydrated with 1 ml PBS buffer (pH 7.2) and sonicated for 3-5 min at 50 ^{0}C using a Branson bath sonicator to obtain 0.1 mM liposomes with an average diameter of 100nm.

Lipid mixing assay

LP_nK_3 decorated liposomes (DOPC/DOPE/CHOL/NBD-DOPE/RHD-DOPE; 50/24.75/24.75/0.5/0.5 mol %) were mixed with LP_nE_3 (1 mol%) decorated liposomes (DOPC/DOPE/CHOL; 50/25/25 mol %). Fluorescence measurements for lipid mixing were performed using a luminescence spectrometer LS50B (Perkin Elmer). All spectra were obtained at room temperature using a cuvette with a 1 cm path length. The NBD fluorescence was used to calculate the lipid mixing percentage with time. Fluorescence measurements were started immediately after mixing 750 μL of the fluorescent-labelled LP_nK_3 liposome suspension with 750 uL of unlabelled LP_nE_3 liposome suspension in the cuvette. The NBD fluorescence intensity at 530 nm was monitored in a continuous fashion. To calculate the percentage of fusion the following equation (1) was used:

$$F_{(\%)} = [\,(F_{(t)} - F_{(0)}] \,/\, [(F_{(max)} - F_0)] \times 100 \qquad (1)$$

where F(t) is the fluorescence intensity measured at time t, F_0 is the 0% fluorescence and F_{max} is the fluorescence intensity measured using 1:1 lipid mixing.

Content Mixing assay

Content mixing experiments were carried out as reported earlier.[27] A dried film containing DOPC/DOPE/CH (50:25:25 mol%) and the corresponding LP_nE_3-peptides (1 mol%) were hydrated and sonicated (5 min at 50°C) with a sulforhodamine B (20 mM) containing HEPES buffer solution (20 mM HEPES, 90 mM NaCl, pH 7.2). The final lipid concentration was 1 mM. To remove non-encapsulated dye the liposomal solution was subjected to Sephadex column (G50, Superfine, 15 cm in length) using HEPES (20 mM HEPES-Na, 90 mM NaCl) buffer as eluent. The fraction containing the liposomes was collected and diluted to a final liposome concentration of 0.1 mM. 600 μL of the LP_nE_3 containing liposomes with encapsulated sulforhodamine B were added to a small volume disposable

cuvette. Fluorescence measurements for content mixing were performed using a luminescence spectrometer LS50B (Perkin Elmer). All spectra were obtained at room temperature using a cuvette with a 1 cm path length. The fluorescence signal of the Sulforhodamine (λ_{em} = 580 nm) was detected and 600 µL of the corresponding LP_nK_3 containing liposomes (0.1 mM) in HEPES-buffer were added and the increase of sulforhodamine B fluorescence, due to a relief of self-quenching, was detected. After 30 min, 120 µL of 10% (v/v) solution of Triton X was added to lyse the liposomes and reach maximum fluorescence. To calculate the percentage of fusion, equation (2) was used:

$$F_{(\%)} = [(F_{(t)} - F_{(0)}] / [(F_{(max)} - F_0)] \times 100 \quad (2)$$

where F(t) is the fluorescence at a certain time, $F_{(max)}$ is the fluorescence after lysis of the liposomes with Triton X and F(0) is the starting fluorescence after addition of the K-peptide containing liposomes.

DLS Measurements

Particle size distributions were obtained with the aid of a Malvern Zetasizer Nano ZS which was equipped with a peltier controlled thermostatic holder. The laser wavelength was 633 nm and the scattering angle was 173º. To obtain an estimation of the hydrodynamic radius, D_h, the Stokes-Einstein relation (3) was used:

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

Here, k_B is the Boltzmann constant and η is the viscosity of the solvent. Measurements were carried out at room temperature.
For the DLS measurements, 0.5 mM liposomes bearing 1 mol% of LP_nK_3 or LP_nE_3 were prepared. LP_nK_3 liposomes were mixed with LP_nE_3 liposomes and size was measured for 30 min at 25 °C.

CD measurements

CD spectra were obtained using a Jasco J-815 spectropolarimeter equipped with a peltier-controlled thermostatic cell holder (Jasco PTC-423S). 0.5 mM liposomes of 100 nm size were decorated with 1 mol% LP_nK_3 or LP_nE_3 lipopeptides. Spectra were recorded from 260 nm to 200 nm in a 5.0 mm quartz cuvette at 25 °C. Data was collected at 0.5 nm intervals with a 1 nm bandwidth and 1 s readings. Each spectrum was the average of 5 scans. Measurements were started immediately after mixing. All spectra were recorded in 20 mM phosphate buffer at pH 7.4. For analysis each spectrum had the appropriate background spectrum (buffer or liposomes of 100 nm in size) subtracted. The ellipticity is given as mean residue molar ellipticity, [θ] (10^3 deg cm^2 dmol-1), calculated using equation (4)

$$[\vartheta] = (\vartheta_{obs}\ MRW) / (10lc) \tag{4}$$

Where θ_{obs} is the ellipticity in millidegrees, MRW is the mean residue molecular weight (i.e. the molecular weight of the peptide divided by the number of amino acids residues), l is the path length of the cuvette in cm and c is the peptide concentration in mg/mL. From this mean residue molar ellipticity, the ellipticity ratio for the peptide could be calculated, using equation (5)

$$\text{Ellipticity Ratio} = [\vartheta]_{224} / [\vartheta]_{211} \tag{5}$$

Control Experiment for Lipid mixing:

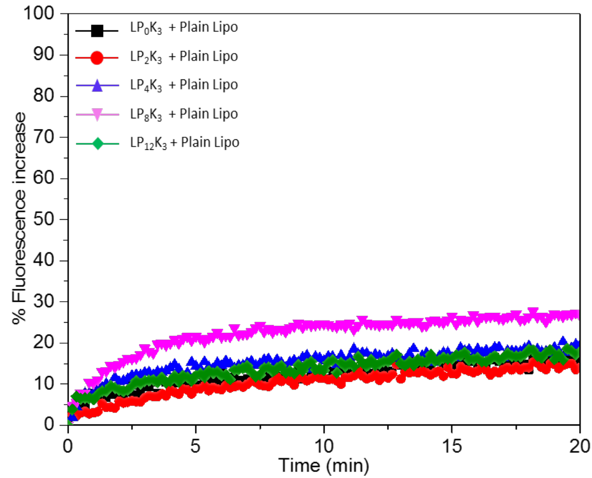


Figure A2. *Lipid mixing between K decorated liposomes and plain liposomes, as indicated by an increase in NBD fluorescence. Non-fluorescent liposomes (0.1 mM) were added to fluorescent K liposomes (0.1 mM, 1% LP_nK_3).*

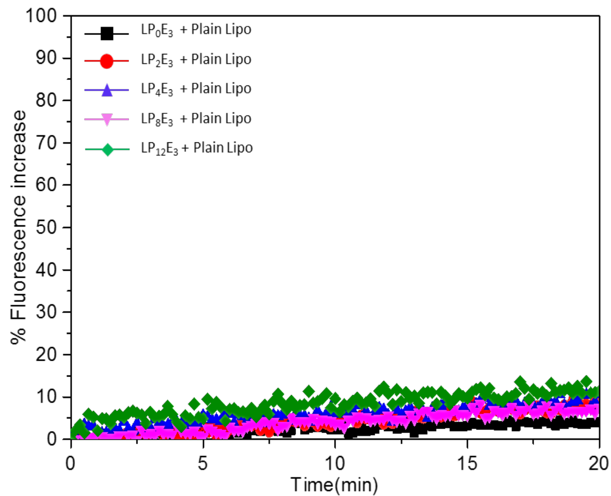


Figure A3*: Content mixing between LP_nE_3 and LP_nK_3 modified liposomes (0.1 mM total lipid concentration, 1 mol% LP_nE_3 peptides). To test no specific leakage during fusion LP_nE_3 decorated liposomes (0.1 mM total lipid concentration, 20 mM sulphorhodamine B) were added to the plain liposomes (0.1 mM total lipid concentration).*

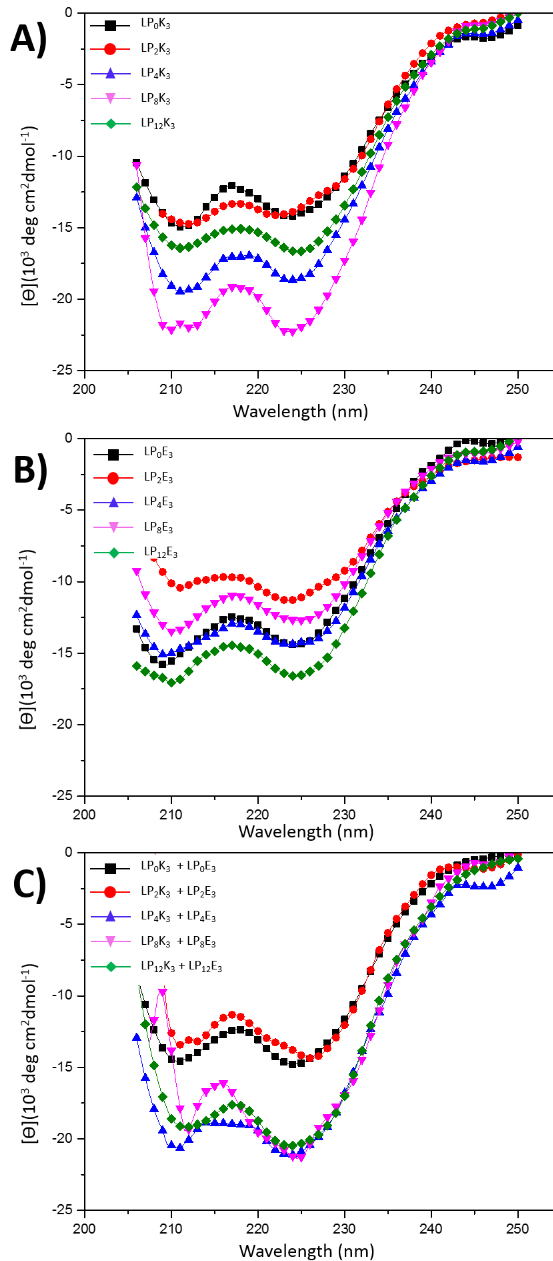


Figure A4: *Circular Dichroism spectra of A) LP_nK_3 liposomes B) LP_nE_3 liposomes and C) (1:1) mixture of LP_nK_3 liposomes and LP_nE_3 liposomes where the baseline was corrected with plain liposomes of 100nm in size. 0.5 mM liposomes in PBS were decorated with either 1 mol% LP_nK_3 or LP_nE_3.*

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