Supporting Information
Controlling the rate of coiled coil driven membrane fusion

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1. Materials and Methods

1.1 Materials

Fmoc-protected amino acids and Sieber Amide resin were purchased from Novabiochem. Fmoc-NH-PEG_{12}-COOH was purchased from IRIS Biotech. DOPC was purchased from Avanti Polar Lipids, DOPE was purchased from Phospholipid, and cholesterol was obtained from Fluka. DOPE-NBD and DOPE-LR were obtained from Avanti Polar Lipids, All other reagents and solvents were obtained at the highest purity available from Sigma-Aldrich or BioSolve Ltd. And used without further purification. Milli-Q water with a resistance of more than 18.2 MQ cm-1 was provided by a Millipore Milli-Q filtering system with filtration through a 22 um Millipak filter. Phosphate buffered saline, PBS: 5 mM KH_2PO4, 15mM K_2HPO_4, 150 mM NaCl, pH 7.4.

1.2 Abreviations

Fmoc:	fluorenylmethoxycarbonyl
DOPE:	1,2-dioleoyl-sn-glycero-3-phosphatidylcholine
DOPC:	1,2-dioleoyl-sn-glycero-3-phosphatidylcholine
DOPE-NBD:	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt)
DOPE-LR:	1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-lissamine-rhodamine
HCTU:	1H-benzotriazolium-1-[bis(dimethylamino)methylene]-5-chloro-hexafluoro- phosphate-(1-),3-oxide
DIPEA:	N,N-diisopropylethylamine
DCM:	dichloromethane
MALDI-TOF:	matrix-assisted laser desorption-ionization time-of-flight
LCMS:	Liquid Chromatography-Mass Spectrometry
RP-HPLC:	reversed-phase high-pressure liquid chromatography
TEA:	triethanolamine
TIS:	triisopropylsilane
ACH:	α-cyano-4-hydroxycinnamic acid
CAN:	acetonitrile

1.3 General Methods

RP-HPLC was performed with a Shimadzu HPLC system with two LC-8A pumps, and an SPD-10A VP UV-VIS detector, Sample elution was monitored by UV detection at 214nm and

256nm. Sample elution was monitored by UV detection at 214nm and 256 nm. Samples were eluted with a linear gradient from A to B, A being ACN, and B 0.1% (V:V) TFA in H2O. Purification of the peptides and hybrids was performed on a C18 Vydac Column with a flow rated of 15ml/min. Sample purity was verified by LCMS. MALDI-TOF mass spectra were acquired using an Applied Biosystems Voyager System 6069 MALDI-TOF spectrometer with an ACH matrix. Samples were dissolved in 1:1 (v/v) 0.1% TFA in water:acetonitrile(TA), at concentrations of ~0.3mg/ml for K and E. Solutions for spots consisted of (V/V) 1:10 sample solution: 10 mg/ml ACH in TA. Phosphate buffered saline, PBS: 5 mM KH_2PO_4, 15 mM K_2HPO_4, 150 mM NaCl, pH 7.4.

2. Experimental details

2.1 Peptide Synthesis:

The peptides E_x and K_x were prepared using standard Fmoc-chemistry on a Syro-1 peptide synthesizer (Biotage). The peptides were synthesized on Sieber-Amide resin (0.62 mmol/g of NH_2). HCTU was used to activate the amino acids derivatives. The peptides were acetylated. Cleavage and de-protection was carried out using 95:2.5:2.5 (V:V) TFA:H_2O:TIS for 1 hour. The cleavage mixture and three subsequent rinses of the resin with the TFA mixture were added drop-wise to cold diethyl ether. The white precipitate was compacted with centrifugation and the supernatant removed. This was repeated three times with the addition of fresh diethyl ether. The pellets were dried in air or under reduced pressure.

The crude products were purified by RP-HPLC. ACN used as mobile phase A, H_2O with 0.1% TFA as mobile phase B. Samples were eluted with a linear gradient from 90% to 10% B (V/V). After purification all compounds were lyophilized from water to give white material with typically a yield of 40% for all the peptides.

2.2 Lipopeptide synthesis:

The peptide components of LPE and LPK were prepared with standard solid-phase peptide synthesis protocols using Fmoc-chemistry on a Syro-1 automated peptide synthesizer (Biotage), with a PL-sieber Amide resin on a 0.25 mmol scale. The peptide coupling reagent was HCTU. The N-terminal Fmoc was removed with 20% (V/V) piperidine in NMP. After the peptide

component was prepared, the resin was removed from the reaction vessel and Fmoc-NH-PEG_{12}-COOH was coupled to the immobilized peptides. The resin was swollen in NMP for 1 hour. 2.5 equivalents of Fmoc-NH-PEG_{12}-COOH and 2.5 equivalents of HCTU were dissolved in NMP(20ml) and mixed with 5 equivalents of DIPEA. After pre-activation for 1 minute the mixture was added to the peptide-resin and shaken for 20 hours. The uncoupled amines were capped with 0.05 M acetic anhydride, 0.125 M DIPEA in NMP. The N-terminal Fmoc was removed with 20% (V/V) piperidine in NMP. The resin was washed thoroughly with 10×10 ml DCM. Next, succinic anhydride was coupled to the immobilized peptide-PEG. The resin was swollen in NMP. 5 equivalents of succinic anhydride were dissolved in NMP (20mL) and mixed with 6 TEA. The mixture was added to the resin and shaken for 15 hours. The resin was washed thoroughly with 10×10mL NMP, and 10×10mL DCM. DOPE was coupled to the immobilized peptide-PEG_{12}-succinic acid in the same way, except that 3 equivalents of DOPE, 3 equivalents of HCTU, and 6 equivalents of DIPEA were used, and 1:1 (V/V) NMP: DCM was used to swell the resin and to couple the DOPE. After the peptide synthesis and after each subsequent coupling step the synthesis was tested by MALDI-TOF mass spectroscopy. Cleavage from the resin and deprotection was carried out by shaking 15 mg resin with 95: 2.5:2.5 (V/V) TFA:H_2O:TIS for one hour. The cleavage mixture and three subsequent rinses of the resin with the TFA mixture were added drop-wise to cold diethyl ether. The white precipitate was compacted with centrifugation and the supernatant removed. This was repeated three times with the addition of fresh cold diethyl ether. The pellets were dried in air or under reduced pressure. Bulk cleavage of the compounds was performed in the same way except using Bulk cleavage of the compounds was performed in the same way except using 47.5: 47.5: 2.5: 2.5 (V/V) TFA: DCM: H_2O: TIS for one hour. The crude products were purified by RP-HPLC, the yield of LPE_2 and LPK_2 are 40%, LPK_4 30%, LPE_4 20%. For each compound the purity was estimated from RP-HPLC to be greater than 95%, with a mobile phase of 0.1% TFA ACN, and H_2O.

2.3 Mass spectrometry and HPLC

Lipopeptide	Mass (calcd.)	Mass (found)	HPLC purity
LPE_2	2952.8	2951.74	> 95%
LPK_2	2950.9	2949.74	> 95%
LPE_3	3706.2	3706.62	> 95%
LPK_3	3703.4	3703.68	> 95%

LPE_4	4461.6	4463.42	> 95%
LPK_4	4458.3	4458.21	> 95%

Table S1: Overview about calculated and found masses via MALDI-MS

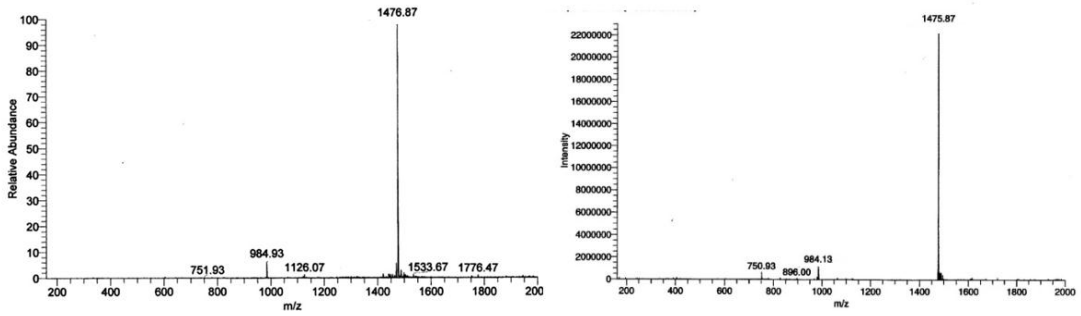

Fig.S1: Maldi-MS spectra of LPE_2 (left) and LPK_2 (right)

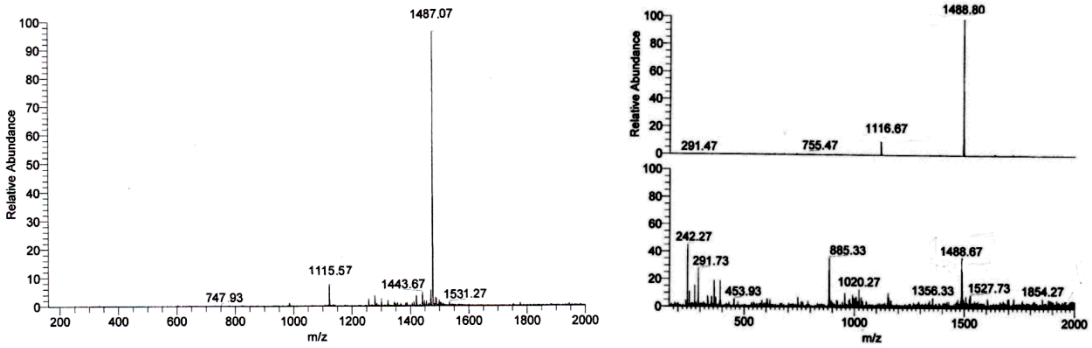

Fig.S2: Maldi-MS spectra of LPK_4 (left) and LPE_4 (right)

3. Liposomes

3.1 Liposome preparation:

1mM lipid stock solutions were made in chloroform with the composition DOPC/DOPE/CH 50:25:25 mol%. 1mM lipopeptide stock solutions were made in 1:1 (v/v) chloroform: methanol. Unless otherwise stated, liposome solutions are 1 mM in PBS. Three types of liposome solutions were prepared: plain liposomes, liposomes with 1 mol% LPE (99:1 (v/v) lipid stock solution: LPE stock solution), and liposomes with 1 mol% LPK (99:1 (v/v) lipid stock solution: LPK stock solution). To prepare small unilamellar vesicles the solvent was removed from the stock solution

(2 mL) using a rotary evaporator to get a lipid film. Following this PBS (2 mL) was added to prepare a 1 mM liposome solution. The sample was vortexed for 1 minute and sonicated at 50 °C to form large unilamellar vesicles (it takes approximately 5 minutes for plain liposomes and 2 minutes for decorated liposomes respectively). The hydrodynamic diameter was approximately 100 nm as determined by DLS.

3.2 Content Mixing

Content mixing experiments were carried out as follows: A dried film containing DOPC/DOPE/CH 50:25:25 mol% and the corresponding E-Peptides (1 % of either LPE_2, LPE_3 or LPE_4) 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) at pH 7,2. The final lipid concentration was 1 mM. To get rid of non-encapsulated dye the liposomal solution was subjected to Sephadex (G50, Superfine) using HEPES (20 mM Hepes-Na, 90 mM NaCl) buffer as eluent. The fraction containing liposomes was collected and diluted to a final liposome concentration of 0.1 mM. 400 µL of the E-Peptide containing liposomes with encapsulated sulforhodamine B were added to a small volume disposable cuvette. The fluorescence signal of the Sulforhodamine (λ_{em} = 580 nm) was detected and another 400 µL of the corresponding K-Peptide containing liposomes (0.1 mM) in HEPES-buffer at pH = 7,2 were added and the increase of sulforhodamine B fluorescence, due to a relief of self-quenching, was detected. After a certain time 100 µL of 10% (v/v) solution of Triton X was added to lyse the liposomes and reach the maximum dilution. To calculate the percentage of fusion the following equation was used:

$$F\% = (F_{(t)} - F_{(0)} / (F_{(max)} - F_0) \times 100$$

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

3.3 Lipid Mixing.

All spectra were obtained at room temperature using a quartz cuvette with a 1 cm path length. Liposomes consisting of DOPC/DOPE/CHOL/NBD-DOPE/RHD-DOPE (49.5/24.75/24.75/0.5/0.5 mol %) and 1 % of LPK_x where mixed with liposomes consisting of

DOPC/DOPE/CHOL (59/25/25 mol %) and 1% of LPE_x. The NBD fluorescence was used to calculate the lipid mixing percentage with time. Fluorescence time series measurements were started immediately after mixing 750 µL of the fluorescent-labeled liposome suspension with 750uL of unlabeled liposome suspension in the cuvette. The NBD fluorescence intensity at 530 nm was monitored in a continuous fashion for 3000 seconds. After that the liposomes were lysed by the addition of 150 µL of 10 wt % Triton X-100 in PBS to obtain 100 % increments.

The values measured after lysis were multiplied by 1.82 to take into account the effect of Triton X-100 on the NBD fluorescence and dilution, which was obtained from a separate lysis experiment of a liposome solution that only contained DOPE-NBD. The percentage of fluorescence increase (%) is calculated as:

$$F(\%) = (F(t) - F0) / (F_{max} \times 1.82 - F_0) \times 100$$

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 after addition of Triton X-100.

3.4 Initial fusion rate

Here we used the initial lipid mixing rate as characterization of the initial fusion rate and is calculated as:

$$R = \Delta F / \Delta t$$

ΔF stands for Fluoresce increase, Δt is time increase after 1:1 equimolar mix fluorescent label K liposome with non-fluorescent label E liposome. The increase in lipid mixing during the first minute of fusion is almost linear, therefore the increase in fluorescence in the first minute is used to calculate the rate of fusion.

4. Additional measurements

4.1 Circular Dichroism Spectroscopy

4.1.1 Peptide conformation and binding energy assay:

CD spectra were obtained using a Jasco J-815 spectropolarimeter equipped with a peltier controlled thermostatic cell (Fig. S3,4). The ellipticity is given as mean residue molar ellipticity, $[\theta](10^3 degcm^2dmol^{-1})$, calculated by Eqn. (1),

$$[\theta]= (\theta_{obs}\times MRW)/(10\times lc) \quad (1)$$

Where θ_{obs} is the ellipticity in millidegrees, MRW is the mean residue molecular weight, l is the path length of the cuvette in cm and c is the peptide concentration in mg/mL.

A 1.0mm quartz cuvette and 200 μM concentration of peptide in pH=7.4 PBS were used for detection of the peptide secondary structure. Spectra were recorded from 260nm to 200nm at 25°C. Data was collected at 0.5nm intervals with a 1nm bandwidth and 1s readings. Each spectrum was the average of 5 scans. For analysis each spectrum had the appropriate background spectrum subtracted.

Temperature dependent CD spectra (Fig. S3,4) for calculation of the peptide binding energy were obtained using an external temperature sensor immersed in the sample. The temperature was controlled with the internal sensor and measured with the external sensor. A 10 mm quartz cuvette was used, and the solutions were stirred at 900 rpm. Spectra were recorded from 260 nm to 200 nm, with data collected at 0.5 nm intervals with a 1 nm bandwidth and 1 s readings. Each spectrum was one scan. The temperature range was 6 ºC to 96 ºC with a temperature gradient of 2.0ºC/minute and a 60 s delay after reaching the set temperature. The solutions took 5 minutes to return to 6 ºC. The spectrum of PBS at 6 ºC (average of 5 scans) was subtracted from each spectrum.

The data was analyzed using a two-state unfolding model to determine the fraction folded using Eqn. (2),

$$F_F=([\theta]-[\theta]_U)/([\theta]_F-[\theta]_U) \quad (2)$$

Where $[\theta]$ is the observed molar ellipticity, $[\theta]_U$ is the ellipticity of the denatured state, as determined from the plateau of the ellipticity vs. temperature curve, and $[\theta]_F$ is the ellipticity of the folded state at that temperature as determined from a linear fit of the initial stages of the ellipticity vs. temperature curve.

The fraction unfolded, FU, was calculated by Eqn. (3),

$$F_U=1-F_f \quad (3)$$

The dimer dissociation constant in the transition zone was calculated using Eqn. (4),

$$K_U=2P_tF_U^2/F_f \quad (4)$$

Pt is the total peptide concentration. By taking the derivative of the $\ln(K_U)$ vs. Temperature and using this in the van't Hoff equation, Eqn. (5), the change in enthalpy associated with unfolding with temperature can be plotted:

$$d\ln(K_U)/dT=\Delta H_U/RT^2 \quad (5)$$

The gradient of this plot ΔCp, is the difference in heat capacity between the folded and unfolded forms, and can be used in the Gibbs-Helmholtz equation adapted to monomer-dimer equilibrium, Eqn. (6), to obtain the Gibbs free energy of unfolding as a function of temperature

$$\Delta G_U=\Delta H_m(1-T/T_m)+\Delta C_p[T-T_m-T\ln(T/T_m)]-RT\ln[P_t] \quad (6)$$

T_m and H_m, the temperature and enthalpy at the midpoint of the transition, is determined by the maximum of derivative of the ellipticity vs. temperature graph.

All binding energy calculations were based on the assumption that the peptide pairs form a 1:1 heterodimer complex (1:1 complex of E_x and K_x). We are aware that this is not true for the peptide pairs containing E_4 and/or K_4 and that coiling occurs resulting in higher ordered structures as well. Therefore the calculated binding energies are not to be treated as exact values but more as a qualitative guide for the stability of the resulting coiled-coil motifs, which are in line with the observed Tm's.

With the formula above, we can calculate the binding energy of E/K complex from the graphs (Fig. S3,4) below:

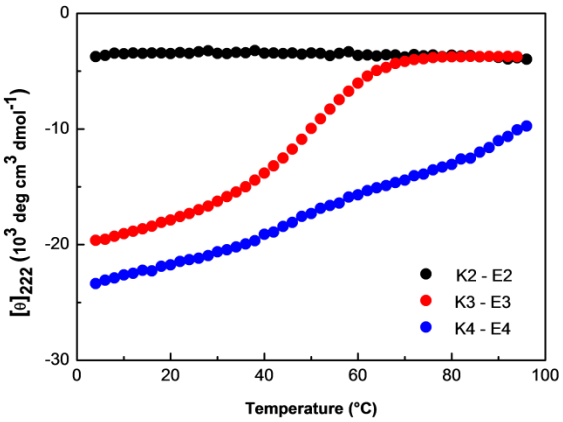

Fig.S3: Temperature dependent CD spectra monitored molar ellipticity changing at 222nm. K_m/E_n pairs in 20mM phosphate, 150mM NaCl, pH 7.4, [Total peptide] = 40 µM.

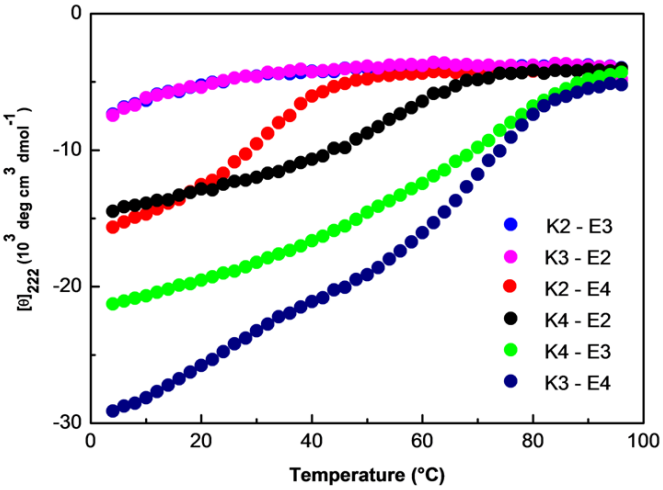

Fig.S4: Temperature dependent CD spectra monitored molar ellipticity changing on wavelength 222nm. K_m/E_n pairs in 20mM phosphate, 150mM NaCl, pH 7.4, [Total peptide] = 40µM.

Figure S5 and S6 show the temperature dependent CD-spectra for E_3-K_3 and with E_4-K_4. With increasing temperature the E3-K3 coiled coil dissociates, but the E_4-K_4 coiled coil still exists even when the temperature reaches 96 °C.

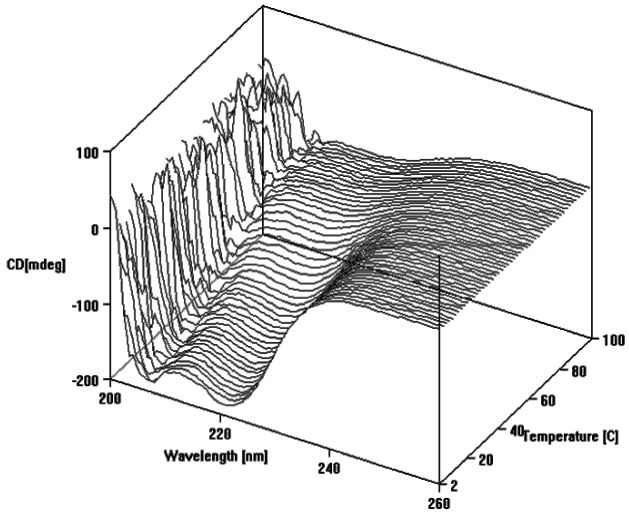

Fig.S5: 3D spectrum of the E_3/K_3 coiled-coil complex ellipticity upon increasing the temperature from 2-96°C.

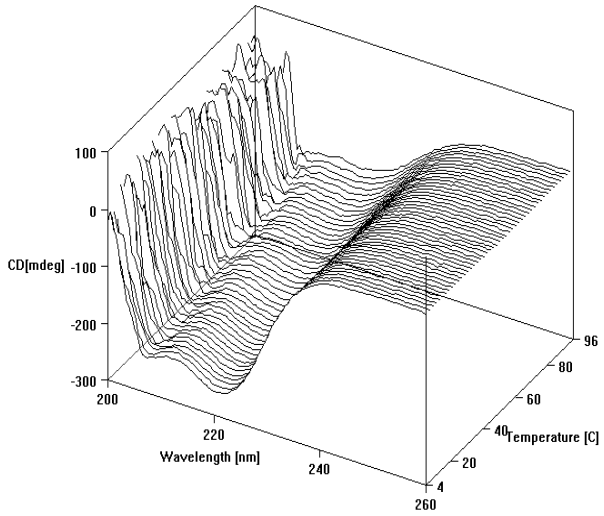

Fig.S6: 3D spectrum of E4/K4 coiled coil complex ellipticity following by the temperature increasing from 2-96°C.

Details for CD measurement (table 1)

Calculation of approaching value for E2-K2 and E4-K4 fraction fold curve.

The fraction folded in percentage of E2-K2 and E4-K4 was calculated by using the value for E3/K3 as reference. [1].

4.1.2 Determination of the percentage α-helix and the confirmation coiled-coils

The percentage α-helicity is the ratio of the observed $[\theta]_{222}$ to the predicted $[\theta]_{222}$ for an α-helical peptide of n residues ×100. The predicted $[\theta]_{222}$ = -40000×(1-4.6/n).[2] Peptide interactions were further confirmed by TFE-CD measurements. TFE is known to enhance intramolecular α-helicity but decrease intermolecular interactions.[3]. We measure equimolar E and K mixture in first PBS, then TFE:PBS 1:1 (v/v). If there is a significant decrease in the in the $[\theta]_{222}/[\theta]_{208}$ ratio from PBS to 50% TFE in PBS, one can assume, that there is a destruction of the coiled-coil binding motif. Here total peptide concentration is 1mg /mL in 50mM phosphate, 150mM NaCl, pH 7.4, 25°C.

Peptide	Theta222		%alpha-helix		Theta222/Theta208		Coiled-coil
	PBS	50%TF	PBS	50%T	PBS	50%TFE	
E2	-2490	-17718	9	66	0.50	0.73	-
K2	-1876	-14142	7	53	0.32	0.73	-
E2+K2	-3561	-17786	13	66	0.37	0.73	-
E3	-5819	-22465	19	72	0.59	0.84	-
K3	-6638	-23139	21	74	0.73	0.84	-
E3+K3	-24705	-23277	79	75	1.10	0.90	+
E4	-22173	-23176	66	69	1.43	0.86	+
K4	-24714	-25812	74	77	1.25	0.86	+
E4+K4	-31066	-31341	93	94	1.11	0.90	+

Table S2: Concentrations of the peptides: 1mg/ml, Buffer 50mM phosphate, 150mM NaCl, pH 7.4, 25°C.

4.1.3 Comparison of secondary structure of E_2, K_2 and liposomes modified with LPE_2 and LPK_2.

Peptide	% alpha-helicity		Peptide	% alpha-helicity
Ac-E2	9		LPE2	20
Ac-K2	7		LPK2	19
Ac-E2+Ac-K2	13		LPE2+LPK2	40

Tab.S2:Comparision of acetylated E2,K2, E2+K2 percentage of alpha-helix changed from uniform disperse in buffer with fixed on surface of liposome. Acetylated peptide were measured in pH=7.4 PBS buffer (PBS buffer as baseline), 25℃. LPE2, LPK2 and LPE2+LPK2 were decorated on surface of liposome to make them water-soluble (plain liposome in same buffer as baseline). All the acetylated peptide were measured in 1mg/ml concentration, 1 mm cuvette was used, 4 scans for each peptide, while all the lipopeptide were decorated 1% on liposome surface which compose of 0.5mM lipid in pH=7.4 PBS in 5mm cuvette on 25℃, 6 scans for each lipopeptide.

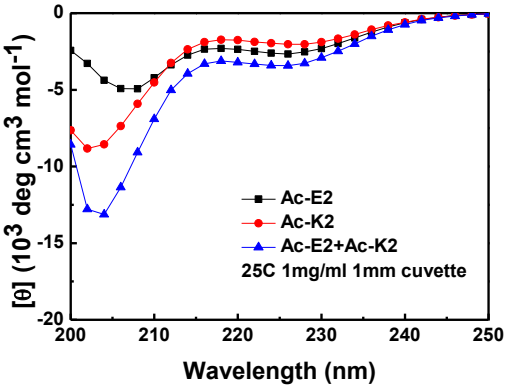

Fig.S7: CD spectroscopic data of acetylated E2, K2 and E2-K2 complex. 1mg/ml peptides in pH=7.4 PBS (50mM phosphate, 150mM NaCl) were measured with 1mm cuvette on 25℃.

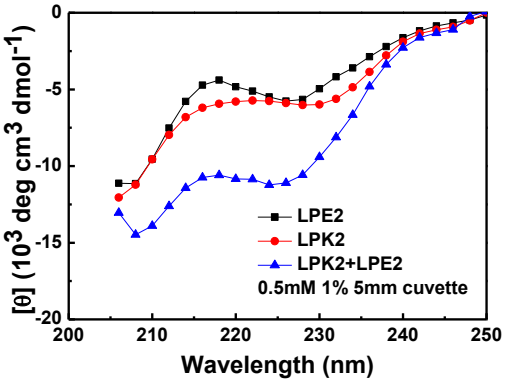

Fig.S8: CD spectroscopic data of LPE2, LPK2, LPE2+LPK2 complex. All lipopeptide were decorated on surface of liposome, to make them water soluble, meanwhile use plain liposome as baseline during all the lipopeptide-liposome measurements. All the samples content 0.5mM lipid and 1% lipopeptide, and measured by 5mm cuvette was used on 25℃.

4.2 Lipid Mixing (Cross combinations)

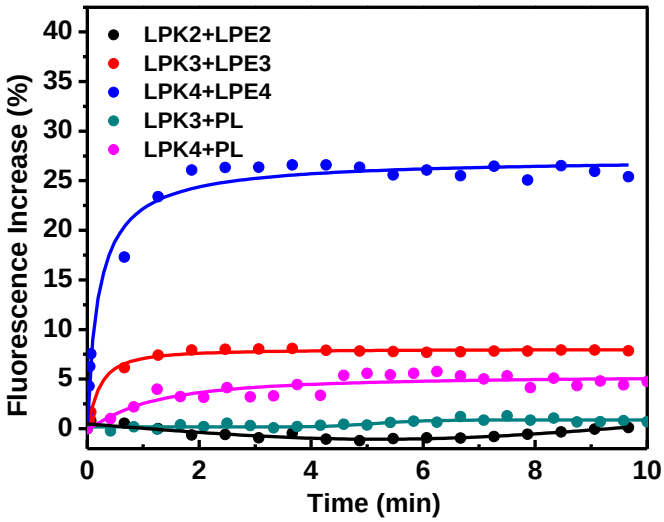

Fig. S9. Fluorescence increase at 60 °C , due to lipid mixing between two batches of liposomes decorated with 1 mol% LPE_2-LPK_2, LPE_3-LPK_3, LPE_4-LPK_4. Two control experiments are shown; lipid mixing between $LPK_{3\ or\ 4}$-decorated liposome with plain liposomes (PL). [lipid] = 0.1 mM.

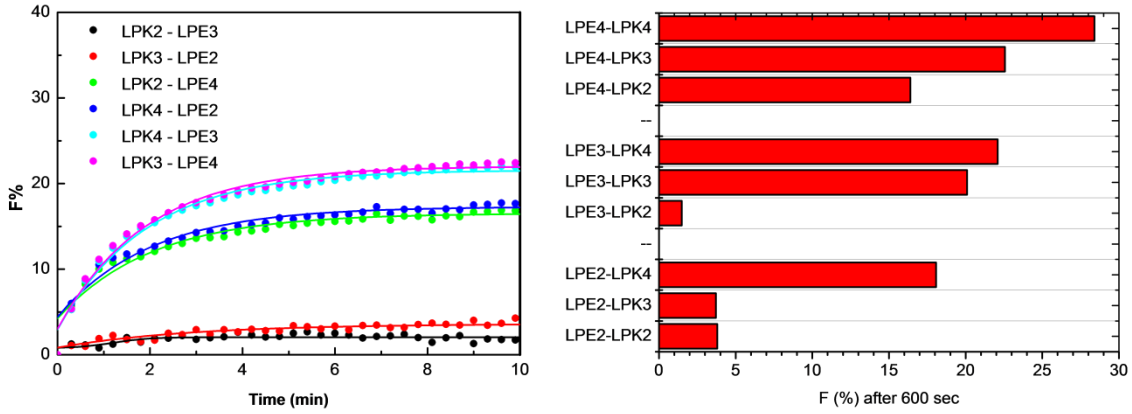

Fig.S10: Lipid mixing based on the fluorescence increase upon mixing LPK_x decorated fluorescent liposomes and LPE_x decorated liposomes

4.3 Content mixing (cross combinations)

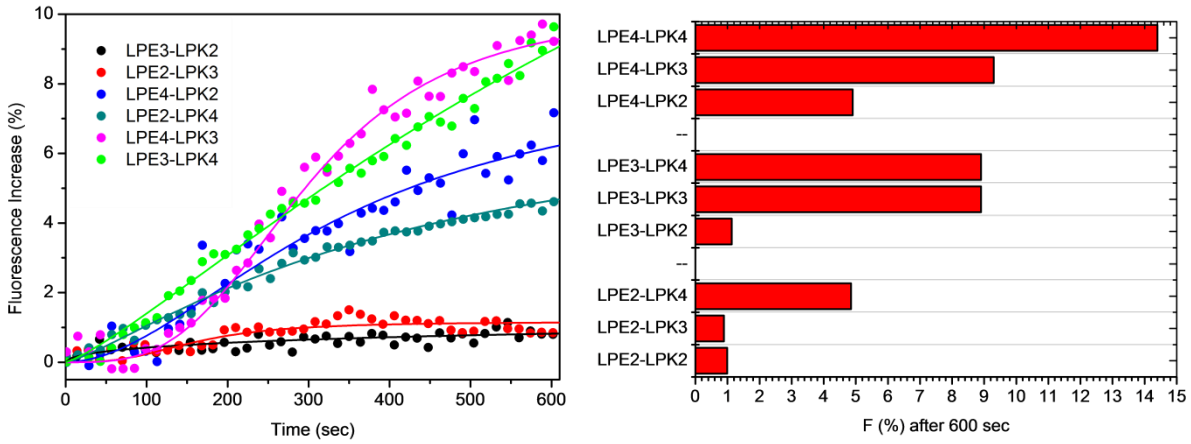

Fig.S11: Lipid mixing based on the fluorescence increase upon mixing LPK_x decorated liposomes and LPE_x decorated liposomes with encapsulated sulphorhodamine (20 mM).

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4.4 Dynamic light scattering (cross combinations)

Hydrodynamic diameters were estimated at 25 °C by dynamic light scattering using a Malvern Zetasizer Nano ZS ZEN3500 equipped with a peltier-controlled thermostatic cell holder. The laser wavelength was 633 nm and the scattering angle was 173°. For individual liposome batches the samples were allowed to equilibrate for 2 minutes. For DLS time series the solutions were mixed in the cuvette for 30 second. Measurements were started immediately after mixing without 2 minutes of sample equilibration, and continued for 1h:

Size increase % = $100*(S_{1h}-S_0)/S_0$

(S_{1h}: Zeta Average diameter after 1 hour mixing, S_0: Z_{av} diameter immediately after mixing).

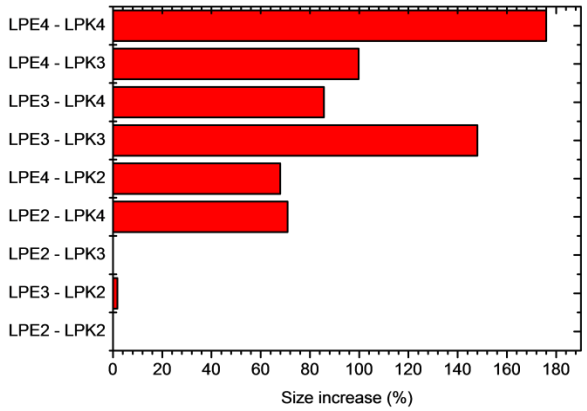

Fig.S12: Size increase in percentage of all cross combination.

4.5 Comparison of different conditions

Influence of temperature:

We followed the same method mentioned in section 3.3 for the lipid mixing, except, that a controllable water bath was used to determine the lipid mixing at different temperature.

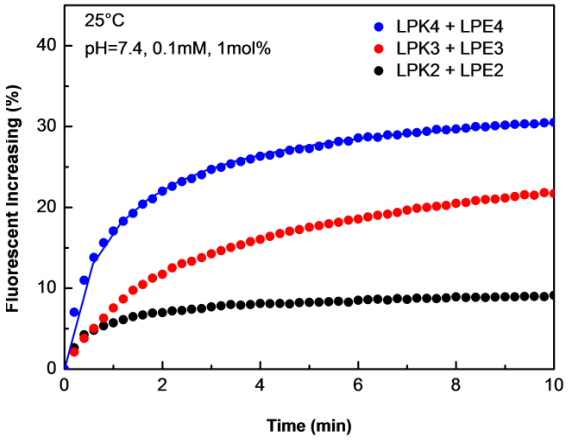

Fig. S13 : Lipid mixing at, pH=7.4.

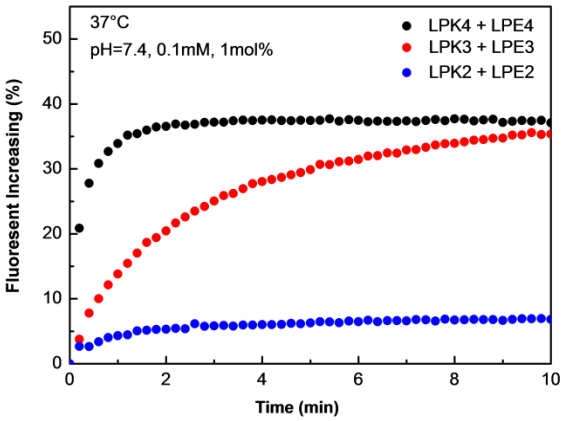

Fig. S14 Lipid mixing at 37°C, pH=7.4.

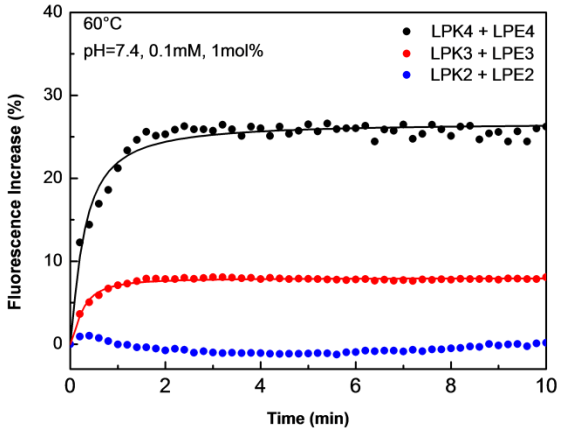

Fig. S15: Lipid mixing at 60 °C, pH=7.4.

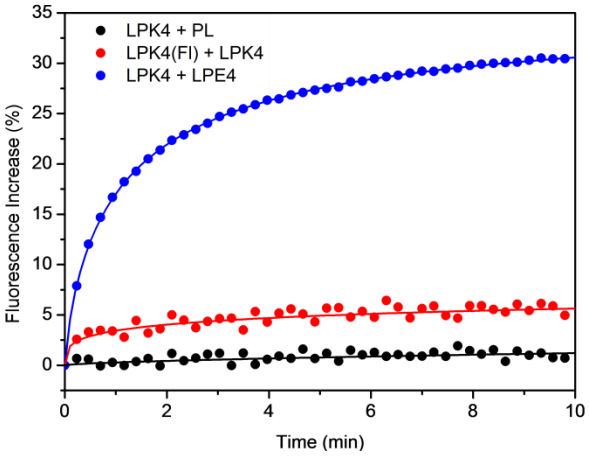

Fig. S16: Lipid Mixing at 25°C, Control experiments, pH = 7,4

Rate of fusion as a function of pH:

We followed the same method mentioned in section **3.3** for the lipid mixing, except, that the pH was varied.

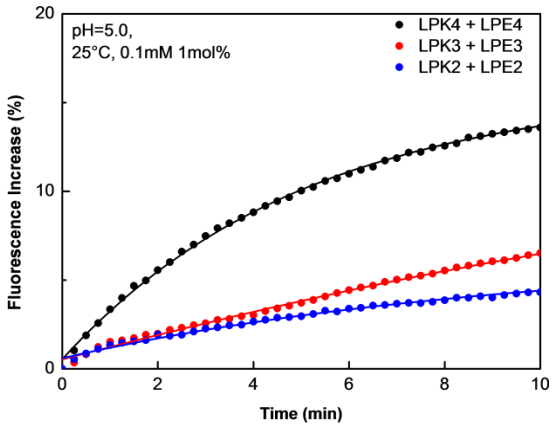

Fig.S17: Liposome fusion at 25°C, pH=5.0.

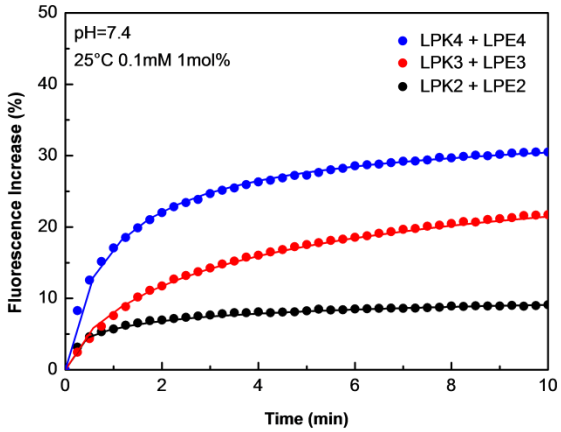

Fig.S18: Liposome fusion at 25 °C pH=7.4.

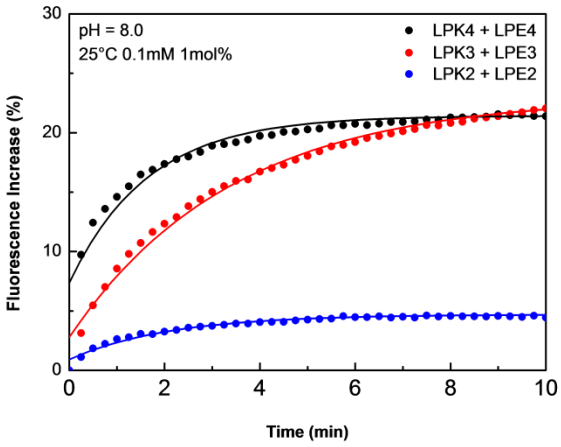

Fig.S19: Liposome fusion at 25°C, pH=8.0.

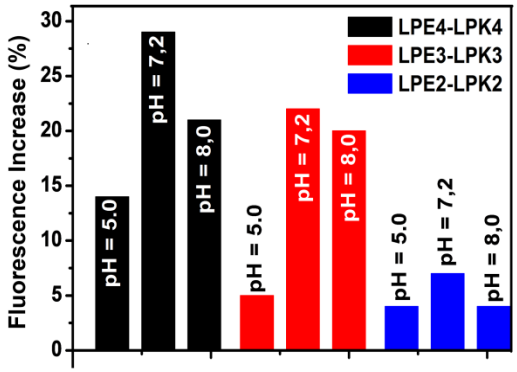

Fig.S20: Comparison of lipid mixing of LPE_x-LPK_x modified liposomes as a function of pH.

References

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Supporting Information

Controlling the rate of coiled coil driven membrane fusion