Peptide Amphiphiles and their use in Supramolecular Chemistry

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Chapter 4

In situ modification of plain liposomes with lipidated coiled coil forming peptides induces membrane fusion

Complementary coiled coil forming lipidated peptides that are embedded in liposomal membranes are able to induce rapid, controlled and targeted membrane fusion. Traditionally, such fusogenic liposomes are prepared by mixing lipids and lipidated peptides in organic solvent (e.g. chloroform). Here, fusogenic liposomes were prepared in situ, i.e. by addition of a lipidated peptide solution to plain liposomes. As the lipid anchor is vital for the correct insertion of lipidated peptides into liposomal membranes, a small library of lipidated coiled coil forming peptides was designed in which the lipid structure was varied. The fusogenicity was screened using lipid- and content mixing assays showing that cholesterol modified coiled coil peptides induced the most efficient fusion of membranes. Importantly, both lipid and content mixing experiments demonstrated that the in situ modification of plain liposomes with the cholesterol modified peptides yielded highly fusogenic liposomes. This work shows that existing membranes can be activated with lipidated coiled coil forming peptides, which might lead to highly potent applications such as the fusion of liposomes with cells.

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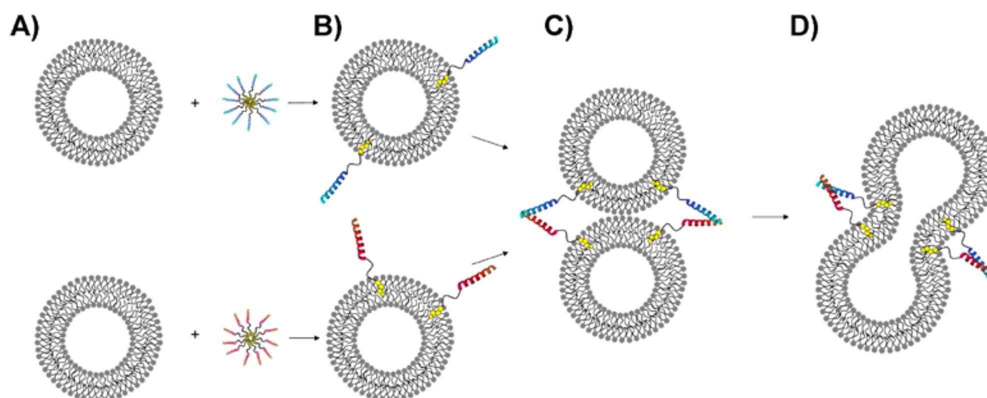
Introduction

The advancement of supramolecular chemistry in recent decades has supplied scientists with new strategies to design functional materials.[1-4] However, the most stunning examples of controlled self-assembly of well-defined architectures are found in nature. In living systems, especially proteins are able to carry out a wealth of processes through their precisely arranged structure. In particular, the well-defined secondary, tertiary and quaternary structures present in proteins allow for the specific recognition of, for example, DNA,[5] RNA,[6] carbohydrates[7] and other proteins.[8] As it is a non-trivial task to manage interactions between complete proteins, chemists have turned to peptide amphiphiles.[9-16] By coupling a hydrophobic element to a peptide, aggregation in aqueous solution is induced and this often results in aggregates in which the peptide moieties display a well defined secondary structure.[17-20] These peptide amphiphiles can be used to mimic naturally occurring, protein driven, processes.[21-23] One example of such a highly specific, regulated process which is based on protein-protein recognition and which can be mimicked by peptide amphiphiles is membrane fusion.[24] This process is defined by the merging of opposing membranes, resulting in content transfer. Proteins located in opposite membranes form a coiled coil motif, thereby forcing the membranes into close proximity. Subsequently, membrane fusion can occur. In living systems this process is vital as it aids, for example, the transport of proteins between intracellular compartments and the controlled release of neurotransmitters. A synthetic supramolecular system was designed which is based on a pair of complementary lipidated peptides and which is capable of inducing rapid membrane fusion, i.e. lipid and content mixing, between liposomes.[25, 26] In the present contribution, the aim was to induce membrane fusion of plain liposomes through addition of aqueous solutions of peptide amphiphiles to pre-formed plain liposomes **(Scheme 1)**. This is in stark contrast to the conventional way to prepare fusogenic liposomes, which is based on the mixing of fusogens (e.g. peptides,[27] carbohydrates,[28] glycopeptides,[29, 30] DNA conjugates[31-33], boronic acid and inositol[34]) and lipids in organic solvent prior to liposome formation. Importantly, this strategy opens up various new applications for our model system, such as the activation of cell membranes in order to induce fusion of liposomes with cells.

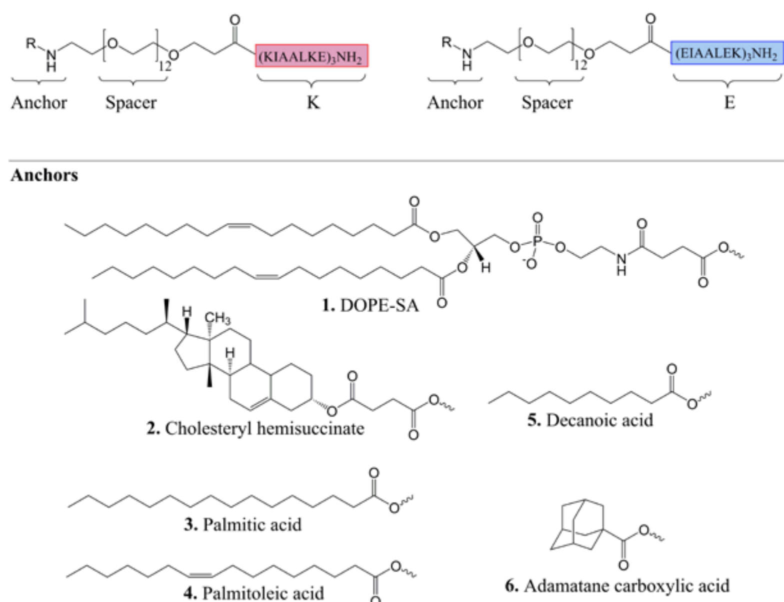


Scheme 1. *Schematic representation of coiled coil driven membrane fusion where the fusogenic liposomes are prepared by in situ modification of plain liposomes with solutions of the peptide amphiphiles (A). Insertion of the lipidated peptides in the liposomal membranes (B) and subsequent coiled coil formation of the complementary peptides (C) leads to membrane fusion (D).*

It was anticipated that the membrane anchor is the key component of the lipidated peptides, as it determines their aggregation behavior and insertion into lipid bilayers. Our strategy was therefore to synthesize lipidated peptides with a variety of membrane anchors **(Scheme 2)** in order to find a set of optimized lipidated coiled coil forming peptides that could 1) spontaneously insert into plain liposomes and 2) trigger membrane fusion once embedded into the liposomal membranes. Furthermore, different hydrophobic anchors have been used in the field of membrane fusion model systems, such as single or double cholesterol moieties,[31, 33] stearic acid,[34] phospholipids,[29, 30] peptides[27] and lipid phosphoramidite.[32] However, no studies have been conducted examining various anchors for their effect on the membrane fusion process. Therefore, the influence of the different membrane anchors on membrane fusion was examined through lipid and content mixing assays. For this screening experiment the conventional preparation route was used. The data then unequivocally shows whether a set of lipidated peptides that was already incorporated into liposomal membranes, could induce fusion.

In our membrane fusion model system, the general structure of the peptide amphiphiles is lipid-spacer-peptide **(Scheme 2)**. A heterodimeric coiled coil forming peptide pair was used, with E $(EIAALEK)_3$ and K $(KIAALKE)_3$[35] denoted as the recognition units. Successful in-situ modification of liposomes by these lipidated peptides is beneficial for several reasons. First and foremost, efficient insertion of

peptides into plain liposomes, which are subsequently available for coiled coil formation, would be a strong indication that the lipidated peptides could also be easily added to natural membranes such as cell membranes. As the coiled coil motif acts as "molecular Velcro", these peptides could then be used as a handle to which the complementary peptide can be attached. When the complementary peptide is anchored into a liposome, this might even lead to fusion of liposomes with cells. Furthermore, efficient encapsulation of biomolecules of interest such as DNA into liposomes typically requires high lipid concentrations.[36] If lipids and peptides are mixed before liposome formation, large amounts of lipidated peptide are required. In situ modification of the liposomes with encapsulated DNA would then be a viable and molecule efficient option.



Scheme 2. *Chemical structures of the lipidated peptides and various membrane anchors used in this study.*

Results and Discussion

Upon mixing two batches of liposomes that bear complementary peptides E and K, heterodimeric coiled coil formation brings opposite liposomes into close proximity, resulting in an increase in particle size, which was investigated by measuring the optical densities at 400 nm. Surprisingly, only the cholesterol, DOPE and palmitic acid anchors induced aggregation of the liposomes **(Figure 1)**. These data were confirmed by dynamic light scattering (DLS) measurements, which showed particle

sizes over 1 µm (after 30 min.) when the cholesterol anchored peptides were incorporated in the liposomal membranes (**Appendix, Figure S1**). It is plausible that the other anchors, which are less hydrophobic, are not embedded into the liposomal membranes firmly enough to hold the liposomes together once coiled coil formation has occurred.[31]

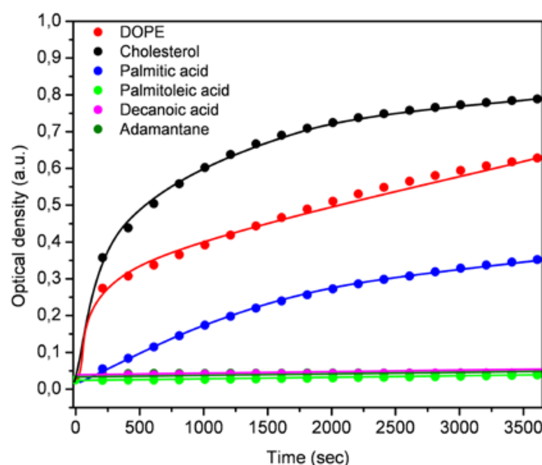


Figure 1. *Optical densities (scattering of 400 nm light) were measured upon combining batches of E and K-decorated liposomes (0.25 mM liposomes with 1 % lipopeptide).*

To support this hypothesis, the hydrophobicity of the lipidated K peptides was determined using RP-HPLC. As the solvent gradient was set to 10-90% actenotrile in H_2O, longer retention times correspond to a more hydrophobic character. As expected, the DOPE peptides yielded the longest retention time, followed by cholesterol, palmitic acid, palmitoleic acid, decanoic acid and adamantane carboxylic acid (**Appendix, Figure S2**). The three most hydrophobic lipidated peptides induced aggregation of liposomes, whereas the more hydrophilic lipidated peptides do not. This might indicate that the membrane anchor needs to be of sufficient hydrophobicity to be able to induce aggregation and subsequent membrane fusion (vide infra). Most likely, the less hydrophobic lipopeptides are rather weakly embedded in the liposomal membranes and are forced out of these membranes upon coiled coil formation.

To assess the efficiency with which the six sets of peptides induce fusion between liposomes, a standard lipid mixing assay was conducted.[26, 37] The peptide K decorated liposomes were also decorated with 0.5 mol% DOPE-NBD (donor) and 0.5 mol% DOPE-LR (acceptor). Upon fusion of these liposomes with non-fluorescent liposomes

bearing the complementary E peptide, an increase in the average distance between the donor and acceptor dyes will ensue, resulting in an increased donor emission. Consistent with the optical density measurements, it was observed that both the cholesterol and DOPE anchor induced rapid and efficient fusion, whereas the other anchors only produced moderate to low levels of fusion (**Figure 2**). Furthermore, control experiments in which one of the peptides was omitted, or when identical peptides were present on the separate batches of vesicles, showed negligible fusion rates (**Appendix, Figure S3-4**). This shows that this is a targeted fusion process and only occurs when both the complementary peptides E and K are present at the surface of liposomes to form heterodimeric coiled coils.

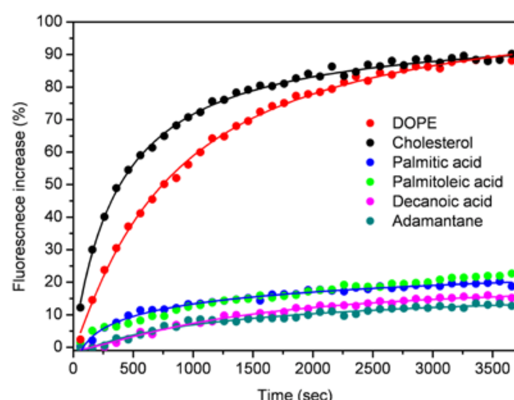


Figure 2. *Lipid mixing between E and K-decorated liposomes as indicated by an increase in NBD emission. Total lipid concentrations were 0.1 mM, with 1% lipopeptide in PBS.*

It should be noted that this lipid mixing assay only detects one round of fusion as additional fusion events between already fused liposomes do not increase the average distance between the donor and acceptor dyes any further. Therefore, the similar fluorescence increase observed for cholesterol and DOPE anchored peptides does not necessarily reflect equal amounts of lipid mixing. Multiple rounds of fusion can be probed using the lipid mixing assay, by adding multiple non-fluorescent liposomes to a single fluorescent liposome. This increases the chance that fusion events will result in an increase of the distance between the donor and acceptor dyes. When the liposome ratio was changed to 1:5 (fluorescent:non-fluorescent), it was observed that the cholesterol anchor induces fusion events more efficiently as compared to the DOPE anchor (**Appendix, Figure S5**).

While lipid mixing is the initial step in the fusion process, full fusion requires mixing of the aqueous compartments of the liposomes. This process can be monitored by encapsulating sulphorhodamine at a self-quenching concentration (20 mM) into one batch of liposomes. Upon mixing with liposomes that do not contain a fluorescent probe in their aqueous interior, content mixing results in relieve of self-quenching of sulphorhodamine and thus gives rise to an increase in fluorescence emission. Consistent with the lipid mixing data, the cholesterol anchor gave the most efficient content mixing, followed by DOPE, whereas all other anchors induced only negligible levels of content mixing **(Figure 3)**. Also, omitting one of the peptides resulted in a low fluorescence increase **(Appendix, Figure S4)**, confirming that coiled coil formation between the lipopeptides E and K is required in order to induce full membrane fusion. Finally, a leakage test was performed, in which both E and K-decorated liposomes were loaded with sulphorhodamine. If leakage occurs, this would result in an increase in rhodamine fluorescence, however this was not observed **(Appendix, Figure S6)**.

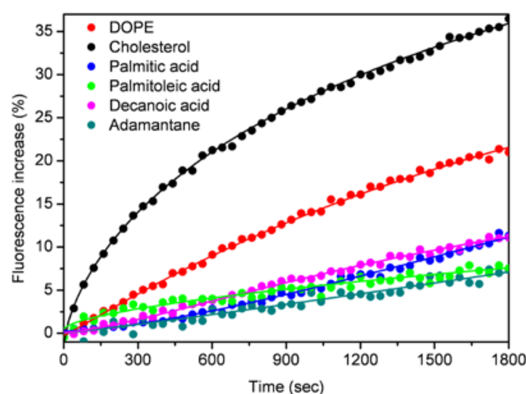


Figure 3. *Content mixing between E and K decorated liposomes as indicated by an increase in sulphorhodamine emission. Total lipid concentrations were 0.1 mM, with 1% lipopeptide in HEPES.*

Although the membrane anchor of the fusogenic peptides is not directly involved in binding the liposomes, this study demonstrates through lipid and content mixing assays that the membrane anchor has a surprisingly large effect on lipid and content mixing rates. As discussed above, the hydrophobicity of the anchor is likely to play an important role. In order to be able to formulate further hypotheses concerning the different results obtained with the various anchors, the effect that the membrane anchor has on the secondary and quaternary structure of the peptides was examined by

conducting circular dichroism (CD) measurements. Upon the combination of E and K-decorated liposomes, coiled coil formation between peptides E and K takes place, which can be measured by examining the ratio of the ellipticity minima at around 222/208 nm. A ratio <1 is considered to indicate single helices, whereas a ratio ≥1 is evidence for interacting helices. However, the use of 222/208 ratios is not uncontroversial, as other possible factors such as scattering might influence this ratio. Nonetheless, ratios >1 were observed for cholesterol and DOPE peptides, while the other lipidated peptides show ratios ~1 (**Appendix, Figure S7 and Table S1**). This indicates that coiled coils were formed between peptides E and K for all the different lipidated peptide pairs. As lipid and content mixing data showed that only cholesterol and DOPE bearing peptides induced membrane fusion, the CD data bolsters the hypothesis that the less hydrophobic anchors are removed from the liposomal membrane upon coiled coil formation. However, further studies are needed to confirm this hypothesized phenomenon. Furthermore, CD data of separate E and K-decorated liposomes **(Figure 4)** reveal that the anchor has a large influence on the helical content of the peptide segments **(Appendix, Table S2)**. When peptides E and K were conjugated to cholesterol and DOPE, the peptides were more helical, compared to less hydrophobic anchors and free E and K peptides in solution (i.e. without a membrane anchor). Furthermore, when the K peptide was anchored in liposomal membranes through a cholesterol or DOPE anchor, the ellipticity ratios of ~1 indicate that homocoiling of the K peptides might occur. It is possible that this homocoiling results in a locally elevated concentration of peptide strands which might be necessary to initiate fusion events as it is very likely that several coiled coils need to be formed to initiate a fusion event. Part of the natural membrane fusion machinery is formed by SNARE proteins and it has been argued that several SNARE complexes are needed for full membrane fusion to occur.[38-40] As the coiled coil motif employed here is much smaller than the natural occurring SNARE complex, it is plausible that aggregates of several E/K coiled coils are required to spark liposome fusion.

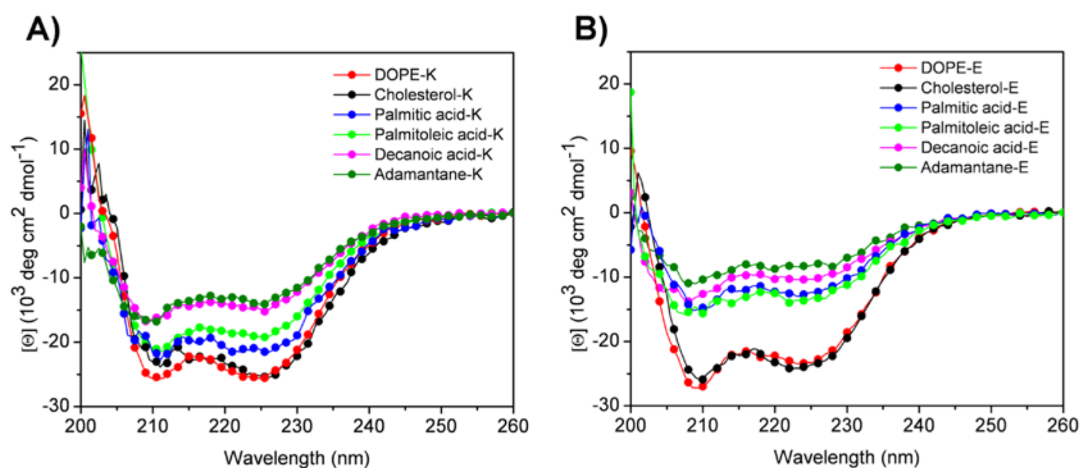


Figure 4. *CD data of A) K decorated liposomes and B) E decorated liposomes. Total lipid concentration 0.25 mM and 3 mol% lipidated peptide, in PBS.*

The in situ modification of plain liposomes with these lipidated peptides was studied next. This modification enables the activation of membranes with coiled coil forming peptides which could lead to future applications such as the fusion of liposomes with cells. To examine whether in situ modification of plain liposomes could indeed induce membrane fusion, plain liposomes were prepared via hydration of a lipid film. Next, a solution of the lipidated peptide (1 mol% with respect to the lipids) in PBS was added to the plain liposomes and left to incubate for 15 minutes at room temperature. Subsequently, in situ modified liposomes (E or K) were mixed with conventionally prepared liposomes (K or E, respectively, **Figure 5**). Also, separate batches of liposomes that were both in situ modified with lipidated peptides E and K were combined. The fusogenity of these systems were first studied with the lipid mixing assay described earlier. As only the cholesterol and DOPE modified peptides showed efficient membrane fusion, these lipidated peptides are tested here.

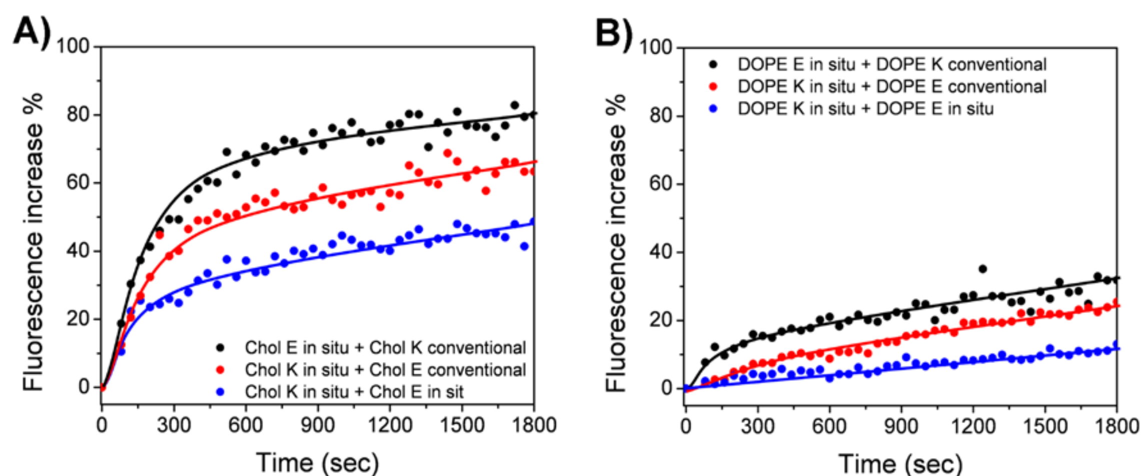


Figure 5. *Fluorescence graphs indicating lipid mixing kinetics between in situ modified liposomes and complementary liposomes (either in situ or conventionally modified). The lipidated peptides were added (1 μM final concentration) to preformed liposomes (0.1 mM). A) Addition of cholesterol-PEG-E (CPE) and/or cholesterol-PEG-K (CPK) to preformed liposomes and B) Addition of (DOPE-PEG-E) LPE and/or (DOPE-PEG-K) LPK to preformed liposomes.*

Figure 5 shows that addition of cholesterol peptides to plain liposomes yields highly fusogenic liposomes, comparable to the traditional liposome preparation method. In contrast, the in situ modification of plain liposomes with the DOPE peptides resulted in much lower lipid mixing rates. Additionally, in situ modification with the E peptides is more favorable as compared to the K peptides **(Figure 5A)**. This was shown by adding liposomes which were postmodified with lipidated E or K to traditionally prepared fusogenic liposomes, bearing the complementary peptide. This phenomenon could be due to the observation that the K peptide interacts more with liposomal membranes then the E peptide.[41] This might inhibit the proper insertion of the cholesterol moiety from inserting into the lipid membrane.

To prove that the fusion events are due to incorporation of the lipidated peptides in the liposomal membranes, an additional content mixing experiment was performed. A solution of lipidated peptides was added to sulphorhodamine loaded liposomes and any non-bound peptide was removed by size exclusion chromatography.

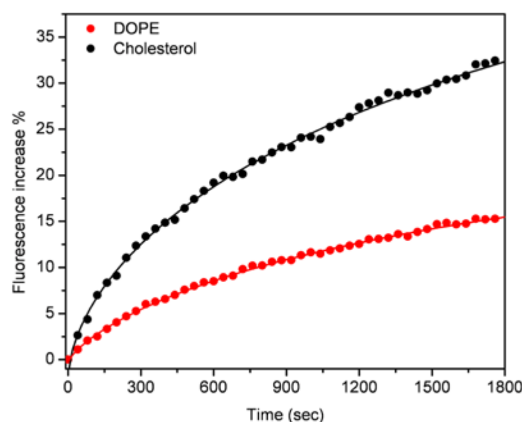


Figure 6. *Fluorescence graphs indicating the rate of content mixing between conventionally prepared cholesterol-PEG-K or DOPE-peg-K liposomes and liposomes to which cholesterol-PEG-E and DOPE-PEG-E were added in situ. Final lipopeptide concentrations were 1 µM and liposome concentrations were 0.1 mM. 20 mM sulphorhodamine B was encapsulated in E-decorated liposomes.*

Both the DOPE and cholesterol modified peptides induced content mixing after in situ modification of plain liposomes with the E peptides. However, the cholesterol modified peptides induced significant more content mixing after in situ modification of plain liposomes with the E peptides (**Figure 6**). This is consistent with the lipid mixing data and it is further evidence that the cholesterol anchor inserts efficiently in the liposomal membranes.

Conclusions

It was shown that the in situ modification of plain liposomes with peptide amphiphiles cholesterol-PEG-E and cholesterol-PEG-K yield highly fusogenic liposomes. The process of membrane fusion is targeted and occurs with efficient lipid and content mixing without leakage. This is a strong indication that both these lipidated peptides spontaneously enter lipid membranes. Consequently, it should now be possible to decorate biological membranes such as cell membranes with these peptide amphiphiles. This opens up the opportunity to form coiled coils at the surface of biological membranes and even induce fusion of liposomes with cells. Furthermore, the hydrophobicity of the membrane anchor was observed to be vital for inducing membrane fusion, since short single alkyl chains were not sufficient to hold the liposomes in close proximity upon coiled coil formation, whereas phospholipid

modified peptides do not readily insert into preformed membranes. Also, the anchor appears to have a large influence on the secondary structure of the peptides at the surface of liposomes, i.e. higher helicity values were obtained for peptide amphiphiles with more hydrophobic membrane anchors. Finally, the cholesterol and DOPE anchored peptide amphiphiles show more homocoiling as compared to the other lipidated peptides. This aggregation of peptides at the surface of liposomes might increase fusion efficiency as it is likely that multiple coiled coil motifs are needed, a phenomenon which is also observed in SNARE induced membrane fusion.

The addition of a set of complementary cholesterol anchored coiled coil forming peptides to separate batches of plain liposomes yielded highly fusogenic liposomes which fused in a targeted and controlled manner. With this fusion machinery in hand, activate pre-existing membranes can now be achieved, which might be used as nano-reactors with controlled mixing of the components, to dock a wide variety of molecular constructs to (natural) membranes and even to induce fusion between liposomes and cells.

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41. In lipid and content mixing assays the mixing of K decorated liposomes with plain liposomes consistently yields higher fluorescence increase than the mixing of E decorated liposomes with plain liposomes. It is likely that this observation is caused by interactions between the K peptide and lipid membranes.

Experimental section

Materials and Methods

Materials

The Fmoc-protected amino acids were purchased from Novabiochem. The Sieber Amide resin was purchased from Agilent Technologies. Fmoc-NH-PEG$_{12}$-COOH was purchased from IRIS Biotech. DOPE and DOPC were obtained from Avanti Polar Lipids and holesterol was obtained from Sigma Aldrich. DOPE-NBD and DOPE-LR were obtained from Avanti Polar Lipids. Palmitic acid, cholesteryl hemisuccinate and adamantane 1-carboxylic acid were purchased from Sigma Aldrich. Solvents were obtained from Biosolve Ltd.

Ge*neral Methods*

The purification of the hybrid peptides was performed by RP-HPLC with a Shimadzu system with two LC-8A pumps and a SPD-10AVP UV-VIS detector. UV detection was performed at 214 nm. The peptide hybrids were dissolved in a mixture of tert-butanol:acetonitril:water (1:1:1 v/v) and eluted with a flow rate of 20 mL/min.and with a linear gradient from A to B, where A was H_2O with 0.1 vol% TFA and B was acetonitrile with 0.1 vol% TFA. For lipopeptides with a phospholipid tail, purification was performed on a Vydac C4 column (214TP54, 4.6 mm, 250 mm length, 10.00 µm particle size). For the other peptide hybrids, purification was performed on a Gemini C18 column. Initially, samples were eluted with a linear gradient from 10% to 90% B over 3 column volumes. After this initial run, the product peak was identified and the gradient was adjusted to run from x% to x + 10%. Analysis on the purity of synthesized compounds was performed via LCMS.

Lipopeptide synthesis

The peptide segments (E (EIAALEK)$_3$ and K (KIAALKE)$_3$) of the hybrids were synthesized on a 100 μmol scale using Fmoc chemistry on an automatic Syro peptide synthesizer. A sieber amide resin with a loading of 0.69 mmol/g was used. Amino acid couplings were performed with 4 eq. of the appropriate amino acid, 4 eq. of the activator HCTU and 8 eq. of the base DIPEA, for 1 hour. Fmoc deprotection was performed with piperidine:NMP (4:6 v/v). Subsequent to peptide synthesis, Fmoc-NH-PEG$_{12}$-COOH was coupled to the peptide on the resin. The resin was swollen in NMP for 1 hour. Subsequently, 2 eq. of Fmoc-NH-PEG$_{12}$-COOH, 4 eq. of DIC and 4 eq. of HOBT were dissolved in NMP and left to preactivate for 2 minutes before it was added to the resin. The coupling was performed over night and the Fmoc group was removed.

For the DOPE hybrids, succinic acid (5 eq.) was coupled next, using 6 eq. TEA. The subsequent coupling of DOPE was performed by dissolving 3 eq. of the phospholipid, 3 eq. DIC and 3 eq. HOBT in NMP. This mixture was left to preactivate for 2 minutes and coupling was performed overnight.

For the other hybrids, 4 eq. of the hydrophobic anchor, 4 eq. of DIC and 4 eq. of HOBT was dissolved in NMP. The mixture was left to preactivate for 2 minutes and coupling was performed overnight.

The peptide hybrids were cleaved from the resin by shaking the resin with a mixture of TFA/TIS/H_2O (95:2.5:2.5 v/v) for 1 hour. The cleavage mixture was collected and

after co-evaporation with toluene, the crude product was obtained. Subsequently, the compounds were purified with HPLC

Liposome preparation

To prepare unlabelled liposomes, a 1 mM stock solution with the composition DOPC:DOPE:Cholesterol (50:25:25 mol%) in chloroform was used. For the lipid mixing assay, a stock solution with the compositition DOPC:DOPE:Cholesterol:DOPE-LR:DOPE-NBD (49.5:24.75:24.75:0.5:0.5 mol%) in chloroform was used. The lipidated peptides were dissolved in a mixture of Chloroform:Methanol (1:1 v/v), to a concentration of 50 µM. Typically, liposomes decorated with 1 mol% of the lipidated peptides were used and therefore the peptide hybrid and lipid stock solutions were mixed in equal amounts. The solvent was subsequently removed under a stream of air. For lipid mixing, DLS and CD experiments PBS was added to the dry lipid layer. For the content mixing experiments, HEPES buffer containing 20 mM sulphorhodamine B was added to the dry lipid layer. Subsequent sonication yielded ~100 nm liposomes.

Characterization

Optical density measurements

Optical density measurements were carried out using a Cary UV-Visible spectrometer. A quartz cuvette with a 1cm pathlength was used. The wavelength was set to 400 nm and samples were continuously measured for 30 minutes, subsequent to combining K decorated liposomes with E decorated liposomes (total lipid concentration 0.25 mM and 1 mol% of lipidated peptide).

Circular Dichroism

CD spectra were measured using a Jasco J-815 spectropolarimeter. The observed ellipticity is given in millidegrees, the conversion to the mean residue molar ellipticity is performed by the following equation:

$$[\theta] = \theta_{obs} \frac{MRW}{10lc}.$$

Here, Θ_{obs} is the ellipiticity 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. Spectra were obtained with a total sample concentration of 0.5 mM with 1 mol% peptide in a 0.2 cm quartz cuvette at room temperature. The datapoints were collected at a 0.5 nm interval, at a scanning speed of 100 nm/min and a 1 nm bandwith. Each spectrum was the average of 10 scans.

Helical content was determined using the following formula:

$$f_H = \frac{\Theta_{(225nm)}}{-39500 \times (1 - \frac{2,57}{n})}$$

where f_H is the helical fraction, $\Theta_{(225nm)}$ is the ellipticity at 225 nm and n is the number of peptide bonds.

Dynamic Light Scattering

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 173°. To obtain an estimation of the hydrodynamic radius, D_h, the Stokes-Einstein relation was used:

$$D = \frac{k_B T}{3\pi\eta D_h}.$$

Here, k_B is the Boltzmann constant and η is the viscosity of the solvent. Measurements were carried out at room temperature.

Fluorescence spectroscopy

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.

For content mixing experiments, fluorescence time series measurements were started immediately after mixing 600 μL of the fluorescent-labeled (20 mM sulphorhodamine) liposome suspension with 600 μL of unlabeled liposome suspension in the cuvette. The sulphorhodamine fluorescence intensity at 580 nm was monitored in a continuous fashion for 1800 seconds. After that the liposomes were lysed by the addition of 150 mL of 10 wt% Triton X-100 in PBS to obtain 100% increments. The percentage of fluorescence increase (%F(t)) is calculated as: $\%F(t)=(F(t)-F_0)/(F_{max}-F_0)$ 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.

Fluorescence measurements for lipid mixing were performed on a Tecan Plate Reader Infinite M1000. NBD emission was measured continuously upon mixing fluorescent K decorated liposomes with non-fluorescent E decorated liposomes at 530 nm for 1800s. The 0% value was determined by measuring NBD emission of K liposomes to which an equal amount of PBS was added. The 100% value was determined by using liposomes which contained half the probe (NBD and LR) concentrations. The

percentage of fluorescence increase (%F(t)) was calculated as: $\%F(t)=(F(t)-F_0)/(F_{max}-F_0)$ 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 on liposomes with half the probe concentrations.

Appendix: Supplementary information
DLS data indicating particle size increase upon liposome fusion

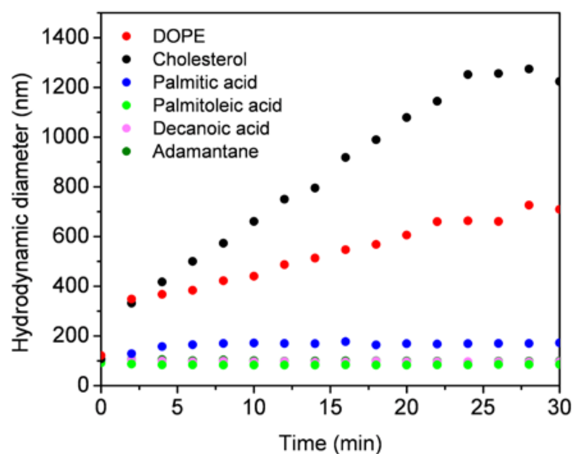

Figure S1. *DLS data were measured upon combining batches of E and K-decorated liposomes (0.25 mM liposomes with 1 % lipopeptide).*

Liquid Chromatography data of lipidated K peptides

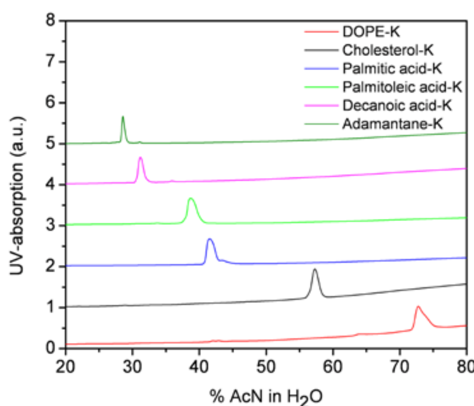

Figure S2. *LC graphs of the lipidated K peptides, revealing the retention time and thus the hydrophilicity. Broad peaks or sholder are a result of the formation of TFA salts of the lipidated peptides, as was revealed by LC-mass spectrometry.*

Control experiments for lipid mixing

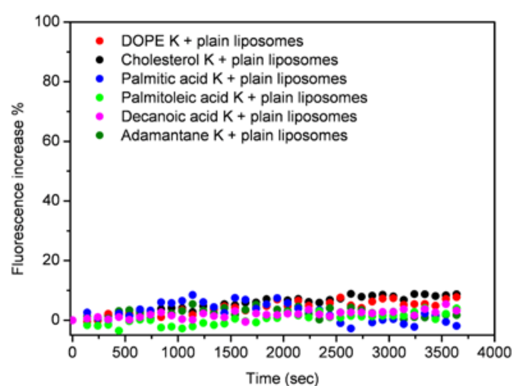

Figure S3. *Lipid mixing between K decorated liposomes and plain liposomes, as indicated by an increase in NBD fluorescence. Non-fluorescent liposomes (0.1 mM) were added to fluorescent K liposomes (0.1 mM, 1% peptide K).*

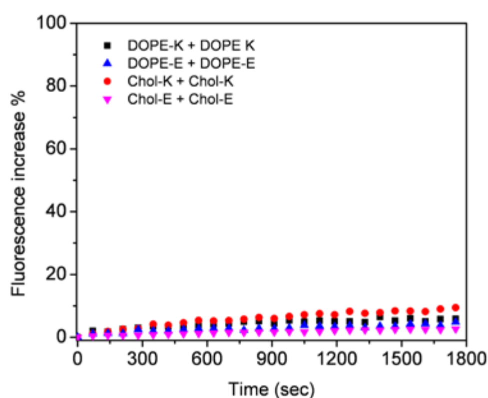

Figure S4. *Lipid mixing between K decorated liposomes with K decorated liposomes, and E decorated liposomes with E decorated liposomes, as indicated by an increase in NBD fluorescence. Total lipid concentration 0.1 mM, 1% peptide K or E.*

Multiple rounds of fusion

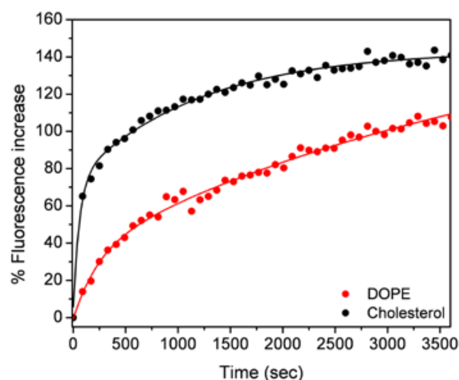

Figure S5. *Lipid mixing between E and K decorated liposomes as indicated by increase in NBD emission. Non-fluorescent E liposomes (0.5 mM, 1% peptide E) were added to fluorescent K liposomes (0.1 mM, 1% peptide K).*

Control experiments for leakage during content mixing

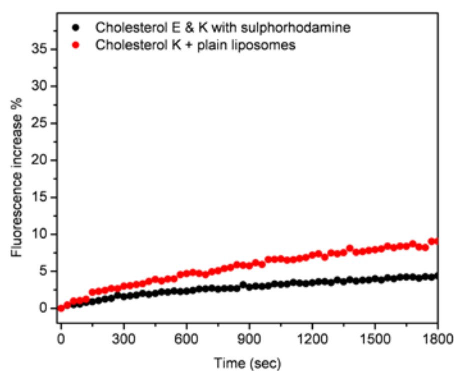

Figure S6. *(black) Content mixing between E and K liposomes (0.1 mM total lipid concentration, 1 mol% lipidated E and K peptides) that were both loaded with 20 mM sulphorhodamine B, to test for leaking during fusion. (red) Control for content mixing; K decorated liposomes (0.1 mM total lipid concentration with 1 mol% cholesterol-K) were added to fluorescently loaded plain liposomes (0.1 mM total lipid concentration, 20 mM sulphorhodamine B).*

Circular Dichroism (CD) data of the lipidated peptides at the surface of liposomes

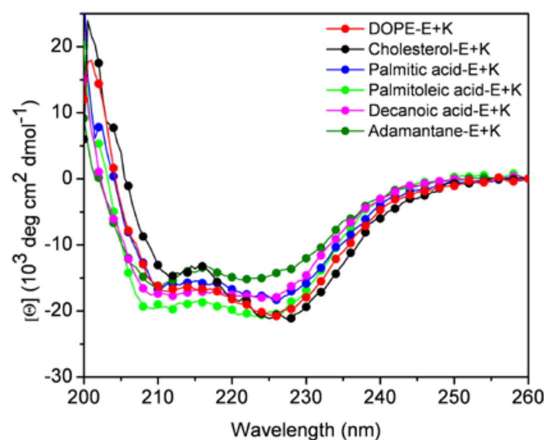

Figure S7. *CD data for a 1:1 mixture of E and K decorated liposomes (15 minutes after mixing). Total lipid concentration 0.25 mM and 3 mol% lipidated peptide, in PBS.*

Table S1. *Ellipticity ratios (~222/208 nm) for E-decorated liposomes, K-decorated liposomes and 1:1 mixtures thereof, as measured with circular dichroism. The total lipid concentration was 0.25 mM, with 3 mol% lipopeptide, in PBS.*

Anchor	K(~222/208)	E(~222/208)	E+K(~222/208)
DOPE	0,97	0,86	1,28
Cholesterol	1,07	0,91	1,37
Palmitic acid	0,95	0,84	1,10
Palmitoleic acid	0,89	0,86	1,08
Decanoic acid	0,93	0,82	1,03
Adamantane	0,83	0,79	0,88
No anchor	0,81	0,74	0,98

Table S2. *Helical content of E and K decorated liposomes (0.25 mM total concentration, 3 mol% lipidated peptide, in PBS) and 1:1 mixtures thereof. Helicities for the mixtures of liposomes with cholesterol and DOPE peptides are lower then expected. This is probably due to the size increase of the liposomes, which causes scattering of the incoming circularly polarized light.*

Anchor	K (% helicity)	E (% helicity)	E+K (% helicity)
DOPE	73	68	60
Cholesterol	74	69	60
Palmitic acid	63	36	53
Palmitoleic acid	56	39	59
Decanoic acid	44	30	52
Adamantane	40	24	41
No anchor	48	39	90