Extending the self-assembly of coiled-coil hybrids

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A REDUCED SNARE MODEL FOR MEMBRANE FUSION

Let's get together: A minimal model system was developed to mimic the SNARE-protein-mediated fusion of biological membranes. Fusion between two populations of liposomes is controlled by a pair of complementary lipidated oligopeptides that form noncovalent coiled-coil complexes and thereby force the membranes into close proximity to promote fusion. The model system displays the key characteristics of in vivo fusion events.

INTRODUCTION

Membrane fusion is a key process in all living cells, as it facilitates the transport of molecules between and within cells. The process is triggered by the specific interaction of fusion proteins. This interaction brings two membranes into close proximity and is followed by local disruption of the lipids and merging of the membranes.[1] The required protein recognition for the fusion of transport vesicles with the neuronal membrane involves the coiled-coil interaction between three complementary SNARE proteins (SNARE=soluble NSF attachment protein receptor; NSF=N-ethylmaleimide-sensitive factor).[2] To induce intracellular transport, a four-helix coiled-coil bundle forms between two membrane-bound SNARE proteins and a cytoplasmic SNARE protein and forces the two membranes within a distance of 2–3 nm from one another (Figure 1c).[3] The exact mechanism and fundamental requirements of fusion are still unknown.[4] Reduced systems have therefore been studied to gain insight into the most important aspects of membrane fusion.[5]

In vivo membrane fusion is a highly controlled process. To mimic this process, model systems must include the following features: Specific molecular recognition must lead to the merging of lipid bilayers, liposome-content mixing must occur without leakage, i.e., with no rupture of the membranes, and there must be an increase in liposome size. Additionally, the fusion process must be inhibited by inverted cone lipids, as these lipids hinder the formation of the low-energy stalk intermediate,[6] and it should not be dependent on membrane curvature stress. However, none of the model systems so far have displayed all of the basic characteristics of native membrane fusion.[5] To date, the major unresolved question has been: What is the minimal machinery required for the controlled fusion of lipid membranes? This question is answered herein with a model system that contains simplified versions of SNARE proteins. These model proteins can cause liposome fusion with the key characteristics of native membrane fusion. In this novel approach, the operative features of SNARE proteins were used to create a simple and applicable membrane-fusion model.

RESULTS AND DISCUSSION

Two lipidated oligopeptide hybrids (LPE and LPK) were designed which possess all of the functional aspects of membrane-bound SNARE proteins (Figure 1). The protein-recognition domain of SNARE proteins is an eight-heptad repeat segment with a high propensity to form coiled coils.[7] This segment is connected through a flexible linker to a transmembrane domain that anchors the protein to the lipid membrane. In the design of the lipidated oligopeptides LPE and LPK, the recognition domain is mimicked by two three-heptad repeat coiled-coil-forming peptides (E and K, Figure 1a). These oligopeptides are the shortest known coiled-coil pair to assemble specifically into a stable heterodimer (Kd $\approx 10^{-7}$ M).[8] In this model system, the formation of the LPE/LPK complex is the driving force to bring two different liposomes close together. The role of the flexible spacer is fulfilled by a short poly(ethylene glycol) chain (PEG_{12}, Figure 1a). This spacer enables extension of the oligopeptide component from the surface of the liposomes. The lipidated oligopeptides are anchored spontaneously in the membrane by means of a phospholipid tail, 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE),[9] which mimics the function of the transmembrane domain of SNARE proteins (Figure 1a).

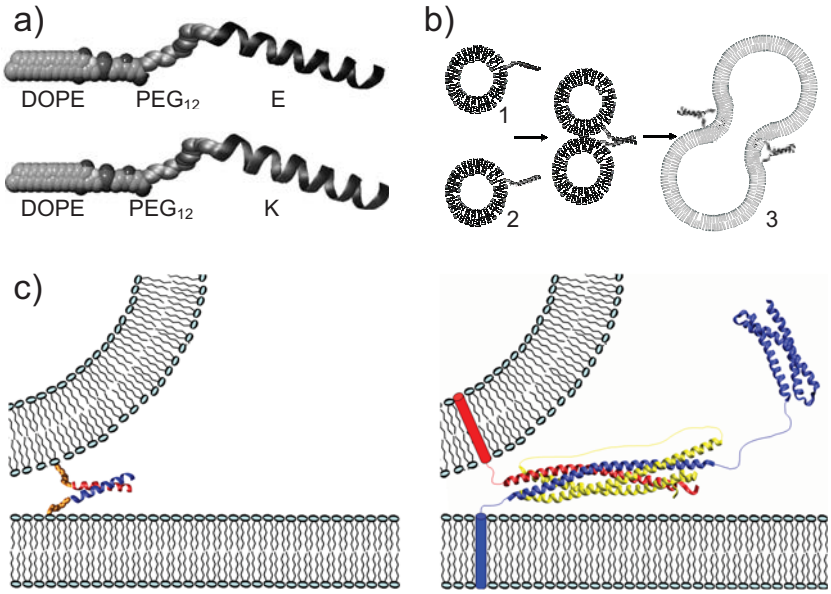

Figure 1. a) Space-filling model of the lipidated oligopeptides LPE and LPK, consisting of a DOPE tail linked through a PEG_{12} spacer to the coiled-coil-forming oligopeptides E and K. The amino acid sequence of E is $G(EIAALEK)_3$-NH_2, and that of K is $(KIAALKE)_3GW$-NH_2. b) The spontaneous incorporation of the DOPE tail in lipid bilayers results in liposomes decorated with either E or K peptides at the surface. When a liposome population carrying LPE (1) is mixed with a liposome population carrying LPK (2), coiled-coil formation (E/K) initiates liposome fusion (3). c) Comparison of the minimal model (left) with the SNARE-protein-based model (right).

Liposomes decorated with either LPE or LPK (3 mol%) were prepared with a nominal hydrodynamic diameter of 100 nm and a polydispersity index of approximately 0.2, as determined by dynamic light scattering (DLS).[10] The peptides were designed in such a way that heterodimers were stable, and homodimers would not form upon mixing.[8] However, as a result of the forced close proximity of the peptides anchored to the surface of the membrane, homocoils were present on these individual liposomes before mixing, as determined by circular dichroism (CD; Figure 2a). Nevertheless, like plain liposomes, LPE- or LPK-decorated liposomes did not show any self-fusion over time (as shown by DLS; Figure 2). Upon mixing of the decorated liposome populations, an immediate change in the peptide quaternary structure was observed by CD. The change in the CD spectrum was indicative of a transition from homocoiled LPE or LPK at the surface of the liposomes to aggregated heterocoils.[11] The observed change in peptide structure was accompanied by an increase in the hydrodynamic radius of the particles from approximately 100 nm to greater than 1 mm within 20 min (Figure 2).

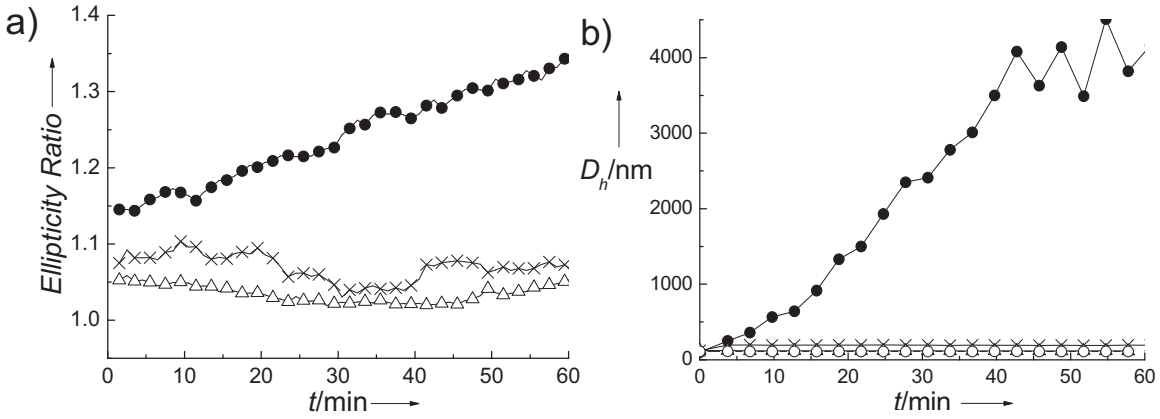

Figure 2. a) Ellipiticity ratios measured by CD of LPE- (Δ) and LPK-modified (×) liposomes. Upon mixing of these liposome batches, the aggregation of coiled coils was observed (●). b) The hydrodynamic diameter, D_h, measured by DLS, increased rapidly when the LPE- and LPK-decorated liposome populations were combined (●). In control experiments (with plain liposomes (○), plain liposomes mixed with LPE-decorated liposomes (Δ), and plain liposomes mixed with LPK-decorated liposomes (×)), no significant change in the hydrodynamic diameter was observed with time.

In the process of liposome fusion, three stages are distinguished, each of which requires energy input to overcome an energy barrier. In the first step, liposomes are brought into close proximity; merging of the outer lipid layers then occurs, followed by mixing of the inner lipid layers. From the initial fusion experiments, it was concluded that the energy gained by the formation of LPE/LPK coiled coils (ca. 14 kT per dimer; see Figure A1 in the Appendices) is sufficient to induce the aggregation of the two liposome populations. However, it is not sufficient to bring membranes into close proximity for membrane fusion to occur;[12] energy must also be provided for the second and third stages. Fluorescence experiments were conducted to determine whether the LPE/LPK interaction also transmits

enough force to the membranes to rearrange the lipids and hence promote lipid mixing followed by content mixing. The efficiency of lipid mixing was determined by using a fluorescence resonance energy transfer (FRET) assay. An LPK-decorated liposome batch was prepared with the donor dye nitrobenzofuran (NBD) and the acceptor dye lissamine rhodamine (LR) attached to the lipid-bilayer surface. A high FRET efficiency resulted from the short fluorophore distance. Upon mixing with a population of LPE-decorated liposomes, an increase in NBD emission was observed as a result of an increase in the distance between the membrane-bound donor and acceptor dyes (Figure 3a). This finding demonstrated that the formation of the LPE/LPK coiled-coil complex is sufficient to overcome the energy barrier that keeps undecorated liposomes apart and also results in at least hemifusion (i.e. lipid mixing of the outer lipid layers). As the fusion process can be halted at the hemifusion stage,[13] further experiments were conducted to ascertain whether the formation of the LPE/LPK complex also leads to mixing of the inner lipid layers. Therefore, the donor fluorophores on the outside of the LPK-decorated liposomes were deactivated to eliminate the FRET effect on the outer layer of the liposomes. Again, upon the addition of LPE-decorated liposomes, an increase in NBD fluorescence was observed. Thus, the merging of both the outer and the inner lipid layer, that is, complete fusion, does indeed occur (Figure 3a). Liposome fusion is used in biological systems to mix the contents of liposomes and hence to transmit chemical compounds and signals. There have been reports of lipid mixing in model systems without content mixing;[14] it was therefore investigated whether the lipid mixing in this system was accompanied by content mixing. The slightly fluorescent complex terbium citrate was encapsulated in LPK-modified liposomes, and the nonfluorescent ligand dipicolinic acid (DPA) was encapsulated in LPE-modified liposomes. When these two batches of liposomes were combined, an increase in fluorescence caused by the formation of the highly fluorescent terbium dipicolinic acid chelation complex[15] was observed (Figure 3b). From this result, it was concluded that membrane mixing resulted in content mixing. The input of too much energy can cause major destabilization of the lipid bilayers and lead to the occurrence of fusion not via the lowest-energy intermediate, but through the uncontrolled rupture of the liposomes accompanied by massive content leakage.[6] To determine whether this model was able to fuse liposomes without content leakage, ethylenediaminetetraacetic acid (EDTA) was added after a fusion experiment between LPK- and LPE-modified liposomes. If fusion proceeds without content leakage, the $Tb(DPA)_3^{3-}$complex should not be quenched by the strong chelator EDTA, which is unable to diffuse into the liposomes. Indeed, no decrease in fluorescence was observed (Figure 3b). These results show that the LPE/LPK-mediated liposome fusion proceeds in a controlled way, similar to the natural SNARE-mediated fusion process. Theoretical and experimental studies indicate that a key feature of all types of fusion between biological membranes is that they proceed through a stalk intermediate, which reduces the number of lipids involved in the fusion intermediates.[6,16] Phospholipids with a packing parameter of less than 0.5[17] have a spontaneous positive membrane curvature, which inhibits the stalk intermediate geometrically and reduces membrane

fusion.[18] To prove that the current model system mimics biological membrane-fusion events, oleoyl lysophosphatidylcholine (packing parameter ≈ 0.4) was added to the LPE- and LPK-bearing liposome populations. Upon mixing, fusion was inhibited, as concluded from the decrease in lipid mixing (Figure 3a). This result indicates that the stalk intermediate is also part of the fusion process in this model system.

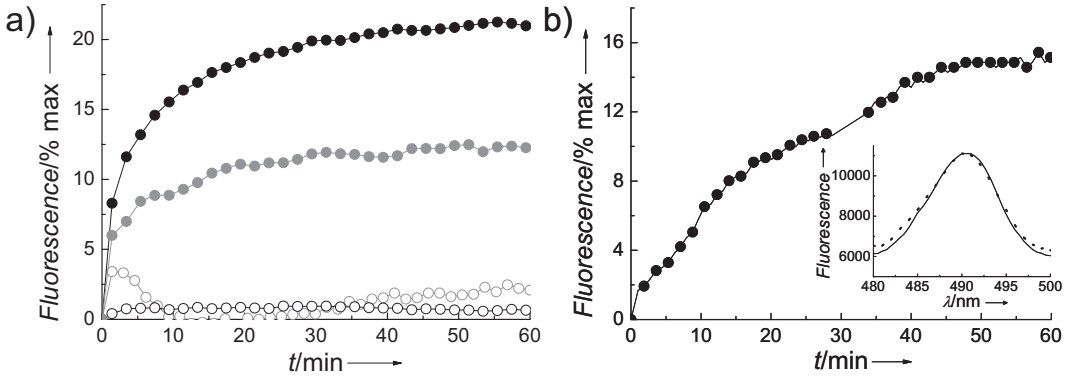

Figure 3. a) Lipid mixing between liposomes as indicated by an increase in NBD emission. Mixing of LPE- and LPK-decorated liposomes: ●; mixing of plain liposomes: ○; mixing of inner-layer lipids when LPE- and LPK-decorated liposomes were combined: ●. Lipid mixing was inhibited when LPE- and LPK-decorated liposomes containing oleoyl lysophosphatidylcholine (15 mol%) were mixed (○). b) Mixing of the aqueous compartments of decorated liposomes with encapsulated $Tb(citrate)_3^{3-}$ and DPA resulted in a fluorescence increase due to the formation of $Tb(DPA)_3^{3-}$. Inset: The content mixing proceeds without leakage, as the fluorescence intensity remains constant before (—) and after (····) the addition of the fluorescent quencher EDTA outside the fused liposomes.

For this system to be a true analogue of SNARE-mediated liposome fusion, it should be shown that curvature stress is not a driving force.[19] Cryo-TEM demonstrated that the fusion of LPE- and LPK-modified liposomes (Figure 4) resulted in the formation of larger liposomes in less than 1 min, a result indicative of fast docking and fusion events. The fused liposomes had complex, highly curved internal membrane morphologies. Thus, the fusion process is indeed not driven by a release of curvature strain in the membrane. The cryo-TEM data also support the observation that no leakage occurs during fusion. In the absence of leakage, the fusion of liposomes does not affect the total amount of lipid or the enclosed volume; therefore, fusion should result in liposomes with an excess of lipid. The observed folding of the bilayers to the interior of the liposomes, in combination with the terbium leakage assay, demonstrates that no or only limited exchange occurs between the interior aqueous phase of the liposomes and the surrounding medium.

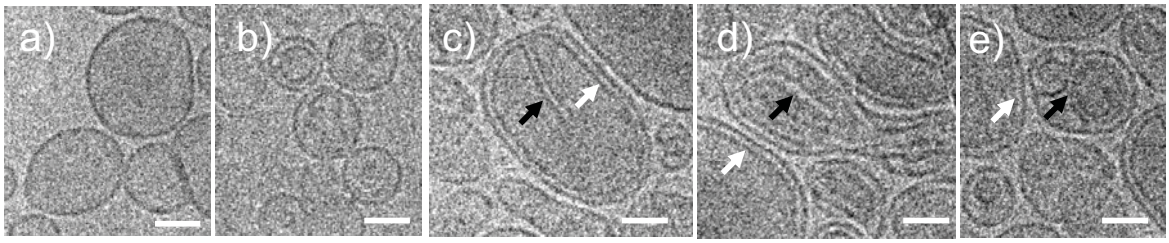

Figure 4. Cryo-TEM images showing fusion: a) LPE- and b) LPK-decorated liposomes; c–e) liposomes with complex membrane morphologies formed 30 s after the mixing of LPE- and LPK-modified vesicles. After fusion, the vesicles show double bilayers (white arrows) and invaginations (black arrows). Scale bars: 50 nm.

To further investigate the hypothesis that the fusion events are not affected by curvature stress, LPE- and LPK-modified liposome populations were prepared with a diameter of approximately 1 mm. These LPE- and LPK-modified liposomes did not change in size with time; however, fusion occurred upon mixing, and liposomes with diameters in the tens of microns were observed by optical microscopy[20] (Figure 5). Further analysis of the optical microscopy data showed that liposomes with a diameter of the order of 10 mm were also able to undergo fusion: a process reminiscent of cell–cell fusion[21] (Figure 5, inset). The specific fusion of liposomes of these dimensions proves that curvature stress is not the driving force for fusion in this model system.

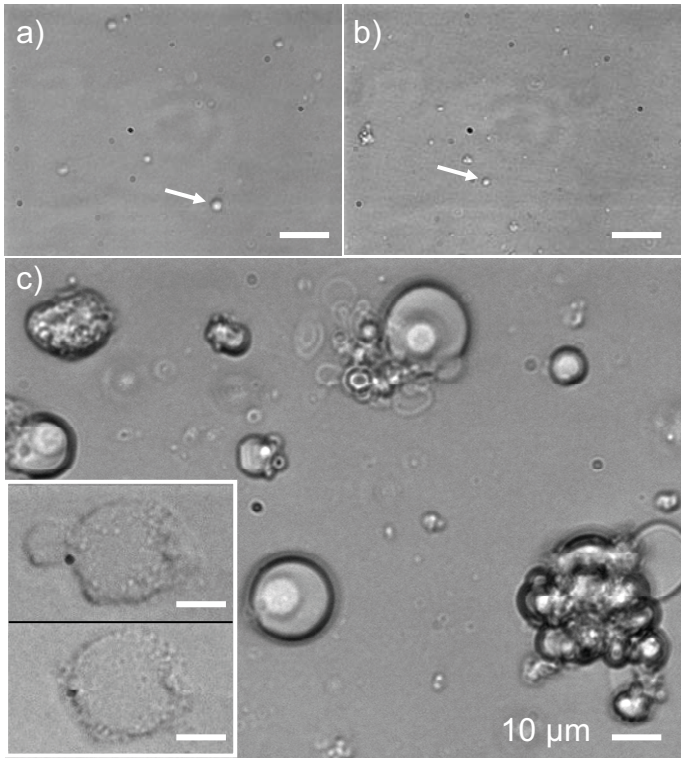

Figure 5. Optical microscopic images of large a) LPE- and b) LPK-functionalized liposomes before fusion. The liposomes are white; examples of liposomes are indicated by arrows. c) Upon mixing, giant liposomes were observed. Inset: Fusion of cell-sized liposomes. Scale bars: 10 mm.

There are two approaches to studying membrane fusion. One is to use known native fusogenic proteins, which can be expressed and studied in vitro or used in native fusogenic systems. Owing to the participation of multiple, often interacting, proteins prior to the actual membrane-fusion step in native systems, it is very difficult to isolate the effect of particular proteins.[22] The other, bottom-up approach enables the determination of the fundamental requirements, the scope, and the limitations of controlled lipid-membrane fusion; however, until now, the mode of recognition has been unrelated to that of natural SNARE-protein-based systems. Furthermore, to date none of the in vitro model systems that have been developed to mimic membrane fusion have met all the key requirements typical for in vivo membrane-fusion systems. The lipidated oligopeptides that were used bridge these two approaches. They are on the one hand analogues of SNARE proteins in the sense that membrane fusion occurs by the same recognition mechanism, and they display the same key characteristics. Therefore, the results are more directly applicable to native membrane fusion. On the other hand, as the shortest known hetero coiled coil is used in this model, they are the most reduced form of SNARE proteins possible, which makes them simple enough to enable ready synthesis and the use of a range of physical organic techniques to study their behavior. The reduced SNARE model presented herein has been shown to meet all of the characteristics of native membrane fusion, and this similarity combined with the ease of use makes the system a true minimal model for SNARE mediated membrane fusion. This fusion system extends the realm of synthetic biology and enables us to understand an aspect of nature—liposome fusion in eukaryotic cells— through mimicry. It may also lead to new functions, such as the directed delivery of encapsulated reagents to cells or liposomes.

EXPERIMENTAL SECTION

Materials and Methods

Materials

Fmoc-protected amino acids and Sieber Amide resin (0.62 mmol of NH_2 g^{-1} of 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. $TbCl_3 \cdot 6H_2O$ (99.9% pure) and sodium citrate, were purchased from Sigma-Aldrich. Dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid) (≥99.5%) and octaethylene glycol monododecyl ether were obtained from Fluka. 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 MΩ cm^{-1} was provided by a Millipore Milli-Q filtering system with filtration through a 0.22 μm Millipak filter. Phosphate buffered saline, PBS: 5 mM KH_2PO_4, 15 mM K_2HPO_4, 150 mM NaCl, pH 7.4.

General Methods

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 22.5% (v/v) H_2O, 0.1% (v/v) TFA in CH_3OH, and B 10% (v/v) CH_3OH, 0.1% (v/v) TFA in acetonitrile. Purification of the peptides and hybrids 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^{-1}. For verification of sample purity a reversed phase Vydac C4 column (214TP54, 4.6 mm diameter, 250 mm length, 5.00 μm particle size) was used with a flow rate of 1 mL min^{-1} and a gradient of 10% – 100% B over 60 minutes. Sample elution was additionally monitored by an evaporative light scattering detector. MALDI-TOF mass spectra were acquired using an Applied Biosystems Voyager System 6069 MALDI-TOF spectrometer. Samples were dissolved in 1:1 (v/v) 0.1% TFA in water:acetonitrile (TA), at concentrations of ~0.3 mg mL^{-1} for K and E and ~3 mg mL^{-1} for LPE and LPK. Solutions for spots consisted of (v/v) 1:10 sample solution: 10 mg mL^{-1} ACH in TA. ^{1}H-NMR spectra were recorded on a Bruker DPX300 spectrometer using the residual proton resonance of deuterated methanol for calibration. FPLC was performed with an Äkta prime, Amarsham Pharmacia Biotech apparatus with a Pharmacia XK 26 column (135 mm x 25 mm) packed with Sephadex G50-fine. PBS was used as the eluent. The flow rate was 5 mL min^{-1}, UV sensitivity was set on 0.1 AU, 1%, the conductivity was set on 15-20 mS cm^{-1} and the wavelength for UV recording was 254 nm. Differential interference contrast (DIC) optical micrographs were recorded with a Zeiss axiovert-200 inverted microscope equipped with a 63 x objective long-range working lens. The images were recorded with a black and white CCD camera (AxioCam MRm) connected to an image-recording and –processing system (Axiovision 4.4).

Lipopeptide synthesis

The peptide components of LPE and LPK were prepared with standard solid-phase peptide synthesis protocols using Fmoc chemistry on an Applied Biosystems 431A automated peptide synthesizer, 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 (20 mL) 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 x 10 mL NMP, and 10 x 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 (20 mL) and mixed with 6 TEA. The mixture was added to the resin and shaken for 15 hours. The resin was washed thoroughly with 10 x 10 mL NMP, and 10 x 10 mL 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:water:TIS for one hour. The cleavage mixture and three subsequent rinses of the resin with the TFA mixture were added drop-wise to cold diethylether. The white precipitate was compacted with centrifugation and the supernatant removed. This was repeated three times with the addition of fresh diethylether. The pellets were dried in air or under reduced pressure. 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:water:TIS for one hour.

The crude products of were purified by RP-HPLC, with gradient elution 30% to 80% B over 15 minutes for LPE, and 10% to 60% B over 15 minutes for LPK. After purification the compounds were lyophilized to give white powders. LPE had a yield of 7%, MALDI-TOF MS: M_n = 3768.9 g mol^{-1}. LPK had a yield of 22%, MALDI-TOF-MS: M_n = 3951.9 g mol^{-1}. The purity of the compounds was confirmed by means of RP-HPLC, MALDI-TOF MS, and ^{1}H-NMR. For each compound the purity was estimated from RP-HPLC to be greater than 95%.

Liposome Preparation

1mM lipid stock solutions were made in chloroform with the composition DOPC/DOPE/CH 50:25:25 mol%. For fluorescence studies a 1 mM lipid stock solution was made in chloroform with the composition DOPC/DOPE/CH/DOPE-LR/DOPE-NBD 49.5:24.75:24.75:0.5:0.5 mol%. To investigate the effect of lipids with positive curvature on lipid mixing 1 mM lipid stock solutions were made in chloroform with the composition DOPC/DOPE/CH/OLPC 42.5:21.25:21.25:15 mol% and DOPC/DOPE/CH/OLPC/DOPE-LR/DOPE-NBD 42.08:21.04:21.04:15:0.43:0.43 mol%. 1mM hybrid 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 3 mol% LPE (97:3 (v/v) lipid stock solution: LPE stock solution), and liposomes with 3 mol% LPK (97:3 (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 in a bath sonicator at 45 °C to 50 °C to get small unilamellar vesicles (this takes approximately 5 minutes and 2 minutes for plain and decorated liposomes respectively). The hydrodynamic diameter as determined by DLS was approximately 100 nm.

Liposome preparation for lipid mixing assay

The mixing of lipids between batches of liposomes was assayed according to (Struck, D Biochemistry 20, **1981**, 4093-4099). Fluorescence experiments were carried out with the Fluorescent Resonance Energy Transfer (FRET) pair DOPE-NBD (λ_{ex}= 460 nm λ_{em}= 534 nm) and DOPE-LR (λ_{ex}= 550 nm λ_{em}= 590 nm). Fluorescent LPK decorated liposomes were prepared (DOPC/DOPE/CH/DOPE-LR/DOPE-NBD 49.5:24.75:24.75:0.5:0.5 mol%). LPE decorated liposomes had no fluorescent labels.

Decorated liposome preparation for inner leaflet mixing assay

Reduction of DOPE-NBD/DOPE-LR containing vesicles with sodium dithionate ($Na_2S_2O_6$) resulted in liposomes with selectively eliminated fluorescence from the outer leaflet. For this assay, 2.5 mM fluorescent liposomes decorated with LPK were prepared. The fluorescence intensity of the sample was measured with a Varian UV-visible Spectrophotometer model Cary 3 Bio that excited the NBD (λ_{ex}= 460 nm) at 460 nm. Then, 10 vol% of the sample was replaced by a 20 mM solution of $Na_2S_2O_6$ in PBS. Hereafter, the fluorescence intensity was monitored with the UV Spectrophotometer and 2 minutes after equilibration, the $Na_2S_2O_6$ was removed by injecting the sample into an FPLC column. The liposome concentration was corrected for the column dilution, and the fluorescence intensity at 460 nm was measured by UV Spectroscopy and the CD intensity of the sample was measured. The final liposome concentration was typically 0.3 mM. The 10 vol% non-reduced liposome sample was diluted to the same final concentration. LPE decorated liposomes without the FRET pair as well as undecorated liposomes were made with the same final concentration for the mixing experiment.

Decorated liposome preparation for contents mixing assay

The mixing of aqueous contents between batches of liposomes was assayed according to (Wilshut, J Biochemistry **1980**, 26, 6011-6021). For this assay, 2.5 mM liposomes were prepared. LPK decorated liposomes were prepared in PBS containing 50 mM DPA, 20 mM NaCl and 100 mM NaOH (to prepare the sodium salt of DPA). LPE decorated liposomes were prepared in PBS containing 2.5 mM $TbCl_3$ and 150 mM sodium citrate. A sample of undecorated liposomes was prepared with the Tb solution. The Tb and DPA solutions had been filtered over a 200 nm filter. Liposomes were prepared from the films by first adding 500 μL of the Tb or DPA solutions in PBS and after 3 minutes of sonication 1.5 mL more was added. These solutions were added in two steps to promote a higher concentration of DPA or Tb encapsulation in the liposomes. Immediately after preparation, the unencapsulated solution was removed by passing the liposomes over the FPLC column that used a PBS buffer. Hereafter, the liposome concentration was corrected for the column dilution and the CD intensity of the sample was measured. The three different batches were taken to the same final concentration with PBS to allow 1:1 mixing for the experiments. The final concentration of the samples was typically 0.4 mM.

Preparation of large unilamellar vesicles/ large multilamellar vesicles (LUVs/LMVs). LUVs/LMVs were prepared according to a slight variation of the solvent evaporation method of (Moscho, A.; Orwar, O.; Chiu, D. T.; Modi, B. P.; Zare, R. N *Proc. Nat. Acad. Sci. U.S.A.* **1996**, 93, 11443-11447). In a 50 mL round bottom flask 1970 μL lipid stock solution (1 mM), 100 μL methanol, 30 μL hybrid stock solution (1 mM) were combined. 3 mL of 17 mM PBS was carefully added. The organic solvents were evaporated for 2 minutes using a rotary evaporator at 40 rpm and with the water bath at 40 °C. This yields 0.33 mM liposomes that are approximately 500 – 1000 nm in diameter.

Characterization of Liposomes

Circular Dichroism

CD spectra were obtained as detailed in the experimental section of Chapter 2. For CD time series measurements were started immediately after the solutions were mixed (1000 rpm for 30 seconds). 60 CD spectra were obtained at intervals of 1 minute. Each spectrum was one scan. 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 in buffer) subtracted.

Dynamic Light Scattering

Dynamic light scattering was conducted as detailed in the experimental section of Chapter 3. For individual liposome batches the samples were allowed to equilibrate for 2 minutes. For DLS time series the solutions were mixed in the cuvette (1000 rpm for 30 seconds).

Measurements were started immediately after mixing and without 2 minutes of sample equilibration. 20 consecutive scans were measured in 1 hour.

Fluorescence Spectroscopy

Fluorescence measurements were performed using a FS920 fluorometer from Edinburgh Instruments with a DTMS-300X excitation monochromator and a peltier-controlled thermostatic cell. All spectra were obtained at 25 °C using a quartz cuvette with a 1 cm path length. The step size was 0.5 nm, with a sampling time of 0.5 s at each wavelength, and 1 scan was measured for each spectrum. For the lipid mixing assays excitation and emission slits were 1 nm. Emission spectra were measured from 490 nm to 700 nm at a fixed excitation wavelength of 460 nm. Fluorescence time series measurements were started immediately after mixing the solutions in the cuvette (30 seconds stirring at 1000 rpm). NBD fluorescence increase was measured in a continuous fashion for 1 hour (each scan was measured in 2 minutes). The NBD fluorescence was calibrated to enable calculation of the change in fluorescence percentage with time. The 0% fluorescence intensity was measured by diluting the fluorescent-labeled liposome batch 1:1 (v/v) with PBS to get the same overall fluorophore concentration as one would get after mixing the fluorescent-labeled liposomes 1:1 with unlabeled liposomes. After the one hour time series the 100% value was obtained. The liposomes were lysed by addition of 1% (w/v) Triton X-100 in PBS (10% (v/v) of 10% (w/w) Triton X-100 in PBS). The value that was obtained was multiplied by 1.55 to take into account the effect the Triton X-100 has on the NBD fluorescence. This value was obtained by preparing a 1 mM liposome solution that only contained the fluorophore NBD (DOPC/DOPE/CH/DOPE-NBD 49.75/24.875/24.875/ 0.5 mol%, 1mM). First, an emission spectrum was recorded for these liposomes to measure the NBD fluorescence intensity. Hereafter, the liposomes were lysed by adding 1% (w/v) Triton X-100 in PBS and an emission spectrum was recorded to determine the effect that Triton X-100 has on the NBD fluorescence. The value that was obtained was also multiplied by 1.1 to take into account the loss of 10 volume percent of sample that was replaced with Triton X-100. The percentage of fluorescence is calculated using equation 8, $F(\%) = (F(t) - F_0) / (F_{max} \times 1.55 \times 1.1 - F_0) \times 100$ (8) In this equation, F(t) is the fluorescence intensity measured at time t, F_0 is the 0% fluorescence and F_{max} is the 100 % fluorescence. The spectra were corrected by subtraction of buffer or liposome spectra. Content mixing is preferred as a technique to demonstrate the fusion event. The internal content mixing assay monitors the fusion of the aqueous compartments of the liposomes using molecules that are encapsulated into the liposomes. In this experiment, the molecules $Tb(citrate)_3^{3-}$ and dipicolinic acid (DPA), are used. Tb^{3+} ions are weakly fluorescent at λ_{ex} = 276 nm λ_{em} = 545 nm[3] and DPA does absorb but does not fluoresce at these wavelengths. Upon fusion (i.e. contents mixing), the highly fluorescent $Tb(DPA)_3^{3-}$ complex is formed that will show an increase in fluorescence intensity. For the content mixing assays excitation and emission slits were 5 nm. Emission spectra were measured from 480 nm to 520 nm at a fixed excitation wavelength of 276 nm. Fluorescence time

series measurements were started immediately after mixing the solutions in the cuvette (30 seconds stirring at 1000 rpm). The $Tb(DPA)_3^{3-}$ fluorescence increase was monitored for 1 hour (each scan was measured in 48 seconds). For calibration, the 0% fluorescence intensity was measured by diluting the Tb containing liposome batch 1:1 with PBS. After the one hour time series the 100% value was obtained. The liposomes were lysed by addition of 0.8 mM octaethylene monododecyl ether in a solution containing 20 μM free DPA (1% (v/v) 80 mM octaethylene monododecyl, 2 mM DPA in PBS). The percentage fluorescence is calculated according to equation 8. To see if there is any leakage of the contents, 1 mM ETDA can be added to the sample. The EDTA chelates to any Tb that has leaked, hereby disrupting the highly fluorescent $Tb(DPA)_3^{3-}$ that will be formed with any leaked DPA. In this way any increase in fluorescence signal is caused by pure content mixing. After the time series 1 mM EDTA (1% (v/v) 100 mM EDTA) was added to the mixture and the Tb fluorescence was recorded. Following this the liposomes were lysed with the addition of 0.8 mM octaethylene monododecyl ether, releasing the contents of the liposomes. All Tb fluorescence was quenched.

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APPENDICES

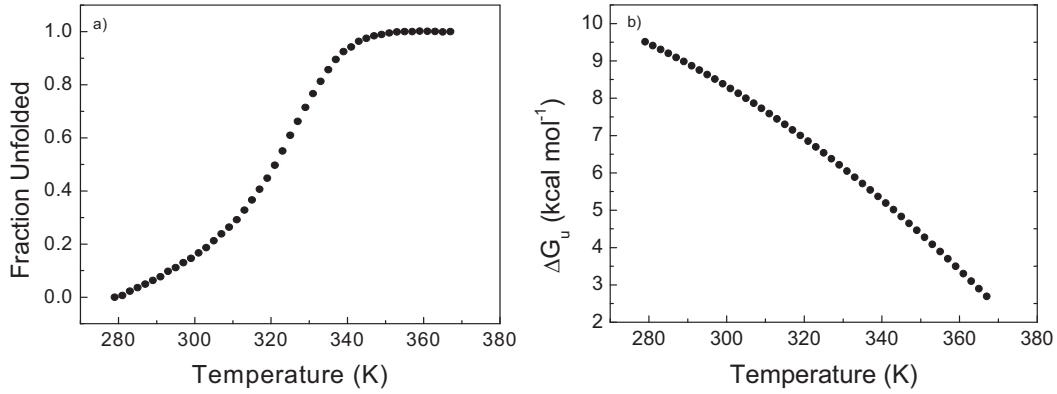

Figure A1.a) The thermal unfolding curve of E/K[i], derived from the temperature dependent ellipticity at 222 nm as followed by CD. (Heating and cooling rate 2 °C / min. [Total Peptide] = 40 μM, 50 mM phosphate, 100 mM KCl, pH 7.0 buffer). b) Free energy associated with the unfolding of E/K[i] as a function of temperature. At 25 °C the value of ΔG_u is 8.5 kcal mol^{-1}, or 14.4 kT. The dissociation constant at 25 °C calculated from this ΔG^{PBS} value is 5.6 x 10^{-7} M.

[i] The amino acid sequences of E and K are exactly the same as those of the peptide components of LPE and LPK except that for the temperature dependent data K does not have the C-terminal tryptophan residue.

Variations from the standard conditions

All of the results presented in Chapter 7 use 3 mol% lipopeptide incorporated into liposomes, and where possible 1 mM liposome concentrations are used. Each decorated liposome population is equimolar, and the experiments are conducted at 25 °C. The value of 3 mol% lipopeptide was chosen so that the CD signal would be large enough to be reliably interpreted. For the observed liposome sizes this corresponds to ~ 1600 peptides on the outside of the liposomes. After the characteristics of lipopeptide induced liposome fusion were established using these conditions, four variables were changed in order to investigate their effect on liposome aggregation and fusion, namely: liposome and lipidated peptide concentrations, ratios of LPK and LPE decorated liposomes, and the temperature at which the experiments were conducted. Initial information on the effects of these variables on liposome aggregation and fusion were obtained by DLS, and by using the total lipid mixing assay.

Liposome concentration

In the experiment in which the liposome concentrations were changed, 3% LPK modified fluorescent liposomes and 3% LPE modified liposomes were made with liposome concentrations of 0.2, 0.4, 0.6 and 0.8 mM. DLS time series were recorded to follow the change of liposome size with time caused by liposome aggregation or fusion (Figure A2a). All liposome concentrations result in particle size increases. For 0.2 mM decorated liposomes there is a linear increase in particle size with time, whereas for the more concentrated samples there is exponential size growth, with the more concentrated samples containing particles of many microns. As the number density of lipopeptide

modified liposomes in solution increases there is a corresponding increase in particle size because the liposomes encounter each other more often due to Brownian motion.
Fluorescence time series were also recorded to follow the total lipid mixing of these mixtures containing 0.2, 0.4, 0.6 and 0.8 mM liposomes (Figure A2b). The fluorescence increase reaches 17-22% for all these mixtures and is therefore comparable to the 20% obtained for mixing the 1 mM liposome solutions. There is a trend of increasing total lipid mixing with decreasing liposome concentration, although the absolute values were not fixed, as seen by the two values for 0.8 mM samples at one hour. These results suggest that the concentration of modified liposome does not dramatically affect the fusion event. Contrary to lipid mixing, liposome clustering is strongly dependent on the liposome concentration, therefore the liposome cluster size does not influence total lipid mixing to a large extent. These results also suggest that the larger size increase observed in Figure A2a for higher liposome concentrations is the result of aggregation of liposomes rather than fusion of liposomes.

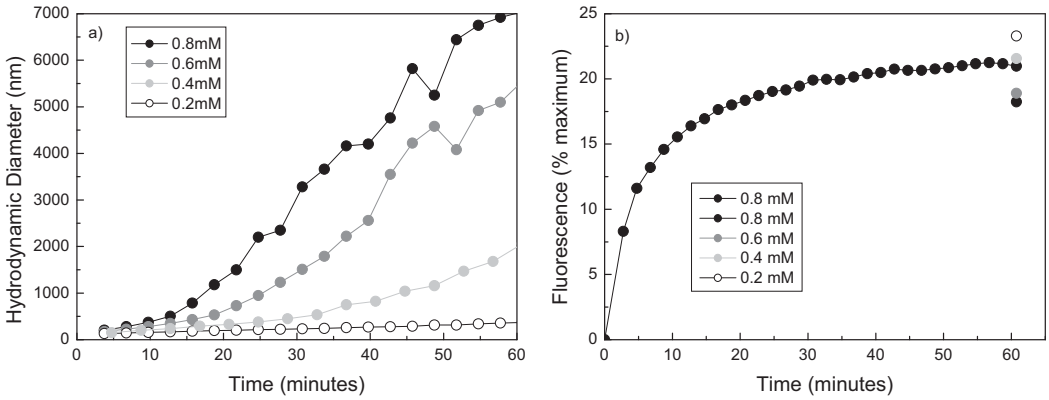

Figure A2. a) DLS time series recorded for mixtures of 3% LPK modified fluorescent liposomes and 3% LPE modified liposomes with liposome concentrations of 0.2, 0.4, 0.6 and 0.8 mM. b) Total lipid mixing as monitored by fluorescence spectroscopy for mixtures of 3% LPK modified fluorescent liposomes and 3% LPE modified liposomes with liposome concentrations of 0.2, 0.4, 0.6 and 0.8 mM.

Lipopeptide concentration

To evaluate the effect of lipopeptide concentration, samples were made with LPK modified fluorescent liposomes and LPE modified liposomes with 1, 0.5 and 0.1 mol% lipopeptide in place of the standard 3 mol%. The lipid concentration used was the standard 1 mM. A decrease in the peptide density on the membrane of the liposomes is expected to influence the bending and distortion of the membrane and the numbers of peptides bridging different liposomes, and hence the aggregation and fusion events.
For these samples, a DLS time series was recorded which showed a larger size increase and hence more aggregation or fusion with time for liposomes decorated with higher percentages of lipopeptide (Figure A3). The 4000 nm hydrodynamic diameter after 1 hour observed for 1 mol% hybrid is comparable with the size increase observed for the 3 mol% decorated liposomes. However, a significant decrease in size growth is observed for the

lower lipopeptide proportion of 0.5% and especially for 0.1%. There may be a hybrid density that leads to the largest clusters of liposomes determined by the balance of attractive forces originating from hetero coiled-coil formation and steric forces resulting from very high peptide densities. The threshold in the peptide density in order to induce aggregation and fusion appears to be close to 0.1 mol% hybrid in a 1 mM liposome sample.

The initial rate of decorated liposome aggregation and/or fusion is much faster when the liposomes are modified with 1 mol% lipopeptide rather than 3 mol% lipopeptide, but both reach the same order of initial liposomes fused after 1 hour. This difference may originate from the more accessible peptides that are not homocoiled on the liposome surface at densities of 1 mol%. The initial rate for the 0.5 mol% hybrid is more comparable in the first 20 minutes with the 1 mM liposome 3 mol% lipopeptide samples. As the peptide density was very low in these samples no information could be obtained using CD spectroscopy.

Total lipid mixing was monitored with time by fluorescence measurements as can be seen in Figure A3b. The samples with 1 mol% and 0.5 mol% hybrid sedimented, therefore the duration plotted is reduced. As the DLS sample for these mixtures did not show any precipitation, an explanation for this is probably the difference in stirring strength and further sample preparation. The 0.1% sample showed a 7% increase in fluorescence. This is significantly lower than the 20% increase in fluorescence observed when liposomes are decorated with 3 mol% lipopeptide, confirming that the lipid mixing depends on the hybrid concentration.

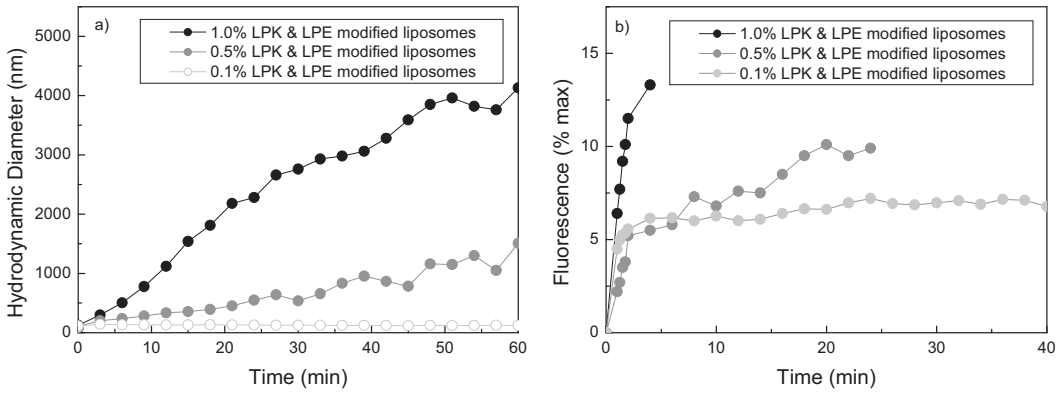

Figure A3. a) DLS time series for mixtures of LPK modified fluorescent liposomes and LPE modified liposomes with 1, 0.5 and 0.1 mol% lipopeptide. b) Total lipid mixing recorded by fluorescence spectroscopy for liposomes modified with 1, 0.5 and 0.1 mol% LPK and LPE.

Ratios of decorated liposome populations

Different ratios of LPK and LPE decorated liposomes were mixed together and the size increases were recorded with DLS, as seen in Figure A4. As the amount of one type of modified liposomes outstrips the other fewer liposomes cluster together. The size increase is comparable for LPK:LPE ratios of 2:1 and 1:2, so the peptide that is in the majority

does not influence the cluster size. As the amount of LPK-liposomes decreases and that of LPE-liposomes increases, the probability of finding a LPK decorated liposomes near a LPE decorated liposomes is reduced, and there are fewer aggregation possibilities. In the extreme situation, the LPK decorated liposomes may be surrounded by LPE decorated liposomes, and no more K is available or accessible to E for coiled-coil folding. The extent of lipid mixing is greater when LPE and LPK ratios of 1:2/4/8 are employed rather than 1:1, so although fewer liposomes cluster the number that proceed over the energy barrier is greater.

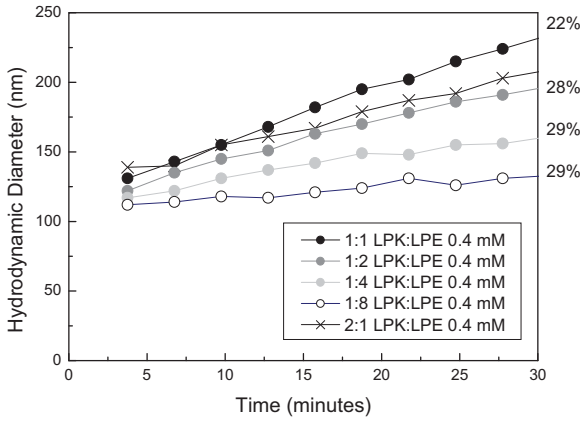

Figure A4. DLS time series for the assay in which 3 mol% LPK and LPE decorated liposomes (0.4 mM) were mixed in different ratios, i.e. 1:1, 1:2, 1:4, 1:8 and 2:1. Smaller LPK:LPE ratio resulted in smaller aggregates. The increase in fluorescence which accompanies lipid mixing is given at the right of the graph.

Temperature

The particle size and total lipid mixing experiments were repeated at 37 °C rather than at 25 °C. At 37 °C the modified liposomes cluster together very quickly in comparison to the increase observed under all conditions at 25 °C. The extent of lipid mixing was also greater, rapidly reaching a plateau of 30% fluorescence increase (Figure A5). This is the expected effect of increasing the temperature of the system to 37 °C because at this temperature E/K association still occurs (see Chapter 2), and the lipid membrane has greater fluidity, so the energy barrier to fusion is lowered.

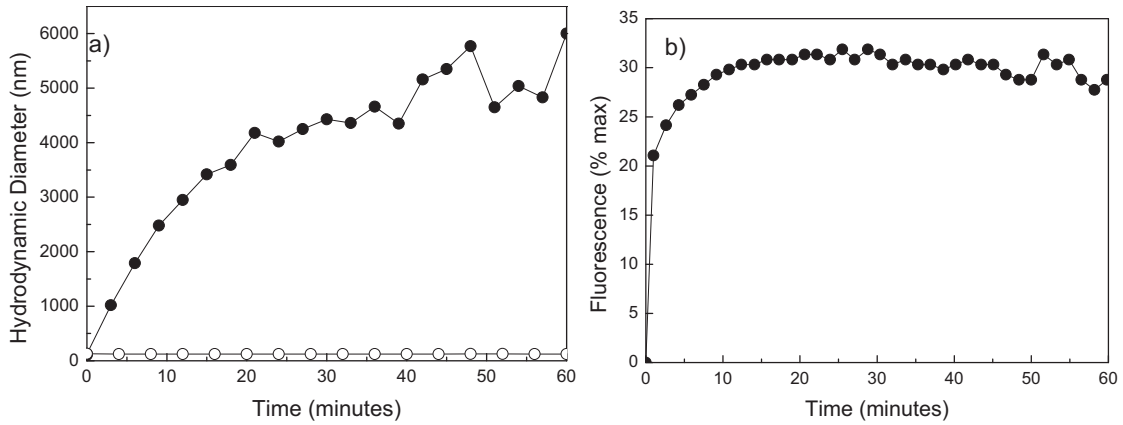

Figure A5. a) Change in particle size with time upon combining 3 mol% LPK decorated fluorescent liposomes with 3 mol% LPE decorated liposomes at 37 °C (●), and the equivalent without the lipopeptides (○), as monitored with DLS. b) Total lipid mixing upon combining 3 mol% LPK decorated liposomes with 3 mol% LPE decorated liposomes at 37 °C, as monitored by fluorescence spectroscopy.

Summary

LPK- and LPE-modified liposome clustering increases with increasing liposome concentration, with increasing lipopeptide concentration, when the two forms of modified liposomes are equimolar, and at 37 °C. That is, the greater the number of encounters between E and K the more decorated liposomes cluster together.

Large clusters of liposomes are not required for lipid mixing, and larger clusters of liposomes do not mean that the amount of lipid mixing is greater, therefore liposome aggregation is not the rate limiting step in the fusion process.

Lipid mixing still occurs with 80 rather than 1600 peptides anchored to the outer surface of the liposomes, analogous to the number of SNARE proteins on transport vesicles.

The rate and extent of lipid mixing is sharply increased by elevating the temperature to body temperature.