Synthetic Biology

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A Reduced SNARE Model for Membrane Fusion**

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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, that is, 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]

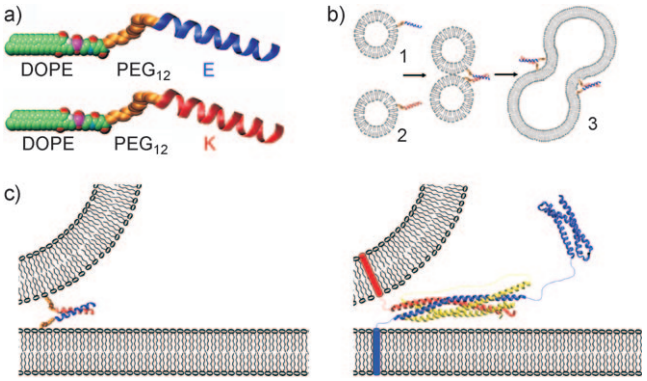

Figure 1. a) Space-filling model of the lipidated oligopeptides LPE and LPK, consisting of a DOPE tail linked through a PEG_{12} spacer to the coiled-coil-forming oligopeptides E and K. The amino acid sequence of E is $G(EIAALEK)_3$-NH_2, and that of K is $(KIAALKE)_3GW$-NH_2. b) The spontaneous incorporation 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).

To date, the major unresolved question has been: What is the minimal machinery required for the controlled fusion of lipid membranes? We answer this question 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.

We designed two lipidated oligopeptide hybrids (LPE and LPK), 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 ($K_d \approx 10^{-7}$ M).[8] In our 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

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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).

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 μm within 20 min (Figure 2).

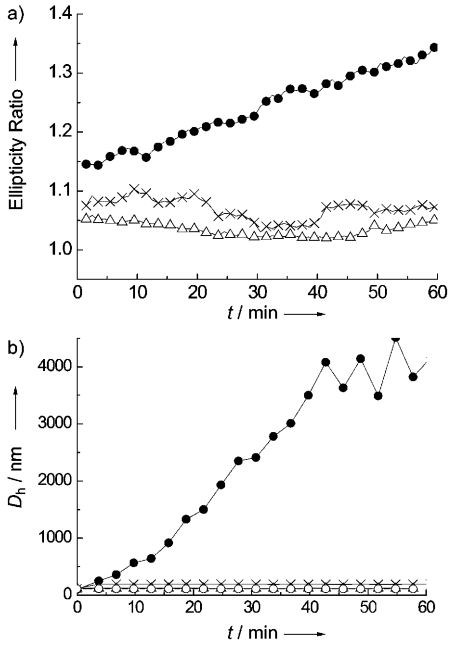

Figure 2. a) Ellipiticity ratios measured by CD of LPE- (△) and LPK-modified (×) liposomes. Upon mixing of these liposome batches, the aggregation of coiled coils was observed (●). b) The hydrodynamic diameter, D_h, measured by DLS, increased rapidly when the LPE- and LPK-decorated liposome populations were combined (●). In control experiments (with plain liposomes (○), plain liposomes mixed with LPE-decorated liposomes (△), 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, we concluded that the energy gained by the formation of LPE/LPK coiled coils (ca. 14 kT per dimer; see Figures S1–S3 in the Supporting Information) 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] we therefore investigated whether the lipid mixing in our 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, we 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

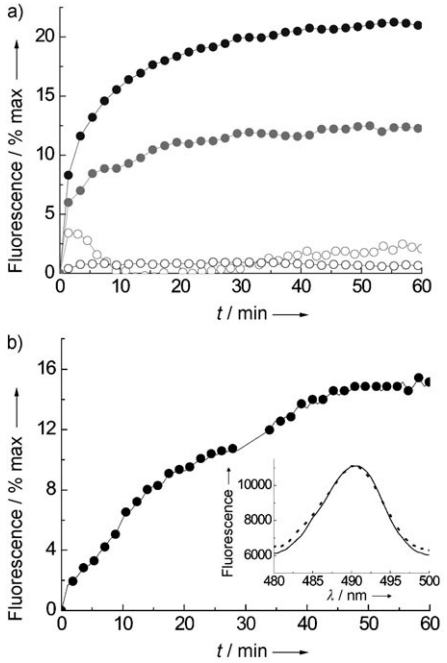

Figure 3. a) Lipid mixing between liposomes as indicated by an increase in NBD emission. Mixing of LPE- and LPK-decorated liposomes: ●; mixing of plain liposomes: ○; mixing of inner-layer lipids when LPE- and LPK-decorated liposomes were combined: ●. Lipid mixing was inhibited when LPE- and LPK-decorated 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.

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, we added ethylenediaminetetraacetic acid (EDTA) 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 our model system mimics biological membrane-fusion events, we added oleoyl lysophosphatidylcholine (packing parameter ≈ 0.4) 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 our model system.

For our 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)

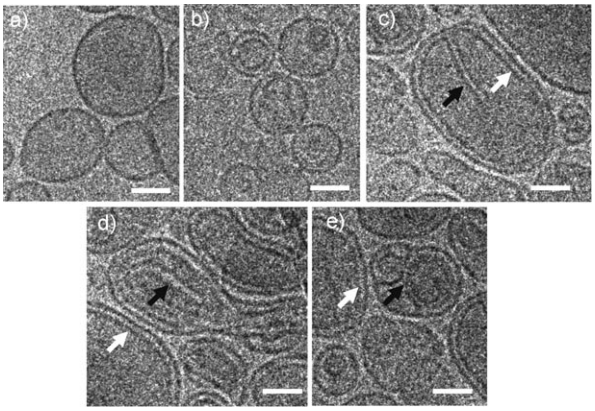

Figure 4. Cryo-TEM images showing fusion: a) LPE- and b) LPK-decorated liposomes; c–e) liposomes with complex membrane morphologies formed 30 s after the mixing of LPE- and LPK-modified vesicles. After fusion, the vesicles show double bilayers (white arrows) and invaginations (black arrows). Scale bars: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

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.

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 μm. 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 μm 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.

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

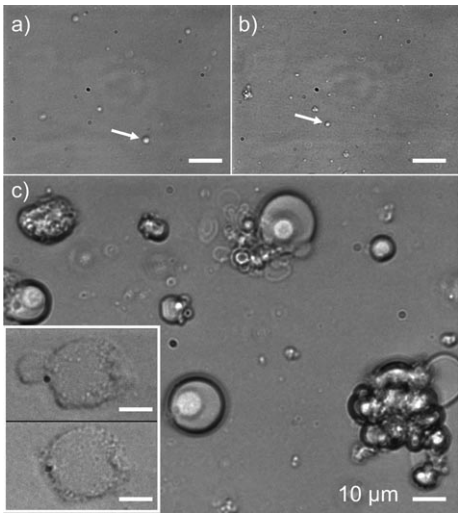

Figure 5. Optical microscopic images of large a) LPE- and b) LPK-functionalized liposomes before fusion. The liposomes are white; examples of liposomes are indicated by arrows. c) Upon mixing, giant liposomes were observed. Inset: Fusion of cell-sized liposomes. Scale bars: 10 μm.

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 we use 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.

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