Chapter I

General Introduction

NATURAL AND ARTIFICIAL MEMBRANE FUSION

Membrane fusion, i.e. the merging of two initially separate lipid bilayers into one continuous bilayer is a key process in all living systems. It ranges in its versatility from fusion of enveloped viruses with cells, to fusion of vesicles with the synaptic membrane upon neurotransmitter release, and to the fusion of cells, for instance myoblasts during the formation of muscular tissue. However all these processes sha*re* the same basic procedure at the membrane level starting with the approach, followed by merging of the bilayers and finalized with opening and expansion of the fusion pore.[1] During these processes, the lipids in the bilayers have to rearrange which involves intermediate states that might be non-bilayer structures and are unfavorable as they require high local curvatures (Figure **1**A-D). Therefore, spontaneous vesicle fusion does not take place in biological systems and fusion proteins are employed to guide this process.[1-4]

One of the most studied protein complexes that plays a central role in intracellular and neuronal vesicle fusion consists of the SNARE (soluble NSF attachment protein receptor) proteins.[5] These proteins are known to dock vesicles to the target membrane by the formation of a 4-helix coiled-coil complex (Figure 1E).[6,7] However, especially in neuronal vesicle fusion the SNARE proteins are not the only proteins involved, other

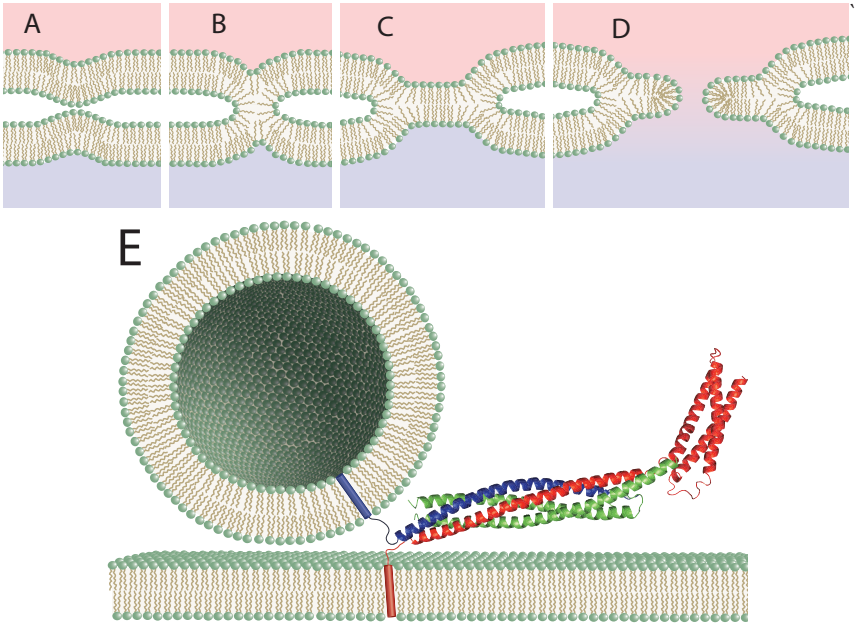

Figure 1. Intermediate states of lipid reorientation during membrane fusion: (A) point-like protrusions, (B) stalk, (C) hemifusion diaphragm, (D) fusion pore opening. (E) Illustration of the cis-SNARE complex with syntaxin (red), synaptobrevin (blue), and SNAP-25 based on X-ray structures of the SNARE complex PDB-ID: 1SFZ;[6] and N-terminal segment of syntaxin (red) PDB-ID: 1BR0.[7]

important proteins for instance SM (Sec1/Munc18-like), complexin, and the calcium sensor syntaxin assemble to a complex protein machinery that enables specific, quick Ca^{2+} triggered fusion.[8-10] Although, most of the involved proteins are known, the precise series of molecular events that results in intracellular fusion are a matter of ongoing debate.

In their important review, Chernomordik and Kozlov[1] summarized and analyzed the different tasks fusion proteins have to fulfill in order to enable full fusion of membranes. First, the membrane compartments that are supposed to fuse must be brought together; this process requires a high degree of specificity. In SNARE mediated fusion this specificity is achieved due to the fact that complementary SNARE proteins are specifically incorporated in the vesicles (v-SNAREs) or the target membrane (t-SNAREs).[5] Next, the lipid interfaces must be brought into close proximity; almost zero distance, which is hindered by strong intermembrane repulsions. Several mechanisms have been proposed for how proteins might accomplish this task. It was proposed that a force is generated and transferred to the membrane via the SNARE linker regions resulting in the membranes being pulled together.[8,9,11] This force might also create a bulge in the membrane.[12,13] Likewise bulges, point like protrusions, or fluctuations in the prefusion membranes might be caused by incorporation of synaptogagmins C2B domain and this promotes the lipid interface approximation (Figure 1A).[12-16] Subsequently, the first connection between the two membranes is obtained in the fusion stalk (Figure 1B).[17,18] In this intermediate state the monolayers are strongly bent and lipids have to tilt and splay to prevent vacuum voids. Negative spontaneous curvature promotes this intermediate, thus for DOPE the stalk was predicted to be energetically favourable, while for DOPC energy has to be expended, which might be curvature strain generated by the fusion proteins.[1] Next, the stalk extends into a hemifusion diaphragm in which the acyl chains of the distal lipid monolayers are in contact (Figure 1C). Finally, a fusion pore has to open in the hemifusion diaphragm to allow the mixing of the two initially separated aqueous compartments (Figure 1D). Chernomordik and Kozlov hypothesized that in these stages the task of fusion protein might be the generation of a pulling force that increases the diameter of the hemifusion diaphragm, as this would increase the probability of a spontaneous fusion pore opening.[1] Other models suggested the existence of complex proteinaceous fusion pores, i.e. a bridging of the intermembrane gap by protein channels.[17,19] However, these models circumvent the hemifusion diaphragm intermediate which has been observed experimentally for instance in SNARE mediated fusion.[20-22]

The hallmark of biological membrane fusion, meaning the specific content mixing between two different enclosed compartments without leakage, makes this process an interesting target for supramolecular and biomaterials chemists as it opens a route to applications in biotechnology and drug delivery. Consequently,

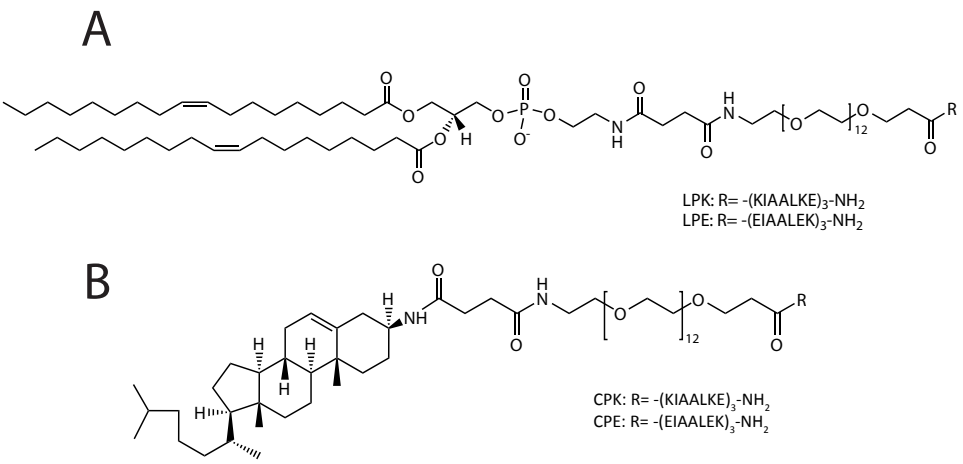

Figure 2. Chemical structures of (A) LPE and LPK, (B) CPE and CPK.

simple model systems have been designed with the aim to enable membrane fusion by means of simple, synthetic molecules.[23,24] Despite the immense efforts in this field, the creation of a targeted, and effective model system that shows lipid mixing and non-leaky content mixing remains a challenge. One system showing these properties was inspired by the SNARE proteins and designed in the Kros laboratory.[25] In the original system two heterodimeric coiled-coil forming peptides, termed E and K are covalently linked to lipid membrane anchors via a polyethyleneglycol (PEG_{12}) linker, yielding the lipopeptides LPE and LPK (Figure 2A). Incorporated into DOPC : DOPE : Cholesterol (2 : 1 : 1) vesicles of ~100 nm diameter these lipopeptides initiated efficient lipid- and content mixing, upon combination of the vesicles (Figure 3).[25,26] The E/K coiled coil formation is thought to be responsible for the specific molecular recognition and the vesicle docking. Further semi-rational improvement of the system showed that using a cholesterol anchored version of the lipopeptides CPE and CPK, permits a post modification of preformed vesicles and yields efficient membrane fusion (Figure 2B).[27] Studies on the peptide molecular recognition units of the lipopeptides revealed that the binding orientation has no influence on the fusion,[28] while an increase of the binding strength was reported to increase fusion efficiency.[29] The systems was also shown to enable the specific targeting of living cells and zebrafish skin.[30]

In light of the challenging tasks demanded from fusion proteins, the effective lipid and content mixing displayed by these systems appears astonishing. The simple docking model as proposed by Marsden et. al.[25,26] gives no answer to the question of what promotes the lipid reorientation (Figure 3), hence a further role of the lipopeptides LPE and LPK beyond molecular recognition and vesicle docking

appears reasonable. This role is to date unknown and is the central study of this thesis. It is expected that a detailed understanding of the mechanism of lipopeptide mediated membrane fusion will help to rationally improve this system.

COILED COILS

The term coiled-coil refers to several interacting peptide helices winding around each other. These structures are an abundant motif for protein oligomerization domains and can be found in proteins with different functions such as DNA transcription factors,[31,32] ion transporters,[33] viral fusion proteins,[34,35] or intracellular fusion proteins.[6] The underlying sequence motif termed the heptad repeat, consists of seven polar (*p*) or hydrophobic (*h*) amino acids in the pattern *hpphppp*. The positions in this pattern are usually denoted *abcdefg*. Hydrophobic interactions between the *a* and *d* positions of adjacent peptide chains leads to a tight knobs-into-hole packing of these side chains (Figure 4). Furthermore electrostatic interactions between the *e* and *g* positions contribute to the binding and stability of these peptide oligomers.

The amino acid sequence of coiled-coils determines their oligomeric state, orientation and stability and analysis of natural sequences has led to sets of design rules that enable the de novo design of coiled coils.[36] For instance the de novo design of specific heterodimeric coiled coil pairs based on leucine residues in the *d* position and glutamic acid and lysine residues in the *e*, *f*, and *g* positions were reported by Litowski and Hodges.[37] Amongst the studied peptides the pair with only three heptad repeats of IAAL in the *a-d* positions was found to form conformationally stable, fully helical, specific, and dimeric hetero coiled coils. The two resulting peptides $(EIAALEK)_3$ and $(KIAALKE)_3$ are denoted as peptides E and K within this thesis. Fletcher et al. reported the targeted design of a basis set of peptide oligomers with defined oligomeric states from dimer up to a tetramer, of which the trimer CC-Tri-N13 is studied, amongst others, in C*hapter II*.[38]

PEPTIDE MEMBRANE INTERACTIONS

The interactions of peptides with lipid membranes are manifold in terms of the effects in biological systems. One can for instance distinguish antimicrobial, cell penetrating, or cytolytic peptides.[39-42] The property of many of these peptides to induce leakage or lysis of lipid membranes has been explained with different models including the carpet-, barrel stave- or torroidal pore mechanisms.[43,44] All these mechanisms describe the disruption of the membrane integrity as the result of the concerted action of folded membrane-bound peptides. A common principal in these models is that, prior to the joint peptide action, a threshold concentration of lipid bound peptides has to be exceeded.

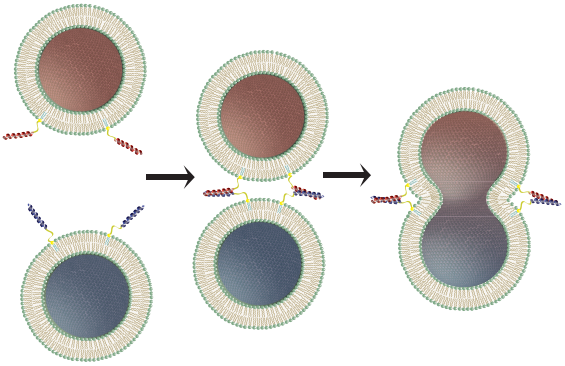

Figure 3. Model of vesicle fusion mediated by lipopeptides LPE and LPK as proposed by Marsden et. al.[25]

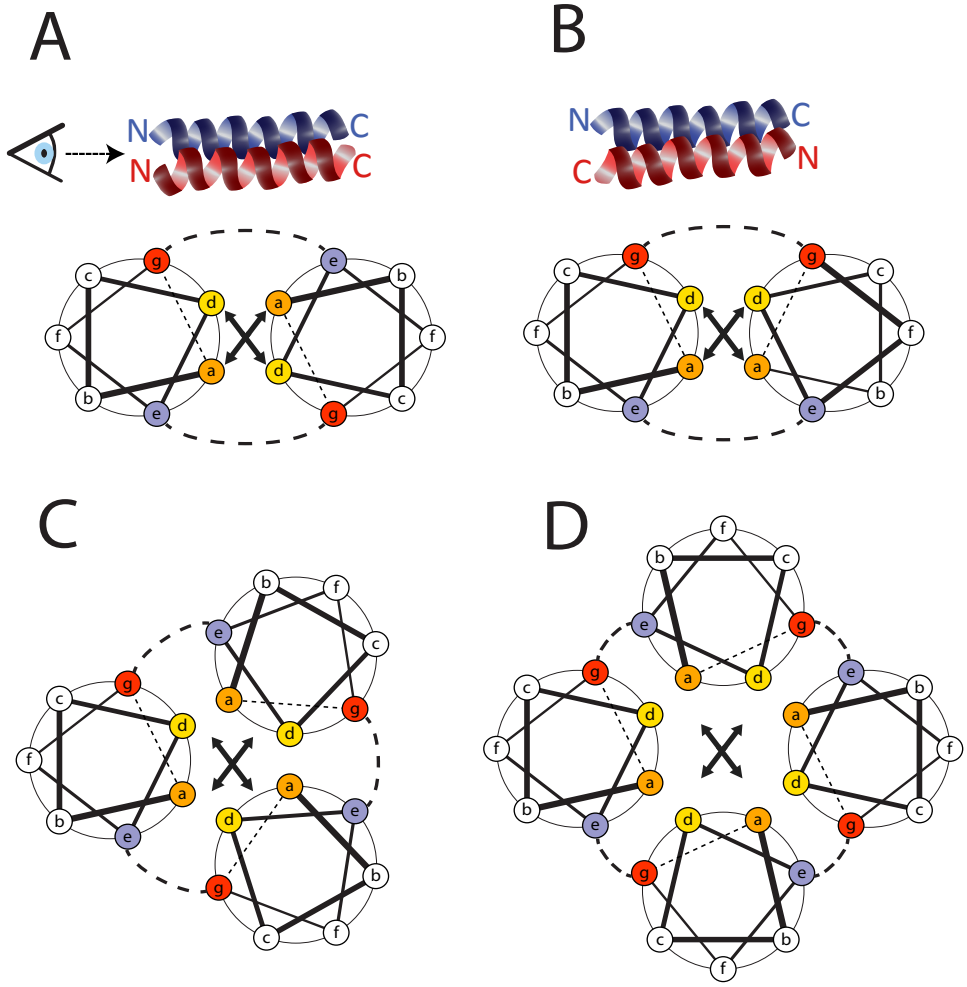

Figure 4. Positions of heptad repeat *abcdefg* projected in helicel wheels of coiled coil complexes. (A) Parallel dimer; (B) antiparallel dimer; (C) parallel trimer; and (D) parallel tetramer. Hydrophobic interactions are highlighted with arrows, salt bridges with dashed lines.

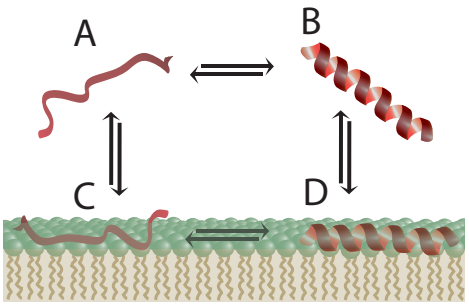

Figure 5. Illustration of equilibria in partitioning-folding coupling. Peptide are in (A) solvated, unfolded; (B) solvated, (C) folded; membrane bound, unfolded (C); and (D) membrane bound, folded states.

The initial step, the peptide binding to the membrane, is relatively well understood and depends on the specific equilibrium between hydrophobic and electrostatic interactions. Some examples of peptides exist that bind to membranes in an unordered state as in the case of strongly positively charged cell penetrating peptides[42] interacting with negatively charged lipid membranes or very short tryptophan and proline rich peptides.[45] However, a majority of peptides fold into a distinct structure upon membrane binding and unfold upon membrane unbinding, which is generally referred to as partitioning-folding coupling (Figure 5).[46-50] In this case, the membrane bound structures are amphipathic in nature, i.e. they comprise a spatial separation of hydrophobic and polar amino acid residues which are partitioned in the amphipathic membrane environment. The separation of hydrophobic and polar residues results in a hydrophobic moment in the peptide molecule.[51,52]

White and coworkers studied the thermodynamics of peptides that exhibit partitioning-folding coupling in detail.[48-50] They found that binding of unstructured peptides to membranes is usually very weak because the energetic costs for partitioning of solvated peptide bonds into the hydrophobic environment exceed the Gibbs energy gained from partitioning of the hydrophobic side chains (state C in Figure 5).[48] Compared to that, the energetic costs of partitioning a hydrogen bonded peptide bond of a folded peptide into the bilayer is drastically reduced (state D in Figure 5). Thus, the formation of hydrogen bonds between peptide bonds poses the strongest Gibbs free energy contribution to the folding process and hence drives the membrane binding of the peptide.[48-50,53]

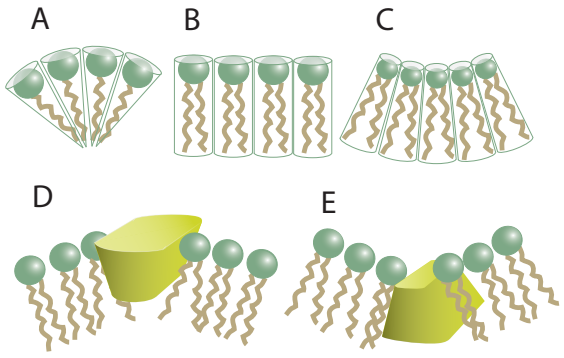

Figure 6. Illustration of spontaneous curvature of lipid interfaces. (A) Positive curvature from inverted cone shaped lipids such as lyso-PCs; (B) planar interface from cylindrically shaped lipids such as PCs; (C) negative curvature from inverted, truncated cone shaped lipids such as PEs. (D) Models of wedge like insertion into monolayers: (E) positive curvature caused by preferential insertion in headgroup region and negative curvature from preferential insertion in acyl chain region.

AMPHIPATHIC α-HELICES

One common structural motif in membrane active peptides, as well as in membrane associated proteins is the amphipathic α-helix (AH).[54-56] In these structures the regular arrangement of the hydrophobic and polar amino acids on separate faces of the helix results in a hydrophobic moment perpendicular to the helical axis. In 1990 Segrest et. al. proposed a classification of naturally occurring AHs in proteins based on the analysis of the hydrophobic moment and the distribution of the amino acids on the helical wheels of these peptides.[55] The membrane interacting AH were divided in classes A: apolipoproteins; H: polypeptide hormones; L: lytic polypeptides; and M: transmembrane helices. These peptides generally have positive charges on the polar face, but differ in the size of the hydrophobic face, the charge distribution, and the charge density. Further classes were defined for AHs that prefer protein-protein interactions, such as class G: globular proteins; K: calmodulin-regulated protein kinases; and C: coiled-coil proteins. Such a classification comes in useful for the identification of AHs in databases, their computational analysis, and the discovery of an intriguing effect, the so called 'Snorkel' mechanism in class A AHs.[46,55-58] However, the boundaries between the different classes are blurred and often a distinct helix can show hallmarks of different classes at the same time.[56] Also, recent views on membrane proteins include a more dynamic picture with different equilibria between multiple membrane associated and soluble states, which renders a too rigid classification inconvenient.[59] A new type of AH, the so called ALPS motif (ArfGAP1 lipid packing sensor) with polar but uncharged serine and threonine residues on the polar face was reported from the Antonny group.[60,61] The ALPS motifs have the remarkable property of being able to bind preferably, strongly curved membranes as found in small vesicles. Although it was already known that the interaction of AHs with membranes can create curvature strain, this sensing ability was a new perspective.

CURVATURE IN MEMBRANE INTERACTIONS

The effects that peptide interactions have on the global properties of membranes are manifold and depend also on the studied membrane system. For instance cell lysis and vesicle leakage,[62,63] pore formation in bilayer stacks,[64,65] tubulation from vesicles or supported lipid bilayers,[66,67] or stabilization of vesicles[63] have been reported. However, most often a common effect is employed to explain these observations: the induction of curvature strain that is a direct consequence of peptide insertion into the bilayer.[40,54,63-69]

By general convention, lipid interfaces curved towards the acyl chains are termed positively curved, while curving towards the headgroup is termed negative. The spontaneous curvature of a lipid interface as well as the distinctive lipid mesophase

behavior is determined by the intrinsic molecular shape of the lipids (Figure 6).[70,71] Inverted cone shaped molecules prefer positively curved interfaces and micelles, while more cylindrical ones tend to form planar bilayers, and truncated cones tend to form negatively curved interfaces (Figure 6A-C). These intrinsic parameters can influence the global and the local properties of lipid mixtures. For instance DOPC/DOPE mixtures form globally small unilamellar vesicles (SUV) upon sonication, with the more cylindrically shaped DOPC enriched in the outer leaflet and DOPE with an inverted cone shape enriched in the inner leaflet.[72] An example for a local curvature effect is the enrichment of DOPC in highly curved lipid tubules pulled from giant unilamellar vesicles (GUV) made of DOPC, brain sphingomyelin and cholesterol.[73]

Several reasons are generally considered to cause the creation of curvature strain by peptides in bilayers: hydrogen bonding, electrostatic repulsion, monolayer surface area, and lateral pressure.[40] AHs are thought to employ the two latter principles which can be understood by the concept of molecular shapes (Figure 6D-E). An incorporation of a wedge with a cone-shaped or inverted cone-shaped cross section into a monolayer results in different lateral pressures on lipid headgroup and acyl chain regions which can cause positive or negative curvature of the lipid interface.[63] A more general, but nonetheless representative, model assumes that the incorporation into one leaflet of an elastic lipid bilayer can increase the area of this monolayer, to avoid the creation of empty volumes between the two leaflets the bilayer reacts by creation of curvature.[74,75] The multiple incorporation of several wedges in a close proximity is known to create local areas of very high curvature and can for example cause membrane bulging and vesicle budding in cells.[12,13,74,75]

AIMS AND GENERAL OUTLINE OF THIS THESIS

Starting with the working hypothesis that the docking of vesicles by LPE and LPK is not sufficient to yield full membrane fusion, the work reported here aims to study the properties of the lipopeptides LPE and LPK as well as similar systems in detail, and to develop reasonable hypotheses regarding modes of action of these lipopeptides that enable fusion, and to elaborate methods to test the hypotheses. These peptides are able to form amphipathic helices, thus detailed studies of the peptide-peptide and peptide-membrane interactions in different membrane model systems are conducted for this purpose. Initially a new tool is developed to study peptide-peptide interaction by means of spectroscopic unfolding curves in *Chapter II*. The thermodynamics of folding of coiled coil complexes of any oligomeric state in aqueous environment can be investigated by this method. It will prove useful in the characterization of newly designed variants of the original peptides E and K, in the following chapters. Next, the interaction of lipopeptides LPE, LPK and the

free peptides E and K with lipid monolayers are studied in *Chapter III*. The insights and hypothesis gained from the simplified monolayer system are extended towards bilayers, in the form of vesicles and tested experimentally in *Chapter IV* and *Chapter V*. The data collected in these chapters indicate that distinctive peptide membrane interactions might promote the lipopeptide mediated fusion. To test this hypothesis a targeted inhibition of these interactions is attempted in *Chapter VI*. Based on the collected data and the conclusions a summary and perspectives for further research on this topic are given in *Chapter VII*.

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