Barbara Elisabeth Hubrich

Synthesis of Model Systems for SNARE Mediated Membrane Fusion Based on PNA/PNA Base Pair Recognition





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Synthesis of Model Systems for SNARE Mediated Membrane Fusion Based on PNA/PNA Base Pair Recognition

Dissertation

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within the doctoral program *Physics of Biological and Complex Systems* of the Georg-August University School of Science (GAUSS)

> submitted by Barbara Elisabeth Hubrich from Bonn

> > Göttingen 2017



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Abstract

SNARE proteins are regarded as key players in membrane fusion. They reside on both sides of opposite membranes and specifically interact via their characteristic SNARE motifs. The interaction leads to the formation of a stable SNARE complex which pulls the membranes together and eventually results in membrane merger. The exact mechanism of SNARE mediated membrane fusion, however, is still of a matter of debate. Therefore, the development of SNARE model systems is a valuable tool to study membrane fusion by mimicking the action of SNARE proteins *in vitro*. Model systems contain fusogenic peptides that exhibit a less complex structure compared with that of the native models. This allows easy modifications of the structure. In this way, the influence of essential SNARE domains on distinct steps of the fusion pathway can be examined.

In this thesis, model peptides are developed that exhibit artificial peptide nucleic acid (PNA) hybrid recognition units. These are made of N-(2-aminoethyl)glycine-PNA and alanyl-PNA, which feature different duplex formation rates due to different topologies. With this, it is intended to achieve a directed duplex formation of the PNA hybrid recognition units so that the model peptides mimic the assumed SNARE zippering.

The model peptides are synthesized by means of solid-phase peptide synthesis. The transmembrane domains of two neuronal SNAREs are taken to anchor the peptides into the membrane of large unilamellar liposomes. The fusion behavior of the model peptides is then comprehensively analyzed via fluorescence spectroscopy in bulk lipid mixing assays, via fluorescence cross-correlation spectroscopy, and via dynamic light scattering. The strengths and weaknesses of these experimental techniques are examined and discussed. Only the combination of all of them enables to obtain a detailed picture of the fusogenicity of the model peptides. It is shown that a directed duplex formation does not occur between the SNARE analogues. Instead, additional alanyl-PNA in the recognition unit reduces the extent of fusion. By studying a variety of peptides it is found that model peptides with a recognition unit made of pentameric aeg-PNA strands exhibit the highest fusogenicity. With that, they represent useful and easily accessible alternatives to previously reported model peptides with a decameric aeg-PNA recognition unit.

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motifs,^[15,16] deoxyribonucleic acid (DNA)^[17,18] and peptide nucleic acid (PNA)^[19,20] strands and even small molecules^[21–23]. The achievement of full fusion or at least hemifusion has been reported in all cases. The SNARE zippering, however, has not yet been specifically addressed in artificial model systems. One possibility to do so is to equip SNARE model peptides with a recognition unit that is made of two parts. If these parts differ for example in the rate of dimerization, a directionality in complex formation ought to be achieved.

The intention of this work was to design and analyze SNARE model peptides containing recognition units that are made of two different types of PNA. PNA is a DNA analogue, in which the nucleobases are attached to a backbone based on peptide bonds.^[24] This makes PNA neutral and resistant towards enzymatic cleavage. By using PNA, the recognition unit can be designed in a highly predictable fashion concerning the stability and orientation of the strands. The two PNA types are *N*-(2-aminoethyl)glycine (aeg)-PNA and alanyl (ala)-PNA, which differ in their backbone structure. This results in different topologies of the double strands and different dimerization kinetics. The assembly of helical aeg-PNA duplexes is fast,^[25] whereas the complex formation of linear ala-PNA oligomers in kinetically hindered and thus slow.^[26] Combining aeg-PNA and ala-PNA within one recognition unit thus aims at achieving a directionality in duplex formation, which starts with fast aeg-PNA dimerization followed by ala-PNA interaction. With this, the minimal structural requirements for mimicking the presumed SNARE zippering are probed.

This thesis targets at the following two main points: First, implementation of the synthesis of model peptides with a PNA hybrid recognition unit. This is accomplished by using Fmoc-based solid-phase peptide synthesis (SPPS). The PNA monomers for the recognition unit are assembled stepwise in a continuous fashion on the resin containing the native SNARE transmembrane domain sequences. Making use of these does not only ensure a stable anchorage in the membrane but also takes account of the assumed active role of the TMDs during fusion.^[27] Purification of these kinds of peptides is challenging. Therefore, different strategies based on high performance liquid chromatography (HPLC) and size exclusion chromatography (SEC) are tested elaborately.

Second, analysis of the model peptides regarding their fusogenicity, which is the capability to fuse membranes. Are the peptides with a PNA hybrid recognition unit in general capable of liposome fusion? Does the PNA hybrid recognition unit constitute the minimal structural requirement for mimicking the SNARE zippering? How is the extent of liposome fusion compared to other model systems? To obtain results that are as differentiated



as possible, various fusion assays are applied, which are based on two different principles. The first principle is detecting liposome fusion by making use of fluorophore-labeled liposomes. Depending on the position of the fluorophores-they are either located on one liposome population or are separated on two different liposome populations-the change in their distance is expressed by either a decrease in Förster resonance energy transfer (FRET) or an increase in FRET. This is monitored in bulk lipid mixing assays, of which two options are applied, total lipid mixing (TLM) and inner lipid mixing (ILM) assays. Whereas with TLM assays it is possible to detect lipid mixing in general, ILM assays allow the specific detection of the mixing of the inner leaflets.^[28] Therefore, they indicate whether the liposome fusion process proceeds completely or is arrested in the hemifusion stage, a step in the fusion process in which only the outer leaflets of the liposomes have merged.^[29,30] In addition, fluorescence cross-correlation spectroscopy (FCCS) is employed. With this technique the interaction of fluorophore-labeled liposomes can be determined in more detail as it allows distinguishing between docking and fusion of liposomes. The second principle is detecting liposome fusion by applying dynamic light scattering (DLS). DLS gives quantitative information on the size distribution of particles. Therefore, it is a valuable supplement to the fluorophore-based assays as it provides the size of interacting liposomes, a quantity that is not accessible by lipid mixing assays.

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2. Membranes and Membrane Fusion

2.1. The Structure of Biological Membranes

Biological membranes constitute the boundaries of cells and cell organelles and ensure the spatial separation of cellular processes.^[31,32] Apart from that they are a place where a multitude of reactions occurs, made possible by various attached proteins. The essential components of biological membranes are lipids.^[33] They shape the basic membrane framework by being ordered into a lipid bilayer. Their polar headgoups point to the outside whereas their unpolar alkyl chains are oriented inward (see Figure 2.1). Due to their amphipathic character, the lipid bilayer is formed spontaneously in an aqueous environment driven by non-covalent interactions among the hydrophobic alkyl chains.



Figure 2.1. Schematic view of the composition of biological membranes. Usually, the membrane components are not evenly distributed but form membrane patches (often denoted as "lipid rafts"), in which saturated phospholipids, glycolipids, sphingolipids, lipidated proteins and glycosylphosphatidylinositol (GPI)-anchored proteins segregate from areas with unsaturated phospholipids and other membrane components. Lipid rafts likely participate in various physiological functions. Cortical actin is thought to mediate the lateral distribution and to support domain formation. Reprinted by permission from Macmillan Publishers Ltd: NATURE REVIEWS MOLECULAR CELL BIOLOGY (Ref. [34]), copyright (2017).



2. Membranes and Membrane Fusion

In addition to lipids, biological membranes host a wealth of proteins fulfilling various tasks, thus providing different types of membranes with different functional properties. Accordingly, the membrane composition is organelle-specific and can differ wildly.^[35] Membrane proteins are involved in processes such as transport of particles across the membrane, signal transduction via receptors, enzymatic activities for membrane-associated reactions or intercellular recognition.^[36] Proteins are often classified as integral or peripheral membrane proteins, depending on how they are associated with the lipid bilayer. Integral proteins exhibit segments that are inserted into the lipid bilayer. Transmembrane proteins, for example, span the entire membrane via single or multiple helices or as β -barrels.^[37,38] Proteins can also be embedded in the membrane via a lipid anchor or the glycosylphosphatidylinositol anchor, which is an oligosaccharide linker (see also Figure 2.1). Peripheral proteins are bound to one side of the membrane without being embedded in the hydrophobic core of the lipid bilayer. Instead, they interact with the membrane via binding to integral proteins or via association with the polar lipid headgroups.

In the early 1970s, Singer and Nicolson developed the fluid mosaic model, which made an essential contribution to understanding the structure of membranes.^[39] It describes membranes as a two-dimensional sea of lipids in which proteins are evenly distributed in a low concentration. Proteins and lipids rapidly diffuse within the membrane which is referred to as lateral diffusion. Up to now, however, a huge amount of investigations suggests that the fluid mosaic model is not as generally applicable as it seemed to be at the time it was proposed. Over the past decades, the concept of lipid rafts emerged, ensuing from various observations that cell membranes are highly heterogeneous and can be separated into different fractions (see Figure 2.1).^[34] According to this concept, sterols and sphingolipids self-ensemble into microdomains ("rafts") which are separated from the other membrane components.^[40] Rafts are commonly described as small dynamic assemblies being about 10–200 nm in size and containing lipids and proteins.^[41] The formation of rafts is based on the liquid-liquid immiscibility of different lipid species, and proteins associate with rafts according to their affinity for these lipid patches. Proceeding from the first hypothesis that rafts play an important role in membrane-associated signalling processes,^[42] there is growing evidence that rafts are revelant for physiological functions.^[34] Proper detection of lipid rafts, however, is difficult, especially in living cells. From the very beginning of its postulation, the lipid raft model has therefore been discussed controversially.^[43] Though hints that lipid rafts do exist increase,^[40] alternative models explaining how the plasma membrane is organized are discussed as well.^[44] For example, the segregation of lipids and proteins into distinct domains can also be mediated by charge.^[45] The concept of lipid shells hypothesizes that proteins are surrounded by lipids, conceptionally analogously to the hydration shell of molecules in water. Lipid shells are assumed to be the smallest entity of domains in the lipid bilayer and formed by specific lipid–protein interactions.^[46] On top, heterogeneity in the membrane composition is achieved by the actin cytoskeleton. Cortical actin is supposed to modulate the lateral distribution of lipids and proteins in the membrane by anchoring proteins via an actin cytoskeleton "fence" which hinders other proteins and lipids from diffusing by.^[47]

2.2. Concepts of Membrane Fusion

Membrane fusion is the merger of two opposing lipid bilayers to form one continuous lipid bilayer. Already in 1968, Palade and Bruns studied vascular tissues with electron microscopy and described fusion of membranes and intermediates therein.^[48] Remarkably, this was done even before basics of the structure of membranes were known. The fluid mosaic model by Singer and Nicolson, for example, did not come up until 14 years later (see Section 2.1).

Today, mainly two mechanisms regarding membrane fusion are distinguished, depending on whether proteins ("direct fusion") or lipids ("fusion-through-hemifusion") form the fusion pore.^[49,50] In the direct fusion pathway, proteins from both membranes assemble and a proteinaceous fusion pore is formed upon a conformational change of the protein complex. The fusion pore is believed to be surrounded by a ring of proteins. In a second step, the fusion pore widens when lipids replace the proteins.^[51] This mechanism implies that content mixing takes place before lipid mixing. Details, however, are unknown to date.^[50] A more often discussed pathway of membrane fusion is the mechanism of fusion-through-hemifusion, in which lipids shape the fusion pore (see Figure 2.2).^[49] Proteins may ensure that the membranes are located closely next to each other, but the pore formation is thought to be brought about solely by lipids. It is assumed that if the opposing membranes are in close proximity (Figure 2.2, step i) a point-like protrusion of several lipids reduces the hydration repulsion (ii) so that a hemifusion stalk (iii) can form. In the hemifusion stage, the outer leaflets of the lipid bilayer have merged, but the inner leaflets are still separated. From this stalk, the formation of the fusion pore (v) can take place, possibly via an extended hemifusion diaphragm (iv). The fusion pore establishes an aqueous connection between the formerly separated bilayers so that contents can be exchanged. Contrary to the proteinaceous pore formation, lipid mixing preceeds content mixing here.





Figure 2.2. Schematic view of steps in the fusion-through-hemifusion pathway of membrane fusion. The details are explained in the text. Reprinted by permission from Macmillan Publishers Ltd: NATURE STRUCTURAL & MOLECULAR BIOLOGY (Ref. [30]), copyright (2008).

The stalk model was originally described by Kozlov and Markin in 1983.^[52] They theoretically studied the formation of the stalk by implying mathematical calculations of the transition states during membrane fusion. Although it became apparent that the elastic energy was overestimated due to an incorrect assumption of the curvature of the stalk—a refinement of the model was done in 2002,^[53,54] the stalk model profoundly contributed to the understanding of membrane fusion.

Hemifusion diaphragms have been observed directly^[29,55] and there are studies that consider the hemifusion diaphragm a dead-end state in membrane fusion.^[56] Calculations showed that it is an unusually stable intermediate and that its formation as well as the sub-sequent dilation into the fusion pore are energetically costly.^[57] Especially if the length of the hemifusion diaphragm increases, it is very unlikely that the formation of a fusion pore occurs spontaneously due to a decreasing lateral tension.^[57,58] Consequently, only a small frame remains in which the hemifusion diaphragm is short enough for a fusion pore to efficiently increase in size.^[56] It is thought that proteins prevent the extension of the hemifusion diaphragm and thus ensure fast membrane fusion.^[58] This was also shown by experiments in which protein-free and protein-containing liposomes were examined.^[29] The extent of observable extended hemifusion diaphragms was significantly higher in the case of protein-free liposomes indicating that proteins suppress the formation of elongated hemifusion diaphragms.

The tendency of membranes to fuse is crucially influenced by their lipid composition. Depending on the ratio of the area required by headgroups and alkyl chains, lipids adopt different shapes (see Figure 2.3a). For example, lipids are cone-shaped if the mean diameter of the headgroup is smaller than that of the area occupied by the alkyl chains, like in unsaturated phosphoethanolamine (PE). The shape determines the spontaneous curva-

ture of the monolayers. The curvature is defined as positive if the monolayer's surface is bent into the direction of the lipid headgroups. Respectively, it is defined as negative in the opposite case.^[59] As the stalk has a negative curvature, cone-shaped lipids like PE promote its formation. Inverted-cone-shaped lipids like lysophosphatidylcholine (LPC), on the contrary, inhibit stalk formation.^[30] Figure 2.3 illustrates these relationships. In contrast to that, adding LPC to the distal leaflets supports fusion pore formation whereas PE inhibits it. This confirms that the rims of the fusion pore are positively curved.^[30]



Figure 2.3. Illustration of lipid shapes. (a) Inverted-cone-shaped lipids like LPC (red) form a positively curved monolayer, whereas cone-shaped lipids like PE (green) lead to a negatively curved monolayer. (b) The distal leaflets in the stalk intermediate are negatively curved, which is why cone-shaped lipids promote stalk formation. Inverted-cone shaped lipids would disturb the arrangement and thus inhibit stalk formation.

The extent of curvature is relevant as well. Highly curved membranes fuse more readily than less curved membranes.^[30] Accordingly, the smaller the liposomes the more fuso-genic they are. This is because a high curvature implies a high tension and thus a high readiness to fuse.

2.3. Neuronal Exocytosis

Since the seminal work by Katz and Miledi, who discovered the fundamental pathways of synaptic transmission,^[60] neuronal exocytosis is one of the best studied membrane fusion processes in nature. Neuronal exocytosis happens at the conjunction sites of two nerve cells. Briefly, nerve cells consist of the soma, *i.e.* the cell body that contains nucleus and cell organelles and ramifies into dendrites (see Figure 2.4a). The axon is an elongated appendix of the soma along which an electrical pulse is transmitted. The termini of the