Voltage dependent anion channel: Interaction with lipid membranes.

Dissertation

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“Anyone who has never made a mistake has never tried anything new.”
Albert Einstein
Abstract

Evidence has accumulated that the voltage dependent anion channel (VDAC), located on the outer membrane of mitochondria, plays a central role in apoptosis. The involvement of VDAC oligomerization in apoptosis has been suggested in various studies. However, it still remains unknown how exactly VDAC supra-molecular assembly can be regulated in the membrane. Previous studies suggested the possible influence of various proteins on the formation of VDAC oligomers, but the important issue of the VDAC oligomeric state regulation by lipids has not been studied so far. Nevertheless, the effect of lipids on the oligomerization of several membrane proteins has been mentioned in the literature and in general, protein-lipid interactions are under extensive investigation.

In the present work, I addressed the influence of lipids on VDAC oligomerization experimentally by reconstituting the fluorescently labelled VDAC in giant unilamellar vesicles (GUVs)—a chemically well defined, cell-free minimal model system. Fluorescence cross-correlation spectroscopy was performed to determine the oligomeric state of VDAC. I investigated the effect of important for apoptosis anionic lipids, phosphatidylglycerol and cardiolipin, on VDAC oligomerization. I demonstrated that phosphatidylglycerol significantly enhances VDAC oligomerization in the membrane, whereas cardiolipin disrupts VDAC oligomers. These results suggest that up- or down- regulation of these lipids in mitochondria during apoptosis can tune VDAC oligomerization in the membrane.

Thus, this study sheds light on the role played by the above-mentioned lipids in the regulation of VDAC oligomerization during apoptosis and provides additional information on the molecular mechanisms of the programmed cell death.

Another objective of this work was to investigate the partitioning of VDAC into liquid disordered or liquid ordered lipid phases. The existence of lipid domains or the lipid rafts in mitochondria and VDAC enrichment in these rafts is still under debate. Additionally, mitochondrial VDAC was recently found in the plasma membrane. The role of this VDAC is not known, however, it was shown to be located in caveolae (specialized lipid rafts) and play an important role in neuronal apoptosis and Alzheimer’s disease. Therefore, VDAC partitioning to the lipid rafts is an interesting question for investigation.

The possibility to reconstitute VDAC into minimal model systems—GUVs with phase separation, allowed to reveal the preferential partitioning of VDAC into liquid disordered lipid domain, which suggests either non-raft localization of VDAC or the requirement of the other factors for the recruitment of VDAC into lipid rafts.
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Chapter 1

Introduction

1.1 Mitochondria

1.1.1 Mitochondria: structure and function.

Mitochondria are organelles playing the central role in energy generation within the cell. The word “mitochondria” originates from the Greek “mitos” (thread) and “chondros” granule. These organelles were first discovered by Altman in 1890 and were called “bioblasts” [1]. Altman described mitochondria as an elementary organisms living inside the cell. Such description can be used even several decades later as it clearly emphasizes the complexity of these cellular compartments. Unlike any other organelle in eucariotic cell, mitochondria have an ability to reproduce independently from a cell. They contain their own DNA [2], as well as transcriptional and translational machineries [3, 4]. Such properties of mitochondria make it similar to bacteria and provide an evidence of the importance of this organelle in the evolution of complex organisms. Mitochondria have diameter between 0.5 and 1 µm and length up to 10 µm. These organnels form tubular networks in many cell types. The number of mitochondria in the cell and their shape can vary depending on the tissue type and metabolic state of the cell [5]. However, the main features of mitochondria are always preserved. As was shown by electron microscopy studies [6] mitochondria contains an outer membrane and an inner membrane that create two separate mitochondria compartments: intermembrane space and matrix (Fig. 1.1).

Inner and outer mitochondria membranes have very different protein-lipid composition and function. The outer membrane is considered to be widely permeable to ions and larger molecules because of the presence of transport proteins called porins or voltage-dependent anion-selective channels (VDAC). Porins are the most abundant proteins in the mitochon-
drial outer membrane. These molecules are important for mitochondria metabolism as they allow exchange of various metabolites such as NADH [7], ATP and ADP [8] between mitochondria and cytoplasm. However, evidence accumulates, that porins play a more complex role in mitochondria function and might be involved (together with the other components of the inner and the outer mitochondria membranes) in the process of the programmed cell death or apoptosis. The involvement of porins or VDACs in apoptosis will be discussed in more details in the chapter 1.2.4. Other important proteins in the outer mitochondria membrane include enzymes, involved in synthesis of mitochondrial lipids, and protein import machineries, providing the pathway for the mitochondria proteins synthetized in the cytoplasm [5]. The inner mitochondria membrane has much lower permeability towards small molecules than the outer membrane and, therefore, creates the physical and chemical barrier separating mitochondria matrix from the cytosol. The electrochemical gradient across mitochondria inner membrane is crucial for the energy production by mitochondria. The transport of metabolic products through the inner mitochondrial membrane is accomplished by specific ion transporters, e.g., adenine nucleotide translocase (ANT), which specifically exchanges ADP for ATP. The inner membrane also contains proteins responsible for oxidation reactions of the respiratory chain. Proteins involved in these reactions are organized into the so called enzymatic complexes (I–IV).
Mitochondria

These complexes pump protons from the matrix to the intermembrane space and thus, create an electrochemical gradient across the inner membrane. This gradient is used by the enzyme complex ATP synthase, located on the inner mitochondrial membrane, to generate ATP – the energy molecule of the cell. The inner membrane creates folds or wrinkles called the cristae. In this way the total surface area of the inner membrane is increased, allowing for the higher protein content in the membrane. The mitochondrial matrix contains mitochondria DNA as well as ribosomes, tRNAs and enzymes that are required for gene expression and protein synthesis. Enzymes involved in the major metabolic pathways such as citric acid cycle, lipid and amino acid oxidation also reside in the matrix of mitochondria. The inner and outer mitochondrial membranes are separated by the intermembrane space. This compartment contains not only enzymes involved in nucleotide phosphorylation and components of the electron transport chain, but also some signaling molecules, like apoptogenic factors—the molecules involved in programmed cell death or apoptosis. Among these factors are: AIF [9], endonuclease G [10] as well as an essential component of electron transport chain—cytochrome c [11] The inner mitochondria membrane dynamically interacts with the outer membrane, forming the contact sites [12]. These contact sites are created by specific interaction of inner and outer membrane components such as VDAC and ANT (Fig. 1.2). Mitochondria contact sites were proven to play an important role in mitochondria metabolism and apoptosis [13].

![Figure 1.2](image)

Figure 1.2: Schematic representation of the contact sites of mitochondria. VDAC in the outer mitochondrial membrane and ANT in the inner mitochondrial membrane are believed to colocalize in the mitochondrial contact sites.

Mitochondria are crucial for cell function and apoptosis, therefore any disorder in the structure and function of mitochondria can lead to serious diseases. Among these diseases are schizophrenia, Alzheimer’s disease, cardiovascular disease and many others.
Understanding the processes taking place in mitochondria is important for development of the therapeutic approaches for the treatment of mitochondria associated disorders and cancer. Though, mitochondria research goes back to 19th century, there are still many open questions in this field.

1.1.2 Mitochondria in apoptosis.

Cell death is one of the major events for multicellular organism. There are two distinguished cell death mechanisms: apoptosis and necrosis. In contrast to necrosis, apoptosis is a strictly regulated or programmed process, which is required for the removal of aged, damaged or infected cells [14]. The abnormal regulation of apoptosis leads to a variety of severe diseases like autoimmune diseases, cancer, infarction, and AIDS. The major requirement for apoptosis induction is activation of caspases, a group of cysteine aspartate-specific proteases, which cleave cellular substrates [15] causing the degradation of the cell: cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomcal DNA fragmentation. The activation of caspases can be executed by either extrinsic or intrinsic apoptotic pathways.

The extrinsic pathway is triggered in response to stress stimuli (UV light, irradiation, viral infection) by the activation of death receptors on the cell membrane surface such as FAS or TNF which, in their turn, transfer the death signal to effector caspases, cleaving the cell components. The intrinsic apoptotic pathway is also called mitochondria pathway, since this organelle plays the central role in this type of cell death [16]. In mitochondria associated apoptosis, the proteins initiating caspase activation (apoptogenic factors) are released from the mitochondria intermembrane space. The release of these proteins is a highly regulated process in which different anti- and pro-apoptotic proteins of Bcl-2 family are involved.

One of the best described apoptogenic factors is cytochrome \( c \) located between the inner and outer mitochondria membrane. Upon apoptosis induction it is released to the cytoplasm where it interacts with its receptor Apaf-1 allowing its oligomerization and binding to Cspase-9 [17]. Caspase-9 then activates caspase-3 and caspase-7, which are responsible for cell destruction. It has been more then a decade since the central role of cytochrome \( c \) in apoptosis has been discovered, however it still remains unknown how this protein is released from mitochondria. Nevertheless, numerous studies strongly suggest the involvement of Bcl-2 family apoptotic proteins Bax, tBid, Bcl-xl and mitochondrial porin (VDAC) in cytochrome \( c \) release. Various models concerning the formation of the
pore and subsequent cytochrome c release were proposed among which are (for review see [18]):

![Diagram of mitochondrial mechanisms](image)

**Figure 1.3:** Models of cytochrome c release from mitochondria. In models (a) and (b), the outer mitochondrial membrane ruptures as a result of swelling of the mitochondrial matrix, allowing cytochrome c to escape from mitochondria. Model (a) involves PTP opening and rupture of mitochondrial membrane. In model (b) VDAC is closed, what induces mitochondrial-matrix swelling. Models (c)–(e) involve the formation of a large channel in the outer mitochondrial membrane, allowing cytochrome c release without the rupture of the mitochondrial membrane. Taken from [18].

1) Opening of the permeability transition pore (PTP)—megachannel spanning both mitochondria membranes. A major components of PTP are: ANT in the inner and VDAC in the outer mitochondrial membrane. PTP opening leads to mitochondria swelling (Fig. 1.3 (a), (b)).

2) Formation of a big pore created by Bax oligomers (Fig. 1.3 (c)).

3) Formation of lipiddic pore or pore created by lipids and proteins together (Fig. 1.3 (d)).

4) Pore formation by heterooligomers of VDAC and Bax (Fig. 1.3 (e)).

The origin of the pore for cytochrome c release still remains unknown and there are
a lot of open questions regarding proteins involved in the process of apoptosis. In this dissertation, I will focus on VDAC as an important player in the cell death.

1.2 Voltage dependent anion channel (VDAC)

VDAC is the major component of the mitochondria outer membrane (> 50% of total protein), which serves as the pathway for metabolite exchange between cytosol and mitochondria. It was first identified in 1976 in crude extracts of mitochondria from *Paramecium Aurelia* upon reconstitution into planar lipid bilayers [19]. Since then, VDAC has been purified from mitochondria of various organisms, e.g., yeast, *N.crassa* and rat liver cells. Three different VDAC isoforms exist in mammals: VDAC1, VDAC2 and VDAC3 [20]. The physiological role of these isoforms has yet to be elucidated. VDAC1—the frequently studied isoform, also considered in this dissertation, is the most abundant one and overcomes VDAC2 of one order of magnitude and VDAC3 of two orders of magnitude by expression levels in mitochondria. In addition, it was shown that the content of VDAC2 and VDAC3 is constantly changing in the cell, while VDAC1 has a steady level of expression, suggesting a very important role VDAC1 plays in apoptosis [21]. VDAC1 will be referred as VDAC in this work.

1.2.1 Structure of VDAC

Recently, the crystal structures of the mouse and human VDACs were solved by two independent research groups using NMR spectroscopy and X-ray crystallography [22, 23]. Both works show, that VDAC adopts a β-barrel architecture composed of 19 β-strands and one α-helix. All β-strands create an antiparallel pattern with the exception of 1st and 19th strands that associate in a parallel manner (Fig. 1.4).

The pore created by β-strands has slightly concave shape with horizontal dimensions 3.5×3.1 nm and the length of 4 nm. The inner diameter of the pore is 1.5×1 nm which allows the passage of small metabolites through the channel [22]. The exterior of VDAC mainly consists of hydrophobic residues, whereas the interior is hydrophilic and slightly positively charged, what makes it conductive for metabolites and anion-selective. The N-terminus of VDAC is hydrophilic and forms a distorted α-helix. This charged α-helix is ideally positioned in the interior of the VDAC pore to regulate the permeability of the channel towards metabolites [23].

There is no doubt that the solved 3D structure of VDAC provides very important infor-
mation in the structural level, however there is an evidence, that the presented structure of native VDAC in the mitochondria membrane can slightly differ from the one presented above. The described crystal structures of VDAC are in conflict with the results from biochemical and functional studies published in the last years [24].

The crystal structure of the human VDAC was determined in detergent, environment, which is very different from the native lipid membrane. For crystallography studies with mouse VDAC the so called lipid bicells formed from the mixture of lipid and detergent were used. This system better mimics the native conditions, however still might be different from the surrounding provided by the phospholipid bilayer. Studies indicate that the folding patterns of membrane proteins in general, and VDAC in particular, are different in the detergent and in the membrane. The detergent mole fraction variation in the detergent/lipid mixtures containing VDAC have been shown to induce dramatic change in the protein structure as was observed by circular dichroism spectroscopy [25]. In the same study, it was demonstrated, that VDAC inserts and spontaneously folds into phospholipid bilayers. These results indicate that the lipid environment plays a very important role in the structure and function of VDAC.

1.2.2 Electrophysiological characteristics of VDAC

VDAC is not easily accessible for electrophysiology studies, because of its location in mitochondria. To investigate the properties of this channel, reconstitution approach is used.
Purified VDAC is reconstituted into black lipid membrane (BLM) and the conductivity of the channel is investigated under various conditions. BLM separates two aqueous compartments and allows to control the composition of the solution and the potential across the membrane. The properties of VDAC purified from different species (e.g. potato, rat, sheep) are highly conserved [26]. The main characteristic of this channel is voltage dependence. At low voltages (10 mV) applied across the membrane, VDAC channel is mainly in its open state and at higher voltages (above 20 mV, either positive or negative), it undergoes transition to the lower or “closed” conductive state. Generally, the gating does not involve the transition to the completely closed states, but rather the conductance is decreased approximately twice. The average VDAC conductance value in its fully open state is 4 ns in 1 M KCl whereas in closed state it is about 2–2.5 ns. Interestingly, the open state of the channel shows preference for anions and the closed state is favorable for cations [27]. Therefore, large anions such as ATP can not pass through the closed channel, which becomes cation selective. As a consequence, closure of VDAC channels regulates metabolite flux in and out of mitochondria [28].

The voltage dependence of VDAC was discovered 30 years ago, however the physiological relevance of the voltage-gating and the factors regulating channel’s open and closed states in vivo, still remain unknown. The main question arises: does mitochondria outer membrane, which is completely permeable to ions have a membrane potential? Some hypothesis of the existence of such a potential were proposed. Among them are: the existence of Donan potential created by charged macromolecules in mitochondria and cytosol [29], the potential generated by mitochondria metabolites, because of differential permeability of VDAC towards anions and cations [30] and the potential across the outer membrane generated due to the proximity of the inner membrane [31]. These membrane potentials can reach 20–25 mV, what would be in the switching region for the VDAC permeability. Recently, a new hypothesis about the regulation of the outer mitochondria membrane permeability and respiration was suggested. Cytoskeletal protein tubulin was shown to regulate VDAC conductivity and decrease the respiration rate of isolated mitochondria [32], therefore suggesting the important role of this protein in regulation of VDAC function and mitochondria metabolism.

1.2.3 VDAC oligomerization

VDAC oligomerization plays an important role in apoptosis, which is discussed in details in the next chapter. The existence of VDAC dimers and higher order oligomers were
Voltage dependent anion channel (VDAC) mentioned for the first time almost 30 years ago in studies on cross-linked water-soluble VDACs from N. crassa [33]. Cross-linking experiments on VDAC in yeast [34] and rat liver [35] mitochondria outer membranes also indicate the existence of VDAC oligomeric complexes. Biochemical and nuclear magnetic resonance (NMR) studies have shown, that purified VDAC forms dimers and higher oligomers in detergent solutions [36, 37]. Recent atomic force microscopy (AFM) studies on native mitochondrial membranes confirm the ability of VDAC to oligomerize [38, 39]. VDAC in the purified outer mitochondria membranes of potato tubers [38] and yeast [39] exists in different oligomeric states: monomers, dimers, trimers, hexamers and even arrays of up to 20 molecules (Fig. 1.5). In addition, the oligomers were shown to be highly dynamic and able to rearrange into mobile groups with highly variable numbers of VDAC molecules [39].

**Figure 1.5:** Frequency-modulation AFM image of VDAC in the outer mitochondrial membrane of potato tubers. VDAC forms monomers and various oligomers. The figure is made by B. Hoogenboom, taken from http://www.bristol.ac.uk/nsqi-centre/events/2010/74.html.

The analysis of the recently obtained crystal structure of mouse VDAC reveals an antiparallel dimer that further assembles as hexamers [40], which is in agreement with AFM studies on native membranes. Packing analysis of VDAC grown in lipidic bicells suggests that the dimer is formed by β-strands 1–4, 18 and 19. All contacts are formed by van der Waals interactions except one hydrogen bond located between Ser-43 of each monomer. This tightly packed antiparallel dimer can interact with surrounding lipids and associate further to create higher order oligomers [40]. Authors also suggest that lipids may play a critical role in VDAC oligomerization.
1.2.4 The role of VDAC and its oligomerization in apoptosis

VDAC plays an important role not only in the cell life, but also in the cell death: accumulating evidence indicates that VDAC is involved in mitochondria mediated apoptosis [41, 42, 43, 44]. It was demonstrated that siRNA mediated down-expression of VDAC prevents cisplatin and endostatin induced cell death [45, 41]. On the other hand, overexpression of VDAC in various species causes apoptosis [46, 47, 48]. Several studies suggest the interaction of VDAC with Bcl-2 family apoptotic proteins—regulators of cytochrome $c$ release from mitochondria during apoptosis. The interaction of VDAC with anti-apoptotic protein Bcl-xl was shown by biochemical studies and NMR [36, 49]. However, the effect of Bcl-xl on VDAC function and the role played by the interaction of these proteins in cytochrome $c$ release remains controversial. According to one report, Bcl-xl induces closure of VDAC and therefore prevents the formation of permeability transition pore during apoptosis [49]. Another study suggests that Bcl-xl maintains VDAC in its open state allowing the efficient function of mitochondria and, thus prevents the cell death [50]. Other controversial results connect VDAC with pro-apoptotic protein t-Bid. t-Bid was reported to increase VDAC pore size in several electrophysiology studies [51, 52], whereas in the work of Rostovtseva et al. t-Bid was reported to induce irreversible closure of the channel [53]. In vivo experiments demonstrate that VDAC is involved in the release of apoptogenic proteins from mitochondria through the interaction with Bax [42, 43]. In vitro studies with permeabilised liposomes show that pro-apoptotic Bax directly interacts with VDAC and forms a novel large cytochrome $c$ permeable pore [49, 51, 54]. However, a direct interaction of these two proteins has not been confirmed. Electrophysiology studies investigating the formation of VDAC/Bax large pore are also in disagreement [53, 51, 52].

Therefore, there are still many open questions regarding the interaction of VDAC with apoptotic proteins and the origin of the pore for cytochrome $c$ release. Nevertheless, numerous studies on VDAC, proposing different models for cytochrome $c$ release from mitochondria, have the common observation: VDAC oligomerization is significantly enhanced during apoptosis [48, 55, 36, 56, 57]. Apoptotic cell death induced by various stimuli, like chemotherapy agents, UV and H$_2$O$_2$ is accompanied by up to 20-fold increase in VDAC oligomerization. Overexpression of VDAC, which is known to induce cell death, also leads VDAC oligomerization enhancement. In addition, apoptosis inhibitor factors prevent VDAC oligomerization [48]. Similarly, the apoptosis inducing effect of As$_2$O$_3$ was reported to mediate VDAC dimerization that was prevented by overexpression of Bcl-xl [57]. Experiments in living cells, using bioluminescence resonance energy transfer
also confirm that cytochrome \( c \) release and apoptosis are coupled to VDAC oligomerization, suggesting that apoptosis induction shifts the dynamic equilibrium between VDAC monomers and oligomers towards the formation of dimers, trimers, tetramers and higher order multimers. [44, 48]. The oligomerization of VDAC is an important process in cell death, however the factors regulating VDAC oligomerization in the mitochondrial membrane still have to be identified.

### 1.3 Principles of membrane organization

#### 1.3.1 Functions of cellular membranes

The biological membrane is an important and indispensible structural element of the cell. Except the plasma membrane, which separates the interior of the whole cell from the external medium, there is a number of intracellular membranes creating physico-chemical barrier between cellular organelles and the cytoplasm. The major components of membranes are proteins and lipids. According to the fluid-mosaic model suggested by Singer and Nicolson in 1972 [58] the biological membrane represents a two-dimensional liquid, in which integral membrane proteins are embedded into phospholipid membrane and freely move in the plane of the membrane (Fig. 1.6). The lipid molecules in the membrane are arranged into a double layer which is 3–5 nm thick and creates a relatively impermeable barrier for the passage of molecules. Lipids can freely diffuse in the plane of the membrane. For a long time lipids were considered just as a passive environment for membrane proteins—the active regulators of the cell membrane function. Nowadays, it becomes obvious that lipids are as important constituents of the biological membrane.

![Figure 1.6: The structure of a cell membrane. The proteins (blue) are embedded inside the phospholipid bilayer (green).](image-url)
as proteins. Only collectively, lipids and proteins can fulfil many complex functions of
the cell membrane: maintaining gradients of ions, pH and electrical charge, accomplishing
the signal transduction, biochemical reactions, the recognition between cells, and helping
the membrane to adopt certain shapes. The cell membranes have very different lipid
and protein content what plays a crucial role for the proper functioning of the particular
membranes and the whole cell.

1.3.2 The phospholipid bilayer

The phospholipid bilayer is a thin film created by two layers of lipid molecules. Eucar-
iotic cells have thousands of different lipids. Each cellular organelle has a specific lipid
composition, what is important for their structure and function. The most abundant are
phospholipids. All phospholipids share some structural similarities, namely, they have
so called “hydrophilic (polar) head group” and “hydrophobic (nonpolar) tails”. The head
groups usually contain glycerol and phosphate group esterified to an alcohol, e.g. choline,
serine or ethanolamine. Hydrophobic tails of phospholipids are made of fatty acid chains
which contain 14–24 carbon atoms. Fatty acids can be either saturated or unsaturated,
meaning they contain one or more double bonds [5]. In figure 1.7, the chemical structure of
one of the most abundant phospholipids 1,2-dioleoyl-sn-glycero-3-phosphocholine—DOPC
(PC) is presented.

![Figure 1.7: The structure of the common phospholipid, phosphatidylcholine.](image-url)
Cell membranes in addition include several types of lipids other than phospholipids. An important examples of such lipids are cholesterol and sphingomyelin. These lipids are constituents of the so called “lipid rafts” (discussed in the next chapter)—specialized membrane microdomains, which play an essential role in cellular processes, e.g. signaling, protein trafficking and neurotransmission [59].

Lipids have an ability to self-associate into various structures. This self association is driven by hydrophobic, van der Waals and electrostatic forces between the amphipathic lipid molecules. If lipids are surrounded by aqueous solution, it becomes entropically favorable for these molecules to assemble so that the contact between hydrophobic part of the lipid and water is reduced whereas their polar headgroups are exposed to the exterior. In this way, lipids, depending on their shape, create different structures (Fig. 1.8). Lipids can arrange themselves into bimolecular sheets called bilayers, or spherical micells made of one layer of lipid molecules. Lipid bilayers tend to close themselves to eliminate the contact of hydrophobic tails with the water at the edges, giving rise to other lipid structure—vesicle or liposome [5].

![Figure 1.8: Lipid structures.](image)

The shape of the lipid is determined by the surface area occupied by the headgroup, the length of the acyl chains and the volume occupied by hydrophobic tails. For example, double chained lipids with small headgroup areas like phosphatidylethanolamine (PE) and phosphatidylserine (PS) have cylindrical shape and therefore, tend to form planar bilayers. PC has a truncated cone shape, determined by the bulky head group and therefore it favours to associate into vesicles. Lipids with a small head group or bulky tails, like cardiolipin (CL), can create nonlammelar structures, e.g. inverted micelles [60].
1.3.3 The lipid raft concept

Lipid rafts are small, heterogeneous, highly dynamic membrane domains stabilized through protein–protein and protein—lipid interactions. These domains compartmentalize an important processes taking place in cellular membranes, e.g. protein and receptor trafficking, signal transduction, regulation of neurotransmission and endocytosis. Lipid rafts were shown to be enriched in cholesterol and sphingolipids such as sphingomyelin (SM). Cholesterol preferentially interacts with sphingolipids, because of the structure of their hydrocarbon chain. Being saturated, sphingolipids create more tightly packed bilayer. It is more favorable for rigid sterol group to partition into rigid bilayer, then in more fluid membrane formed by unsaturated lipids like PC [59]. Lipid rafts can include or exclude other lipids and membrane proteins (Fig. 1.9).

Figure 1.9: Schematic representation of lipid rafts. Some membrane proteins are excluded from lipid rafts, whereas some integral membrane proteins, receptors and GPI-anchored proteins partition into rafts.

The key concept of the raft hypothesis is that phase separation in cell membranes is driven by lipid—lipid interactions. In this case, it should be possible to observe the
phase separation in the model membrane systems, composed only from lipids. The first visualization of raft like domains in model membranes such as giant unilamellar vesicles or supported lipid bilayers was achieved in 2001 [61]. The so called liquid ordered (Lo) and liquid disordered lipid (Ld) domains were obtained not only in the membranes composed of equimolar mixtures of phosphatidylcholine, sphingomyelin and cholesterol but also in the membranes prepared from natural lipid extracts. Liquid ordered phase was shown to be enriched in sphingomyelin and cholesterol and exhibited gel-like short range order, but translational mobility typical for the liquid crystalline state.

The experimental observation of lipid rafts in the cell membranes is more difficult, because of their dynamic structure and small sizes. Instead of the straightforward visualization of lipid domains, often some indirect biochemical methods are used. The first studies on the lipid rafts in the cell membranes were performed by detergent extraction of the lipid rafts from a plasma membrane, using non-ionic detergents like Triton X100. After such treatment the fluid membrane is dissolved but lipid rafts stay intact and can be extracted. Because of this fact, lipid rafts are also sometimes called Detergent Resistant Membranes (DRMs) [62]. With this method association of variety of proteins with lipid rafts were shown, among the first raft proteins identified by this method are Glycosylphosphatidylinositol (GPI) anchored proteins and placental alkaline phosphatase (PLAP). The validity of the detergent resistance methodology to study membrane domains has recently been questioned, because the perturbation caused in the membrane by the detergent can create a number of artifacts. Nevertheless, DRM method still remains to be a valuable biochemical technique to study the affinity of proteins towards the lipid rafts [63].

The sizes of lipid rafts in native membranes were proven to be below the resolution limit of the light microscopy. The implementation of new biophysical techniques like FRET [64] and STED-FCS [65] allowed for more direct detection of lipid rafts and moved further our understanding of membrane heterogeneities. These studies suggest that rafts are highly dynamic structures 10–20 nm in size, and single molecules such as GPI-anchored proteins remain associated with rafts for several ms.

1.3.4 Lipid rafts and VDAC

The possibility of the existence of lipid rafts in mitochondria was suggested in several studies [66, 67, 68]. In these studies VDAC was co-purified with DRMs and was shown to colocalize with GD3 ganglioside (a ceramide based glycolipid) in microdomains on mitochondrial outer membrane. However, some contradictory results denying mitochondria
lipid rafts were also obtained [69]. One of the hypothesis provided by authors about the observation of mitochondria proteins, including VDAC, in DRMs, is the possibility that the localization of this proteins is not restricted to mitochondria. Indeed, according to the recent investigations, mitochondrial VDAC was also found on the plasma membrane (pl-VDAC) (for review see [70]). The role of pl-VDAC is not known, however, it was shown to be located in caveolae (specialized lipid rafts) [71] and play an important role in neuronal apoptosis [72] and Alzheimer’s disease [73].

Interestingly, lipid rafts from plasma membrane were found to be recruited to mitochondria during apoptosis where they contribute to the events required for the cell death [74, 75] and were VDAC was shown to partition [67]. Therefore the raft partitioning of mitochondrial and plasma membrane VDAC requires further investigation.

1.3.5 Mitochondrial lipids

Mitochondria, as well as other organelles in the cell, has unique lipid composition. Though, two mitochondria membranes—outer and inner, are in close contact with each other, they also have very different lipid and protein contents.

Mitochondria are known to be partially autonomous organelles. This is also related to the lipid synthesis. Part of the mitochondrial lipids is produced directly in the organelle. However, another part is synthesized in the endoplasmic reticulum and transferred to the mitochondrial membrane.

Similar to membranes of other cellular compartments, PC is the main lipid in the mitochondrial membrane. Another lipid found in high quantities in this organelle is PE. The important characteristic of the mitochondrial membrane lipid composition, making it significantly different from the lipid composition of any other sub-cellular membranes, is that it contains high amounts of cardiolipin (CL) (10–20 % of total lipids)—lipid found almost exclusively in mitochondria, and relatively small quantities of sterols and sphingolipids. Cardiolipin is a unique lipid, which has four acyl chains instead of two as other phospholipids do. Because of this feature, CL has inverted cone shape and can adopt nonlamellar hexagonal phases [60]. The precursor lipid for CL synthesis is phosphatidyl-glycerol (PG) and in physiological conditions its content in mitochondria is relatively low [76]. The presence of PG and CL as well as high PE/PC ratio in mitochondria suggests the bacterial origin of this organelle [77].

CL is considered to be enriched in the inner mitochondrial membrane (18 wt.% of the total lipid content). The amount of CL in the outer mitochondrial membrane is lower.
Table 1.1: Lipid composition of mitochondrial membranes and mitochondrial contact sites. Values are presented in percent by weight of total lipids. OM, outer membrane; IM, inner membrane. Adapted from [78].

<table>
<thead>
<tr>
<th>Components</th>
<th>OM</th>
<th>OM contact sites</th>
<th>IM contact sites</th>
<th>IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acids</td>
<td>8.2</td>
<td>3.4</td>
<td>5.6</td>
<td>7.6</td>
</tr>
<tr>
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<td>5.8</td>
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<tr>
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<td>5.1</td>
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<tr>
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<td>9.4</td>
<td>0.4</td>
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<td>Phosphatidylcholine</td>
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<td>25.9</td>
<td>25.8</td>
<td>35.1</td>
</tr>
<tr>
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<td>3.1</td>
<td>2.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
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<td>7.7</td>
<td>7.7</td>
<td>5.0</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
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<td>21.5</td>
<td>21.7</td>
<td>26.5</td>
</tr>
<tr>
<td>Cardiolipin</td>
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<td>24.4</td>
<td>18.0</td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

(around 4 wt.% by weight), however it is found in high concentrations in the contact sites between the inner and the outer mitochondrial membrane (27 wt.%) [78].

For a long time, the composition of mitochondrial contact sites remained unknown. In 1990, the mitochondrial contact sites were successfully purified and separated from the inner and the outer mitochondrial membrane fractions. The major components of contact sites were found to be PC, PE and CL. Contacts also contained relatively high amounts of PI and cholesterol [78]. For more detailed information about the composition of the inner and the outer mitochondrial membranes as well as their contact sites see table 1.1.

The particular content of mitochondrial membranes is very important for its proper functioning. The variations in the amounts and structures of mitochondrial lipids can lead to various severe deceases. For example CL alterations are associated with Barth Syndrome, Heart Failure, Ischemia and Reperfusion (for review see [79]).

Interestingly, apoptosis also induces changes in mitochondrial lipids. The alterations in the amounts of lysophosphatidyl-choline, GD3, PG and CL during apoptosis were reported in several studies [80, 81, 82, 83, 84].

1.4 Synthetic model membrane systems

Synthetic model membrane systems provide an important tool for the investigation of properties of cellular membranes, membrane protein function, protein—lipid and lipid—lipid interactions. Model membranes are systems of reduced complexity, where the chemical
composition and the environment can be precisely controlled, which allows the determination of various parameters related to membrane functions those can not be accessed and discriminated in complex native membranes.

A model lipid bilayer can be made of synthetic or natural lipids. The simplest lipid bilayer contains only pure synthetic lipid of one type. More physiologically relevant artificial membranes can be made of several types of lipids. The physical properties of such bilayers like tension, lateral organization and phase separation can be studied by different methods. The complexity of model membrane systems can be further increased by incorporation of membrane proteins. Such membranes can be used to investigate particular protein—protein and protein—lipid interactions.

Various types of model membranes are existing. Among them are supported lipid membranes, bicelles, micelles, nanodiscs, droplet interface bilayer systems and discussed here black lipid membranes and giant unilamellar vesicles.

1.4.1 Black lipid membranes

Black lipid membrane (BLM) is one of the first model bilayer systems. It was introduced in 1962 by Mueller et. al [85]. The bilayers were called black, because of their optical appearance—as a result of the interference of the reflected light on the thin lipid film. This type of bilayer is “painted” across the small aperture by applying the solution of lipids dissolved in organic solvent using brush or pipette tip. The method was later modified to eliminate the amount of the organic solvent in the bilayer. In this approach black lipid membrane was obtained from lipid monolayers created on the surface of two fluid chambers from both sides of the aperture (Montal-Mueller method) [86]. The disadvantage of BLMs is that this system contains residual organic solvent and membranes are unstable, however the big advantage is the possibility to easily control the solution composition from both sides of the membrane and to place electrodes in the compartments separated by the bilayer. This makes the black lipid membranes very useful in electrophysiology applications. Electrical properties of lipid bilayer were accessed in this systems [87, 88]. In addition, a number of ion channels, e.g., mitochondrial ATP-dependent potassium channel, VDAC and bacteriorhodopsin were successfully incorporated into BLM and their properties were investigated [89, 19, 90].
1.4.2 Giant unilamellar vesicles

Giant unilamellar vesicles (GUVs) are the largest class of liposomes formed from a double layer of lipid molecules. The sizes of GUVs can reach several hundreds of micrometers. This means that they can be observed by the light and fluorescence microscope and therefore represent a valuable system to study fundamental features of lipid membranes. GUVs were first described in 1969 [91] and are still very popular in the membrane research field. The currently and widely used protocol, which is relatively easy and reproducible for preparation of unilamellar vesicles 15–70 µm in diameter was suggested by Angelova et al. [92]. In this protocol, dried lipid film is rehydrated in the presence of a slowly alternating electrical field. However, this method cannot be used to grow GUVs in a high salt buffer. Protocols for the preparation of GUVs in physiological conditions were just recently developed [93], but they are not so effective as the one proposed by Angelova et al.

Initially, GUVs were used for biophysical investigations of vesicle morphology, polymorphism of lipid systems and elastic properties of lipid bilayers [94, 95, 96]. Later, after introduction of “lipid raft” hypothesis, GUVs were extensively employed to study membrane domains [97, 61, 98, 99]. The large scale phase separation, observable by means of fluorescent microscope can be achieved in this system.

GUVs are an attractive systems to study membrane deformations and transformations, e.g., tubulation and budding induced by specific proteins [100, 101, 102].

These model membranes also represent a valuable platform for membrane protein incorporation and investigation of protein function and protein—protein interactions within the membrane. A number of membrane proteins e.g. bacteriorhodopsin [103, 104], the sarcoplasmic reticulum Ca\(^{2+}\) ATPase [104] and SNARE proteins [105] were successfully introduced to GUVs and several reconstitution protocols were developed.

Nevertheless, the incorporation of proteins requires an individual approach and still remains a challenge. However, once this goal is achieved, protein—protein and protein—lipid interactions in a controlled conditions can be accessed by various techniques.

1.5 Fluorescence correlation spectroscopy (FCS)

Fluorescence correlation spectroscopy (FCS) is a versatile single molecule technique to study membrane dynamics, protein—protein and protein—lipid interactions. It can provide information about diffusion coefficients, concentrations, and molecular interactions.
of proteins and lipids in the membrane. The main parameter of interest in FCS, in contrast to other fluorescent techniques is not the fluorescent emission intensity, but the fluctuations of the intensity signal caused by different physical-chemical factors. The first theoretical and experimental realization of FCS was described by Magde et al. in 1972 [106]. This publication was followed by a number of studies in this field. However, the early FCS measurements were suffering from the low detection efficiency, because of the large number of molecules in the detection volume. To overcome this problem, Rigler and co-workers introduced the confocal detection to the FCS technique [107]. The possibility of limiting the detection volume, so that the molecule concentration in nanomolar range can be used for FCS measurements, allowed to increase the signal fluctuations induced by the fluorescent molecules in the observation volume and therefore, significantly improved the signal-to noise ratio.

Another important break-through was the development of fluorescence cross-correlation spectroscopy (FCCS) technique to study the molecular interactions. The first experimental realization of this technique was achieved by Schwille et al. [108]. Nowadays, FCCS is very popular biophysical method to study interactions in both in vitro and in vivo and the field is constantly developing.

Protein—protein interactions in general and protein oligomerization in particular can be studied by FCCS. Oligomerization properties of Bacteriorodopsin [109], epidermal growth factor receptor [110], interleukin-4 and erythropoietin receptors [111] were investigated by this method. The influence of lipid membrane on interactions between apoptotic proteins was also studied by FCCS [112].

1.5.1 Principles of fluorescence

Fluorescence is the emission of light from any substance which occurs as a result of molecule relaxation from electronically excited state to the ground state. Return of the electron from the excited singlet state to the ground state is spin-allowed and followed by the emission of a photon. The main way to excite the molecules is the absorption of light by the fluorophore. The process of fluorescence absorption and emission is usually presented by a Jablonsky diagram, which is shown in figure 1.10. Fluorophores at electronic energy levels $S_0$, $S_1$, $S_2$ have various vibrational energy levels (0, 1, 2, etc.). The light absorption often induces several processes. Usually fluorophore is exited to some higher vibrational level, but sometimes molecules relaxation to the lowest vibrational level of the excited state occurs. This process is called internal conversion and is generally much faster than
the fluorescence emission. Fluorescence generally occurs as a result of the transition from the lowest vibrational level state of $S_1$ to the higher vibrational state of $S_0$. Therefore, the energy of the emission is less than that of the absorption what leads to the shift of the fluorescence emission to the longer wavelengths. This phenomenon is called Stokes-shift and makes it possible to separate the fluorescent light from the excitation light by dichroic mirrors.

![Jablonski diagram. Adapted from [113].](image)

Molecules in the $S_1$ state can also undergo transition to the first triplet state by the so called intersystem crossing. Transition from the triplet state to the singlet ground state is forbidden, therefore the emission rate is several orders of magnitude smaller compared to the rate of fluorescence.

Fluorescence is generally presented by the emission spectrum, which is a plot of the fluorescence intensity versus wavelength or wavenumber. The representative absorption and emission spectra of fluorophore Alexa 488 is presented in figure 2.6 (chapter 2.9).

The most important characteristics of the fluorophore are the fluorescence lifetime and the so called quantum yield. The fluorescence quantum yield is the ratio of the number of emitted photons to the number of absorbed photons. If the emission rate of the fluorophore is $\Gamma$ and its rate of nonradiactive decay to the ground state is $k_{nr}$, then the quantum yield is given by,

$$Q = \frac{\Gamma}{\Gamma + k_{nr}}$$

(1.1)
The life time of the excited state $\tau_n$ is the average time spent by the molecule in the excited state prior to return to the ground state and is given by,

$$\tau_n = \frac{1}{\Gamma + k_{nr}}$$ (1.2)

In general fluorescence lifetime is around 10 ns [113]. Characteristics of fluorophore such as fluorescence life time and quantum yield are very important to consider while choosing the appropriate fluorescent labels for FCS measurements.

### 1.5.2 Principle of FCS

The standard FCS setup consists of a confocal inverted microscope equipped with a high-numerical aperture objective, appropriate dichroic mirrors and emission filters (Fig. 1.11).

![Figure 1.11: Principle of FCS. (A) FCS and FCCS setup. The laser light is reflected by a dichroic mirror into a high numerical aperture objective and is focused to the sample. Fluorescent molecules in the sample are excited and detected only if they diffuse through the confocal volume (B). The light emitted by the molecules is collected by the objective, and after passing the emission filter (or beamsplitter and two emission filters in case of FCCS) and pinhole is detected by an APD (or two APDs in case of FCCS), giving rise to a fluctuating intensity signal (C) which can be correlated in time to obtain the autocorrelation or cross-correlation function (D).](image)
To apply FCS either in vivo or in vitro, the molecules of interest have to be fluorescently labelled. For FCS measurements the laser is focused to the area of interest (a lipid bilayer or solution with labelled molecules) and fluorophores diffusing in and out of the detection volume induce fluctuations in the fluorescence intensity signal, which is collected by the microscope objective and detected by means of avalanche photodiode (APD) giving rise to a fluctuating intensity trace \( F(t) \) (Fig. 1.11). By temporally correlating this trace in time, or by other words, measuring the self similarity of the obtained intensity signal, one can obtain the auto-correlation curve:

\[
G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t + \tau) \rangle}{\langle F(t)^2 \rangle} \tag{1.3}
\]

\( \langle F(t) \rangle = \frac{1}{T} \int_0^T F(t) dt \) is the time average of \( F(t) \) with measurement time \( T \). \( \tau \) is called “lag time”. The experimental autocorrelation curve is fitted to a mathematical model which is suitable for the given experimental situation (e.g., free diffusion or flow) and important quantities are obtained as fitting parameters. The basic mathematical models used to fit the autocorrelation curves for the molecules diffusing in 2D (membrane) or 3D (solution) dimensions are:

\[
G_{3D}(\tau) = \frac{1}{N} \cdot \frac{1}{1 + \tau/\tau_d} \cdot \frac{1}{\sqrt{1 + \tau/S^2} \cdot \tau_d} \tag{1.4}
\]

\[
G_{2D}(\tau) = \frac{1}{N} \cdot \frac{1}{1 + \tau/\tau_d} \tag{1.5}
\]

Here \( S = w_z/w_0 \) is the so-called structural parameter, which describes the ratio of axial, \( w_z \) and radial, \( w_0 \) dimensions of the detection volume. The decay time of the autocorrelation curve is the diffusion time \( \tau_d \) – the time which the molecule spends in the detection volume.

At the high laser powers applied for FCS, the probability of the transition between the first excited singlet and triplet state increases in most fluorescent dyes. The excited state lifetime for the triplet state is in microseconds range. Fluorophores start to blink on and off because of intersystem crossing. Intersystem crossing changes the quantum efficiency of the system. The fluorescent molecules, in this case, can be divided to two fractions: nonfluorescent fraction, \( \alpha \), and fluorescent fraction:

\[
X_{\text{triplet}}(\tau) = \frac{1 - \alpha + \alpha \cdot e^{-\tau/\tau_{\text{triplet}}}}{1 - \alpha} \tag{1.6}
\]
The correlation function must be multiplied by this fraction and the expressions for correlation function will be:

\[
G_{\text{total}}(\tau) = X_{\text{triplet}}(\tau) \cdot G_{3D}(\tau) \quad (1.7)
\]

\[
G_{\text{total}}(\tau) = X_{\text{triplet}}(\tau) \cdot G_{2D}(\tau) \quad (1.8)
\]

If the dimensions of the detection volume are known, one can obtain the diffusion coefficient \(D\):

\[
D = \frac{w_0^2}{4 \tau_d} \quad (1.9)
\]

As can be seen from the equations 1.4–1.5 the number of particles \(N\) in the detection volume is inversely proportional to the amplitude of the autocorrelation curve:

\[
G(0) = \frac{1}{N} \quad (1.10)
\]

The concentration of labelled molecules can be calculated as:

\[
C = \frac{N}{V_{\text{eff}}} \quad (1.11)
\]

where the effective detection volume \(V_{\text{eff}}\) for 3D Gaussian is determined as:

\[
V_{\text{eff}} = \pi^2 w_0^2 w_z \quad (1.12)
\]

Therefore, if the dimensions of focal volume are known, the concentration and the diffusion coefficient of the molecule can be determined. These values can be obtained from calibration measurements, using a dye with known diffusion coefficient. Therefore, such FCS measurements will yield only relative values with respect to the calibration. FCS variants for calibration free concentration and diffusion coefficient determinations were also developed. Among them is the described later scanning FCS technique.

Another important parameter in FCS is effective molecular brightness \(\eta_{\text{eff}}\) which is measured in counts per particle per second (cpps) and is determined as:

\[
\eta_{\text{eff}} = \frac{\langle F(t) \rangle}{N} = \langle F(t) \rangle G(0) \quad (1.13)
\]

where \(\langle F(t) \rangle\) is the averaged fluorescence intensity and \(N\) is the number of particles.

Measurements of the diffusion time by FCS can access the binding of molecules of
interest, however, only substantial increase in particle mass of binding partner will pro-
duce a significant effect on the diffusion time. Diffusion coefficient is connected to the
hydrodynamic radius, \( r \), of a particle by Stokes-Enstein relation:

\[
D = \frac{k \Theta}{6 \pi \eta r}\quad (1.14)
\]

where \( k \) is Boltzmann constant, \( \Theta \) is the temperature and \( \eta \) is the viscosity. The
hydrodynamic radius for globular particles usually scales with the cubic root of the molec-
ular mass, a globular protein would therefore need to gain eight times its own mass to
provide the change in the diffusion coefficient of a factor of two, which is often not the
case in protein—protein interactions. To overcome this problem, two-color Fluorescence
cross-correlation spectroscopy (FCCS) was developed to study molecular interactions.

### 1.5.3 Fluorescence cross-correlation spectroscopy (FCCS)

FCCS method was developed to study molecular interactions between the molecules of
similar size. In this case the interacting molecules are labelled with spectrally separated
dyes and the fluorescence is detected in two separate channels (Fig. 1.11). Similar to
the autocorrelation curve, the spectral cross-correlation curve can be obtained from the
intensity traces \( F_i(\tau) \) and \( F_j(\tau) \) measured in different spectral channels:

\[
G_{ij}(\tau) = \frac{\langle \delta F_i(t) \rangle \cdot \langle \delta F_j(t + \tau) \rangle}{\langle F_i(t) \rangle \cdot \langle F_j(t) \rangle}
\quad (1.15)
\]

If the number of interacting molecules increases, the amplitude of the cross-correlation
curve also increases (see curves 1, 2, 3 on the Fig. 1.13). Under the ideal conditions
where both channels have the complete overlap of the detection volumes and fluorophores
have fully separated emission spectra (negligible cross-talk) the following auto- and cross-
correlation functions can be used:

\[
G_i(\tau) = \frac{\langle C_i \rangle M_i(\tau) + \langle C_{ij} \rangle M_{ij}(\tau)}{\langle C_i \rangle + \langle C_{ij} \rangle} V_{eff}
\quad (1.16)
\]

\[
G_{ij}(\tau) = \frac{\langle C_{ij} \rangle M_{ij}(\tau)}{\langle C_i \rangle + \langle C_{ij} \rangle} V_{eff}, \quad i, j = r, g, i \neq j
\quad (1.17)
\]

Where \( M_{ij}(\tau) \) is the motional part of the correlation curves coinciding in the case of
translational diffusion with \( G_{3D}(\tau) \) and \( G_{2D}(\tau) \) in equations (1.4), (1.5). Usually, the
relative cross-correlation amplitude which can be obtained from the amplitudes of the correlation curves is used as a measure of molecular interactions:

$$\frac{G_{ij}(0)}{G_j(0)} = \frac{C_{ij}}{C_{ij} + C_i} , i, j = r, g, i \neq j, (4)$$

(1.18)

1.5.4 Two-focus scanning FCCS

In the standard implementation of the FCS or FCCS technique to study diffusion of molecules in GUVs, the confocal detection volume is parked on the upper pole of a GUV sitting on top of a lower coverslip of a flow chamber. However, such an approach has several disadvantages. Among them are photo bleaching and problems with the stable positioning of the focal spot on the membrane. The latter one can be particularly dramatic, if the membranes are not stable and move out of focus during the measurements, this can significantly change diffusion times and introduce false positive cross-correlation between differently labeled fluorescent species. The introduction of the scanning FCS technique allowed to overcome these difficulties. In this method, the detection volume is repeatedly scanned through the membrane (Fig. 1.12). The emitted fluorescence is detected and the photon arrival times are recorded. The obtained data is binned and arranged in a matrix with rows corresponding to one line scan. This matrix can be displayed as an image, where membrane movements are visualized as a deviation of the image trace from the straight line. To correct the membrane instabilities, the position of the maximum signal is found and all line scans are shifted relative to each other to align all maxima in one line. Membrane contributions are summed up for each line scan and the intensity trace $F(t)$ is obtained. This intensity trace is used to calculate the auto-correlation curves.

As it is mentioned in the chapter 1.5.2, the determination of absolute values of diffusion coefficient $D$ and concentration of molecules $C$ requires calibration measurements using free dye in solution with known diffusion coefficient to define the dimensions of the detection volume. To overcome this problem, the calibration free, two-focus FCS technique was introduced, which allows the determination of $C$ and $D$ as a parameters of the fitting of auto- and cross-correlation curves. In this method, two spatially separated detection volumes are created and the distance $d$ between them is determined. Two intensity traces $F_1(t)$ and $F_2(t)$ measured in two foci give rise to the two autocorrelation curves (see equation (1.3)) and two spatial cross-correlation curves $G_{12,21}(\tau)$ are obtained:

$$G_{12}(\tau) = \frac{\langle \delta F_1(t) \rangle \cdot \langle \delta F_2(t + \tau) \rangle}{\langle F_1(t) \rangle \cdot \langle F_2(t) \rangle}$$

(1.19)
Fluorescence correlation spectroscopy (FCS)

Figure 1.12: Scanning FCS perpendicular to the membrane plane. (A) For one-focus scanning FCS, a single line is scanned repeatedly through the membrane, for two-focus scanning FCS, two lines separated by a known distance are scanned through the membrane. (B) The data obtained for each focus is arranged in a matrix with rows corresponding to one line scan. (C) The maxima signals are shifted relative to each other along one line to correct for membrane movements. (D) Photons for each line scan are summed up to obtain an intensity value. (E) The intensity trace is correlated to obtain auto-correlation curve (circles) and one cross-correlation curve (squares) (in case one-color two-focus FCS was used).

Two-focus FCS can also be used for the cross-correlation measurements. In this case, in addition to two spatial channels, two spectral channels are used and four intensity traces are obtained: $F_{r1}(t), F_{r2}(t), F_{g1}(t), F_{g2}(t)$. (Here and after two spectral channels will be referred as red ($r$) and green($g$)). From these intensity signals, 16 auto- and cross-correlation curves can be calculated, however only 6 of them will be distinct (Fig. 1.13).

Two-focus FCCS measurements also can be carried out in a scanning regime (as described above). In this case, two lines perpendicular to the membrane plane are scanned in two spectral channels (Fig. 1.12). The following equations are used to fit corresponding auto- and cross-correlation curves [114]:
CHAPTER 1. INTRODUCTION

Figure 1.13: Combination of two-color and two-focus FCS measurements. Four fluctuating intensity traces give rise to 6 distinct correlation curves: autocorrelations of red and green species (1) and (2); cross-correlation between two colors in the same focus position (3); spatial cross-correlation of red (4) and green (5) species; two color cross-correlation between the two focus positions (6). d reflects the distance between focuses.

\[
G_i(\tau) = \frac{1}{(C_i + C_{ij}) \pi w_i w_z} \left( 1 + \frac{4D_i \tau}{w_i^2} \right)^{-1/2} \left( 1 + \frac{4D_i \tau}{w_z^2} \right)^{-1/2},
\]

\(i = r, g\)  

for single-color red (r) and green (g) auto-correlation curves;

\[
G_{ij}(\tau) = \frac{2C_{ij}a(\tau)b(\tau)}{\pi (C_i + C_{ij}) (C_j + C_{ij})} \exp \left[ -2d_{ij}^2b(\tau)^2 - 2d^2a(\tau)^2 \right],
\]

\(i, j = r, g; \ i \neq j\)  

\[a(\tau) = \left(8D_{ij}\tau + w_i^2 + w_j^2\right)^{-1/2}\]

\[b(\tau) = \left(8D_{ij}\tau + w_i^2S_i^2 + w_j^2S_j^2\right)^{-1/2}\]

for two-focus two-color (red-green, rg) cross-correlation curves;

34
The aim of this work

Despite numerous publications on the involvement of VDAC oligomerization in the process of apoptosis, the factors that might regulate VDAC oligomeric state in mitochondria are still unknown.

In light of the recent studies on the importance of lipids for the function of membrane proteins, one can suggest that mitochondrial lipids can be involved in the oligomerization of VDAC. Taking into account the changes in mitochondria lipid composition during apoptosis, the possible relevance of these alterations to the VDAC oligomerization, cytochrome c release and cell death represent an intriguing possibility.

However, mitochondria is a very complex organelle of a small size located inside the cell, therefore the direct investigation of VDAC interactions with phospholipids as well as other components of mitochondria membrane is very complicated. Using in vitro reconstitution
as a bottom-up strategy to study the processes taking place in mitochondria membrane, represents a very useful approach. The aim of this work is to develop an experimental assay that allows for the reconstitution of VDAC into the membrane model system to study its interaction with phospholipids.

GUVs have been proven to be a very useful model suitable for fluorescence microscopy studies of protein—lipid interactions.

Incorporation of fluorescently labelled VDAC into GUVs, would allow for the investigation of VDAC oligomerization in a controlled lipid environment using fluorescence cross-correlation spectroscopy measurements.

This model system is also very useful to study VDAC interaction with lipid rafts. The possible partitioning of VDAC into lipid rafts can be investigated in GUVs with phase separation using fluorescence microscopy approach.

The presented here approach to investigate VDAC oligomerization and raft association can also be used further to study the effect of other mitochondrial lipids and proteins on the properties of VDAC.
Chapter 2

Materials and methods

2.1 Generation of single cysteine mutant of VDAC

For the fluorescence correlation spectroscopy measurements, molecule of interest has to be labeled with a single fluorophore. Specific labeling on cysteine residues in a protein is possible, however VDAC has two cysteins, therefore one of the cysteines has to be replaced by another amino acid in order to get single labeling.

A single cysteine mutant of hVDAC1 was generated from plasmid pTMVDAC1 (a generous gift of Dr. Jörg H. Kleinschmidt, University of Konstanz, Germany) using a PCR Quick change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Cysteine at the position 232 was replaced by serine using sense strand primer GCACATTAACCTGGGCagcGACATGGATTTCGACATTGC and anti-sense strand primer GCAATGTCGAAATCATGTCgctGCCCAGGTTAATGTGC (lowercase letters show mismatched codons). For the successful PCR reaction, primers had to be between 25 and 45 bases in length, and the melting temperature ($T_m$) of the primers had to be greater than or equal to 78°C. The following formula was used to estimate the $T_m$ of primers:

$$T_m = 81.5 + 0.41(\%GC) - 675/N - \%\text{mismatch} \quad (2.1)$$

where $N$ is the primer length in base pairs.

The length of both primers was 39 base pairs and melting temperature was 81°C. Parameters for PCR reaction are given in Table 2.1. The obtained clones were sequenced in DNA sequencing facility (MPI-CBG, Dresden, Germany) and the desired single cysteine mutation was confirmed. Plasmids were stored at $-80$ °C.
### Table 2.1: Parameters for PCR reaction.

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</tr>
<tr>
<td>3</td>
<td>1</td>
<td>68°C</td>
<td>7 minutes</td>
</tr>
</tbody>
</table>

#### 2.2 Expression and purification of VDAC protein

VDAC and its single cysteine mutant were purified, following the protocol described by Schanmuqavadi et al. [25] with the minor modifications.

Plasmids were transformed into expression bacterial strain *E. coli* BL21(DE3) using electroporation technique. Bacteria were grown overnight in a shaking flask at 37°C in 50 ml of LB medium containing 1 % glucose and 0.1 g/l ampicilin. 15 ml of this preculture was transferred into a 500 ml of LB-ampicilin medium and was grown until the absorption measured at 600 nm reached 0.9. To induce expression of the protein, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM and cells were incubated at 37°C for 5–6 hours. Cells were centrifuged at 500 rpm for 10 minutes, resuspended in 2× PBS buffer and disrupted in three passages at 1.5 kBar using EmulsiFlex-C5 microfluidizer disruption system (Avestin Europe GmbH, Mannheim, Germany). To obtain inclusion bodies of hVDAC1, cells were centrifuged at 11000 rpm for 30 minutes and the pellet was resuspended in a buffer containing 25 mM Tris pH 8, 150 mM NaCl and 0.4 % Chaps using Dounce homogenizer. Homogenized pellet was centrifuged again and solubilized in a buffer containing 8M urea, 100 mM Tris pH 8, 10 mM DTT, 1 mM EDTA overnight with constant stirring. Solubilized inclusion bodies were loaded on Diethylaminoethyl (DEAE) cellulose column in a buffer containing 50 mM Tris pH 7.5, 8 M urea and eluted in 50 mM MES, pH 6, 8 M urea, 0.5 M NaCl buffer. DEAE column is positively charged. VDAC protein also has a positive charge at pH 7.5, because its pK value is 8.6 [115]. Therefore, the protein was not binding to DEAE column and was eluted. The flow through pool from DEAE column was dialyzed overnight against 50 mM MES, pH 6, 8 M urea buffer and loaded on a Carboxymethyl (CM) cellulose column. This column was negatively charged and therefore VDAC bound to it. The elution was carried out by applying a linear gradient of 0–500 mM NaCl. The purity of the protein was estimated by SDS-PAGE gel stained with Coomassie Blue (Fig. 2.1). As expected, the band at 31 kDa, corresponding to
VDAC protein was detected. The concentration of the protein was determined by Lowry method or by absorption measurements at 280 nm, using the extinction coefficient of 1.25 l/(g·cm) [115] (both methods gave similar values). Proteins were stored at −80°C.

By this purification procedure VDAC in unfolded form can be obtained with a high yield (several milligrams).

![Coomassie stained polyacrilamide gel of the VDAC protein after purification in DEAE and CM columns.](image)

**Figure 2.1:** Coomassie stained polyacrilamide gel of the VDAC protein after purification in DEAE and CM columns.

### 2.3 Protein labeling with fluorescent dyes

Unfolded mutated VDAC in urea was labelled on the single accessible cysteine with thiol-reactive dyes: Alexa Fluor 488 C5-maleimide (Invitrogen GmbH, Darmstadt, Germany) and ATTO 655-maleimide (ATTO-TEC GmbH, Siegen, Germany). Labeling procedure was carried out according to manufacturer instructions. Protein was concentrated in YM-10 centrifugal filter device (Millipore Corporation, Billerica, MA) to a concentration of 50–100 µM. To prevent the formation of disulfide bonds between cysteine residues of VDAC molecules, a 10-fold molar excess of dithiothreitol (DTT) was added to the protein solution.
followed by an incubation for 2 hours. After that, DTT was removed by dialysis against 50 mM MES pH 7, 8 M urea, 500 mM NaCl buffer. A 15 to 20-fold molar excess of fluorescent dye was added to the solution in an eppendorf with constant stirring. The solution was incubated in argon gas atmosphere overnight in dark at 4°C with stirring. The excess dye was removed from the protein by allowing the solution through Sephadex G-25 desalting column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The fractions were collected and the degree of labeling was determined by measuring the absorption spectrum of the labelled protein, using the following formula:

\[
\frac{\text{moles of dye}}{\text{moles of protein}} = \frac{A_{\text{dye}}}{\varepsilon_{\text{dye}}} \times \frac{\text{MW}_{\text{protein}}}{\text{mg/ml protein}}
\] (2.2)

where \(A_{\text{dye}}\) is the absorbance value of the dye at the absorption maximum wavelength, \(\varepsilon_{\text{dye}}\) is molar extinction coefficient of the dye at the absorption maximum wavelength. The concentration of the protein was determined from the absorption value at 280 nm and corrected for the dye absorption at this wavelength using the formula:

\[
\text{mg/ml protein} = \frac{A_{280} - k \times A_{\text{dye}}}{\varepsilon_{\text{protein}}}
\] (2.3)

where \(\varepsilon_{\text{protein}}\) is a molar extinction coefficient of the protein and \(k\) is the correction factor. For ATTO 655, \(k = 0.08\) and for Alexa 488, \(k = 0.11\). The degree of labeling was found to be 90% for VDAC-Alexa 488 (VDACgreen) and 70% for VDAC-ATTO 655 (VDACred).

Labelled protein was again concentrated and stored at −80°C.

### 2.4 Refolding of VDAC

The purified labelled VDAC was in the unfolded state. Therefore, the proper refolding procedure was required in order to obtain an active VDAC protein. The refolding procedure includes 40 times dilution of VDAC-urea solution in citrate buffer (pH 3 with 2 mM EDTA) with the following incorporation of VDAC into N,N-dimethylododecilamine N-oxide (LDAO) detergent micelles [25]. VDAC was incubated in 13 mM LDAO for 5 hours at 25°C. The residual urea was removed by dialysis against citrate buffer with three buffer replacements. The proper folding of VDAC was confirmed by circular dichroism, fluorescence spectroscopy and electrophysiology measurements (see Chapter 4). The refolded VDAC in detergent was stable for one week, if kept at 4°C.
2.5 Preparation of model membranes

2.5.1 Lipids and lipid markers

The lipids used in this study were purchased from Avanti Polar Lipids (Alabaster, AL) and are listed below:

- 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DiPhPC)
- n-stearoyl-d-erythrosphingosylphosphorylcholine (sphingomyelin 18:0; SM)
- cholesterol (ovine wool, >98%) (Chol)
- 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, PC),
- 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt)(DOPG, PG),
- 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DOPS, PS),
- 1,2-dioleoyl-sn-glycero-3-phospho-(1′-myo-inositol) (ammonium salt) (DOPI, PI),
- Cardiolipin (heart, bovine) (sodium salt) (CL),
- 3, 3′-dioctadecyloxycarbocyanine perchlorate (DiO)
- 1,1′-dioctadecyl-3, 3, 3′, 3′ tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD)

2.5.2 Incorporation of VDAC into proteoliposomes

First, lipid mixtures (DOPC, DOPC:SM:Chol, DOPC:CL, DOPC:PG, DOPC:PI, DOPC:PS, DOPC:PG:CL) in chloroform were prepared at desired lipid ratios. Lipids were dried under nitrogen flow to evaporate the solvent and then 1 hour in vacuum desiccator, to remove chloroform traces. Dried lipids were dissolved in 2 mM Mops-Tris buffer, pH 7 at a total lipid concentration of 4 mg/ml and mixed vigorously until lipids were completely dissolved and solution became opaque. The obtained multilamellar vesicles were sonicated in the water-bath sonicator for 15 min, or until the solution became transparent indicating the formation of small unilamellar vesicles (SUVs). SUVs were solubilized in detergent at the lipid/detergent (LDAO) ratio of 2 w/w to prepare mixed micelles. VDAC in LDAO was added to the mixed micelles mixture at the lipid/protein ratio 40 w/w and incubated for 30 min. To remove the detergent, 30 mg of Bio-Beads SM-2 (Bio-Rad Laboratories, Hercules, CA) per 1 mg of detergent were added to the mixture followed by incubation for 4 hours. Proteoliposomes were saved at −80°C.
2.5.3 Preparation of black lipid membranes (BLM)

The technique developed by Montal and Mueller was used in this work to produce BLMs. In this method, bilayer is made from two monolayers on two sides of an aperture in a teflon film separating two aqueous compartments (Fig. 2.2).

![Figure 2.2](image.png)

**Figure 2.2:** (A) An aperture in a teflon film. (B) The chamber for electrophysiology measurements. Two aqueous compartments are separated by the teflon film with an aperture.

To produce stable bilayers, the apertures in the teflon film should be round and have smooth edges. This was achieved by using an electric discharge of high frequency generator (BD-10 ASV, Chicago, IL). The discharge took place between two syringe needles: one connected with the electrode and the other with the earth of the socket. Apertures of 50 to 150 µm in diameter in a 0.025 mm thick teflon film were obtained by this method (Fig. 2.2).

The chamber was assembled, the aperture was pretreated with hexadecane and dried with a stream of air. Both compartments of the chamber for BLM production were filled with 10 mM Tris, 5 mM CaCl$_2$, 1 M KCl, pH 7.4 to a level just below the aperture in the partition. The monolayers were produced in both compartments by spreading approximately 20 µl of lipids dissolved at concentration 5 mg/ml in pentane on the surface of the aqueous solution.

In 5–15 min, when the evaporation of the organic solvent was complete, the levels of the aqueous solutions were subsequently raised and the bilayer was established over the aperture. In figure 2.3 the production of the folded bilayer from two monolayers is presented schematically. The formation of the bilayer was monitored by the capacitance measurements.
2.5.4 Preparation of giant unilamellar vesicles (GUVs)

GUVs containing VDAC were produced from proteoliposomes in a custom-made perfusion chamber by the electroformation method [92]. Proteoliposomes obtained in the above-described procedure (see chapter 2.5) at a concentration of 2 mg/ml of lipids were deposited on indium tin oxide (ITO) coated glass slides (GeSiM, Grosserkmannsdorf, Germany) in the form of several 2 \( \mu \)l droplets. Proteoliposomes were dried for 30 min in a desiccator. The copper wire was attached to the conductive side of ITO coverslips and the perfusion chamber was assembled (Fig. 2.4).

The ITO with dried lipids was placed on the lower part of the chamber to be able to observe the vesicles with the inverted microscope. The parts of the chamber were sealed with vacuum grease (Glisseal, Borer Chemie, Switzerland). The chamber was filled with a buffer containing 300 mM sucrose, 1 mM Mops-Tris, 2 mM KCl, pH 7. Copper wires were connected to the function generator and sinusoidal alternating current electric field with the RMS field strength of 240 V/m at a frequency of 12 Hz was applied for 3 h (Voltcraft 8202, Conrad Electronic SE, Germany). The sucrose in the chamber was carefully exchanged to 10 mM Tris, 150 mM KCl, pH 7.2 using syringe and polyethylene tubing system. The osmolarity of this buffer was measured using osmometer and it was equal to the osmolarity of the buffer inside the vesicles.

This GUV electroformation procedure from proteoliposomes was simpler than the one described in [104] and was sufficient for producing good-quality GUV samples with GUV diameter in the range of 10–100 \( \mu \)m.

GUVs with phase separation were produced in a custom-made teflon chamber with two
CHAPTER 2. MATERIALS AND METHODS

Figure 2.4: ITO chamber for GUV formation. (A) Metal chamber, plastic spacer with polyethylene tubing and ITO covered coverslides with attached copper wire. (B) Assembled chamber for GUV formation: ITO slides are attached to the plastic spacer which is positioned between two parts of the metal holder. Solution is injected using a syringe, other inlets are closed by metal clips.

electrodes made of platinum wires. The composition of the lipid mixture was DOPC/SM(18:0)/Chol 2/2/1. 5 µl of lipid mixture (1 mg/ml) in chloroform was spread on each wire. The concentration of DiD was adjusted to obtain an optimal number of molecules in FCS measurements and was 0.005 mol % in the case of measurements in Ld phase and 0.15 mol % for FCS in the Lo phase and imaging. After evaporation of the solvent, the chambers were assembled and filled with 300 mM sucrose solution. Electroformation was performed in two chambers in parallel at 65 °C and at room temperature by applying alternating electric current (2 V electric field corresponding to 400 V/m, 10 Hz) for 1.5 h. In the final step, the frequency was decreased to 2 Hz to detach the vesicles from the electrodes. 50 µl of the solution were transferred to one well in a 8-well chamber (Nunc, MatTeck) containing 800 µl of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4; PBS).

2.6 Circular dichroism measurements

CD spectra was recorded on Jasco 715 CD spectrometer (Jasko, Tokyo, Japan) as described earlier [25]. I performed these measurements in University of Konstanz (Germany), group of Dr. Kleinschmidt. The scans were performed from 190 to 250 nm with a response time
8 s, a bandwidth of 1 nm and a scan speed of 20 nm/min. The recorded CD spectra was normalized to the mean residue molar elipticity \( \lambda \), given by:

\[
[\Theta](\lambda) = 100 \frac{\Theta(\lambda)}{c \cdot l \cdot n},
\]

where \( l \) is the path length of the cuvette in cm, \( \Theta(\lambda) \) is the recorded elipticity in degrees at wavelength \( \lambda \), \( c \) is the concentration in mol/l, and \( n \) is the number of aminoacid residues of VDAC (282).

### 2.7 Electrophysiology measurements

Electrophysiology measurements were conducted in a specific chamber with two compartments separated by the teflon film with an aperture. The membrane was formed in this aperture as described in the chapter 2.5.3. The compartments were filled with the buffer containing KCl salt and AgCl electrodes were immersed into the solution from both sides of the membrane (Fig. 2.5). The voltage was applied across the membrane and the electrical current was measured. Cis-compartment of the chamber was connected to the virtual ground and the voltage signs are refereed to it.

![Figure 2.5:](image)

**Figure 2.5:** The schematic representation of electrophysiology measurements and methods of VDAC incorporation into BLM. The electrodes (A) are placed into the cis and trans compartments of the electrophysiology chamber and the current flow through the membrane is measured under the voltage clamp conditions. (B) The BLM with VDAC incorporated using two different methods: (C) Proteoliposomes containing VDAC fuse with the BLM, (D) VDAC inserts to the membrane from the detergent solution.
The simplified electrical equivalent of this system is a parallel circuit of a capacitor and a resistor. The resistance describes the electrical conductance of the bilayer, which is very low for the pure lipid membrane. In this case, the membrane can be approximated by a capacitor. The capacitance is given by

\[ C = \frac{\varepsilon_0 \varepsilon_r A}{d}, \]  

(2.5)

where \( C \) is the capacitance in Farads (F), \( \varepsilon_0 \)-absolute dielectric constant, \( \varepsilon_r \) relative dielectric constant of the membrane (approximately 2–3), \( A \) is the membrane area and \( d \) is the thickness of the membrane. The current across a capacitor is determined as:

\[ I = C \times \frac{dU}{dt}, \]  

(2.6)

where \( I \) is the current in Amperes (A) and \( U \) is applied voltage in Volts (V).

If \( \frac{dU}{dt} = 1 \), then the capacitance value is equal to the current value (1A=1F). In practice, triangle-wave voltage with the peak-to-peak amplitude of 20 mV and frequency of 25 kHz can be used to determine the capacitance of lipid bilayer from the current value. Determination of the capacitance value is important for the evaluation of the bilayer quality. The capacitance of pure lipid bilayer should be approximately 0.8 \( \mu F/cm^2 \) [116].

Two slightly different systems were used to check the activity of VDAC refolded in detergent LDAO and VDAC reconstituted into proteoliposomes.

VDAC in detergent micelles was added to the cis-compartment of the chamber depicted in figure 2.5 at a concentration of 0.2–0.3 \( \mu g/ml \). BLMs were made of DiPhPC lipid. Currents were recorded under the voltage clamp conditions using an Axopatch 200B patch clamp amplifier (Axon Instruments Inc, Union City, CA). Data were acquired with the help of a Digidata 1440A board and analysed using the pClamp 10 software (Axon Instruments Inc, Union City, CA).

Electrophysiology measurements on proteoliposomes were carried out using a Bilayer Explorer (Ionovation GmbH, Osnabrück, Germany). This system is similar to the system described above, but the bilayer is horizontal. In addition, BLM is produced automatically, the volumes of the chambers are very small, and the setup has a perfusion system, which allows efficient buffer exchange in both compartments without breaking the bilayer. These characteristics are very important for the fusion of the proteoliposomes with BLM.

In this work, proteoliposomes made of DOPC containing VDAC were added to the cis compartment and fused to the BLM made of DOPC lipid in presence of a salt gra-
Confocal laser scanning microscopy

After protein insertion (from detergent micelles or from proteoliposomes), the potential across the membrane was increased in increments of 10 mV from $-60$ mV to $+60$ mV, and conductance $G$ was determined from the recorded current as a function of the voltage applied across the membrane:

$$G = \frac{I}{U} \quad (2.7)$$

The maximum conductance $G_{\text{max}}$ was determined and the relative conductance $G/G_{\text{max}}$ was plotted as a function of the applied voltage.

2.8 Confocal laser scanning microscopy

The imaging was performed on LSM 510 Meta confocal microscope (Zeiss, Jena, Germany). The light was collected using 40× NA 1.2 UV-VIS-IR C-Apochromat water immersion objective (Zeiss, Jena, Germany). The sample was excited by the 488-nm line of an Argon-ion laser and the 633-nm line of a He-Ne laser. The laser power was adjusted depending on the fluorescence intensity of the sample. A HFT 488/543/633 dichroic mirror was used to separate the excitation from the emission, and HFT 545 dichroic mirror, BP 500-550 and BP 650-710 band-pass filters to separate the emission from two spectral channels.

2.9 Fluorescence correlation spectroscopy

Fluorescent dyes Alexa 488 and ATTO 655 were used for microscopy imaging and for Fluorescence cross-correlation spectroscopy (FCCS) experiments. These dyes not only have a high quantum yield, but are very photostable. In addition, their emission spectra are well separated (Fig. 2.6), what is very important for FCCS measurements, because it allows to avoid spectral crosstalk while using the appropriate fluorescence filters.

FCCS measurements were carried out on a LSM 510 Meta system (Zeiss, Jena, Germany) using a home-built detection unit at the optical fiber output channel. All experiments were carried out at $21\pm0.5^\circ$C. The light was collected using 40× NA 1.2 UV-VIS-IR C-Apochromat water immersion objective (Zeiss, Jena, Germany). The sample was ex-
cited by 488-nm line of an Argon-ion laser (6 µW) and 633-nm line of a He-Ne laser (15 µW). A 570DCXRU V dichroic mirror, and HQ 520/40 and HQ700/75 band-pass filters (AHF analysentechnik, Tübingen, Germany) were positioned behind a collimating achromat to split the emission for the dual-color detection and to reject the residual laser light. Fluorescence was detected by avalanche photodiodes (PerkinElmer, San Jose, CA). Photon arrival times were recorded in the photon mode of a Flex 02-01D hardware correlator (correlator.com, Bridgewater, NJ).

Two-focus two-color scanning FCCS measurements perpendicular to the GUV equator were carried out as described in [114] (see also chapter 1.5.4). This is a calibration free mode of FCCS, which allows for the determination of diffusion coefficients and molecular interactions. Membrane movements can be corrected for, so that false positive cross-correlation is avoided. The auto- and cross-correlation curves were obtained from fluorescence intensity traces. Irregular curves resulting from experimental instabilities were excluded from the data sets. The global analysis of auto- and cross-correlation data was carried out by nonlinear least squares fit using the equations (1.20)–(1.22), assuming a zero cross-talk between the red and green detection channels. Additionally, we assumed that the diffusion coefficients of the red-labeled, green-labeled, and red-green-labeled species are all equal and independent of the protein oligomerization degree. The latter assumption is justified because of the very weak dependence of the translational diffusion coefficient on the membrane inclusion size for the expected monomer and oligomer sizes [117]. Struct-

Figure 2.6: The fluorescence absorption (dash lines) and emission (solid lines) spectra of Alexa 488 (blue) and ATTO 655 (red) fluorescent dyes.
ture parameters of the red and green detection volumes required for the data analysis were determined by calibration measurements in solutions of the Alexa 488 and ATTO 655 fluorophores and fixed during data fitting.

In our experiments, the amplitude of the two-focus two-color red-green channel cross-correlation was on the level of experimental noise, and it was not included in the data sets subjected to quantitative analysis.

As a result of the analysis of a set of auto- and cross-correlation curves, surface concentrations of single-color \( C_r \) and \( C_g \) and two-color particles \( C_{rg} \) were determined.

Oligomerization of VDAC should not depend on whether it is labeled with a red or green fluorophore, and therefore formation of two-color (red-green) and single-color (red or green) oligomers is possible. Therefore, \( C_r \) (\( C_g \)) will account not only for red (green) monomers, but also for the corresponding single-color oligomers. At the same time \( C_{rg} \) will describe the concentration of oligomers containing both red- and green-labeled molecules. As a result, \( C_{rg} \) will generally underestimate the total concentration of oligomers (a fraction of which will be single-color), but this discrepancy vanishes fast upon an increase in the degree of oligomerization.

To quantitatively characterize the oligomerization of VDAC in the membrane, we therefore, define the (approximate) oligomer fraction as follows:

\[
f = \frac{C_{rg}}{C_{rg} + \min (C_r, C_g)}.
\] (2.8)
Chapter 3

VDAC activity measurements

3.1 Introduction

VDAC is a channel, which has very specific voltage dependent properties when reconstituted into BLMs. It stays in the open state at low voltages, whereas at high voltages (either positive or negative) the channel undergoes transitions to a lower conductive states [19]. These important characteristics of the channel are preserved among VDACs of various species [26].

Investigated in this study human VDAC, was cloned in E. coli and purified in its unfolded form from inclusion bodies. The refolding procedure was required to obtain an active form of this protein. To check if the structure and function of the refolded protein were consistent with the literature data, circular dichroism, fluorescence and electrophysiology measurements were performed with refolded VDAC in detergent. In addition, single labeling of VDAC was achieved, by introducing cysteine mutation. The activity of mutated VDAC and its labelled versions also had to be proven by electrophysiology measurements, since the presence of fluorescent dyes on the VDAC molecule could alter the function of the channel.

It was also important to check if the electrophysiological characteristics of VDAC are preserved after the reconstitution of the protein from detergent micelles into proteoliposomes.

Another valuable information about the protein folding was obtained while incorporating VDAC from its unfolded form without any detergent into the BLMs. It appeared, that in case of particular lipid composition of BLM, some fraction of the protein is able to spontaneously fold from its unfolded state into the functional protein in presence of the
lipid membrane.

### 3.2 Results

#### 3.2.1 Activity of refolded, labeled VDAC incorporated into BLM

Unfolded VDAC, denatured in urea was refolded in detergent LDAO. In order to check the insertion of VDAC into detergent micelles, fluorescence spectra of the protein was measured. The spectra of unfolded VDAC in urea had a maximum at 347 nm. After urea dilution and protein insertion into detergent micelles, the spectra maximum was blue shifted to 335 nm and the fluorescence intensity increased (Fig. 3.1). These changes in VDAC spectra indicated that the average microenvironment of VDAC became more hydrophobic, suggesting the protein incorporation into detergent micelles. These results are in agreement with the literature data [25].

![Figure 3.1](image)

**Figure 3.1:** The fluorescence spectra of VDAC in urea (black) and in LDAO detergent micelles (red).

The secondary structure of VDAC in LDAO was examined by CD measurements. CD spectra of VDAC refolded in LDAO matched with the one reported previously [25] indicating the formation of 16% α-helix, 32% β-strand and 23% β-turn structure (Fig. 3.2).

Therefore the structure of VDAC suggested the proper folding of the protein, however the functionality of the refolded VDAC channel can be proven only after the electrophys-
Figure 3.2: Normalized CD spectra of VDAC in detergent LDAO micelles.

It is known that when VDAC in detergent solution is added to the BLM, it spontaneously inserts into the bilayer and creates ion-permeable conductive pores permeable to ions. Therefore, in a voltage clamp conditions, each channel insertion leads to a stepwise increase in the current through the membrane. VDAC from various spaces has a single channel conductance of 3.9–4.5 nS in 1 M KCl solution [118]. Refolded from the unfolded state human VDAC was also shown to have a conductance of 4 nS [119, 25].

To find out if VDAC purified and refolded in this study was functional, the protein solution in detergent was added to BLM and channel insertions were followed by recording the current trace. The single channel conductance values were determined as described earlier [118] while measuring a current at a potential of 10 mV applied across the membrane. At this potential, the channel is in its fully open state. As can be seen from the histogram in figure 3.3 (A), most of the channels indeed had conductance values of 4–4.5 nS.

In this work, mutation was introduced in VDAC, by the replacement of the cysteine residue with a serine at the position 232, so that the fluorescent label can be attached to the remaining single cysteine residue in the protein. The alterations in the amino acid sequence of the protein can change its properties. To investigate the effect of the mutation on electrophysiological properties of VDAC, mutated VDAC was also refolded and incorporated in BLM. Its single channel conductance was similar to the one of the wild type protein (Fig. 3.3 (B)). Therefore the mutated VDAC can be used for the labeling. VDAC was fluorescently labelled and also refolded in LDAO. As shown on the figure
Results

3.3 (C), the introduced label did not had any significant effect on VDAC single channel conductance.

It is interesting to mention, that Hiller et al. [119] were not able to obtain functional VDAC refolded in LDAO, unless cholesterol was introduced into BLM. In this work, however, no cholesterol was required for the proper folding of VDAC while following the protocol described by Shanmugavadiyu [25].

Figure 3.3: VDAC single channel conductances plotted versus the number of events N for (A) wild type VDAC, (B) Single cystein mutant of VDAC, (C) fluorescently labeled VDAC.

Figure 3.4: A record of ion current through the membrane containing several VDAC channels. An increase in the voltage above 30 mV leads to the transition of the channels to various low-conductance “closed” states.

Another very important characteristic of the functional VDAC channel is its voltage dependency. Although, the exact values of the conductance at different voltages can slightly vary for different VDAC spaces and experimental conditions, the typical bell-shaped curve is always obtained for the conductance-voltage dependence of these channels.

This means that the channels are mainly in the open state at low voltages (10–20 mV), whereas upon increasing the voltage above 30 mV (either positive or negative), channels
undergo transitions to various closed conductive states. Such a behaviour can be clearly seen in figure 3.4, which shows the stepwise decrease in the currents through the BLM containing multiple VDAC channels, when the voltages above 30 mV are applied.

At high voltages (around 60 mV), the fast fluctuations between the open and the closed state of the single channel were observed (Fig. 3.5). To obtain the dependence of the channel conductance on the applied voltage, the voltage was increased with a step size of 10 mV and a switching to 0 mV before each step. The current through the membrane was recorded for several minutes and the conductance of the single channel at the particular voltage was determined as described in the literature [120]. Conductance was normalized for the maximal value. The voltage dependence or open probability of wild type VDAC and its fluorescently labeled mutants (VDAC ATTO 655 and VDAC Alexa 488) is presented in figure 3.6. The similar voltage dependent behaviour was obtained for wild type protein and its labelled mutants.

These results are in agreement with the recently published data [121], showing that VDAC cysteine residues are not important for the voltage gating of the channel. Electrophysiology measurements have shown that the fluorescently labeled VDAC is functional and can be used further to investigate VDAC properties by fluorescence-based techniques.
3.2.2 Spontaneous folding of VDAC from its unfolded state in lipid membrane containing cardiolipin

One of the aims of this dissertation, was the incorporation of VDAC into GUVs. As it will be shown in the next chapter, unfolded VDAC without any detergent was able to bind to the membrane of GUVs containing lipid cardiolipin. It was interesting to check, if VDAC incorporated into GUVs in this way is just peripherally bound to the membrane, or it can insert into the lipid bilayer and thereafter, properly fold and function?

Several studies have shown, that some proteins are able to spontaneously fold in the membrane bilayer. Bacterial porins, OmpA [122], OmpF [123] and FomA, [124] can insert into the membrane in their unfolded state without detergent. The previous results obtained by circular dichroism studies also demonstrate that unfolded in urea VDAC can incorporate into large unilamellar vesicles made of phosphatidylcholines with saturated acyl chains of different hydrophobic lengths. The structure of the protein matched with the one of refolded VDAC incorporated into the membrane from detergent solution [25]. However, the electrophysiology measurements proving the activity of the folded protein were not performed in these studies.

In order to determine the functional state of VDAC incorporated from its unfolded state into cardiolipin containing membranes, I conducted electrophysiology experiments. Surprisingly, some channels indeed inserted into the BLM containing cardiolipin and even

Figure 3.6: Multi-channel conductance of VDAC in BLM as a function of applied voltage. The results for VDAC reconstituted from detergent LDAO: wild-type VDAC (black), VDACred (red) and VDACgreen (green) into a DPhPC membrane. Data are averages from three to eight independent experiments. Error bars represent the standard deviation.
had a voltage dependent behaviour similar to the VDAC incorporated into BLM from the preliminary folded state in detergent solution (Fig. 3.7). This indicates that VDAC can properly fold in the lipid bilayer containing cardiolipin from the unfolded aqueous form.

![Normalized conductance vs Voltage](image)

**Figure 3.7:** Multi-channel conductance of VDAC in BLM as a function of applied voltage. The results are for wild type VDAC (black) and VDACred (red) reconstituted from unfolded state into DPhPC membrane. Data are averages from three to eight independent experiments. Error bars represent the standard deviation.

The obtained results might be relevant to the VDAC folding *in vivo*. VDAC is synthesized in the cytosol and imported in its unfolded state to the mitochondria. The insertion and folding of proteins into outer mitochondrial membrane are poorly understood (for review see [125]). Evidence accumulates that not only protein chaperons can assist the proper folding of integral membrane proteins, but also lipids may play a role of molecular chaperones in this process [126]. The most prominent example of lipid playing a chaperone role is PE, which is required for proper folding of the protein LacY from *E. coli* [127]. It was also suggested that not only structure and chemical properties of individual lipids are important for protein folding, but also the collective behavior of lipids, determining the physical properties of the bilayer.

Cardiolipin, which promoted VDAC folding in this study, is found in high amounts in mitochondria and especially in the contact sites between the inner and the outer mitochondrial membrane [78], where VDAC is believed to localise [128]. It can also induce the local changes of the physical properties of the lipid bilayer, because it is known to adopt nonlamellar hexagonal phases under specific conditions [60]. Therefore the role of cardiolipin in VDAC folding can be physiologically relevant. In addition, VDAC can serve as a useful model to study protein folding and investigate the role of lipids in this process.
3.2.3 Activity of VDAC incorporated into BLMs from proteoliposomes

Formation of proteoliposomes, is a general step in preparation of GUVs containing protein of interest. In this work, VDAC refolded in detergent LDAO was incorporated into proteoliposomes by detergent removal in presence of small lipid vesicles (see chapter 2.5.2). This procedure has to be carefully optimized in order to avoid protein misfolding. Also the folding pattern of VDAC in LDAO and in obtained proteoliposomes can be different. For example, VDAC structural investigations show that the structure of this protein in LDAO and in the lipid membrane is not exactly the same [25]. In order to check the functional state of VDAC incorporated into proteoliposomes, electrophysiology studies were performed. However, in this case a slightly different setup (Bilayer Explorer) was used to prepare BLMs. This system has been chosen, because it allows for automated buffer exchange from both sides of the membrane without its breaking. In order to obtain VDAC incorporated from proteoliposomes into BLM, liposomes have to fuse with the membrane. The fusion process requires the presence of salt gradient across the bilayer and an open ion channel in the vesicle to provide the pathway for the ions. The vesicles are added to the compartment with high salt concentration. When the proteoliposomes get in contact with the membrane, the water starts to flow across two bilayers from the compartment with the low salt concentration into the vesicle and on the other hand, the high salt concentration around the vesicle promotes the ion flow through the membrane. These processes lead to the swelling of the proteoliposomes with their rupture and fusion with the bilayer. Using the described protocol, proteoliposomes containing fluorescently labeled VDAC were fused to the BLM made of DOPC and electrophysiology measurements under voltage clamp conditions were conducted. As can be seen from the figure 3.8, the VDAC incorporated in this way had a voltage dependent conductance similar to the VDAC reconstituted into BLM from detergent solution (see Fig. 3.4 for comparison). However, the rate of the proteoliposome fusion with the membrane is much lower than in the case when the protein is incorporated from detergent micelles, therefore the number of performed experiments was lower in this case. This might explain a slightly lower values for the VDAC conductance at $-40$ and $-50$ mV. Another difference was that the channel was undergoing transitions to the closed states even at the low voltages (around 10 mV) whereas the VDAC reconstituted from LDAO was mainly in the open state at these voltages. These minor differences could be explained by the slightly different structure of VDAC folded in proteoliposomes. However, the protein clearly shows the voltage depen-
3.3 Discussion

The electrophysiology measurements conducted in this study allowed to confirm that human VDAC cloned in *E. coli*, purified from inclusion bodies and refolded in LDAO was active. In addition, single cysteine mutation and fluorescent labeling of the VDAC protein do not affect the VDAC electrophysiological activity, therefore, making it possible to use fluorescent techniques to investigate VDAC properties. Electrophysiology measurements have suggested that two different methods can be potentially used to incorporate VDAC into GUVs: addition of unfolded VDAC to GUVs containing cardiolipin or preparation of GUVs from proteoliposomes. The addition of unfolded VDAC to the membranes containing cardiolipin was shown to induce the spontaneous folding of VDAC to its active state. Incorporation of VDAC into proteoliposomes was also achieved, so that the protein was functional. Therefore, both methods ensure the functional state of VDAC in the membrane, however, as it will be shown in the next chapter, only the fluorescent microscopy studies on GUVs containing fluorescently labelled VDAC incorporated by these methods...
will allow to choose the most appropriate approach to reconstitute VDAC into GUVs, so that the fluorescence measurements to study VDAC interaction with lipid membrane are possible.
Chapter 4

Reconstitution of VDAC into Giant Unilamellar Vesicles

4.1 Introduction

One of the goals of this work was to reconstitute VDAC into the cell free, chemically well defined model system—GUV. The lipid composition of this system can be precisely controlled and therefore, the protein–lipid interactions can be efficiently studied (see chapter 1.4.2). The oligomerization of VDAC in various lipid environments can be investigated in such system. In addition, the preferential partitioning of VDAC into liquid ordered or liquid disordered domains in GUVs, with a large scale phase separation was easily accessed by fluorescence techniques.

Earlier, different reconstitution protocols for membrane proteins were developed [104, 105, 103, 104], however, these protocols still have to be optimized for the particular protein.

Recently, the novel protocol for VDAC reconstitution, involving spontaneous fusion of proteoliposomes containing VDAC with GUVs, was reported [129]. The functionality of the protein was proven by electrophysiology measurements. Nevertheless, the concentration of the protein in the membrane was very low and protein was not fluorescently labeled in these studies. GUVs were imaged using the fluorescence of the lipid dye. Therefore, the method did not provide the information about the protein distribution in the membrane, which is of a crucial importance for fluorescence studies.

In this work, two different approaches for reconstitution of fluorescently labelled VDAC into GUVs are presented. In the first approach, VDAC is incorporated in its unfolded state into GUVs containing CL. The second reconstitution procedure involves the production of
Results

GUVs with various lipid composition from VDAC containing proteoliposomes. As it was shown in the previous chapter, the both approaches allow for the functional protein incorporation into lipid membrane, however only the second method provides the homogenious distribution of the fluorescently labeled protein in the membrane, what is very important for the FCS measurements and studies concerning protein oligomerization.

The reconstitution of VDAC into GUVs requires careful control of the physical-chemical conditions in order to avoid protein misfolding and degradation. One of the parameters attracting a special attention during the incorporation of VDAC into GUVs with phase separation is temperature. The preparation of the phase separating vesicles, containing lipid components of the raft mixture—sphingomyelin and cholesterol, traditionally involves heating of the lipid mixture above the transition temperature (65 °C) what can be easily applied to the pure lipid mixtures, but may be deleterious for membrane proteins like VDAC. Therefore, in this study, GUVs with phase separation were prepared at room temperature. In order to check if the vesicles prepared in this way can be used as a platform for the reconstitution of membrane proteins, the morphology of the obtained vesicles, their size distribution, the number of vesicles with phase separation and diffusion coefficients in liquid disordered and liquid ordered phases were compared to the values characteristic for the vesicles prepared at 65 °C.

4.2 Results

4.2.1 Reconstitution of VDAC from unfolded state into GUVs containing cardiolipin

It was previously shown, that VDAC in urea is completely unfolded and does not form α-helix or β-sheet structures, however, once transferred to an aqueous solution, the protein develops 39 % β-strand structure. When such a partially folded VDAC is added to the large unilamellar vesicles made of phosphatidylcholine, it can fold into its native form composed of 11 % α-helix, 36 % β-strand and 23 % β-turn structure [25]. To this end, it was interesting to check, if unfolded VDAC, upon urea dilution, can bind and fold in GUVs with various lipid composition. VDAC was fluorescently labeled and therefore protein binding to the membrane of GUVs can be easily followed by the fluorescence imaging.

No VDAC binding was observed when GUVs made of neutral lipids, DOPC, or mixture of DOPC:DOPE 80:20 were used. To promote electrostatic binding of a positively
CHAPTER 4. RECONSTITUTION OF VDAC INTO GIANT UNILAMELLAR VESICLES

charged VDAC to the membrane, negatively charged lipids were introduced to the system. Namely, PG and CL were included into GUV composition as these anionic lipids represent the important components of the mitochondrial membrane. VDAC was binding to the GUVs containing CL (Fig. 4.1). Nevertheless, no binding was observed in case, when PG (the other negatively charged lipid, precursor of CL) was included into the lipid mixture. Another negatively charged lipid PS also did not promote VDAC binding to the membrane.

Figure 4.1: Representative confocal fluorescence microscopy images of GUVs with reconstituted from an unfolded state red-labeled VDAC: (A) red (ATTO 655–VDAC) channel, (B) green (DiO-lipid marker) channel, (C) merge of (A) and (B), scale bar 50 µm. (D) red (ATTO 655–VDAC) channel, scale bar 10 µm. GUV composition: DOPC:CL 80:20.

Therefore, the specific binding of the unfolded VDAC to the GUVs containing CL was observed. These findings suggest, that, not only electrostatic forces are important for the binding of unfolded VDAC to the membrane, but also, the local properties of the membrane, like the spontaneous curvature of the lipid bilayer may play a role in this process. CL is known as a cone shaped lipid with four acyl chains, which promotes the formation of a negative spontaneous membrane curvature and non-bilayer lipid structures, like inverted hexagonal phase. This lipid is especially enriched in the contact sites between the inner and the outer mitochondrial membrane [78] and VDAC was suggested to preferentially insert into CL-rich domains [130].

The specific requirement of CL for the proper folding of another membrane protein–
mitochondrial matrix enzyme rhodanese was also reported earlier [131] and evidence accumulates, that lipids, in general, may play a role of molecular chaperones in protein folding (for review see [127]).

The results of the previous chapter suggest, that VDAC in the membranes of GUVs, containing CL, is not only peripherally bound, but also inserted to the membrane and properly folded. However, the folding conditions are unfavourable in the case when the protein is incorporated into the lipid bilayer from the aqueous solution without the help of any detergent. Therefore, only the small fraction of the protein can bind to the membrane and spontaneously fold. As shown in figure 4.1 (D), some fractions of the fluorescently labelled protein are homogeneously distributed on the membrane of GUV, whereas some are misfolded and form big aggregates on the membrane surface. These aggregates, in their turn, influence the morphology of the GUV membrane, creating small vesicles and tubules (Fig. 4.1 (A, B, C)). Such membrane morphology changes and clustering of the protein on the GUV surface, indicating the presence of high level of the protein aggregation, makes it difficult to use FCS in studying protein oligomerization. However, two-focus scanning FCS measurements were performed on this system on the areas of the GUV membranes with the homogenous protein distribution. Diffusion coefficient of VDAC in the membrane was found to be $5.5 \pm 0.78 \, \mu m^2/s$. This value was also similar to the one obtained for VDAC reconstituted into GUVs from proteoliposomes (see Table. 4.1). Nevertheless, another reconstitution protocol was required in order to reduce protein aggregation and enhance FCS measurements on the membrane.

### 4.2.2 Reconstitution of VDAC into GUVs from proteoliposomes

As it was described in the previous chapter VDAC can be successfully reconstituted from detergent solution into proteoliposomes, where it remains active and adopts its natural conformation in a lipid environment. These proteoliposomes can be further used to generate GUVs, using traditional electroformation method (see chapter 2.5.4). In such reconstitution protocol VDAC is first refolded in detergent, which is further slowly removed in the presence of small lipid vesicles. Thus, this procedure protects protein from denaturation. Therefore the conditions for protein reconstitution are more mild compared to the protocol using an unfolded VDAC. Representative confocal fluorescent images of GUVs containing fluorescently labeled VDAC reconstituted from proteoliposomes are shown in figure 4.2. As can be seen from the image, the protein and the fluorescent lipid marker were homogeneously distributed in the membrane, showing no significant aggregation and membrane
morphology changes, which allowed us to perform FCS measurements with high accuracy. To study VDAC oligomerization by FCCS technique VDACred and VDACgreen were reconstituted to GUVs (Fig. 4.3). The protein was found to freely diffuse in the membrane, with the diffusion coefficient in a DOPC environment similar to the one reported previously for trimeric glutamate transporter [132]. We found, however, that the diffusion coefficient showed slight variations depending on the lipid composition of the membrane of GUVs (Table 4.1).

![Figure 4.2](image)

**Figure 4.2:** Representative confocal fluorescence microscopy images of GUVs with reconstituted from proteoliposomes red-labeled VDAC: (A) red (ATTO 655–VDAC) channel, (B) green (DiO–lipid marker) channel, (C) merge of (A) and (B), scale bar 10 µm. GUV composition: DOPC:CL 80:20.

**Table 4.1:** Translational diffusion coefficients of VDAC in GUVs with different lipid compositions obtained from the analysis of FCCS data. Measurements were carried out at 21 ± 0.5 °C. Mean values and standard deviations obtained from measurements on 10 to 30 GUVs.

<table>
<thead>
<tr>
<th>Lipid composition</th>
<th>$D$ (µm²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>DOPC:CL 80:20</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td>DOPC:DOPG 90:10</td>
<td>5.2 ± 1.0</td>
</tr>
<tr>
<td>DOPC:DOPG 80:20</td>
<td>5.8 ± 0.8</td>
</tr>
<tr>
<td>DOPC:DOPI 90:10</td>
<td>6.1 ± 0.4</td>
</tr>
<tr>
<td>DOPC:DOPI 80:20</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>DOPC:DOPS 90:10</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td>DOPC:DOPS 80:20</td>
<td>4.8 ± 1.0</td>
</tr>
<tr>
<td>DOPC:DOPG:CL 80:15:5</td>
<td>6.3 ± 0.7</td>
</tr>
<tr>
<td>DOPC:DOPG:CL 80:10:10</td>
<td>5.4 ± 0.8</td>
</tr>
</tbody>
</table>
4.3 Discussion

GUVs containing incorporated proteins are very useful models to study protein-lipid interactions and protein-protein interactions in the membrane. However, the reconstitution of membrane proteins requires careful following and sometimes modification of established protocols.

In this work, the protocols for the reconstitution of VDAC into GUVs were developed. The labeling of the VDAC with fluorescent dyes allowed to follow the distribution of the protein on the membrane and evaluate the protein area concentration. The reconstitution of VDAC from proteoliposomes using a protocol by Girad et al. [104] with a minor modifications was found to be the most appropriate approach to incorporate fluorescently labelled VDAC and investigate its properties using fluorescence techniques. VDAC was homogeniously distributed in the membrane, showing no significant protein aggregation, and was freely diffusing in the lipid bilayer as was shown by FCS measurements.
Chapter 5

VDAC in Giant Unilamellar Vesicles with phase separation

5.1 Introduction

Several proteomics investigations of DRMs have found VDAC to be present in preparations (for review see [133]). VDAC is the major protein in the outer membrane of mitochondria, therefore, this observation suggests a possible partitioning of VDAC into lipid rafts, which might be present in mitochondria. The existence of lipid rafts in mitochondria is still under debate [66, 67, 68, 69]. Nevertheless, some studies indicate that even, if there are no lipid rafts in mitochondria under normal physiological conditions, they can be recruited to mitochondria during apoptosis [74, 75].

The other explanation for the observation of VDAC in DRMs would be, that VDAC localization is not restricted only to mitochondria. Indeed, VDAC was recently found in plasma membrane. In particular, VDAC was suggested to be localized in caveolae (specialized lipid rafts) [71]. Therefore, VDAC partitioning into lipid rafts requires farther investigation.

Reconstitution of fluorescently labeled VDAC into GUVs with phase separation can be a useful model to study preferential localization of VDAC to lipid domains. A large scale phase separation can be obtained in such model system and VDAC distribution can be followed by fluorescence microscopy.

Ternary mixtures containing saturated sphingomyelin, cholesterol and unsaturated phosphatidylcholine lipids, are typically used to mimic the membrane raft nanodomains by the lipid organization in fluid liquid-disordered (Ld) and liquid-ordered (Lo) phases.
Introduction

(for a review see [134] and references therein), which coexist below a miscibility transition temperature, dependant on ternary mixture composition [135]. Lipid dynamics in Lo and Ld phases has been studied by means of fluorescence correlation spectroscopy (FCS) [97, 136, 137, 138], single particle tracking and imaging [61], fluorescence recovery after photobleaching (FRAP) [139] and pulse gradient NMR [140]. Apart from the studies devoted exclusively to lipid mixtures, GUVs with phase separation were also used as a platform for membrane protein reconstitution from previously prepared proteoliposomes [105]. However, GUV electroformation from proteoliposomes poses several limitations. In case of phase separating vesicles grown from proteoliposomes, one of such limiting factors is the temperature. It is advised that at each preparation step, the temperature is chosen to ensure melting and mixing of all lipids, followed by a slow cooling to achieve near-equilibrium states for phase behavior studies [141]. Indeed, it was reported that for phospholipids with high gel-to-liquid phase transition temperatures, the electroformation did not result in GUV production when performed at room temperature [142]. However, incubation at high temperatures seems to be potentially deleterious for membrane proteins reconstituted in proteoliposomes. Multi-span transmembrane proteins usually unfold irreversibly, showing aggregation at high temperatures in the most severe cases [143]. Therefore, the reconstitution of eukaryotic membrane proteins in PC/SM/Chol GUVs, which are traditionally prepared at high temperature, must be optimized.

Here, I address the influence of temperature on GUV preparation for the DOPC/SM/Chol (2/2/1) mixture. The characterization of this system prepared at room temperature, is important, because it can serve as a platform for membrane protein reconstitution in general, and VDAC in particular. The vesicles prepared at room temperature and at 65°C were compared in a series of confocal fluorescence images with respect to their morphology, size, and the yield of phase separating liposomes observed at room temperature. To specifically examine the dynamic character of Lo and Ld phases in liposomes prepared at different temperatures, two-focus scanning FCS measurements were conducted and the absolute diffusion coefficients were determined. After showing the validity of the preparation of GUVs with phase separation at room temperature, VDAC was successfully reconstituted into this system and preferential partitioning of VDAC into one of the lipid phases was shown. The results are discussed in the context of benefits and limitations of using GUVs as a versatile platform for membrane protein reconstitution.
5.2 Results

5.2.1 Preparation of GUVs with phase separation at room temperature

Vesicle morphology

To examine the effect of growth temperature on the morphology of GUVs, a series of confocal images were collected and analysed. The electroformation performed on the same amount of lipids at room temperature and at 65°C in parallel, yielded comparable number of liposomes. In both cases, the obtained vesicles were spherical, without any shape deformations (Fig. 5.1).

![Figure 5.1: Giant unilamellar vesicles prepared at (A) room temperature and (B) 65°C. Three-dimensional image reconstructions (upper panels) and single confocal slices (lower panels). Scale bars are 10 µm, except the lower right images in both (A) and (B) for which the bars are 20 µm.](image)

To assess whether the temperature of preparation may affect the size of the GUVs, their diameters in the equatorial plane were measured. To determine the boundaries of the vesicles, the fluorescence intensity profiles were analysed and the diameters were calculated from the pixel size (Fig. 5.1 (A, B)). The distributions of GUV diameters prepared at low and high temperatures have a very similar non-Gaussian shape with a mean and SD values of 28±10 µm and 25.3±9.6 µm for RT and 65°C, respectively (Fig. 5.2, Table 5.1).

From hundreds of vesicles analysed by imaging, one can conclude that the lower prepa-
Table 5.1: Size, phase separation percentage and diffusion coefficients in GUVs prepared at room temperature (RT) and 65°C. Numbers of vesicles analyzed (n). Errors are given as standard deviation (SD). Fields of view (FV).

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Diameter (µm)</th>
<th>Phase separating GUVs (%)</th>
<th>D in Ld (µm²/s)</th>
<th>D in Lo (µm²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT</td>
<td>28±10 (n=146)</td>
<td>59±28 (n=449 (94 FV))</td>
<td>5.3±0.7 (n=17)</td>
<td>1.5±0.3 (n=23)</td>
</tr>
<tr>
<td>65°C</td>
<td>25.3±9.6 (n=145)</td>
<td>79±22 (n=332 (79 FV))</td>
<td>6.0±1.1 (n=23)</td>
<td>1.3±0.2 (n=25)</td>
</tr>
</tbody>
</table>

Figure 5.2: (A, B) Image of a vesicle (scale bar 10µm) with a corresponding intensity profile used for the diameter determination. (C) Histograms of the diameters of phase separating vesicles for the room temperature preparation (RT) (light grey) and 65°C (dark grey).

The temperature did not affect the domain shapes, which were always circular. In some samples prepared at RT, little vesicles or aggregates inside as well as outside the GUVs were present (Fig. 5.1 (A)). Less frequently, long fragments of tubulated membranes were
observed, often linking two vesicles or being located at the top surface of the vesicle (Fig. 5.1 (A)). Such artefacts were found also in the samples prepared at higher temperature, however, substantially to a lower extent.

**Percentage of phase-separating vesicles**

To focus in more detail on the Lo/Ld phase separation in the GUVs prepared at different temperatures, I performed a quantitative analysis of confocal images. In each batch of electroformation, the bottom surface of a chamber containing GUVs with a field of view adjusted to observe few vesicles at the same time was scanned. The occurrence of separate phases in whole, single vesicles was carefully checked and the ratio between the number of phase-separating vesicles and their total number was calculated. The above analysis was repeated to obtain statistically comprehensive data sets from hundreds of vesicles collected from three independent electroformations (Table 5.1). The average percentages of phase separating vesicles depends on the growth temperature. In the pool of GUVs prepared at 65°C, 79±22% GUVs exhibiting Lo/Ld phase separation were observed, whereas this value was reduced to 59±28% for the vesicles prepared at room temperature.

**Diffusion coefficient of the lipid dye in Lo and Ld phase**

A question on the influence of the preparation temperature on the lipid microenvironments in the membranes of GUVs may be raised. To address this problem, I performed a series of two-focus scanning FCS measurements and determined the diffusion coefficients of the fluorescent dye (DiD) in Ld and Lo phases in the membrane of GUVs prepared at different temperatures. Typical auto- and cross-correlation curves for the Lo and Ld phases in vesicles prepared at different temperatures are presented in figure 5.3 (A, B) and the corresponding averaged fitting values of diffusion coefficient are collected in Table 5.1. The values of diffusion coefficients for both disordered and ordered phases are in agreement for the two preparations (P-values calculated from the two-tailed T-tests were 0.97 and 0.95 for Ld and Lo, respectively). Thus, it may be concluded that the preparation temperature did not affect the diffusion coefficients in fluid phases in GUVs, as an indicator of similar viscosities.

To quantify the diffusion coefficient reduction in the Ld phase of DOPC/SM/Chol vesicles compared to the fluid phase unmodified by the presence of cholesterol, the mobility of DiD in GUVs composed exclusively of DOPC was measured. The electroformation performed at room temperature ensures the liquid state of DOPC, for which the gel-liquid
Results

Figure 5.3: Typical two-focus scanning FCS correlation curves for a fluorescent lipid analogue diffusing in the Ld and Lo phases in the vesicles prepared at (A) room temperature and (B) 65°C. Autocorrelation curves are depicted in dark grey, cross-correlation curves between the two foci are in light grey.

phase transition temperature is −20°C. By means of the same FCS technique variant, the determined diffusion coefficient was 10.7±1.0 µm²/s (average from n=14 measurements), which is approximately 2 times larger than the diffusion in the Ld phase and 7–8 times larger than the diffusion in the Lo phase of the studied tertiary mixture. It is worth to mention that the relative error of the diffusion coefficient values in the case of monocomponent, DOPC membrane did not exceed 10%, whereas for the tertiary mixture it was larger than 15%, for both phases (Table 5.1).

Concentration of lipid dye in GUVs

To illustrate the extent of composition distribution in single GUVs, I used the amplitudes of registered autocorrelation curves, which correspond to the average number of dye molecules diffusing through the focal volume. Figure 5.4 (C, D) illustrates the histograms of DiD concentration in single vesicles, recalculated as the number of molecules per unit area in the Lo and Ld phases. It can be seen that the lowered growth temperature did not affect the distribution widths which means that both preparation conditions lead to similar composition fluctuations at the single vesicle level. It is interesting to observe, that the DiD concentration distributions in the DOPC GUVs has also similar width (Fig. 5.4
5.2.2 VDAC prefers Ld phase

In previous chapter, I have shown that GUVs with lipid mixture DOPC/SM/Chol (2/2/1) prepared at room temperature can be used as a platform for protein reconstitution. There-
fore, I reconstituted VDAC into proteoliposomes with this lipid composition and formed GUVs with phase separation. As before, the lipid domains were visualized by lipid dye

![Confocal fluorescence images of Giant Unilamellar Vesicles with phase coexistence with reconstituted green-labeled VDAC: (A) red (DiD—lipid marker) channel, (B) green (Alexa 488—VDAC) channel. Membrane composition: DOPC/SM/Chol (2/2/1). The liquid disordered phase is labeled with DiD, scale bar 30 µm.](image1)

**Figure 5.5:** Confocal fluorescence images of Giant Unilamellar Vesicles with phase coexistence with reconstituted green-labeled VDAC: (A) red (DiD—lipid marker) channel, (B) green (Alexa 488—VDAC) channel. Membrane composition: DOPC/SM/Chol (2/2/1). The liquid disordered phase is labeled with DiD, scale bar 30 µm.

![Confocal fluorescence images of Giant Unilamellar Vesicles with phase coexistence with reconstituted green-labeled VDAC: (A) red (DiD—lipid marker) channel, (B) green (Alexa 488—VDAC) channel. Membrane composition: DOPC/SM/Chol (2/2/1). The liquid disordered phase is labeled with DiD, scale bar 50 µm.](image2)

**Figure 5.6:** Confocal fluorescence images of Giant Unilamellar Vesicles with phase coexistence with reconstituted green-labeled VDAC: (A) red (DiD—lipid marker) channel, (B) green (Alexa 488—VDAC) channel. Membrane composition: DOPC/SM/Chol (2/2/1). The liquid disordered phase is labeled with DiD, scale bar 50 µm.

DiD, which was partitioning into Ld phase as was confirmed by the diffusion coefficient measurements described in previous section. As can be seen from the figure 5.5, VDAC displays a clear preference for Ld phase and colocalizes with Ld phase marker DiD. The bigger field of view is presented in figure 5.6. One should mention that some GUVs did not have DiD marker, which can be possibly explained by repulsion between positively charged VDAC and DiD (Fig. 5.6, left lower corner).

The diffusion coefficient of VDAC in Ld phase was $3.1 \pm 0.6 \ \mu m^2/s$. This value is approximately 1.5 times lower than the value measured in pure DOPC membranes (table 4.1). The similar reduction in the diffusion coefficient in presence of cholesterol was also
observed for DiD dye: as it was shown in previous sections that DiD diffusion coefficient in GUVs made of DOPC was $10.7 \pm 1 \mu m^2/s$ whereas in Ld phase it was $5.3 \pm 0.7 \mu m^2/s$.

### 5.3 Discussion

Giant unilamellar vesicle electroformation depends on many factors that need to be optimized to produce vesicles having intended properties. Since GUVs are becoming more widely used in the applications involving reconstituted membrane proteins, here I asked the question on the influence of lowered growth temperature on the properties of vesicles composed of DOPC/SM/Chol, commonly used as a mimicry of membrane rafts. Similar kind of general morphology and similar size distributions for the vesicles prepared at different temperatures were observed. The corresponding size histograms had similar shapes and mean values compared to those obtained in former studies of GUVs made of egg phosphatidylcholine (EPC)/lysophosphatidylcholine (LPC) mixtures [144] or from EPC/egg phophatidic acid (EPA) with reconstituted Ca$^{2+}$ ATPase [104]. Despite these similarities, the most pronounced difference between the vesicles prepared below and above the miscibility temperature is the ratio of vesicles showing Ld/Lo phase separation. This fact raises a question on the relevance of using lowered growth temperature and the correspondence to the equilibrium thermodynamics conditions and therefore, requires more detailed discussion in this light. The aforementioned effect may be attributed to an altered composition of vesicles prepared at room temperature, since the overall ratio of Lo/Ld fractions is clearly lower compared to the vesicles prepared at higher temperature. Nevertheless, no dramatic variation in lipid composition is expected, because the coexistence of the fluid Ld/Lo phases for DOPC/SM/Chol mixture can only be observed at the particular range of the concentrations of the components of this lipid mixture, as can be seen from the corresponding ternary phase diagram (Fig. 5.4 (A)). In addition, the mobilities in Lo and Ld phases were not affected by the lower growth temperature and the values of diffusion coefficients were in well agreement with the previous NMR [140] and FCS [136] studies. The difference in lipid mobility between the Lo and Ld phases by a factor of 5 was also revealed by coarse-grained molecular simulations [145]. The similar diffusion coefficient values obtained in this study in the vesicles prepared at different temperatures also indicate that the presence of the cholesterol in these preparations is comparable, since cholesterol changes the diffusion coefficient in Lo phases quite dramatically [136]. The presented here studies on DiD concentration distribution in single vesicles revealed that
the composition variation is, in general, an unavoidable property of giant unilamellar vesicles produced by electroformation, since the DiD concentration distribution showed the fluctuations at the single vesicle level even for the GUVs made of only DOPC lipid. Histograms of DiD concentration in both lipid phases in phase-separating vesicles show that the preparation temperatures did not affect the distribution width, and this width was similar to the one obtained for DiD concentration in pure DOPC membrane.

In summary, obtained results indicate that the giant unilamellar vesicles prepared from DOPC/SM/Chol (2/2/1) mixture at room temperature and at 65°C have very similar physical properties. There were no substantial differences in the lipid microenvironments of ordered and disordered phases, as revealed by two-focus scanning FCS. The preparation of GUVs from raft mixture lipids at room temperature can therefore be justified, especially in the cases of protein reconstitution, requiring lower temperatures for protein stability and biological activity. However, one should be aware of possible composition alterations, which remain to be characterized in the future detailed studies.

Characterization of the GUVs with phase separation prepared at room temperature, allowed the reconstitution of VDAC protein to the system with the well-defined Lo and Ld lipid domains. The experimental results clearly indicate the preferential partitioning of VDAC into Ld domains. This observation either suggests a non-raft localization of VDAC in vivo, in agreement with one of the studies on DRMs [69], or the requirement of other factors which could recruit VDAC to the lipid rafts under specific conditions. Therefore, VDAC reconstituted into GUVs with Lo/Ld phase coexistence is a very useful model system, which can allow the stepwise addition of biological complexity and the further investigation of VDAC raft partitioning in biological membranes.
Chapter 6

The role of lipids in VDAC oligomerization

6.1 Introduction

The mitochondrial VDAC is supposedly of a crucial importance for the homeostasis of living cells, as it provides the main pathway for the exchange of metabolites, such as ATP and ADP, between the mitochondria and the cytosol [27].

Recent studies, however, have shown that this channel also plays an important role in cell death [41, 42, 43]. In particular, apoptosis induction was found to be associated with increased VDAC oligomerization [48, 55, 56]. The existence of VDAC dimers and higher oligomers was mentioned for the first time in 1985 [33]. Recent AFM studies on native mitochondrial membranes, as well as NMR studies on VDAC in detergent solution, confirm the ability of VDAC to form dimers, trimers, tetramers and higher oligomers [36, 38, 39]. VDAC oligomerization during apoptosis has been suggested to result in the formation of large pores, allowing for release of apoptogenic factors, including cytochrome c, from mitochondria, and thus inducing cell death. In particular, the above-mentioned pores are believed to be created by VDAC homo-oligomers [55, 35, 57] or hetero-oligomers of VDAC with apoptotic proteins of the Bcl-2 family [36]. Formation of these oligomers was suggested to be regulated not only by Bcl-2 family apoptotic proteins, but also by hexokinase [55] and cytochrome c [57], although no direct evidence has been presented so far, that these proteins have a direct influence on VDAC oligomerization.

On the other hand, it is known that membrane protein function and structure can be regulated by lipids [146]. Protein—lipid interactions can be either specific or nonspecific;
in particular, they can depend on the properties of the lipid bilayer, including the lipid charge, hydrophobic mismatch, and the presence of nonbilayer lipids (for review, see [147]). Protein oligomerization can thus also be significantly influenced by the lipid environment. In particular, it was shown that negatively charged lipids are important for the stability of the quaternary structure of ADP-ATP carrier [148] and potassium channel KcsA [149].

The anionic lipid cardiolipin (CL) is found almost exclusively in mitochondria [77]. CL is considered as the major negatively charged lipid in the inner mitochondrial membrane (18 wt. % of the total lipid content). The amount of CL in the outer mitochondrial membrane is lower (around 4 wt. %), however it is found in high concentrations at the contact sites between the inner and outer mitochondrial membrane (27 wt. %) [78] where VDAC was shown to primarily localize [128]. CL is synthesized from phosphatidylglycerol (PG) by means of cardiolipin synthetase [150]. Under normal physiological conditions, the amount of PG in mitochondria is relatively low; during apoptosis, however, it can increase as much as 2 fold, whereas the opposite effect is observed for CL, the amount of which is significantly decreased [83, 81]. It was also shown that a knockdown of cardiolipin synthetase increases the level of PG and decreases the level of CL in HeLa cells, leading to an acceleration of stimuli-elicited apoptosis [82]. The influence of CL on VDAC channel activity has been previously demonstrated by electrophysiology measurements, based on which the preferable insertion of VDAC into CL-rich domains was suggested [130]. However, the interaction of VDAC with PG was not addressed before. In addition, whereas the role of CL in apoptosis is emphasized in the literature, the role of PG in the cell death is largely unknown.

Because of the tight relationship between CL and PG, and the important role that CL plays in apoptosis, it is reasonable to suggest that PG can also be involved in controlled cell death by participating in the regulation of VDAC oligomerization. In order to test this hypothesis, a chemically well-defined system that avoids unknown factors and biological off-target effects is required. To this end, I here employ a cell-free minimal model system [151]—giant unilamellar vesicles (GUVs) with incorporated VDAC. To characterize the degree of VDAC oligomerization in various lipid environments, fluorescently labeled VDAC (VDACred and VDACgreen) was reconstituted into GUVs containing CL, PG and two other anionic lipids and two-focus two-color scanning FCCS measurements were carried out as described in the chapter 2.8. The representative auto- and cross-correlation curves obtained by two-color two-focus FCCS measurements for GUV reconstituted VDAC are presented in figure 6.1.

The oligomer fraction was determined as defined in Eq. 2.8.
Based on obtained results, I suggest that VDAC oligomerization in the membrane can be tuned by up- or down-regulation of CL and PG levels in mitochondria during apoptosis, respectively.
6.2 Results

6.2.1 VDAC forms oligomers in detergent which are stable even after reconstitution into the membrane

Previous experiments had shown that VDAC from different species forms dimers and higher order oligomers in detergent and in the membranes of living cells [36, 38, 39, 35, 22, 44].

In order to check the oligomeric state of refolded VDAC in the detergent solution, VDACred and VDACgreen in LDAO micells were mixed together (see the upper part of Fig. 6.2 (A)) and FCCS measurements were carried out on this sample. The estimated oligomer fraction for VDAC in detergent solution clearly indicates the formation of VDACred–VDACgreen complexes (Fig. 6.3).

![Diagram](image)

**Figure 6.2:** Two different methods of reconstitution of fluorescently labeled VDAC into GUVs. (A) VDACred and VDAC green are mixed in detergent solution and incorporated into proteoliposomes, which are further used to form GUVs. (B) VDACred and VDACgreen are incorporated into proteoliposomes separately, and the proteoliposomes are mixed only immediately before the formation of GUVs.

To monitor the oligomeric state of VDAC in the membranes, the same solution was used further to incorporate VDAC into proteoliposomes made of DOPC (Fig. 6.2 (A)). GUVs were grown from these proteoliposomes and two-focus two-color FCCS measurements in the membrane were performed to check whether VDAC oligomers were present in the membrane. The oligomerization level of VDAC in the membrane appeared to be similar
to the one observed in detergent solution (Fig. 6.3). This clearly shows that the VDAC oligomers pre-existing in detergent solution are preserved after reconstitution into the membrane.

The crucial question now is whether the detected oligomers were first formed in detergent and subsequently transferred to the membrane during reconstitution, or whether they were formed in the membrane after protein reconstitution. In order to distinguish between these two scenarios, proteoliposomes containing VDACred and VDACgreen were prepared separately and mixed just before the formation of GUVs, to ensure that only oligomeric complexes newly formed in the membrane are detected by FCCS (Fig. 6.2 (B)). As can be seen from figure 6.3, very few oligomeric complexes were observed in DOPC lipid membrane in this case. Therefore, one can conclude that VDAC forms stable oligomers already in detergent, which are preserved even after incorporation into the membrane. These results confirm suspicions that a particular protein incorporation procedure can strongly affect subsequent experiments and conclusions made on their basis. The goal of the present work was on one hand, to study the stability of existing VDAC oligomers once transferred to the membrane, and their dependence on varying lipid composition. To this end, the preparation procedure depicted in figure 6.2 (A) was applied. Alternatively,
the ability of VDAC to form oligomers after reconstitution to the membrane was studied, using the procedure depicted in figure 6.2 (B).

6.2.2 Cardiolipin disrupts VDAC oligomeric complexes in the membrane

Cardiolipin, a negatively charged lipid with four acyl chains, is an important constituent of the mitochondrial membrane known to be related to mitochondrial metabolism and the process of apoptosis [152]. Cardiolipin is enriched in the contact sites between the inner and outer mitochondrial membrane [78] where VDAC is localized [128]. Therefore, it was straightforward to check whether the VDAC oligomerization state can be affected by this lipid. As can be seen from figure 6.3, very few VDAC oligomers were found in the membrane at the composition DOPC:CL 80:20, similar to the case of the pure DOPC membrane.

In order to check whether CL influences the stability of VDAC oligomers pre-formed in detergent, I carried out experiments on GUVs containing VDACred and VDACgreen incorporated following the procedure depicted in figure 6.2 (A). As it is clear from figure 6.3, the presence of cardiolipin substantially reduced the amount of VDACred–VDACgreen oligomers compared to the pure DOPC membrane. I therefore conclude that cardiolipin is able to disrupt supramolecular assemblies of VDAC in the membrane.

Electrophysiology studies suggest that VDAC can partition into CL-enriched domains [130] formed on mitochondrial contact sites. In view of this model, the implication of the presented here finding is that under normal (non-apoptotic) conditions the relatively high CL level on the mitochondrial membrane precludes VDAC oligomerization.

6.2.3 PG induces VDAC oligomerization

PG is a precursor of CL in mitochondria. Similar to cardiolipin, it carries a negative charge, but, unlike CL, has only two acyl chains. During the programmed cell death, an increase in PG concentration by ca. 30–50 % of the control and the decrease in the CL levels by ca. 15–50 % have been reported [83, 81].

In order to investigate whether the elevated PG levels can influence VDAC oligomerization, VDAC was incorporated into DOPC/DOPG GUVs with the composition DOPC:DOPG 80:20 and the formation of oligomers in the membrane was monitored by means of FCCS (here and elsewhere, if not specified otherwise, VDACred and VDACgreen were reconsti-
tuted into GUVs using Method 2 (Fig. 6.2 (B)). Interestingly, a significantly enhanced level of VDAC oligomerization in DOPC/DOPG membranes was observed compared to pure DOPC membrane. Moreover, an increase in the PG concentration in GUVs leads to a gradual increase in the number of VDAC oligomeric complexes formed in the membrane (Fig. 6.4).

Figure 6.4: Two-color cross-correlation values for fluorescently labeled VDAC in GUVs. GUV composition: DOPC, DOPC:DOPG 90:10, DOPC:DOPG 80:20, DOPC:DOPG:CL 80:15:5, DOPC:DOPG:CL 80:10:10, and DOPC:CL 80:20. Data were obtained in three independent experiments with three independently prepared samples; each point is an average of values measured on 13 to 39 vesicles.

These conclusions are based on the FCCS measurements which provide information on the amount of red-green complexes formed in the membrane. The experiment can be modified such that only one-color species are present in the membrane, and oligomerization of VDAC is followed by the so-called specific brightness of fluorescent particles measured in photon counts per particle per second, which can be extracted from the fluorescence fluctuation data. If single-color particles cluster and diffuse together, the detected specific brightness should increase. The conducted experiments show that the mean specific brightness of fluorescent particles increases roughly by a factor of two in membranes containing PG compared to membranes with CL (Fig. 6.5), which is an additional evidence supporting the formation of oligomeric complexes. The fact that VDAC is able to form not only dimers, but also higher-order oligomers in the membrane [36, 38, 39], does not allow us to draw more quantitative conclusions from these data. The results of the above experiments allow us to conclude that the negatively charged lipid PG induces formation
of VDAC supramolecular assemblies, in contrast to its four-acyl chain analogue CL which, on the opposite, prevents oligomerization in the membrane and even disrupts the oligomers originally formed in solution after they attach to the membrane.

### 6.2.4 Effect of PG on VDAC assembly vanishes in presence of CL

In view of the above findings, it was interesting to check whether the effect of CL on VDAC oligomers would also be detected in the presence of PG in the membrane, and vice versa. The results presented in Fig. 6.4 demonstrate that addition of CL to the lipid mixture disrupts oligomeric complexes of VDAC formed in presence of PG. Virtually no oligomers were detected in GUVs with the compositions DOPC:DOPG:CL 80:15:5 or 80:10:10, similar to what was observed for the pure DOPC membrane (Fig. 6.3).

Thus, I conclude that CL reduces the number of supramolecular complexes of VDAC which can be formed in the membrane in presence of PG. I suggest that under the normal cell conditions, one of the functions of CL in mitochondria consists in keeping VDAC in the monomeric state. During apoptosis, cardiolipin levels in mitochondria decrease [153], the cristae are rearranged, and contact sites between the inner and outer membrane, where VDAC is colocalized with CL, are disrupted [154]. These conditions favour the interaction
of VDAC with PG and subsequent VDAC oligomerization.

6.2.5 Other anionic lipids with two acyl chains also increase the level of VDAC oligomerization

To find out how specific is the interaction of VDAC with DOPG, oligomerization of the protein in membranes containing other negatively charged lipids with two acyl chains, PI and PS, was tested. Figure 6.6 summarizes the results obtained with FCCS. An increase in PI and PS concentrations in the membrane clearly leads to an increase in the amount of oligomeric complexes. Thus, anionic phospholipids PI and PS also induce VDAC oligomerization, although their effect is weaker compared to PG (cf. Fig. 6.4).

![Figure 6.6: Two-color cross-correlation values for fluorescently labeled VDAC in GUVs. GUV composition: DOPC (dark gray), DOPC:DOPI 90:10, DOPC:DOPI 80:20 (light gray) or DOPC:DOPS 90:10, and DOPC:DOPS 80:20 (gray). Data were obtained in three independent experiments with three independently prepared samples; each point is an average of values measured on 13 to 29 vesicles.](image)

6.3 Discussion

To summarize, I have found that PG, PI and PS induce VDAC oligomerization, whereas CL disrupts VDAC supramolecular assemblies in the membrane. I believe that these results can be related to VDAC oligomerization observed during programmed cell death [48, 55, 56]. Indeed, experimental studies show that apoptosis is associated with the
suppressed CL synthesis and an increase in the level of PG [83, 81]. On the other hand, CL deficiency and PG accumulation in cardiolipin synthetase deficient yeast cells were shown to lead to cytochrome c release from mitochondria followed by apoptosis [82]. A decrease in the amount of CL, which interacts with VDAC in the mitochondria contact sites, can also occur as a consequence of rearrangements of cristae observed during cell death [154] and possible disruption of the contact sites. As follows from the obtained here results, a decrease in the amount of CL and increase of PG levels in the membrane can lead to efficient formation of VDAC oligomers. Interestingly, in previous studies [83, 82], CL deficiency was stressed as the most important factor for apoptosis, which was connected only with disruption of interactions between CL and cytochrome c, whereas PG was not even considered as a component playing any significant role in cell death. On the other hand, a recent study [155] has shown that cell lines from patients with the Barth syndrome, which also exhibit low levels of CL, but no increase in PG, are not susceptible to apoptosis. This observation suggests that the level of PG in mitochondria may be a crucial factor regulating apoptosis. As I have demonstrated, an increase in the PG level in the membrane induces VDAC oligomerization which, in turn, is believed to be an important step in programmed cell death. It is known that, in general, lipids can regulate protein oligomerization in different ways, and the literature on this topic is extensive (see, e. g., [147, 156]). Most of the research in this field is concerned with the influence of lipid charge and hydrophobic mismatch on protein association. The importance of negatively charged lipids for protein oligomerization has been discussed widely in the literature. For example, it was shown by in vivo studies that PG is required for the formation and stability of supramolecular assembly of the photosystem I reaction center [157]. Another negatively charged lipid DOPA (dioleoyl phosphatidic acid) was shown to stabilize the tetrameric assembly of KcsA channel [149], whereas DMPS (dimeristoyl phosphatidylserine) and DMPG (dimeristoyl phosphatidylglycerol) were reported to be involved in aggregation of the AβP(25–35) peptide [158]. Other studies also indicate that CL enhances the formation and stability of oligomers of KcsA channel [149] and mitochondrial respiratory chain supercomplexes [159].

Protein—lipid interactions on membranes containing anionic lipids have also been addressed in theoretical studies [160, 161]. The general conclusion is that negatively charged lipids in the membrane migrate towards the positively charged protein, which leads to electrostatics-induced local demixing of charged and neutral lipids, which produces a varying lipid composition profile around the charged protein [161]. In this way lipid—protein complexes can be produced in the form of domains enriched in both positively charged

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protein and anionic lipids.

The presented here results are generally in line with the above-mentioned experimental and theoretical studies [157, 158, 161]. VDAC is positively charged at neutral pH [115] and therefore anionic PG, PS, PI, and CL interact with VDAC, which should result in their local demixing. I found that in agreement with previous findings for other proteins interacting with anionic lipids [149, 157, 158], PG, PI, and PS induce oligomerization of VDAC in the membrane.

On the other hand, I found that, surprisingly, CL which is also negatively charged, does not induce VDAC oligomerization and moreover, precludes the formation of oligomeric complexes in the membrane. The question arises why CL has an opposite effect on VDAC oligomerization compared to the other negatively charged lipids PG, PS and PI.

In my opinion, this difference is most probably related to the different structure of the hydrophobic part of these lipids (CL has four acyl chains compared to two acyl chains in PG, PS and PI), as well as with the character of protein-protein interactions responsible for VDAC oligomerization. The crystal packing analysis of murine VDAC suggests that oligomerization of VDAC is mostly driven by van der Waals interactions [40]. The interaction surface for VDAC oligomers reconstructed in the PyMOL 1.3 software (DeLano Scientific LLC, www.pymol.org) shows areas with strong positive charge (Fig. 6.7 (A)). Negatively charged lipids can interact with the positively charged surface of VDAC, resulting in charge screening, which decreases the distance between VDAC monomers and facilitates the formation of stable complexes due to van der Waals attraction (Fig. 6.7 (B)). If, instead of two-acyl chain lipid (e.g., PG), a substantially more bulky CL is present in the membrane, its local electrostatics-induced demixing should, on the opposite, preclude the close approach of VDAC molecules and thus prevent the formation of VDAC complexes (Fig. 6.7 (C)). In addition, I observed that CL eliminates the effect of PG on VDAC oligomerization. This means that CL competes with PG for binding with VDAC, and interaction of CL with VDAC seems to be energetically more favourable.

Several previous studies suggested that, in general, negatively charged lipids induce aggregation or clustering of transmembrane proteins [149, 158]. While the dependence of the stability of oligomers on the lipid charge and specific properties of the headgroup was emphasized [149], the role of the hydrophobic part of the lipid was usually not taken into consideration in these studies. The experiments presented in this work demonstrate that the shape of hydrophobic part of lipids can play a crucial role in the formation and stability of protein supramolecular assemblies in the membrane. I believe that my results shed light on the role played by the anionic lipids PG and CL in the regulation of VDAC
**Figure 6.7:** The charge-surface (positive, red, and negative, blue) of VDAC monomer responsible for the dimer formation generated using PyMOL 1.3 software (A) and schematic representation of the interaction of VDAC with anionic lipids (B, C). (B) Formation of VDAC dimers in presence of PG, (C) Interaction of VDAC with CL.

oligomerization during apoptosis, and thus provide additional information of the molecular mechanisms of the programmed cell death.
Future directions

In this study, the protocol for VDAC labeling with fluorescent dyes and its incorporation into GUVs was developed. This model system can be successfully used to investigate VDAC properties and its interactions with lipid membranes. Further, the biological complexity of this system can be increased by introducing other membrane proteins. For example, hexokinase, apoptotic proteins and tubulin can be included to this system to investigate their binding to VDAC.

This thesis is concerned with the interaction of VDAC with phospholipids. One of the questions, which still remains under debate, is VDAC localization to lipid rafts. Experimental studies presented here show that fluorescently labelled VDAC incorporated into GUVs with phase separation preferentially partitions into Ld phase. This result suggests that either VDAC is excluded from lipid rafts \textit{in vivo}, or some other proteins can be involved in the recruitment of VDAC into the lipid rafts. Therefore, VDAC reconstituted into GUVs with phase separation can be a valuable system to study VDAC raft partitioning with the possibility of introducing various factors which might be required for VDAC translocation into Lo phase.

In addition, here, the approach was developed to study VDAC oligomerization in the membrane—the phenomenon playing an important role in the programmed cell death. Incorporation of VDAC labelled with two spectrally separated fluorescent dyes, allowed to use FCCS technique to follow VDAC self-assembly. Here, the influence of mitochondria anionic lipids PG and CL on VDAC oligomerization was investigated. The studies revealed that PG can enhance VDAC oligomerization, whereas CL suppresses it. This regulation can be related to the induction of VDAC oligomerization \textit{in vivo} during apoptosis. However, there are a lot of other lipids and proteins which can influence VDAC oligomeric state in mitochondria. The presented model system and FCCS method represent a valuable tools to study VDAC oligomerization and can be used further to reveal proteins and lipids, which might be involved in this process.
Bibliography


(VDAC) precedes caspase activation in neuronal apoptosis induced by toxic stimuli. *Cell Death Differ.* 12:1134–1140.


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formation from lipid mixtures to native membranes under physiological conditions. 


Holand.

137.

zation of lipid bilayer phases by confocal microscopy and fluorescence correlation 


[100] Manneville, J.-B., J.-F. Casella, E. Ambroggio, P. Gounon, J. Bertherat, 
occurs on liquid-disordered domains and the associated membrane deformations are 


[102] Trajkovic, K., C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwille, 
B. Brøgger, and M. Simons. 2008. Ceramide triggers budding of exosome vesicles into 

Reconstitution of membrane proteins into giant unilamellar vesicles via peptide-


Abbreviations

ADP  Adenosine diphosphate
AFM  Atomic force microscopy
AIF  Apoptosis inducing factor
ANT  Aminoglycoside nucleotidyl transferase
APD  Avalanche photodiode
ATP  Adenosine triphosphate
BLM  Black lipid membrane
CD  Circular dichroism
Chol  cholesterol
CL  Cardiolipin
CM  Carboxymethyl
DEAE  Diethylaminoethyl
DiD  1,1'-dioctadecyl-3,3',3',3' tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt
DIGS-BB Dresden international graduate school for biomedicine and bioengineering
DiO  3,3'-Diocotadecylxocarbocyanine perchlorate
DiPhPC  1,2-diphtytyloxytn-glycero-3-phosphocholine
DMPG  1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
DMPS  1,2-dimyristoyl-sn-glycero-3-phospho-L-serine
DNA  Deoxyribonucleic acid
DOPA  1,2-dioleyl-sn-glycero-3-phosphate
DOPC  1,2-dioleyl-sn-glycero-3-phosphocholine
DOPG  1,2-dioleyl-sn-glycero-3-phospho-(1'-rac-glycerol)
DOPH  1,2-dioleyl-sn-glycero-3-phospho-(1'-myo-inositol)
DOPS  1,2-dioleyl-sn-glycero-3-phospho-L-serine
DRM  Deregent-resistant membrane
<table>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FCCS</td>
<td>Fluorescence cross-correlation spectroscopy</td>
</tr>
<tr>
<td>FCS</td>
<td>Fluorescence correlation spectroscopy</td>
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<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
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<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol (GPI)-anchored proteins</td>
</tr>
<tr>
<td>GUV</td>
<td>Giant unilamellar vesicle</td>
</tr>
<tr>
<td>IM</td>
<td>Inner membrane</td>
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<tr>
<td>LB</td>
<td>Lysogeny broth</td>
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<tr>
<td>LDAO</td>
<td>N,N-dimethyldodecylamine N-oxide</td>
</tr>
<tr>
<td>Ld</td>
<td>Liquid disordered phase</td>
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<tr>
<td>Lo</td>
<td>Liquid ordered phase</td>
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<tr>
<td>LSM</td>
<td>Laser scanning microscope</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide hydrate</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>OM</td>
<td>Outer membrane</td>
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<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<tr>
<td>PLAP</td>
<td>Placental alkaline phosphatase</td>
</tr>
<tr>
<td>PI-VDAC</td>
<td>Plasmalemmal VDAC</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PTP</td>
<td>Permeability transition pore</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SM</td>
<td>n-stearoyl-d-erythrosphingosylphosphorylcholine (sphingomyelin 18:0)</td>
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<tr>
<td>SNARE</td>
<td>Soluble NSF attachment protein receptor</td>
</tr>
<tr>
<td>STED</td>
<td>Stimulated Emission Depletion microscopy</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicle</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
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<td>UV</td>
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<tr>
<td>VDAC</td>
<td>Voltage dependent anion channel</td>
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</table>
List of publications

The role of lipids in VDAC oligomerization.
Accepted to Biophysical Journal.

V. Betaneli, P. Schwille, 2011.
Fluorescence correlation spectroscopy to examine protein lipid interactions in membranes.
Methods in Molecular Biology: Lipid-Protein Interactions: Methods and Protocols, in Press.
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Declaration

I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

The thesis work was conducted from 01.02.2008 to 14.12.2011 under the supervision of Prof. Petra Schwille at the Institute of Biophysics, Department of Physics, Dresden University of Technology, Germany.

I declare that I have not undertaken any previous unsuccessful doctorate proceedings.

I declare that I recognize the doctorate regulations of the Fakultät für Mathematik und Naturwissenschaften of the Technische Universität Dresden.
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