THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Methods for elucidating membrane protein structure and function

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Gothenburg, Sweden 2015

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ISBN 978-91-7597-247-3

Doktorsavhandlingar vid Chalmers tekniska högskola
Ny serie nr 3928
ISSN 0346-718X

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Cover:
[A schematic representation of the antibody OTV1 binding to epitopes (red) on the human ion channel TRPV1 together with a visualization of the cellular location of OTV1 using fluorescence measurements, and patch-clamp recordings showing its functional effect on ion channel current response.

The TRPV1 model was used with kind permission from Dr. Gregorio Fernández-Ballester at the Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Spain]

Printed by Chalmers Reproservice
Gothenburg, Sweden 2015

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Abstract

Elucidation of the structure-function relationship of membrane proteins increases our understanding of many biological processes, both physiological and pathological. However, membrane proteins have been notoriously difficult to study, having part of their structure embedded into the lipid cell membrane. The work presented herein describes related functional and structural studies of the human ion channel TRPV1 as well as method development for studies on membrane proteins. First, the effect of membrane cholesterol levels on TRPV1 function was evaluated with patch-clamp recordings and cholesterol-depletion inhibited a time-dependent increase in permeability of large cations. Secondly, a microfluidic flow cell was developed for proteomic studies of membrane proteins where proteoliposomes derived from cell membranes were immobilized and constituted a stationary phase of membrane proteins. In addition, a sequential digestion protocol for limited proteolysis within the flow cell was developed for proteomic profiling and digested peptides were identified with mass spectrometry. Moreover, limited proteolysis was performed on TRPV1 for evaluation of surface exposed regions. Identical digestions were performed during inside-out patch-clamp experiments in order to correlate TRPV1 structure with function. Removal of intracellular peptide regions decreased ion channel activity. Finally, the flow cell was employed for generation of antibody epitopes suitable for development of therapeutic antibodies. Two antibodies were raised against intracellular domains of TRPV1 and were further evaluated for their effect on TRPV1 activity using patch-clamp and fluorescence studies. Both antibodies altered TRPV1 activity. This methodology may thus constitute an effective strategy for generation of therapeutic antibodies.

Keywords: Membrane proteins, ion channels, antibodies, TRPV1, patch clamp, fluorescence, limited proteolysis, proteomics, mass spectrometry, cholesterol.

LIST OF PUBLICATIONS

I Jansson, E. T., Trkulja, C. L., Ahemaiti, A., Millingen, M., Jeffries, G. D. M., Jardemark, K., and Orwar, O. (2013) Effect of cholesterol depletion on the pore dilation of TRPV1. *Mol. Pain. 9,* 1–8.

II Jansson, E. T., Trkulja, C. L., Olofsson, J., Millingen, M., Wikström, J., Jesorka, A., Karlsson, A., Karlsson, R., Davidson, M., and Orwar, O. (2012) Microfluidic flow cell for sequential digestion of immobilized proteoliposomes. *Anal. Chem. 84*, 5582–5588.

III Trkulja, C. L., Jansson, E. T., Jardemark, K., and Orwar, O. (2014) Probing Structure and Function of Ion Channels Using Limited Proteolysis and Microfluidics. *J. Am. Chem. Soc. 136,* 14875–14882

IV Trkulja, C. L., Jardemark, K., Jeffries, G. D. M., Karlsson, A., Karlsson, R., and Orwar, O. Epitope-discovery for sequence-targeting antibody therapeutics. *Manuscript*

My contribution to the papers appended in this thesis is as follows:

I I contributed partially with experiments, and discussion of the results.

II I contributed partially with experiments, and discussion of the results.

III I planned the experiments and contributed partially with the experiments. I wrote the paper.

IV I planned and performed the experiments. I wrote the paper.

TABLE OF CONTENTS

1

INTRODUCTION

The molecular actions of proteins are fundamental to all forms of life. While the genetic code provides a blueprint for each living organism it is the proteins that execute these instructions, e.g. by catalyzing reactions, transmitting signals, transporting molecules, providing defense mechanisms, creating movement and providing structural integrity. Roughly 20–30% of all human genes encodes for membrane proteins [1], [2]. These are proteins performing their functions while being associated with cellular membranes. More than 60% of all drug targets are membrane proteins due to their unique abilities to convey signals from the extracellular milieu to the cell interior [3], [4]. Dysfunctional membrane proteins are implicated in many severe diseases, e.g. cystic fibrosis [5], Alzheimer's disease [6], several cancers [7]–[9] as well as in psychiatric diseases such as schizophrenia, major depression and bipolar disorder [10], [11]. Detailed data that describes protein–protein interactions, conformation dynamics, ligand–protein interactions and posttranslational modifications, that is, information that couples structure to function of these proteins, may aid the discovery of new pharmaceuticals for improved treatment of many diseases. However, the development of novel drugs targeting

membrane proteins is greatly hampered by the lack of structural knowledge. As of mid 2015, the protein data bank [12], [13] contains over 100 000 structural entries while less than 3% are classified as belonging to membrane proteins. The reason for this discrepancy is mainly due to low abundance of membrane proteins compared to cytosolic proteins and their inherent structure with hydrophobic regions spanning the cell membrane, which complicates the use of traditional structural biology techniques such as NMR spectroscopy and X-ray crystallography [14], [15]. Many analytical methods require proteins to be freely dissolved in aqueous solutions. Such conditions will, however, cause extracted membrane proteins to aggregate and precipitate. Detergents, salts and organic solvents are commonly used to maintain membrane protein solubility [16], but it is a delicate balance to keep these hydrophobic proteins in solution while at the same time limit disruption of their function and native structure. Protocols for enrichment of membrane proteins, their extraction from the membrane and subsequent solubilization need to be developed and optimized on a case to case basis.

The importance of this protein class and the demand for functional and structural data has driven the development of novel techniques for membrane protein studies that complement traditional techniques. Large scale proteomic studies have been facilitated by the evolvement of mass spectrometry based techniques [17]–[21]. Mass spectrometry requires less material and can handle complex mixtures of proteins, and provides detailed analysis of purified proteins. The technique has e.g. been utilized to study the interaction of membrane protein complexes by embedding the complex into detergent micelles prior to mass spectrometry analysis. The detergents are subsequently removed upon collision activation, enabling detection of individual subunits [22], [23].

The work described in this thesis has aimed to increase the knowledge of membrane protein structure and function. It includes both functional and structural studies of the human transient receptor potential vanilloid 1 (TRPV1) nociceptive ion channel and the development of methods that were validated using TRPV1 as a model membrane protein. A microfluidic cell was developed where membrane proteins can reside in their native membrane while they are exposed to different chemical treatments. This flow cell was used in combination with mass spectrometry to probe surface topology of the TRPV1 ion channel. The function of these regions were subsequently evaluated using patch-clamp

recording, an electrophysiological technique which is the gold standard for functional studies of ion channels [24]. Patch-clamp recording was also used to study how the cholesterol content of the cell membrane affects the activity of TRPV1. Ion channel activity was found to be dependent on cholesterol content. This information increases our knowledge about pain reception but also highlights the need for a correct lipid composition during membrane protein studies and the benefits of using native membranes in the microfluidic flow cell. Finally, the flow cell was utilized to detect surface exposed regions of TRPV1 which were thereafter used for the development of two antibodies targeting the intracellular side of TRPV1. Both of these antibodies were shown to have the ability to alter TRPV1 response. Antibodies are the fastest growing class of therapeutic drugs [25] due to their ability to bind a drug target (e.g. a membrane protein) with great specificity. Utilizing the microfluidic flow cell to pin-point regions on a membrane protein that can be targeted by an antibody is a completely novel tool which has the potential to greatly aid current techniques for antibody development.

2

MEMBRANE PROTEINS

The cell membrane defines the boundary of a living cell. It acts as a protective barrier, separating the interior of the cell from the extracellular environment while at the same time maintaining the cells' structural integrity. It is comprised of lipids arranged into a bilayer and can be described as a dynamic, thin fluid film. While this structure is provided by the self-assembly of lipid molecules many of its biological functions, such as transport and signaling, are performed by membrane proteins. These are proteins embedded in, or associated with the lipid bilayer and generally constitute 50% of the total membrane mass [26]. Membrane proteins perform a vast variety of essential functions such as mediating communication between cells, transferring signals between the exterior and interior of the cell, cellular respiration, transport of ions across the cell membrane, and promoting adhesion of cells to form larger tissues [26].

2.1 The cell membrane

The main lipid components of the cell membrane are phospholipids, glycolipids and sterols. Phospholipids, the most abundant lipids in the cell membrane, are amphiphilic molecules, consisting of a hydrophilic head group, a glycerol linker, and hydrophobic fatty acid chains. When placed in aqueous solutions, phospholipids will spontaneously arrange themselves into a lipid bilayer (Figure 2.1). The hydrophilic part will face the aqueous surroundings and the hydrophobic parts will be located in the interior of the bilayer, shielded from water [27].

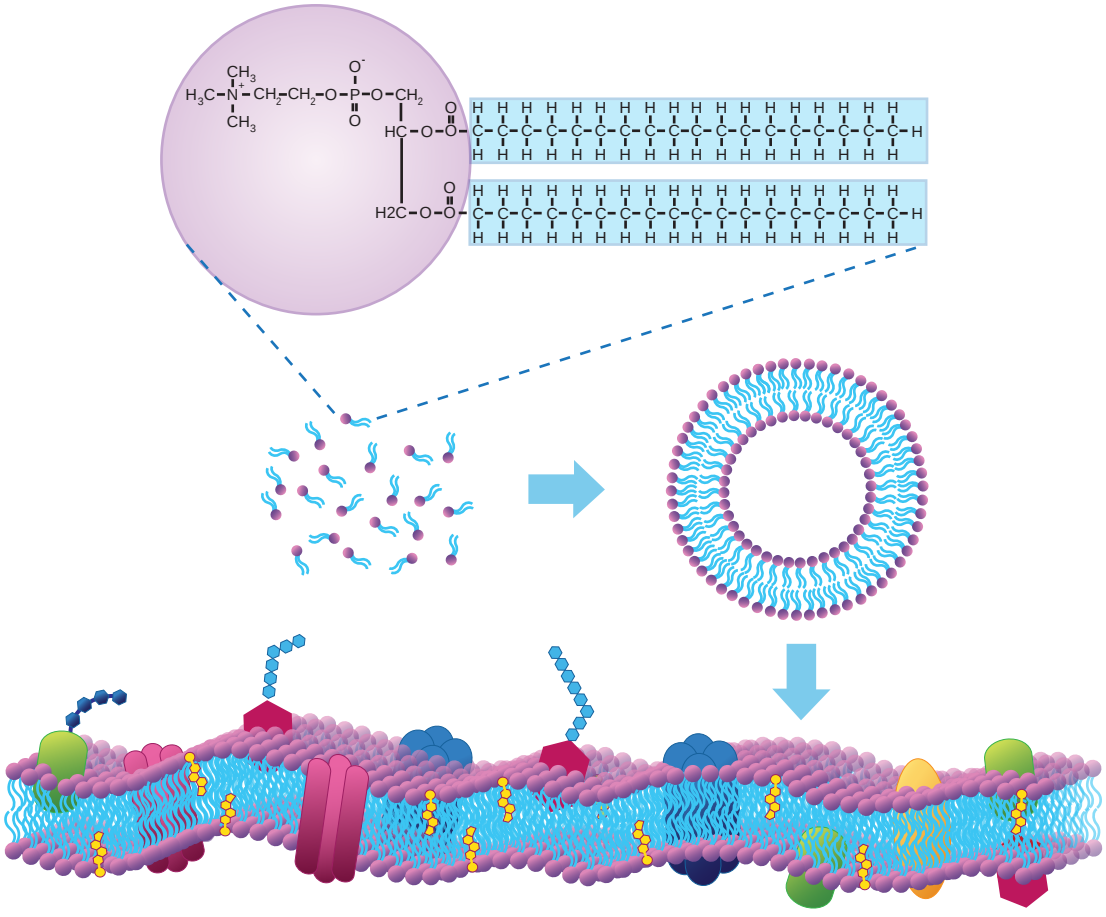

Figure 2.1 A phospholipid is constructed of a hydrophilic headgroup and two hydrophobic hydrocarbon chains (top). Several of these phospholipids will spontaneosly arrange themselves into a spherical bilayer, a liposome, in aqueous solutions (middle). A liposome can be viewed as a simplified comparison of a cell membrane. A biological cell membrane is functionalized with a large amount of membrane proteins (bottom) and contain different lipid molecules.

The lipid bilayer is impermeable to charged molecules and large polar compounds since their passive diffusion is inhibited by the hydrophobic interior of the bilayer. Sterols, such as cholesterol in mammalian cell membranes, increase the packing order of phospholipids within the membrane, which provides mechanical strength and further decreases its permeability [28]. This is an important property of the cell membrane, since an exactly defined ionic composition of the intracellular milieu is crucial for many biological processes. Additional, internal membranes provide further compartmentalization of the cell into different organelles, which enables local environmental differences optimized for various types of biochemical reactions [27].

2.2 Membrane protein structure

Membrane proteins can interact with the cell membrane in several ways. Integral proteins or transmembrane proteins are amphiphilic proteins that have one or several membrane spanning segments as well as regions that are exposed to the aqueous environment on either side of the membrane (Figure 2.2). The amino acid sequences located within the bilayer commonly have hydrophobic side chains in order to be soluble in the hydrophobic membrane core. The peptide bonds are, however, polar and are kept shielded through the formation of secondary structures such as α-helices and β-barrels [29]. Membrane proteins consisting of a β-barrel have alternating hydrophobic and hydrophilic amino acids where the hydrophobic amino acids are facing the lipid bilayer, and the hydrophilic amino acids are facing the interior of the barrel. This creates an hydrophilic cavity which can be used to transport water soluble molecules through the membrane [29]. An example of such a protein class is the porins. Membrane proteins consisting of α-helices typically have one or more α-helices spanning the membrane. Several α-helices are connected with loops that extend on either side of the membrane. The N- and C-termini can be located on both the same or opposite sides of the membrane [27], [29]. A class of membrane proteins that is constituted out of α-helices is G protein-coupled receptors (GPCRs) that have 7 transmembrane α-helices with the purpose of conveying signals from the cell exterior to the interior.

Peripheral membrane proteins or membrane-associated proteins are proteins that function solely on one side of the cell membrane and have a majority of their structure in a

hydrophilic environment. Peripheral membrane proteins may be linked to the membrane by insertion of a hydrophobic region into the lipid bilayer, by covalent attachment of an amino acid to a lipid or by binding to another membrane protein [27], [30].

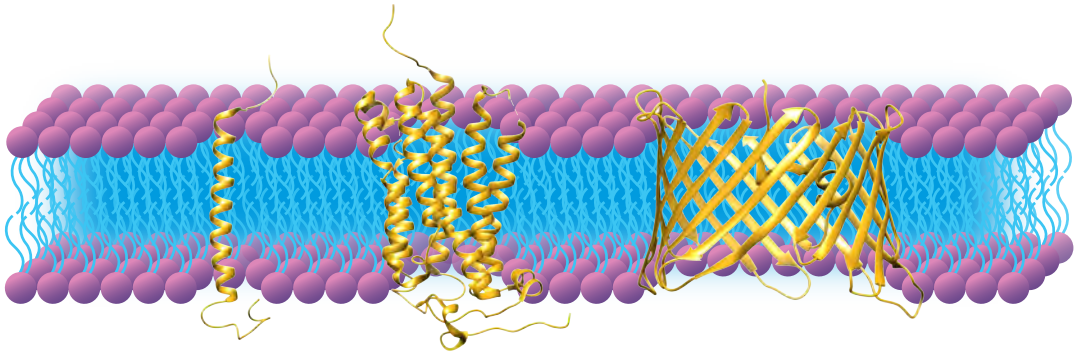

Figure 2.2 Examples of different structural arrangements of transmembrane proteins. Proteins having either one (left) or several (middle) α-helices spanning the cell membrane are the largest class of transmembrane proteins .Proteins consisting of a β-barrel spanning the membrane (right) are less common but comparatively easier to dissolve and study.

2.3 Ion channels

Ion channels have different functions in the human body, e.g. in signal transduction processes leading to the generation of electrical impulses along nerves in the central and peripheral nervous system, the heart and other organs.

The basis of this signal transduction, involving ion channels, is that living cells have a different ionic composition in the cytosol compared to the extracellular environment (Table 2.1), which gives rise to an electrical potential across the cell membrane, defined as the membrane potential [31]. The K^+ concentration is normally higher in the cytosol, and the Na^+ concentration is higher on the extracellular side. This electrochemical gradient is maintained through the activity of membrane proteins, actively or passively transporting ions across the membrane. Two main classes of transmembrane proteins, ion pumps and ion channels, are responsible for this process. Ion pumps transport ions against their concentration gradient using energy. For example, the sodium-potassium pump hydrolyzes ATP to transport three Na^+-ions out of the cell while at the same time transporting two K^+ -ions inside the cell. Thus, it establishes and maintains the difference in K^+/ Na^+ concentrations. While transport of ions using ion pumps is a slow process, ion channels are pore forming proteins that open or close upon stimuli (Figure 2.3) and allow specific ions to rapidly diffuse down their concentration gradient [32]. Rapid transfer of

ions along their concentration gradient will cause a change in membrane potential, a mechanism that is crucial for many biological processes e.g. nerve signaling. During nerve signaling, a local depolarization of the membrane triggers the opening of Na^+-channels, causing an influx of Na^+-ions, which in turn further depolarize the membrane. This causes neighboring Na^+-channels to open and the nerve signal, an action potential, will propagate along the nerve cell. The opening of Na^+-channels is followed by a refractory period in which these channels are inactivated. The refractory period also includes the opening of K^+-channels, causing an efflux of K^+-ions, which hyperpolarize the membrane potential leading to de-inactivation of the Na^+-channels. The sodium-potassium pump exchanges Na^+ for K^+ and restores their initial concentrations. The membrane potential then returns to its original value, i.e. the resting potential. The action potential propagates along the nerve cell at velocities between 0.5 and 150 m/s, depending on the type of nerve cell [33].

Table 2.1. Extracellular and intracellular ion concentrations for a human skeletal muscle cell and their respective thermal equilibrium potential. Adapted from Hille [31]

Ion	Extracellular concentration (mM)	Intracellular concentration (mM)	Equilibrium potential (mV)
Na^+	145	12	67
K^+	4	155	-98
Ca^{2+}	1.5	10^{-7}	129
Cl^-	123	4.2	-90

Ion channels contain many conserved regions and share several structural features. The pore region is usually comprised of multiple membrane-spanning segments, either originating from heterooligomeric or homooligomeric subunits, or from a single polypeptide chain with repeating transmembrane motifs [34]. The membrane-spanning regions are linked by hydrophilic loops that extend either on the intracellular or the extracellular side of the membrane.

Ion channels are often classified depending on their opening mechanism. Resting channels or leak channels are always open, e.g. certain K^+-channels, and they influence the resting membrane potential [34]. Gated channels open upon certain stimuli such as a change in voltage, binding of a ligand, change in temperature or mechanical stress [27].

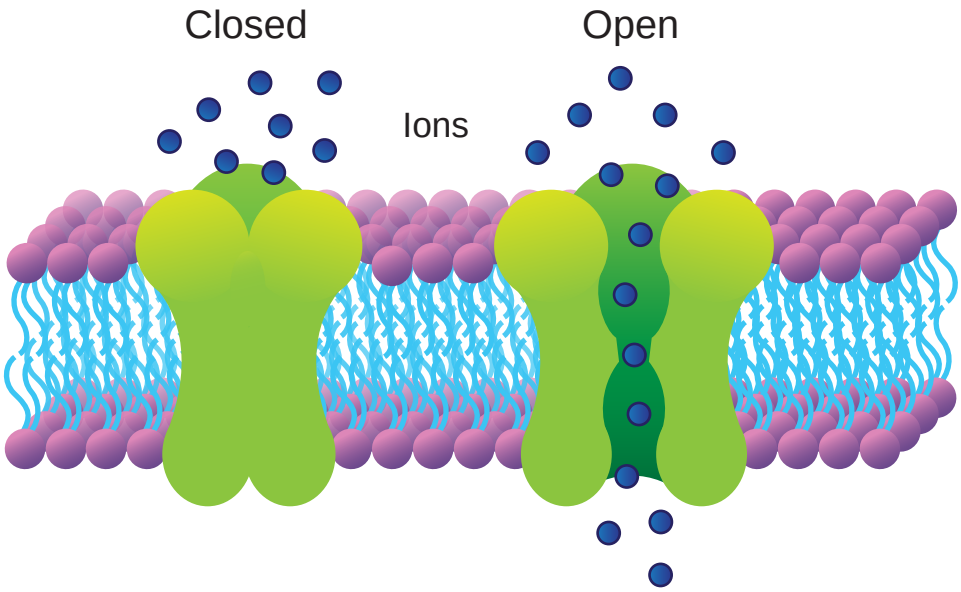

Figure 2.3. The α-helices enclosing an ion channel pore undergo a large structural rearrangement between the open and closed states of the ion channel. During activation, only specific ion species are allowed to pass through the pore.

Many ion channels rely on a specific lipid composition for their function, e.g. cholesterol is important for the function of several types of ion channels [35]–[40]. The effect of cholesterol levels on ion channel function has often been tested with the use of cyclodextrins, cyclic oligosaccharides that can both deliver or extract cholesterol from the cell membrane [41].

2.3.1 Membrane potential

Two main forces are governing the flow of ions across the cell membrane, the electrical potential of the cell membrane and the diffusive force down a concentration gradient [31]. The equilibrium potential when these two forces cancel out and there is no net movement of ions is called the reversal potential or the thermodynamic equilibrium potential. It is given by the Nernst equation:

$$E_S = \frac{RT}{z_S F} ln \frac{[S]_{out}}{[S]_{in}} \qquad \text{Eq. 1}$$

where E_S is the equilibrium potential for the ion S, R is the gas constant, T is the temperature, z_S is the ion-valency, F is Faraday's constant, $[S]_{in}$ is the concentration of S on the intracellular side and $[S]_{out}$ is the concentration of S on the extracellular side [31].

The membrane potential of a cell is a result of the equilibrium potential of all the ions that constitutes the intracellular and extracellular environment, as well as the permeability of the different ion species through the cell membrane which is dependent on the opening and closing of ion channels. The membrane potential can e.g. be calculated using the Goldman-Hodgkin-Katz equation:

$$E_m = \frac{RT}{F} ln \left(\frac{\sum_i^N P_{M_i^+} [M_i^+]_{out} + \sum_j^N P_{A_j^-} [A_j^-]_{in}}{\sum_i^N P_{M_i^+} [M_i^+]_{in} + \sum_j^N P_{A_j^-} [A_j^-]_{out}} \right) \quad \text{Eq. 2}$$

where E_m is the membrane potential, R is the gas constant, T is the temperature, F is Faraday's constant, P is the ion specific permeability and M^+ and A^- are cations and anions, respectively [31].

K^+-channels are generally the only ion channels open during the resting stage of the membrane. Hence, the cell membrane is only permeable to K^+-ions and the resting potential becomes equal to the reversal potential of K^+.

2.3.2 Ion permeability and selectivity

The rate by which an ion species will pass through an open ion channel is given by its permeability. The selectivity of an ion channel is in turn a description of how the permeability of different ions is relative to each other. Ion channels are in general highly selective for only one type of ion, while other ion species have low permeability, and ion channels are as a result often grouped into families based on the main ion which they conduct. The mechanism by which this selection occurs varies between ion species but is mainly accomplished by a sequence of amino acids inside the pore domain, defined as the selectivity filter [32]. The selectivity filter in K^+-channels is the most studied. The pore in a K^+ channel is lined with the amino acid sequence TVGYG which constitutes four binding sites that are selective for K^+ [42], [43]

Relative ion permeabilities can be determined experimentally by measuring and comparing the reversal potentials for two different ions. The reversal potential for the ion X and Y are given by:

$$E_{rev}^{X} = \frac{RT}{zF} ln \frac{P_X[X]_{out}}{P_Z[Z]_{in}} \qquad \text{Eq. 3}$$

and

$$E_{rev}^{Y} = \frac{RT}{zF} ln \frac{P_Y[Y]_{out}}{P_Z[Z]_{in}} \qquad \text{Eq. 4}$$

By subtracting the equation for ion X with the equation for ion Y we obtain how the difference in reversal potential relates to the permeability of ion X compared to ion Y [31].

$$\Delta E_{rev} = E_{rev,X} - E_{rev,Y} = \frac{RT}{zF} ln \frac{P_X[X]_{out}}{P_Y[Y]_{out}} \qquad \text{Eq. 5}$$

where P_Y is the permeability for ion Y, P_X is the permeability for ion X, $[Y]_{out}$ and $[X]_{out}$ are their respective extracellular concentration, ΔE_{rev} is the difference in reversal potential between ion X and ion Y, R is the gas constant, T is the temperature, F is Faraday's constant and z is the ion valence.

In permeability studies of ion channels, the extracellular concentration can commonly be controlled during measurements of ion channel activity and the reversal potential is determined using patch-clamp recording. The ion channel is subjected to a range of different membrane potentials and the potential at zero net current is the reversal potential.

2.3.3 Transient receptor potential vanilloid 1

The transient receptor potential vanilloid 1 (TRPV1) is an ion channel primarily expressed in dorsal root ganglia and trigeminal ganglia where it mediates nociception upon activation with various noxious stimuli. TRPV1 was first cloned in 1997 by Caterina *et al.* [44] after the discovery that certain sensory neurons responded to capsaicin, the pungent compound in chili peppers. It has since then been established that TRPV1 can be directly activated by capsaicin but also by low pH, high temperatures (T>42°C), anandamide, lipoxygenase products and *N*-arachidonyldopamine [44]–[50].

TRPV1 is a polymodal ion channel since it has the ability to respond to a wide range of noxious stimuli, e.g. chemical and thermal. TRPV1 is also responsible for inflammatory hyperalgesia which is an increased pain sensitivity towards noxious stimuli [51]. Inflammatory mediators such as, bradykinin [52], [53], extracellular ATP [54], nerve growth factor [53] and glutamate [55], are responsible for this effect since they can indirectly sensitize TRPV1 and lower its activation threshold toward chemical and thermal stimuli.

TRPV1 is a multimeric protein, consisting of four identical monomers. Each of the monomers has six transmembrane (TM) regions with the fifth and the sixth TM-regions positioned in the protein centre, enclosing the ion channel pore (Figure 2.4). The N- and C-termini of TRPV1 are located on the intracellular side of the cell membrane, comprising roughly 70% of the protein mass. This structural arrangement of TRPV1 has been determined largely based on similarities with the Kv1.2 channel [56], [57]. These models have been further validated with experimental data, using electron cryomicroscopy [58], [59].

The molecular mechanisms for TRPV1-activation with capsaicin, protons and heat are very different. Capsaicin is a hydrophobic molecule that can diffuse through the cell membrane. It activates TRPV1 through interaction with amino acids both in the TM-regions [60]–[62] as well as the N- and C-terminus [63], [64]. Protons activate and potentiate TRPV1 solely on the extracellular side where amino acids in the linker between TM-region 3 and TM-region 4, as well as inside the pore, have been shown to be important [46], [65], [66]. The mechanism by which temperature activation occurs is less characterized, but several regions have been implied to be involved in temperature gating, including parts of the C-terminal and an extracellular region referred to as the pore turret [56], [64], [67], [68].

TRPV1 is not only polymodal in its activation mechanisms but also in its selectivity. TRPV1 is permeable both to monovalent and divalent cations but with higher selectivity for divalent cations, especially Ca^{2+}. The permeability sequence has been determined for TRPV1 as $Ca^{2+} > Mg^{2+} > Na^{+} \sim K^{+} \sim Cs^{+}$ [44].

TRPV1 will desensitize, i.e. lose its activity upon constant application of a stimulus, in the presence of Ca^{2+}. The protein calmodulin has been proposed to be responsible for this mechanism by binding to the intracellular side of TRPV1. Two calmodulin binding sites have been determined, one in the N-terminus and one in the C-terminus [69]–[71].

If there are no Ca^{2+}-ions present, then TRPV1 will undergo a time-dependent increase in permeability towards large cations, which is referred to as pore dilation [72]. This phenomenon is exclusive for a few ion channels, including TRPA1 and P2X [73]–[78].

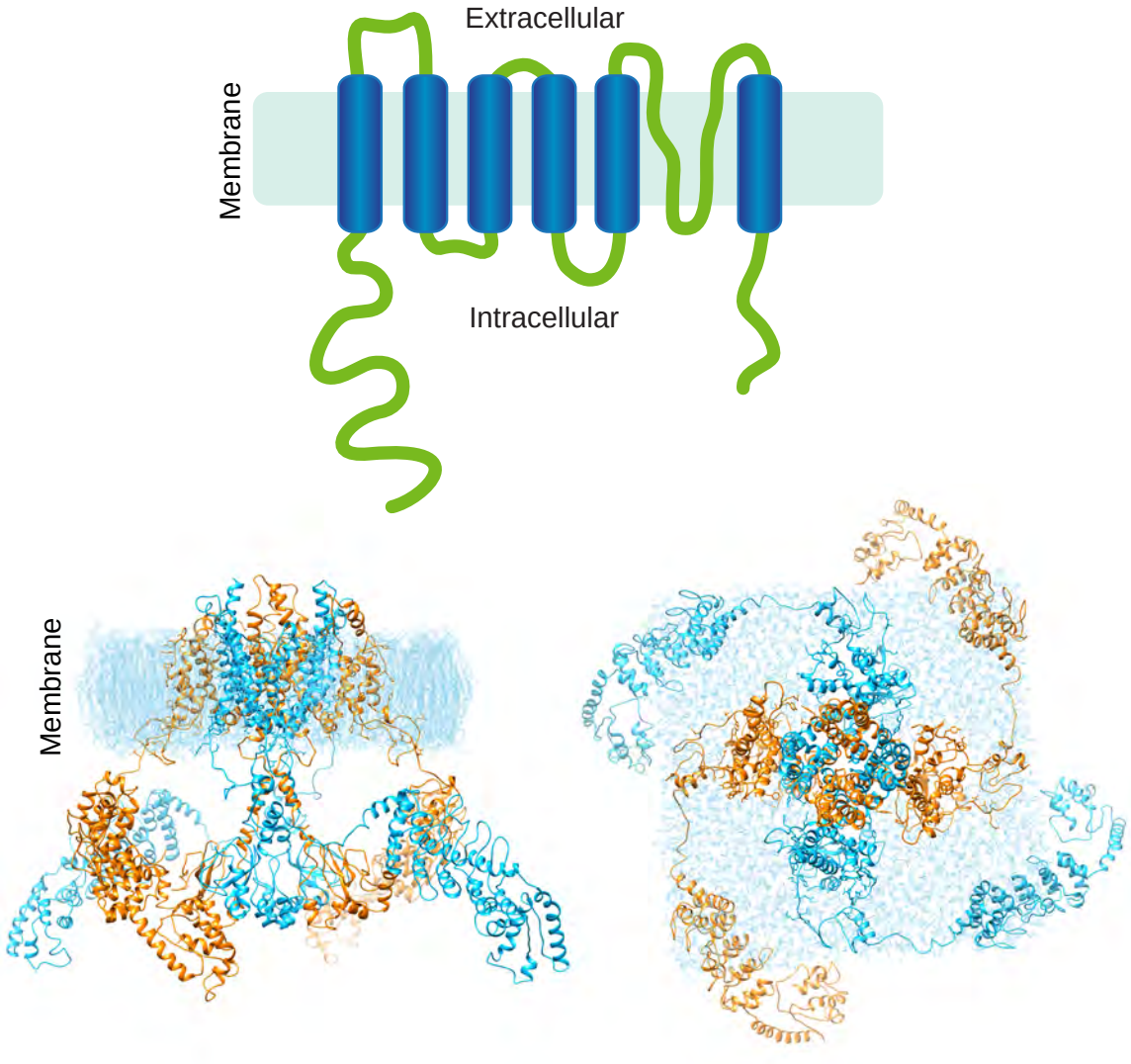

Figure 2.4 A schematic representation of a TRPV1 monomer (top) and their tetrameric arrangement (bottom). TRPV1 consists of four identical monomers, here colored in alternating blue and orange. Their organization residing in the cell membrane are here viewed from the side (bottom left) and from the extracellular side (bottom right)

TRPV1 is regarded to be an important drug target due to its role in pain reception and hyperalgesia [51], [79], [80]. Considerable efforts have been made to characterize its functional regions [81]. Molecules completely blocking TRPV1 activity have, however, so far had little therapeutic success. The ion channels sensitivity toward high temperatures was found to be an important regulator of basal body temperature, because complete blockade of ion channel response causes hyperthermia as a side-effect [82], [83]. More knowledge about this complex ion channel is needed in order to develop drugs that target specific pathways leading to hyperalgesia but not its normal physiological response.

3

ANTIBODIES

Antibodies are large, Y-shaped proteins that bind to and neutralize invading pathogens. They are produced by the adaptive immune system and developed to recognize foreign proteins by binding to specific sequences on the surface of these proteins [84]. Proteins that are capable of triggering an immune response and subsequent binding of antibodies are referred to as antigens, and the region of the antigen that is targeted by the antibody is referred to as an antigenic determinant or epitope [27].

Antibodies have the capabilities, beside binding and recognizing the antigen, to recruit other components of the immune system with the purpose of destroying the foreign entity. The tail of the antibody, called the Fc-region, can bind to receptors on the surface of different effector cells. Examples are effector cells that release toxic molecules and cause lysis of the target cell, or phagocytic cells, like macrophages and neutrophils, that ingest and destroy the antigen upon binding to the antibody. The antibodies can also bind to receptors on mast cells. Mast cells secrete cytokines and amines that dilate blood vessels, increasing the accessibility for immune cells to reach the infected site [27], [85].

The immune system is capable of producing antibodies with a vast diversity in order to be ready for any invading pathogen. Antibodies are synthesized by B-cells with each cell expressing just a single type of antibody with a unique antigen-binding site. In an early stage of B-cell development, the B-cell will express an antibody, targeting a random antigen, located on its cell surface. When a particular antigen invades for the first time, only a few antibody displaying B-cells will have antibodies that matches the structure of the antigen and can bind to an antigenic determinant on its surface. These B-cells will, however, be triggered by the binding and start to differentiate into larger effector cells, capable of producing and secreting large amounts of antibody, up to 2000 molecules per second [27]. Some of the B-cells will differentiate into memory cells. These are cells that will recognize the antigen if it appears a second time and will be able to produce and secrete antibodies at shorter time scales as well as provide immunological memory for a lifetime [27]. This diversity and selectivity of antibodies is crucial for our ability to defend ourselves against infection with harmful pathogens. Severe defects in the adaptive immune system can cause otherwise harmless infections to become lethal.

The immunological memory is the mechanism by which vaccines provide protection against infectious diseases. Vaccines are based on the introduction of a pathogen, or parts of a pathogen, into the body in a form that have been rendered inactive or weakened [86]. The vaccine components are chosen based on their immunogenicity, that is their ability to trigger an immune response but without causing a contagious or harmful version of the disease. The immune system will, as a result, produce antibodies against the pathogen after the vaccination event, and the pathogen will be directly recognized and destroyed during any subsequent infection. The first widespread use of a vaccine began in 1796, when Edward Jenner started performing vaccinations against smallpox using the cowpox virus originating from the same family. This finally led to the eradication of the disease in 1980 [87].

A technique to produce antibodies in host animals was developed in the 1970s [88], which has led to a rapidly growing field where antibodies are used as disease therapies. Such antibody therapies rely on the capabilities of antibodies to selectively bind to a drug target. A method for selection of antigenic determinants within a target protein and

subsequent generation of antibodies for therapeutic purposes was developed within the work that contributed to this thesis.

3.1 Structure

Antibodies belong to a class of proteins called immunoglobulins (Ig). These are globular proteins weighing roughly 150 kDa. The structural arrangement of an antibody is comprised of four monomers, two light chains and two heavy chains, held together in a Y-shaped structure by non-covalent bonds and disulfide bridges (Figure 3.1). Each antibody has two identical antigen binding sites, formed by one light chain and one heavy chain. The heavy chains are longer, 440 amino acids compared to 220 amino acids for the light chains. The exceeding parts of the heavy chains bind together and form the tail part of the antibody, called the Fc-region. This region binds to receptors on mast cells and phagocytes [27], [89]–[91].

The N-termini of the light and heavy chains are located in the antigen binding sites. These four ends each consist of a 110 amino acid-long variable region that are the key parts responsible for antigen binding. They are highly diverse in their sequence, allowing for many structural differences so that a matching antibody will exist for each possible antigen. Three segments on each variable chain serve as the actual binding site. They are called hypervariable loops and are 5-10 amino acids long [27], [89]–[91]. The corresponding binding site on the antigen is small compared to the actual size of the antibody, usually ~25 amino acids [27]. The parts of the antibody not located in the variable regions are called constant regions. There exist five different subclasses of immunoglobulins: IgA, IgD, IgG, IgE and IgM, each with a distinctive constant region of their heavy chains [27], [89]–[91].

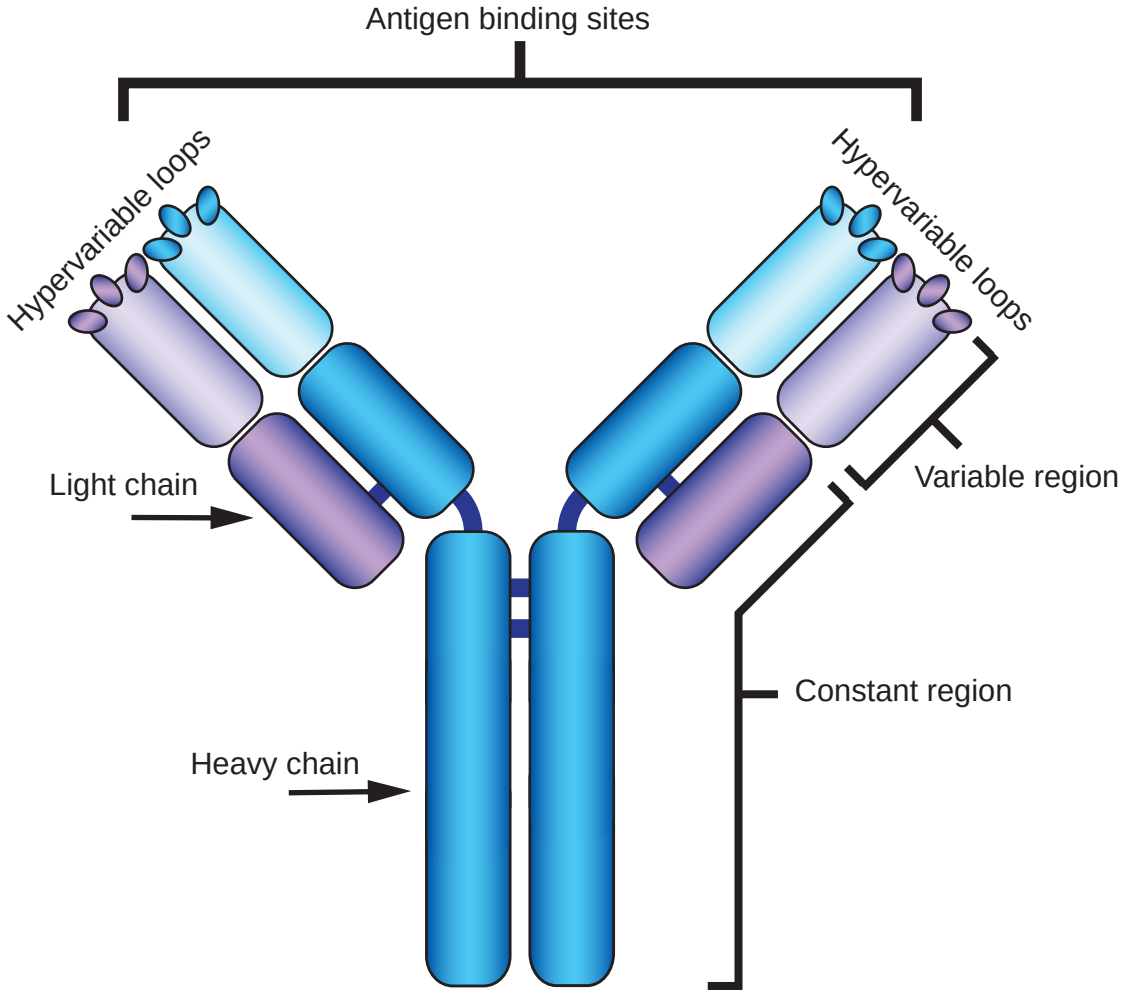

Figure 3.1 Schematic representation of an antibody structure. The heavy and light chains are colored in blue and purple respectively. The variable region of each chain is shaded in a lighter color.

3.2 Antibody therapeutics

Antibody therapeutics utilize nature's own capability to develop highly specific molecules able to bind to any protein target of interest. The first therapeutic antibody, Orthoclone, was FDA-approved in 1985 [92]. Antibodies as therapeutic molecules have since then emerged, being now the fastest growing class of therapeutic drugs [25], which includes successful drugs such as Humira (adalimumab, Abbott), Rituxan (rituximab, Biogen-Idec), Avastin (bevacizumab, Roche) and Remicade (infliximab, Janssen) [93]. These drugs were initially approved for rheumatoid arthritis, non-Hodgkin's lymphoma, colon cancer and Chrohn's disease, respectively, but have all been approved for other indications as well.

The mechanistic action of antibody drugs varies with each antibody and depends on the target protein of interest, e.g. an antibody may be able to abolish or decrease the activity of the target protein by steric hindrance upon binding. The antibody drug can also be designed to utilize the mechanism by which the Fc-region can bind to immune cells and trigger destruction of the antibody target. This is a common strategy for antibody derived cancer therapies where the antibody is designed to recognize and bind to antigens expressed on tumor cells [85], [94].

3.2.1 Development

A method for laboratory production of antibodies was first developed in 1975 and is called the hybridoma technique [88]. Mammals, usually mice, are injected with an antigen that triggers their immune response in order to start producing antibodies targeting the antigen. This process of introducing the antigen is called immunization. B-cells from the animal's spleen are then removed and subsequently fused with immortalized myeloma cells, which are cancerous B-cells. The fused cells are termed hybridomas and they have acquired the ability to proliferate indefinitely from the myelomas. The hybridomas are diluted down to single cells and separated into multi-well plates where they are further cultured into antibody producing colonies. Since one B-cell give rise to each separate colony, the produced antibodies in a single well will be identical, hence, called monoclonal antibodies.

The hybridoma technique will give rise to hundreds of different antibody producing B-cell cultures, with each antibody having a different antigen affinity and biological efficacy. These cultures need to be screened in order to find the most suitable candidate for drug development. The first step is to test the ability of the antibodies to bind to the antigen. This is done using antibody capture/antigen capture techniques, such as the enzyme-linked immunosorbent assay (ELISA), where the antigen is immobilized on a solid support and then subjected to an antibody. Binding between antigen and antibody is confirmed using a secondary antibody: a tagged antibody that is chosen to recognize the constant antibody regions from the species that produced the primary antibody. Other commonly used screening techniques are radioimmunoassays and immunofluorescence

[95]. The antibodies providing the highest ELISA signal are further evaluated for their biological efficacy using methods that are largely dependent on the type of antigen.

The first antibody therapies developed using the hybridoma technique were initially not very successful. Antibody drugs produced in murine hosts contain murine amino acid sequences that are recognized as foreign by the human immune system, thus triggering the production of human anti-mouse antibodies causing mild to lethal side effects [96]. Since then, advances have been made in order to minimize the murine content in therapeutic antibodies. Chimeric antibodies were the first attempt where the Fc-regions of the murine antibody was switched to its human counterpart [97]. This resulted in ~65% human sequences, and less, although not eradicated immunogenicity [98]. Further development have resulted in humanized antibodies with amino acid sequences that are ~95% human [99], [100]. Remaining exceptions are the hypervariable regions of the antigen binding site that remain murine. Fully human antibodies are now also possible. Transgenic mice containing human genes encoding for the heavy and light chains will produce human antibodies upon immunization [101], [102]. These advances in antibody technologies have led to a decrease in immunogenicity, and caused this therapeutic field to grow rapidly.

Today, strategies towards developing and finding successful immunotherapies are not limited to full size monoclonal antibodies. Due to advances in protein engineering, a wide variety of engineered antibody fragments (Figure 3.2) have been derived during the two last decades, including Fab fragments, ScFv fragments, diabodies, tetrabodies, antibody fragments functionalized with protein conjugates as well as bispecific fragments binding to two antigens [103], [104]. These new constructs provide a much larger toolbox when trying to develop antibodies and antibody-derived biologics with high specificity and affinity, deep tissue penetration, high stability and low toxicity.

An advantage with smaller antibody fragments compared to full size antibodies is that they can be produced in different expression systems, e.g. *E. coli*, yeast and mammalian cells, and are no longer limited to production with the hybridoma technique. This enables large scale production at lower cost. and provides many possibilities to genetically modulate antibody properties [105]. Antibody fragments can be displayed on the surface

of the filamentous bacteriophage, a so called phage display, used to create large antibody libraries which are screened against the desired antigen [106]. The screening procedure evaluates which antibody candidates are binding the antigen. It is often repeated in several cycles due to unspecific binding in the first cycles. The conditions during the screening cycles can be changed in order to find the best suitable candidates for a certain environment, e.g. more stable antibodies can be selected by using a harsh environment. Another method to select antibodies with very high affinity is to perform the screening with very low concentration of antigen so that only those antibodies capable of binding during such conditions, remain [107].

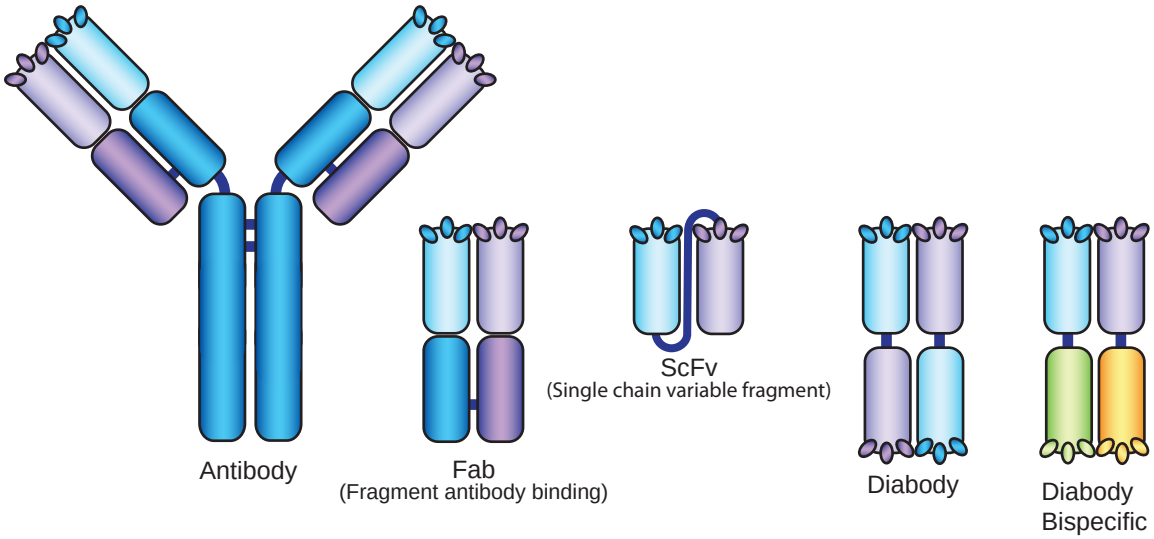

Figure 3.2 A selection of smaller antibody fragments developed from a full size antibody or through genetic engineering.

3.2.2 Antibody delivery

One of the main hurdles with antibody therapies is their restriction to extracellular targets. Antibodies are too large and too polar to enter through the cell membrane and are, generally, unstable in the reducing environment of the cytosol [108]. Several techniques have been developed in order to access intracellular targets, including transport of antibodies across the cell membrane with different transport vectors, e.g. protein transfection (profection) reagents and protein transduction domains (PTDs), as well as the expression of the antibody directly within the target cell, so called intrabodies [108].

Intrabodies can be constructed to target different cellular compartments by fusing the genetic sequence of the intrabody with intracellular trafficking signals. The need for efficient delivery methods is nonetheless a crucial step in intrabody therapy since the genetic material encoding the intrabody still needs to be delivered to the target cell [109].

Delivery of genetic material, drugs and proteins through electroporation have been widely used since its discovery in the 1980s [110]. Electroporation is a method by which transient pores are introduced in the cell membrane when applying an electrical pulse, facilitating uptake of exogenous substances. It has been used clinically for delivery of chemotherapeutic drugs to tumors [111], [112] and several DNA vaccines are in clinical trials using electroporation as the delivery method [113]. Electroporation of antibodies has not been as extensively studied as electroporation of chemicals and genetic material, but several groups have managed to electroporate and deliver antibodies with high viability of the electroporated cells [114]–[116]. Marschall et al., recently compared electroporation of antibodies to internalization through protein transduction domains and profection agents, and found electroporation to be superior [117].

Electroporation was used in the work performed for this thesis for intracellular delivery of antibodies designed to bind to the intracellular part of the ion channel TRPV1.

4

THE PATCH CLAMP TECHNIQUE

The currents mediated by ion channels upon their opening are extremely small, in the picoampere range, and may occur just for a few milliseconds. Patch clamp recording is an electrophysiological technique which was developed with the purpose of studying the electrical properties of ion channels in live cells. The technique is sensitive enough to register the small currents flowing through a single ion channel. It was developed by Erwin Neher and Bert Sakmann in 1976 [118] for which they were awarded the Nobel prize, and was further refined into the technique still used today, in 1981 [119].

Patch-clamp was used within this work to study the function of the TRPV1 ion channel.

4.1 Recording configurations and circuits

The principle of the patch clamp technique is to form a high resistance seal, a gigaseal (> 1 GΩ), between the tip of an electrolyte-filled glass micropipette and the cell membrane. This tight junction, prevents currents from escaping at the micropipette tip and is a prerequisite for low noise recordings of currents flowing through the cell membrane. A patch-clamp amplifier that is connected with the cell through two electrodes, one inserted in the electrolyte-filled micropipette and one reference electrode located in the bath solution surrounding the cell, is used to measure and control the electrical properties of the cell [120].

Patch clamp recordings can be performed in four different configurations, i.e. cell-attached, whole-cell, inside-out and outside-out (Figure 4.1). These different configurations allow for electrophysiological recordings of either a population of ion channels or single ion channels. The cell attached patch configuration is the configuration when a single cell is attached to the micropipette with a gigaseal. The configuration is achieved by approaching the cell with the micropipette, aided by a light microscope. When the pipette touches the membrane, a negative pressure is applied, causing a small part of the membrane to get sucked into the tip, forming a seal. Using this configuration, measurements can be made on ion channels residing in the membrane patch that covers the pipette tip which can be single channels depending on the protein expression level and size of the pipette tip. This configuration allows for recordings to be made of ion channels in their normal cellular environment, but the membrane potential and the cytosolic solution cannot be precisely controlled. The cell attached configuration serves as a starting point for both the whole cell configuration and the inside-out configuration. If the pipette tip is slowly removed from the cell attached configuration, then a part of the membrane will be torn away and remain sealed to the pipette tip. This is called the inside-out configuration, since the inside of the membrane is exposed to the surrounding bath solution. This is also a technique to achieve single channel recordings while the intracellular side of the membrane can be exposed to different types of solutions. Applying a slight suction, or negative pressure, during the cell attached configuration, will produce a rupture of the cell membrane beneath the pipette tip, and the measuring

electrode will be in direct contact with the cytosol via the electrode buffer. This configuration, whole-cell recording, will measure all ion channels simultaneously while the membrane potential can be precisely controlled or clamped. The last configuration is the outside-out configuration. When in the whole-cell configuration, one can remove the pipette tip from the cell. This will tear a part of the membrane away from the cell. This membrane patch will reseal and form a small vesicle at the pipette tip. Now, the extracellular part of the membrane is exposed to the bath solution. This configuration is useful when performing single channel recordings and the ion channel is modulated from the extracellular side [120].

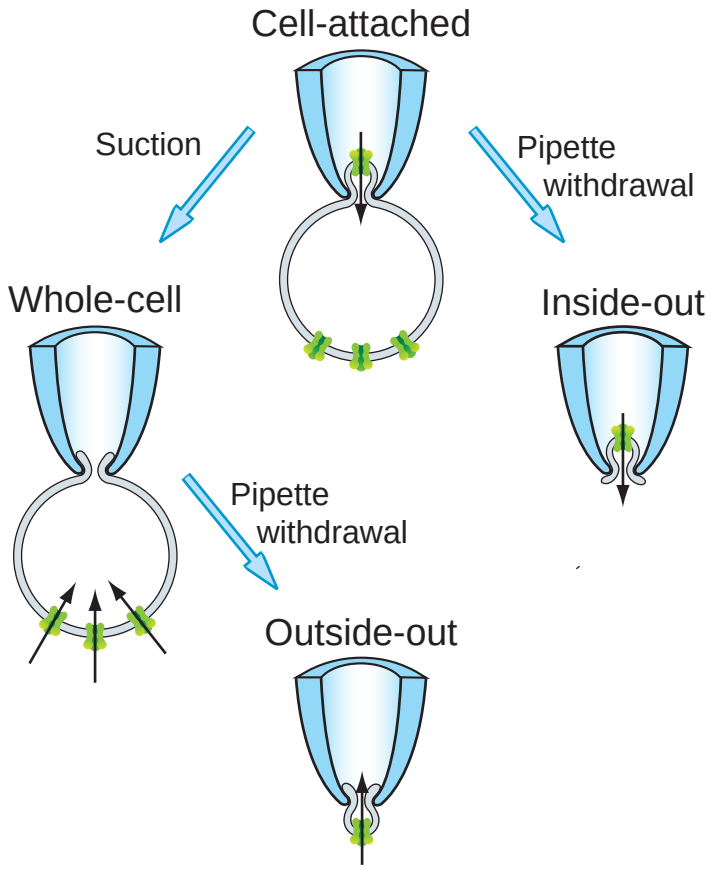

Figure 4.1 Different patch-configurations that can be achieved from the on-cell position by applying suction or by removal of the pipette. The black arrows denote the ion flow direction. Here exemplified for an ion channel which normally cause an influx of ions.

Recordings of ion channel activity can be performed using two different setups; the voltage-clamp or current-clamp modes. In current-clamp mode, the current is held constant while the membrane potential is measured. This method is commonly utilized for the measurement of action potentials mediated by voltage-dependent ion channels. In

voltage-clamp mode the membrane potential is held constant while the current is measured. In order for the membrane potential to be constant, the patch clamp amplifier will continuously measure the potential and compare it to a set value, the holding or clamp potential. If it deviates, the amplifier will inject a small current to compensate for the difference. During activation of an ion channel, the membrane potential will change due to the current. The amplifier will then compensate with the same amount of current, but in opposite direction, in order to keep the membrane potential constant. The injected current is then equal, but with the opposite sign, to the current conducted by the ion channel, and is the current recorded by the amplifier.

Several parameters of the patch clamp setup can influence the recordings and can give rise to errors during the measurements that need to be minimized. The membrane itself acts as an insulator between the cytosol and the bath solution. It then behaves as a capacitor ($C_{membrane}$) that can store charge but also as a resistor ($R_{membrane}$) since it contains ion channels that can transport charges upon opening. An equivalent circuit for a whole cell configuration is shown in Figure 4.2. The interior of the pipette will act as a resistor ($R_{pipette}$) and the glass wall a capacitor ($C_{pipette}$). In addition the pore between the pipette tip and the cytosol will also act as a resistor contributing with the access resistance (R_{access}). The access resistance is configured in series with the pipette resistance and together with the membrane capacitance it forms an RC circuit. These parameters influence the measurements by causing delays during voltage changes. The amplifier has the capability to compensate for some of these factors, but it is important to minimize these resistances, e.g. by using low resistance glass pipettes with a large tip [120].

Two of the configurations described above, i.e. whole-cell and inside-out, were used within the work of this thesis to record current responses mediated by the TRPV1 ion channel. The voltage clamp mode was utilized in order to measure current amplitudes, reversal potentials and EC_{50}-values. The EC_{50} -value for an ion channel agonist is the concentration of this agonist activating a half maximal current response recorded by one of the configurations described above. It is experimentally determined by measuring current responses activated by a number of different agonist concentrations. The currents responses are fitted against a dose-response curve, commonly a four-parameter sigmoidal function.

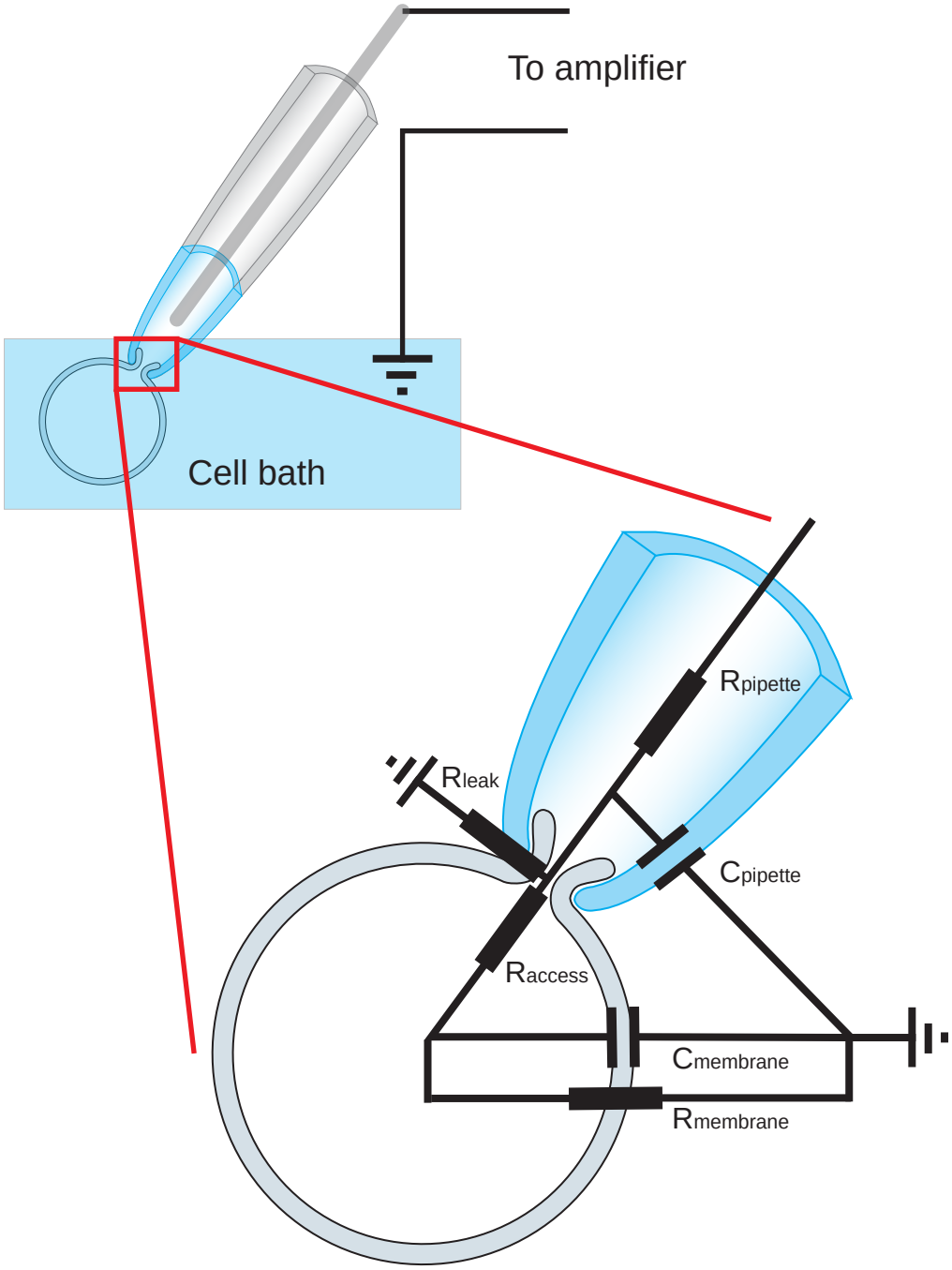

Figure 4.2 Equivalent circuit of a whole-cell patch configuration

5

PROTEOMICS

The proteome of an organism or a cell is its entire set of proteins, expressed at a given time. The term was first coined by Marc Wilkins in the 1990s [121] in alignment with the term used to describe the genetic material of an organism, the genome. While the genetic material of an organism is constant, the proteome is highly dynamic, with the number and identity of proteins being expressed varying greatly with time. The study of the proteome, i.e. how proteins are expressed, how they interact and how they are modified, is called proteomics [122], [123]. One of the first techniques to be used for large scale protein analysis was two-dimensional gel electrophoresis [124]. However, it has now been largely replaced by mass spectrometry as the gold standard for proteomic analysis [125], [126].

Mass spectrometry was used within the work of this thesis to identify proteins and peptides, the principles of which are both described below.

5.1 Mass spectrometry in proteomics

Mass spectrometry is the most widely used technique in proteomics and is used for analysis of protein identity, protein sequencing, studies of post translational modifications (PTMs) and protein interactions [127]–[129].

The mass spectrometer analyzes molecules based on their mass and charge, more specifically their mass to charge ratio (*m*/*z*). Ionization is the first step where the analytes aquire one or more charges. The generated ions are then detected based on their mass to charge ratio inside a mass analyzer. The number of ions for each *m*/*z* is detected and the output is a mass spectrum with the intensity of each ion plotted against its mass to charge ratio. Initially, the method was limited to small volatile compounds but in the mid 1980s two new ionization techniques were developed, matrix-assisted laser desorption ionization (MALDI) [130] and electrospray injection (ESI) [131] that can be used on biomolecules. Since the development of these ionization techniques, the field of mass spectrometry-based proteomics has expanded rapidly, with a continuous development of more accurate and sensitive equipment with higher resolving power.

There are several different types of mass analyzers that can be used for proteomics, such as time of flight (TOF) mass analyzers, quadrupoles, ion traps (quadrupole and linear), orbitraps and Fourier-transform ion cyclotron resonance (FT-ICR) mass analyzers. It is also common to use these instruments in combination with each other [127], [132], [133].

5.1.1 Protein identification and peptide sequencing

Mass spectrometry based proteomics is generally performed either using a top-down or a bottom-up approach. Top-down mass spectrometry analyses entire proteins and is e.g. used for the study of peptide hormones [134] and PTMs [135]. The bottom-up approach, which is more common, performs the analysis on small peptides rather than entire proteins. A protease, commonly trypsin, can be used to degrade the protein into smaller peptides before analysis [133]. The generated peptides are separated, commonly using liquid chromatography (LC), and then identified with the mass spectrometer using a process called peptide mass finger-printing (PMF). Large databases have been established, where all known protein sequences for an organism have been *in silico*, i.e.

theoretically, digested with different proteases. The masses of the peptides are then compared to the *in silico* digested proteins in order to match them to the correct protein. Detection of several peptide masses matched to the same protein is needed for a true identification [136]. An advantage with this technique is that peptides are more easily ionized than entire proteins, thus yielding an increased sensitivity. Peptides are generally easier to dissolve and they are in a mass range suitable for mass spectrometric analysis [137]. However, two peptides of equal masses cannot be distinguished from each other, which can easily occur in digests from protein mixtures.

To further aid the identification of a protein, mass spectrometry can be used to determine the protein amino acid sequence by utilizing a mass spectrometer working in tandem, either with several separate mass analyzers or with one mass analyzer, cycling between detection modes. First, the mass analyzer separates the peptide parent ions based on their mass to charge ratio. One peptide parent ion at a time is then fragmented into smaller ions, often using collision-induced dissociation (CID), a method where the peptides fragment upon collision with an inert gas [138]. The collision causes fragmentation to occur primarily at the peptide backbone and at three different sites. This can yield six different ion types depending on which side the charge remains after fragmentation as can be seen in Figure 5.1. These ions are named a, b, c and x, y, z according to the nomenclature developed by Roepstorff-Fohlmann-Biemann [137]. Using CID will mainly yield b- and y-ions. B-ions have the charge retained on the N-terminal side of the precursor ion and y-ions have the charge retained on the C-terminal side. All the fragments from each peptide type are separated based on their mass to charge ratio in the mass analyzer. The produced spectra can be used to determine the sequence of the specific peptide. In theory, if all possible b- and y-ions are present from a peptide, the sequence can be determined by comparing the mass difference between each peak in the spectrum. Each difference will correspond to the mass of an amino acid. This method of determining the protein sequence directly from the fragment spectra is called *de novo* sequencing [139]. However, all b- and y-ions are in reality rarely present but are accompanied by fragments such as immonium ions and others caused by e.g. neutral loss of water or ammonia [136], [140]. *De novo* sequencing thus requires careful analysis of each spectra and is less suitable for high-throughput screening with bottom-up mass

spectrometry. These limitations can be overcome by matching the mass spectrum with databases of known protein sequences. Even if the sequence of a peptide is incomplete it can still be unique, only matching the sequence of a single protein. The databases are searched using different algorithms such as MASCOT [141], SEQUEST [142] and X!TANDEM [143].

A problem with assigning peptide and protein identities based on partial peptide sequences is the risk of false positives. Several of the search engines score each peptide match based on how well the fragment spectra matches the database spectra. An additional method in order to limit false positives is to search the mass spectra against a decoy database, e.g. using the reversed sequences of the predicted peptides. A threshold for a false discovery rate (FDR) can be applied to the dataset. This gives an estimate on how many fragment spectra that are matched against false peptides. [144], [145].

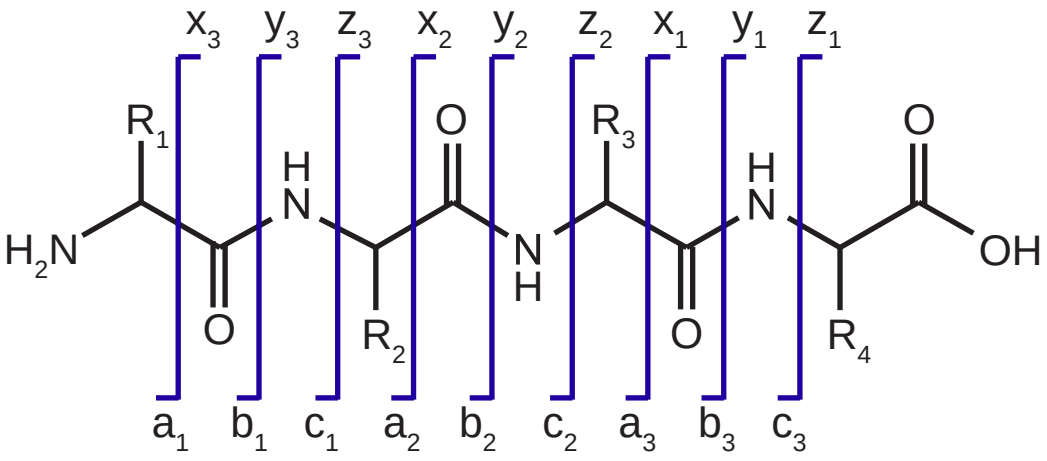

Figure 5.1 Peptide fragments according to the nomenclature developed by Roepstorff-Fohlmann-Biemann

A limitation with bottom-up proteomics is that full sequence coverage of a protein is rarely achieved, leading to loss of information regarding protein isoforms, such as alternative splicing and PTMs. The top-down proteomic approach has instead been utilized for such studies of protein modifications where entire proteins are analyzed and fragmentation of the protein occurs inside the mass spectrometer. This is in contrast to bottom-up proteomics, where proteins are digested prior to analysis [146]. This yields data with potentially full sequence coverage, suitable for investigations of even small

variations in protein structure. The spectra produced after fragmentation of entire proteins are, however, much more complex than spectra achieved from fragmentation of peptides. High resolution mass spectrometers are thus needed for top-down proteomics, and the ability to analyze protein mixtures, as well as high throughput proteomic profiling, is limited [147], [148]. Another drawback is that larger proteins (>50 kDa) are more difficult to fragment [147], [149] which has restricted the use of top-down mass spectrometry to smaller proteins. Bottom-up mass spectrometry has thus been more widely used within the proteomics field but technical advances in instrumentation and separation techniques have led to an increasing interest in top-down mass spectrometry. These advancements resulted in the development of techniques for studies of larger proteins [150], as well as complex protein mixtures [151], using top-down mass spectrometry.

5.2 Limited proteolysis

Membrane proteins are important drug targets, but their hydrophobic parts complicate elucidation of their structure, information that could facilitate drug development to these targets. Several membrane proteins, however, contain hydrophilic regions on one or both sides of the cell membrane. Identification of surface residues within these hydrophilic regions is not only useful when determining protein structure, but also an important parameter when predicting ligand binding sites [152]. Limited proteolysis in combination with mass spectrometry is a suitable method to evaluate surface topology of a protein, by restricting the activity of a protease to digest only the most flexible and surface-exposed parts. This can be achieved, specifically, by controlling the activity of a protease using e.g. low temperatures, low concentrations and/or short digestion times followed by identification of the digested regions with mass spectrometry [153]–[155].

When limited proteolysis has been performed on proteins with known structure, mainly three structural determinants have been recognized as having impact on the sites of proteolytic activity, namely flexibility, surface exposure and local interactions [154], [156], [157]. In order for the peptide chain to enter the active site within a protease, flexibility and the ability to locally unfold is required. Surface exposure renders a cleavage site more likely for proteolysis. This is due to the fact that regions at the surface

tend to more easily unfold as well as impose less steric hindrance on the protease. Finally, the amount of local interactions, like hydrogen bonds and disulphide bridges determines the rate of proteolytic activity. Less local interactions favor proteolysis. All three of these structural determinants are usually correlated within the protein. Hence, limited proteolysis will mainly cleave surface exposed regions given that the protein chain can unfold locally. It has been used as a method to determine flexible regions in proteins with unknown detailed structure [153], [158], [159], as well as to identify stable regions resistant to proteolysis and suitable for crystallization [160], [161].

5.3 Proteoliposomes

The occurrence of a proteolytic event during limited proteolysis is determined by the tertiary structure of the protein rather than the amino acid sequence, hence a native, correctly folded structure is crucial. When performing limited proteolysis on membrane proteins, they need to be situated in a lipid environment in order to be correctly folded and for proteolysis to yield useful structural information. A common method to protect the native structure of membrane proteins is to maintain them in lipid vesicles. Such proteoliposomes can be reconstituted from purified membrane proteins and lipids [162], [163] or be directly derived from the cell membrane e.g. through blebbing [164], [165] or through lysis of the cell [166]. An advantage of using proteoliposomes derived directly from the cell membrane is that they contain the correct lipid composition for membrane protein function as well as other interacting proteins and co-factors. However, when performing limited proteolysis on proteoliposomes, fast and sensitive mass spectrometers are required since the proteolytic peptides will originate from several hundreds of proteins residing in the proteoliposome as well as membrane associated proteins.

Within this work, proteoliposomes were derived from Chinese hamster ovary (CHO) cells, expressing the human TRPV1 ion channel, by lysing the cells with mechanical disruption using a Dounce homogenizer [167], followed by centrifugation steps to remove nuclei and mitochondria. Proteoliposomes (50-150 nm in diameter) were subsequently formed from this membrane fraction by tip-sonication (Figure 5.2). These proteoliposomes were used during functional and structural studies of TRPV1, e.g. evaluation of surface topology, using limited proteolysis.

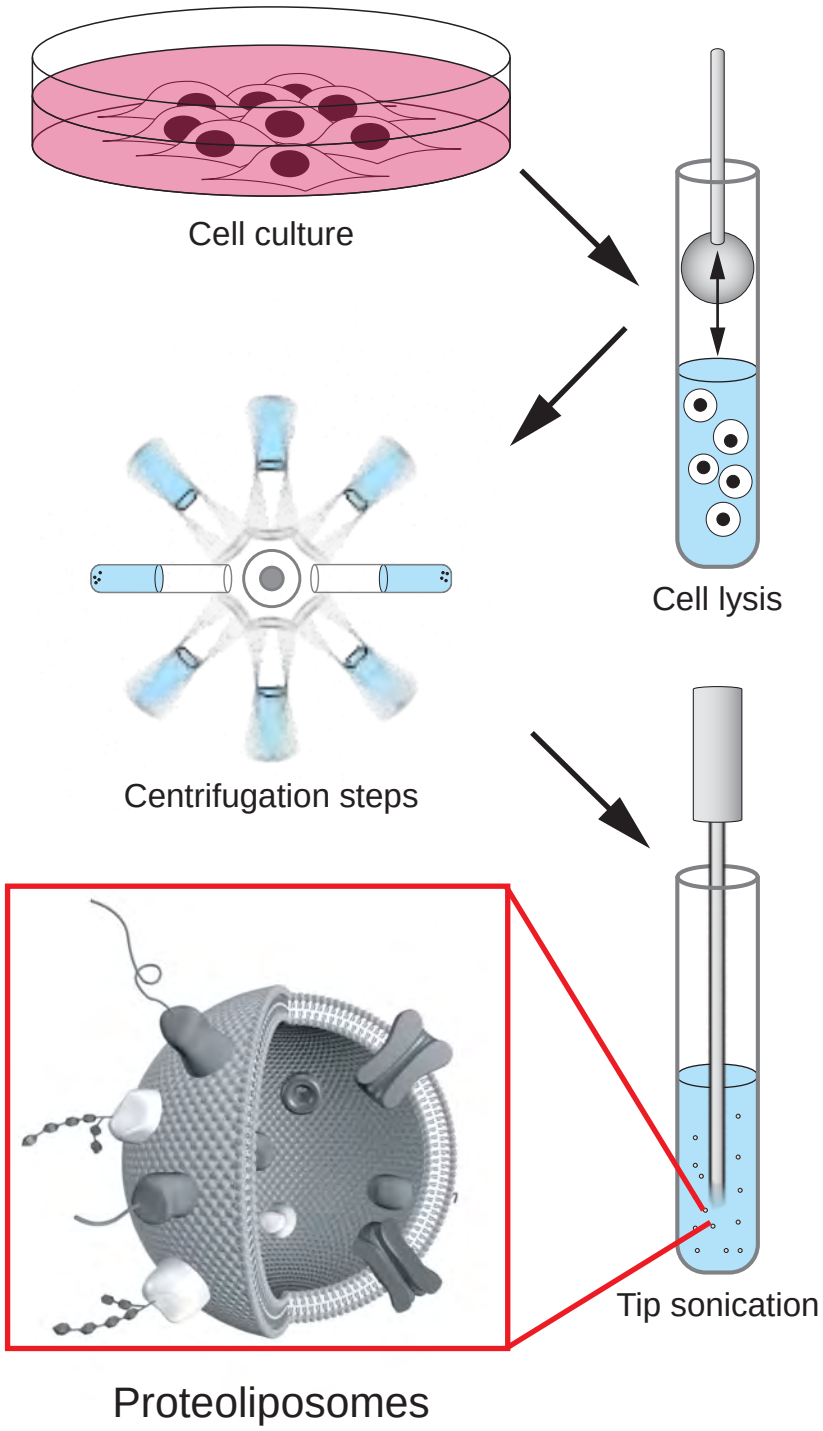

Figure 5.2 Schematic representation of proteoliposome preparation. Cells are cultured and harvested followed by lysis, using osmotic pressure and mechanical disruption. The lysed cells are then centrifuged in several steps to remove nuclei and mitochondria. Finally, proteoliposomes are formed from the remaining membrane fraction, using tip-sonication.

6

OPTICAL MICROSCOPY

Microscopy is the technique of investigating objects that are too small to visualize with just the naked eye. Optical microscopes are the oldest class of microscopes and utilize light and a system of lenses to magnify the microfeatures of the specimen. The lateral resolution of optical microscopes, i.e. the smallest feature that can be distinguished, is dependent on the numerical aperture (NA) of the objective and the wavelength (˜) used to visualize the specimen, according to the Rayleigh criterion [168], [169]

$$R = 0.61\frac{\lambda}{\mathrm{NA}} \qquad \text{Eq. 6}$$

The numerical aperture is given by:

$$\mathrm{NA} = n\sin\theta \qquad \text{Eq. 7}$$

where *n* is the refractive index of the media between the objective and the specimen and θ is the half-angle of the maximum cone of light that can enter or exit the lens. The

refractive index is 1.0 for air, 1.33 for water and 1.56 for oil. Using an oil immersion objective together with a wavelength of 400 nm gives a resolution limit of ~200 nm.

Optical microscopy has been used in this thesis work as visual guidance during patch-clamp experiments and to detect and measure fluorescence with laser scanning confocal microscopy, the principles of which are both described below.

6.1 Fluorescence

Fluorescence is phenomenon where a molecule absorbs light and emits the acquired energy as light of a longer wavelength. The energy of a photon that is absorbed by a fluorescent molecule is inversely proportional to its wavelength and can be described as

$$E = \frac{hc}{\lambda} \quad \text{Eq. 8}$$

where *h* is Planck's constant, *c* is the speed of light and λ is the wavelength.

During absorption of a photon, the energy electronically excites the molecule from a ground state, S_0, to a higher energy state, S_1. At each of these electronic states, the energetically elevated electron in the molecule can exist at several different vibrational levels. These energy sub-states are transient and the molecule will rapidly relax back to the lowest vibrational level of the first singlet state S_1, through vibrational relaxation. To further relax to the ground state S_0, the molecule emits a photon. The emitted photon will have a longer wavelength than the absorbed photon due to loss of energy during vibrational relaxation. This shift in wavelength is called the Stokes shift [170]. The different electronic states of a fluorescent molecule can be visualized using a Jablonski diagram (Figure 6.1).

Molecules that have the ability to fluoresce efficiently are called fluorophores. The efficiency of a fluorophore is given by its quantum yield, defined as the probability of an emitted photon per absorbed photon. It describes how well the energy from the incident light is converted to emitted light. The quantum yield of a fluorophore is generally dependent on its environment. Many fluorophores have a high quantum yield in aqueous solutions but there are also fluorophores that start to fluoresce efficiently, i.e. they exhibit

a rapid increase in their quantum yield, upon binding to other molecules, e.g. different ions.

Fluorophores are used extensively in life science research, where they are mainly used to label different biological structures. Fluorophores that have been utilized in this thesis work are Fura-2, a calcium indicator which shows increased fluorescence upon binding to calcium [171]; YO-PRO, a dye that can pass through active TRPV1 ion channels and increases its quantum yield upon intercalation into DNA [72], [172]; and Alexa 488 [173] which was tagged to goat anti-rabbit antibodies for studies using immunofluorescence.

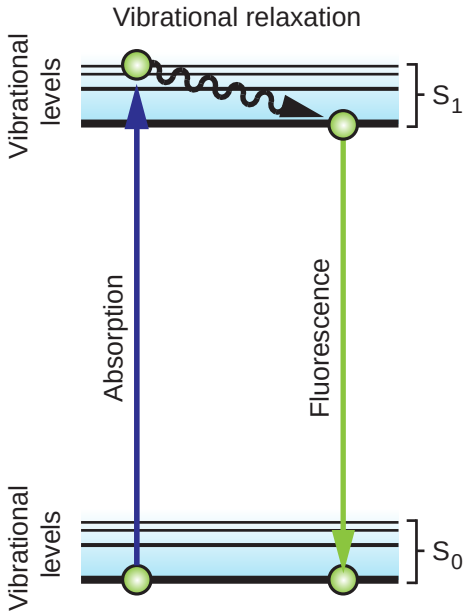

Figure 6.1 A simplified Jablonski diagram showing absorption followed by vibrational relaxation and fluorescence. Electronic excitation to higher states and relaxation routes other than fluorescence are omitted.

6.2 Laser scanning confocal microscopy

A laser scanning confocal microscope is a microscope developed to visualize fluorescent specimens with improved resolution compared to conventional fluorescence microscopes. It is commonly used in biology to study both live and fixed cells. The specimen is excited with a laser that scans the illuminated area in a lateral motion. The light path of both the excitation and emission can be seen in Figure 6.2. The laser is reflected by a dichroic mirror and focused onto the specimen by the objective. The emitted light is collected by the objective, passed through the dichroic mirror followed by a pinhole aperture before it

reaches the detector. During excitation, fluorophores in several layers of the specimen will be excited and emit light. This out-of-focus light blurs the collected image. The pinhole, is positioned in front of the detector so that only light that originates from the focal plane, is allowed to reach the detector. This removes out-of-focus light and facilitates the collection of sharp images, producing thin optical slices or sections of the specimen. A 3D-image of the specimen can be constructed if the optical sections are collected at varying heights thorough the sample and combined [168].

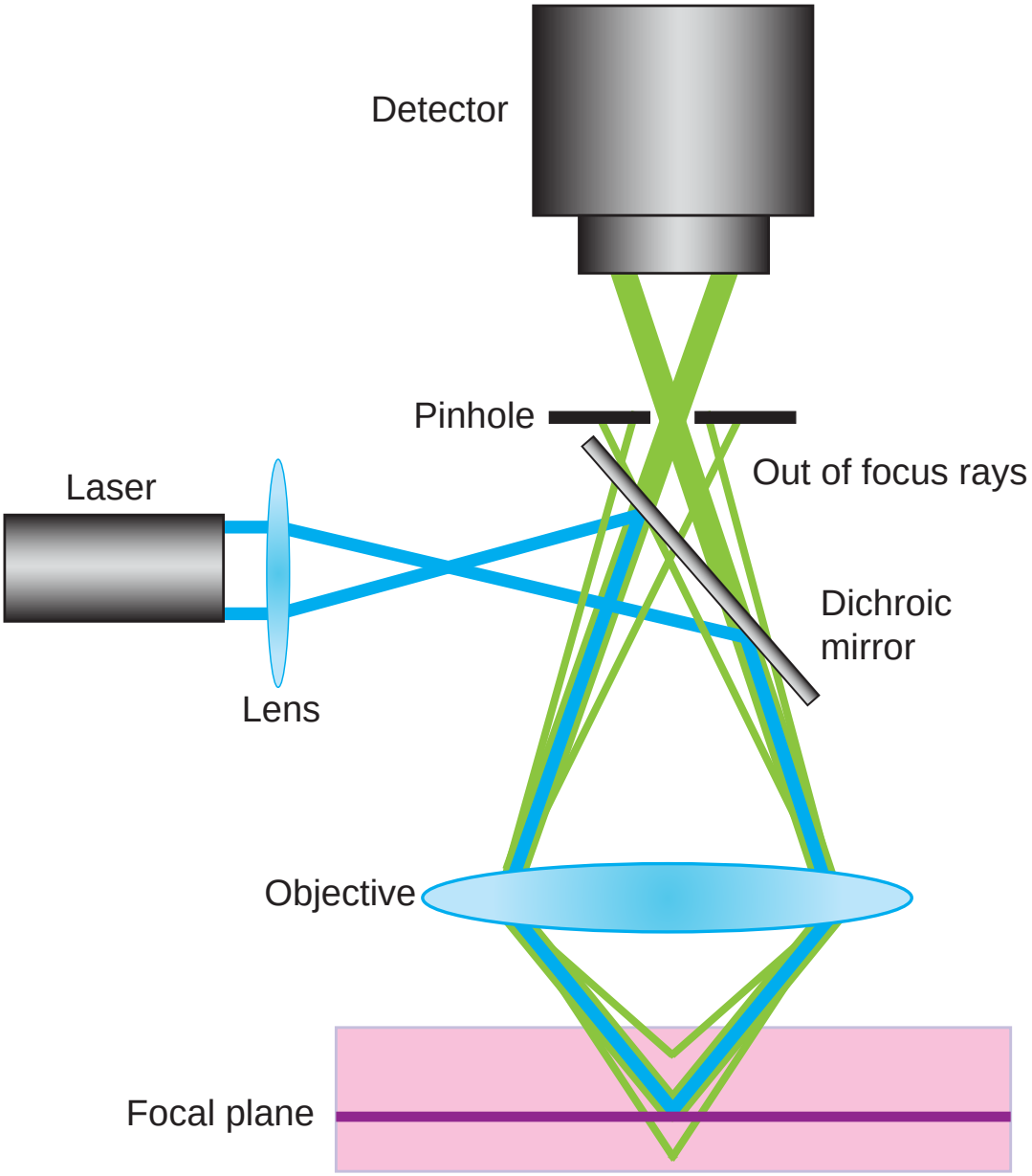

Figure 6.2 A simplified schematic illustration of the beam pathway in a confocal microscope. Only light emitted from the focal plane can pass the pinhole and reach the detector. Out-of-focus light (thin green lines) is blocked by the aperture wall.

6.3 Immunofluorescence

Immunofluorescence is a technique used to visualize different cellular components through targeting with fluorescently labeled primary and secondary antibodies (Figure 6.3). Primary antibodies bind to an antigen within a protein, whereas secondary antibodies bind to another antibody. Visualization of cellular structures can be achieved by directly using primary antibodies with fluorescent tags, or indirectly with untagged primary antibodies that are subsequently recognized with fluorescently tagged secondary antibodies [174]. The secondary antibodies have been designed to bind to the constant region of antibodies from another species, e.g. if the primary antibodies were raised in mouse, then the secondary antibodies are chosen to recognize the constant region of a mouse antibody.

Antibodies cannot cross the cellular membrane so internal structures are difficult to stain in live cells. If the goal is to visualize internal structures, then the cells are commonly fixed [175]–[177], a process that cross-links proteins and permeabilizes the cell membrane.

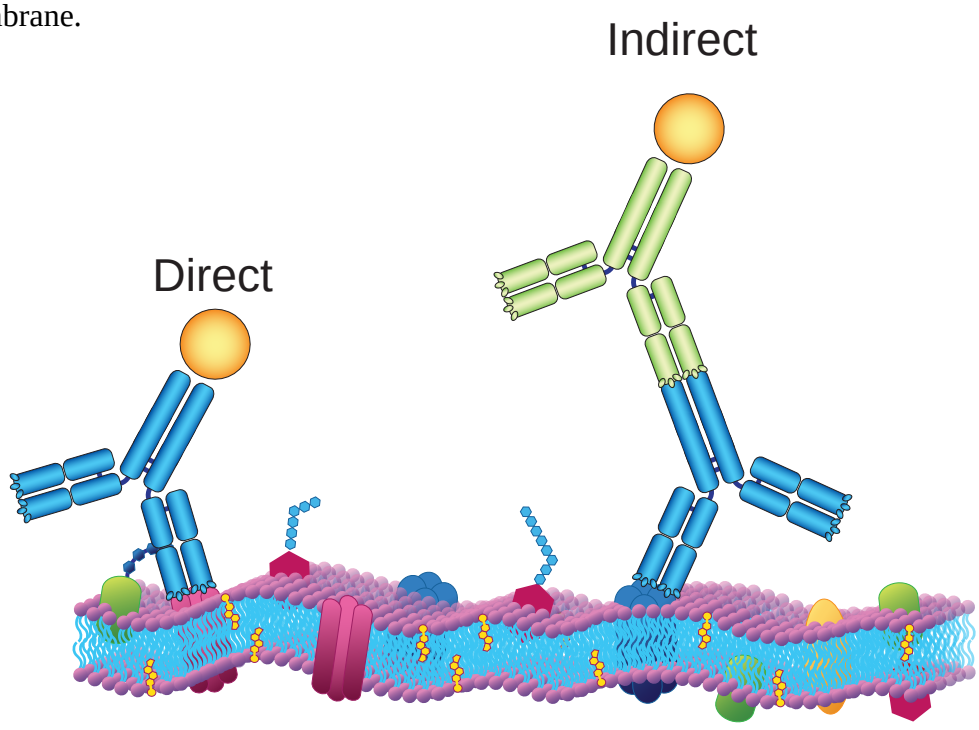

Figure 6.3 Direct and indirect labeling of cellular components using fluorescently tagged antibodies. Primary antibodies binding to the cell component of interest are fluorescently tagged and used for direct labeling. Untagged primary antibodies are used for indirect labeling, where a secondary, fluorescently tagged antibody bind to the primary antibody.

7

MICROFLUIDICS

Microfluidics describes the handling and manipulation of small volumes of fluid, picoliters to nanoliters, inside micrometer scaled channel systems. Fluids at such scales have different characteristics compared to macroscopic systems. Fluids in motion can be described with the Navier-Stokes equation [178]:

$$\rho \left(\frac{\partial v}{\partial t} + v \cdot \nabla v \right) = -\nabla p + \eta \nabla^2 v + F \qquad \text{Eq. 9}$$

where v is the velocity of the fluid,ρ is the density of the fluid, p is the pressure, ˜ is the viscosity of the fluid and F are the external forces acting on the fluid. The Navier-Stokes equation is an application of Newton's second law and describes an incompressible Newtonian fluid. It can be solved into a velocity field for some specific cases. The left hand side of the Navier-Stokes equation describes inertial forces and the right hand side describes forces acting on the fluid such as pressure and viscosity.

When inertial forces are high, the fluid will exhibit chaotic turbulent flow. However when inertial forces are low and viscous forces dominate the flow will become ordered and laminar. The ratio of inertial force to viscous force is described by the Reynolds number:

$$Re = \frac{\rho \upsilon L}{\eta} \qquad \text{Eq. 10}$$

where υ is the velocity of the fluid, ρ is the density of the fluid, η is the viscosity and L is the characteristic length of the channel. The viscous forces dominate and the flow is laminar when the Reynolds number is low. The transition between laminar and turbulent flow in a pipe occurs around Re = 2040 [179]. During laminar flow, the fluid flows in parallel segments and mixing between layers occurs primarily through diffusion [180].

7.1 Superfusion

Superfusion is a technique for delivery of compounds to a tissue or cell through the use of a continuous flow over the tissue or around the cell. Rapid superfusion, i.e. the delivery of active compounds present in the fluid, is crucial for the study of fast biological processes such as kinetic studies of ion channels. The solution exchange around the cell expressing the ion channel needs to be faster than the activation time of the channel, otherwise any measured current will have a rise time that is dependent on the solution exchange. Early devices for fast solution exchange were fabricated from glass pipettes. One such device is the ˜-pipette, which have an internal barrier and are thus capable of deliver two different solutions simultaneously [181]. Rapid solution exchange is achieved either by movement of the cell relative the pipette or movement of the pipette itself. The ˜-pipettes have been extended into a multi-barrel system where several pipettes are used together to deliver multiple solutions. Today, cellular scale superfusion is mainly achieved through the use of microfluidic devices, owed to the precise manipulation of flows in such devices, the ability to handle and test many different solutions, and the small volumes of reactants needed in comparison to bulk experiments.

7.1.1 Dynaflow chip

Superfusion during the patch-clamp experiments described in this thesis was achieved using the Dynaflow chip series, commercially available through Cellectricon AB

(Mölndal, Sweden). They are either fabricated entirely in glass or in polydimethylsiloxane (PDMS) bonded to glass. These microfluidic devices have a pre-defined number of sample reservoirs, 8 and 16 were used through this thesis, with each reservoir entering into an individual channel. The channels are arranged in a closely packed parallel array with exits into an open volume (Figure 7.1). The sample reservoirs are sealed with a lid connected to a pneumatic pump, generating a flow through each channel simultaneously upon application of positive pressure. The flow within the channels is laminar since it is maintained at a velocity keeping the system at low Reynolds numbers. The parallel flows exiting into the open volume then exhibits a pattern with sharp boundaries between each solution, for an extended distance of 10s of microns. The cell is patched inside the open volume and then lifted and translated using the patch pipette to the channel outlets. Rapid solution switching is achieved by movement of the chip relative to the cell, and switching times of ~20 ms are achieved.

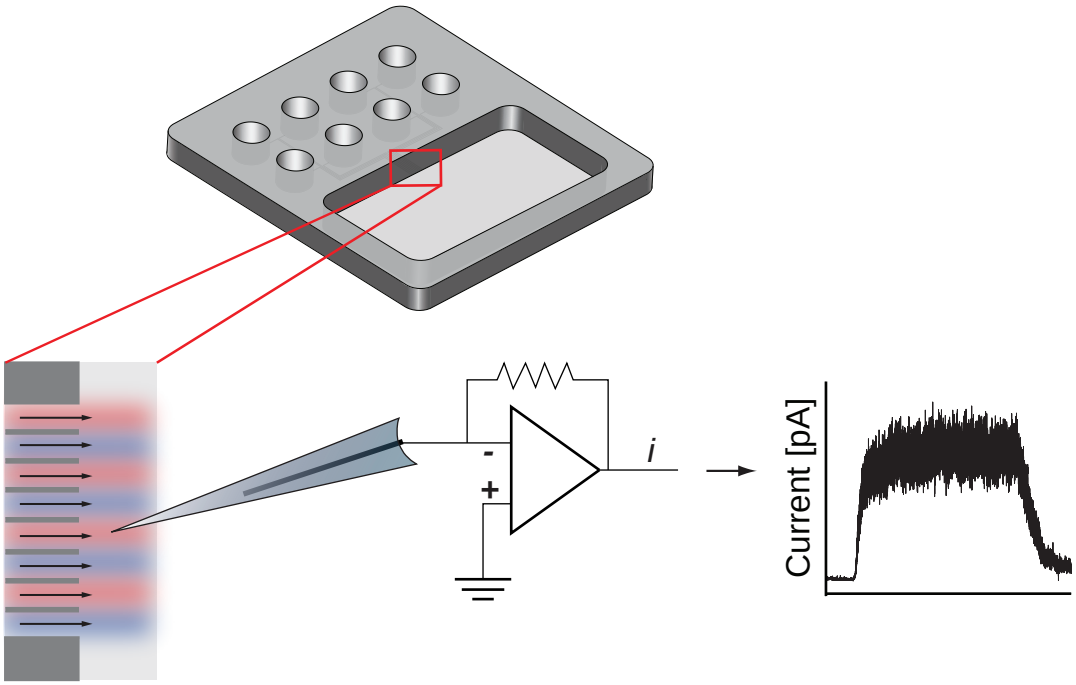

Figure 7.1 Schematic representation of a Dynaflow chip containing eight sample reservoirs with parallel channels exiting into an open volume. Cells are patched inside the open volume with a patch pipette and then moved to the channel outlets for measurements.

7.1.2 LPI flow cell

A microfluidic cell with the aim of performing proteomic profiling of membrane proteins was developed and characterized during the work described in this thesis. The flow cell utilizes a lipid-based protein immobilization (LPI) technology, which enables membrane proteins situated in lipid vesicles to bind to the flow cell surfaces. By deriving proteoliposomes from cells and immobilizing them within the flow cell, a stationary phase of membrane proteins is created, which can be subjected to several sequential solutions and different types of chemical modulations, e.g. by enzymes.

The flow cell is constructed from two gold-coated, parallel, polymer surfaces, separated by a 50 µm thick spacer. Each channel is 50 µm high, 5 mm wide and 95 mm long and has an inlet and an outlet port to be used with either manual pipetting or with a flow generated by, e.g., syringe pumps (Figure 7.2). The flow cell has a large surface to volume ratio that allows for fast solution exchange during laminar flow conditions. Fast solution exchange allows for short, controlled reaction times, which is a requirement for e.g., kinetic studies. The large surface to volume ratio enables much material to be immobilized on the internal surfaces of the cell, while minimizing dilution of, e.g., digested peptides during limited proteolysis of proteoliposomes.

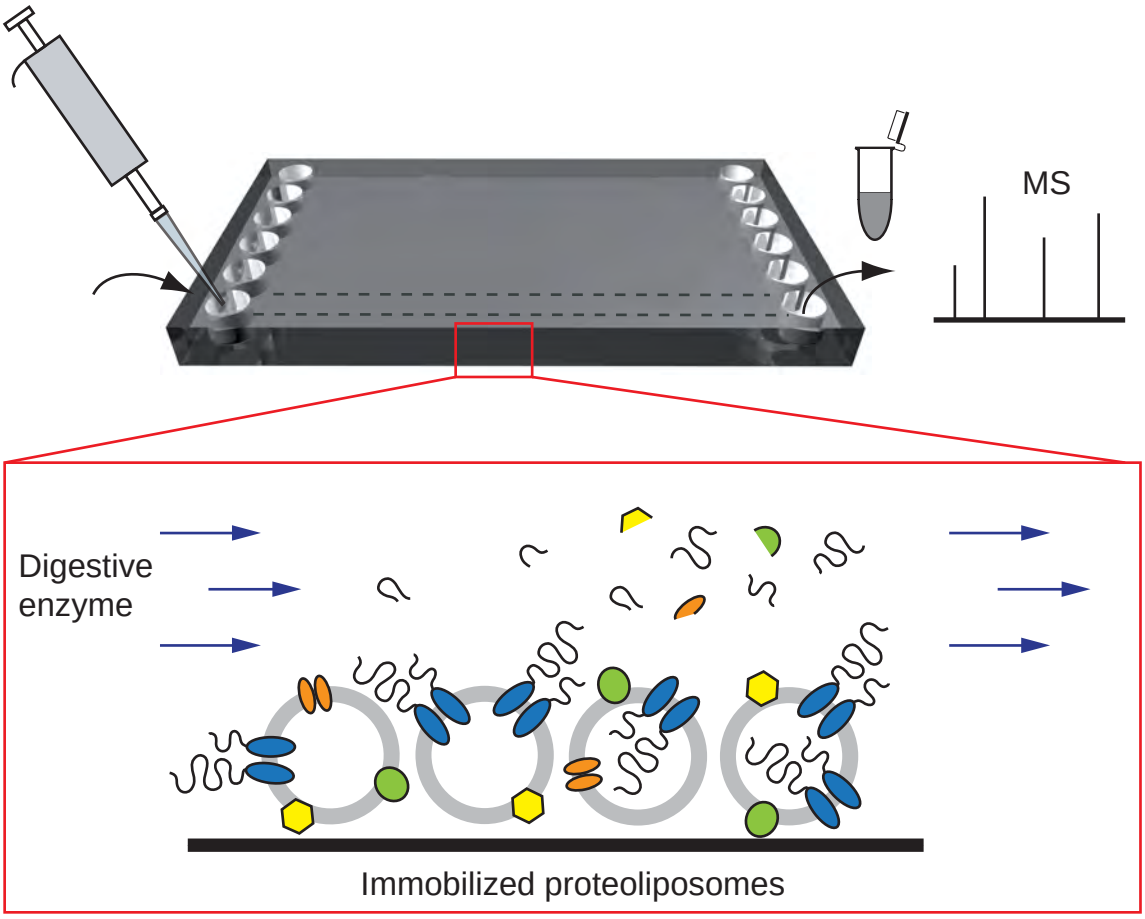

Figure 7.2 Schematic representation of the LPI flow cell containing six different channels to be used for immobilization of proteoliposomes. Only vesicles adhered to the bottom of the cell are displayed.

8

SUMMARY OF RESULTS

The work described in this thesis has aimed to increase the knowledge of membrane protein structure and function. Both directly by performing functional and structural studies of the human nociceptive ion channel TRPV1, and through development of methods that were validated using TRPV1 as a model membrane protein. The technique and associated methods can in principle be used to investigate any membrane protein. Paper I describes a functional study of TRPV1 in which the importance of membrane cholesterol content for ion channel activity was evaluated. Paper II describes the development of a microfluidic flow cell used for both structural and functional studies of membrane proteins. Methods for performing such studies were developed in paper III and further extended for development of therapeutic antibodies in paper IV.

8.1 Paper I

Membrane cholesterol content has been shown to be an important parameter for ion channel activity. TRPV1 has the capability to undergo a time dependent increase in permeability, a phenomenon called pore dilation. The dependence of membrane cholesterol content on this specific property was evaluated in paper I. The cholesterol content was lowered using cyclodextrins, and ion channel permeability was tested with patch clamp and fluorescence.

During pore dilation, which occurs after prolonged activation in hypocalcemic conditions, TRPV1 is permeable to large cations such as N-methyl-D-glucamine (NMDG). This can be measured as an increase in relative permeability of NMDG compared to Na^+ during whole cell patch-clamp experiments. This increase in permeability was found to be impaired in cells with lowered cholesterol content during activation with capsaicin, as the relative permeability was 70% lower in cholesterol depleted cells.

TRPV1-mediated YO-PRO uptake was used to monitor pore dilation with laser scanning confocal microscopy. The effect of cholesterol content on pore dilation was evaluated using YO-PRO uptake with either capsaicin or protons as agonists. A 50% decrease in YO-PRO uptake was observed for cells having 36% less cholesterol during activation with capsaicin. This depletion was, however, not efficient enough to affect activation with protons although an effect on YO-PRO uptake during activation with protons was observed after 54% depletion.

8.2 Paper II

Paper II describes the development of the LPI microfluidic flow cell with the aim of performing proteomic characterization of membrane proteins. Quartz crystal microbalance with dissipation monitoring (QCM-D) was used to characterize the amount of proteoliposomes binding to the flow cell surface. Proteoliposomes derived from red blood cells were used and bound within 5 min of injection until a total of 1 μg proteoliposomes/cm^2 were found to cover the surface. The distribution of the proteoliposomes across the surface was even, as determined using atomic force microscopy. A hydrodynamic analysis was performed in order to determine if the forces

acting on the proteoliposomes during operation would be of an order that has the potential to cause rupture. The forces were evaluated to be in the range of 0.1–1 pN, which are tolerated by the proteoliposomes.

A sequential digestion protocol for limited proteolysis was developed to improve proteomic analysis of complex samples. The membrane proteins were subjected to a stepwise enzymatic digestion as well as single-step digestions. The digested peptides were then analyzed using liquid chromatography with tandem mass spectrometry (LC–MS/MS). The mass spectrometer was able to detect 65% more unique membrane proteins from samples originating from a sequential digestion as compared to a single-step digestion.

8.3 Paper III

In paper III, limited proteolysis was performed within the LPI flow cell developed in paper II, on the human TRPV1 ion channel, in order to gain structural information. Identical experimental conditions were also used during inside-out patch-clamp of TRPV1, where limited proteolysis was performed using the Dynaflow system for fast and controlled delivery of enzyme and agonist. The purpose of this study was to correlate the obtained structural data from using the LPI flow cell with functional information regarding different segments of TRPV1.

The functionality of TRPV1 located in proteoliposomes is a requirement for valid structural studies using the LPI flow cell. The activity of TRPV1 in its native state as well as after removal of different structural segments with tryptic digestion was measured using capsaicin-induced calcium release from proteoliposomes loaded with Ca^{2+}. The fluorescent calcium-binding probe Fura-2 was used to confirm calcium release from proteoliposomes containing active ion channels. Both untreated proteoliposomes as well as proteoliposomes subjected to a short tryptic digestion exhibited active ion channels. Prolonged digestion however, diminished capsaicin response.

A screening of TRPV1 surface topology was performed using the sequential digestion protocol that was developed in paper II. Proteoliposomes containing hTRPV1 were derived from CHO cells and subjected to limited proteolysis with cumulative incubation

times within the LPI flow cell. An increasing number of peptides were detected with time, highlighting regions of the proteins that were accessible and easily digested, as well as more rigid regions. Several of the regions that were observed as cleaved-off peptides after limited proteolysis of TRPV1 correlate with known interaction sites for calmodulin, ATP and PIP_2.

The functionality of the digested regions was further evaluated using patch-clamp recordings and the inside-out patch-clamp recording configuration, allowing the intracellular part of TRPV1 to be exposed to trypsin. A decrease in current response and single-channel conductance as well as an increase in EC_{50}-values, could be observed with increasing trypsin concentrations.

8.4 Paper IV

Paper IV describes the development and characterization of two polyclonal antibodies, OTV1 and OTV2, acting on the intracellular side of the human TRPV1 ion channel. Both antibodies are pharmacologically active, and their targeted epitope regions were chosen based on limited proteolysis of the target protein within the LPI microfluidic platform using two different proteases. The efficacy of the antibodies against native TRPV1 was studied both with inside-out patch clamp, where the intracellular side of TRPV1 could be exposed to antibody solution, and with fluorescence studies where the antibodies were electroporated inside live cells.

Immunocytochemistry was performed in order to visualize the antibody distribution within the cells. Cells were fixed and labeled with OTV1 or OTV2 followed by a secondary goat anti-rabbit antibody tagged with Alexa 488. The fluorescence was visualized with confocal microscopy. Both OTV1 and OTV2 were found in the plasma membrane region.

For OTV1, current amplitudes were measured with the patch-clamp technique by exposing patches to capsaicin, with and without antibody. A ~50% decrease in current response were observed for cells treated with OTV1. For OTV2, current amplitudes were measured by exposing patches to capsaicin, with and without antibody and

calmodulin/Ca^{2+}. A ~45% increase in current response (less desensitization) were observed for cells treated with OTV2.

The efficacies of the antibodies were also tested within whole cells using electroporation to permeabilize the cell membrane, followed by measurement of TRPV1 mediated YO-PRO uptake with confocal microscopy. Cells were electroporated in the presence of either OTV1 or OTV2 and then exposed to YO-PRO and capsaicin. A 60% decrease in uptake rate could be observed during the initial 12 s of activation for OTV1 and an 80% increase in uptake rate could be observed at 15 s of activation for OTV2.

9

CONCLUDING REMARKS

The method developments, and the study protocols, described herein will contribute to an improved understanding of how the lipid composition of the cell membrane as well as how structural manipulations of membrane proteins, and in particular ion channels, modulate functional aspects of this protein class. In addition, the described methodologies will also facilitate the development of novel antibodies for therapeutic purposes.

The studies presented within this thesis have provided additional knowledge of TRPV1 function and its role in pain reception. It has been previously known that the ion permeability of TRPV1 is not constant but increases with time. This phenomenon was shown to be dependent on membrane cholesterol levels, and was abolished by lowering the cholesterol content. A varying permeability can be an underlying mechanism through which TRPV1-mediated hyperalgesia occurs, and modulation of cholesterol content could in turn prove useful in pain treatment. Further studies are needed to support this finding *in vivo* and it would also be of interest to study the effect of increased cholesterol content on TRPV1 function as well as after modification of the content of other lipid species.

This finding also highlights that many membrane proteins require a specific lipid composition for their correct function and that this is a parameter of great importance during structural and functional studies of membrane proteins. The LPI flow cell was developed to facilitate studies on immobilized membrane proteins still residing in their native membrane. It can be used with advantages for large-scale proteomic profiling as well as detailed studies of specific membrane proteins. The analysis can be limited to subcellular compartments by utilizing protocols for subcellular fractionation in order to select for a specific membrane type prior to the formation of proteoliposomes.

The studies herein were performed using limited proteolysis as a tool for structure elucidation, but the use of the flow cell can be extended to any experimental procedure that would benefit from having membrane proteins in a stationary phase in non-denaturing conditions. The environment surrounding the membrane proteins can easily be altered, e.g., to study the effect of different ionic compositions, pH or temperature. The protein of interest can be subjected to different ligands or other modulatory molecules in this context.

The flow cell features a particularly large surface to volume ratio that allows for fast solution exchange during laminar flow conditions. The large surface area can bind a considerable amount of membrane material, which is beneficial due to the low abundance of certain membrane proteins. Fast solution exchange allows for a short digestion time, which is a prerequisite for limited proteolysis experiments. Peptides are preferably digested one at a time in order to rank regions based on their accessibility and surface exposure. This in turn requires precise control of protease activity and solution exchange.

Detailed manipulation of membrane protein environment in combination with kinetically controlled proteolysis enabled evaluation of TRPV1 surface topology. Several flexible, surface exposed regions as well as regions resistant to proteolysis, were discovered using this approach. Inside-out membrane patches were subjected to limited trypsin proteolysis during patch-clamp recordings, causing removal of intracellular peptide regions and alterations in functional properties of TRPV1, such as its channel conductance. The identity of the digested regions could be confirmed with mass spectrometry after using identical digestion protocols within the LPI flow cell. The functional effect of shorter

segments could then be evaluated, which makes this technique more useful and less laborious in comparison to techniques for mutational studies. Here, it is possible to study the effect of removal of single peptide segments on ion channel function by careful selection of protease concentration and digestion time. Proteases with different amino acid preferences could be combined in order to increase sequence coverage.

Characterization of surface exposed peptide regions within the TRPV1 structure further facilitated the discovery of new epitopes for antibody generation. Two regions, one in the N-terminal and one in the C-terminal were both easily digested and located in known functionally important regions, thus chosen for antibody development. The antibody OTV1 binds to an epitope that is important for capsaicin activation of TRPV1 and subsequent capsaicin activation in the presence of OTV1 caused a decrease in current response. Similarly, the antibody OTV2 binds to an epitope that is important for desensitization with calmodulin and treatment with calmodulin in the presence of OTV2 caused less desensitization. Thus, both antibodies caused a predicted alteration in TRPV1 response based on the functional importance of their respective epitope region. This approach can be applied on membrane targets implicated in diseases for the development of new therapeutic antibodies. In cases where diseases are caused by mutated membrane proteins, cell lines expressing these dysfunctional proteins can be used for limited proteolysis and epitope generation. Epitopes generated from such experiments could potentially produce antibodies able to select between functional and dysfunctional proteins.

The established techniques mass spectrometry and patch clamp constitute valuable methodologies for studies of structural and functional aspects of membrane proteins, such as ion channels. These methods were used in conjunction with newly developed techniques which further enables detailed and advanced studies of structure-to-function relationships of this group of membrane proteins. Finally, this combination of novel and established methodologies may also constitute an effective strategy for generating antibodies with therapeutic properties.

10

ACKNOWLEDGEMENTS

To **Owe** for granting me this opportunity and for always being encouraging and optimistic even during less successful days.

To **Aldo** for all support in the lab and solutions to many technical problems.

To **Kent** for your never-ending enthusiasm and happy spirit. I have greatly enjoyed having you as my co-supervisor and you have learnt me a lot.

To **Gavin** for all your invaluable help and for many interesting discussions. You have an answer to every problem.

To **Erik**, **Akram** and **Maria,** it has been a great time working together in the patch lab. I've enjoyed it a lot.

To **Anders** and **Roger** for many fruitful discussions

To **present and former members** of the lab for a super nice working environment.

To **Milovan** the love of my life, and **Thor** my little sunshine

11

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