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Purification and characterization of a pentameric ligand gated ion channel

GLIC

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Abstract

Pentameric ligand gated ion channels are important players in the nervous system due to their role as converters of chemical signals into electrical responses. The malfunction of these important ion channels has been proven to be involved in a variety of neurological diseases. Recently, new crystal structures as well as electron microscopy structures have started to emerge, providing society with new information and coupling structure to function. However, the necessity of studying this important group of ion channels in their natural environment – membranes – is high and thus are the recently emerged structures a bit limited. The project aimed to characterize GLIC's conducting properties with a port-a-patch setup. This was not achieved but initial experiments were performed. However, the purification process of GLIC was successful and optimized during the project. A Histidine-tagged protein with a mass around 37 kDa was observed after western blot, possibly corresponding to GLIC.

Keywords

Pentameric ligand gated ion channels, GLIC, IMAC, SDS-PAGE, western blot, port-a-patch electrophysiology methodology

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Todo es posible
Nada es seguro

(Allt är möjligt, inget är säkert)
Bolivianskt talesätt

DEDICATED TO MY PARENTS
AND MY GRANDMOTHER

PURIFICATION AND CHARACTERIZATION OF A PENTAMERIC LIGAND GATED ION CHANNEL (GLIC)

Anna Isabelle Nerén

POPULÄRVETENSKAPLIG SAMMANFATTNING

Allt i världen är uppbyggt av molekyler – du, jag och marken vi går på. De främsta byggstenarna för oss människor är molekyler som kallas proteiner. Proteiner ger struktur och stadga till vävnader, ansvarar för transport av mindre molekyler, katalyserar kemiska reaktioner och skyddar oss mot infektioner. Proteiner är följaktligen en av de mest biologiskt intressanta molekylerna att studera! Vissa proteiner sitter insprängda i cellmembran. Dessa proteiner brukar kallas för membranproteiner och fungerar ofta som en kanal mellan cellens utsida och insida. De har en förmåga att öppna och stänga sig och på så sätt transportera olika molekyler in och ut ur cellen. Kroppens nervssystem förlitar sig på dessa kanaler då dessa kanaler gör det möjligt att omvandla kemiska signaler till elektriska impulser. Nervssystemet styr det mesta i vår kropp. Det styr vår andningstakt, vårt blodtryck och ansvarar för att registrera nya intryck bland annat.

Studier har visat att vissa membranproteiner är speciellt viktiga för ett funktionellt nervsystem. Dessa kallas för pentameriska ligandstyrda jonkanaler, baserat på deras strukturella komposition. Det finns en stor variation mellan dessa jonkanaler då de både binder till olika molekyler och transporterar olika molekyler, men de viktigaste i mänskliga celler är $GABA_A$-receptorn och glycin-receptorn. Studier har visat ett samband på icke-fungerande $GABA_A$- och glycin-receptorer och olika neurologiska sjukdomar så som epilepsi och depression. Dessa jonkanaler är dessutom målmolekyler för anestetika, sömnpiller och alkoholer vilket är viktigt för framtida läkemedelsutveckling. Idag vet vi mer om de pentameriska ligandstyrda jonkanalerna men det finns fortfarande för lite information tillgänglig rörande de mänskliga $GABA_A$- och Glycin-receptorerna för att man ska kunna utföra experimentellt svåra studier. Man brukar därför använda sig av bakteriella homologa jonkanaler som tros fungera på liknande sätt. I den här rapporten kommer ni få läsa om mitt examensarbete att rena fram och karaktärisera en bakteriell homolog – GLIC – som ett led i att förstå hur dessa jonkanaler fungerar.

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ABBREVIATIONS

ABD	Agonist binding domain
CMC	Critical micellar concentration
ECD	Extracellular domain
GLIC	*Gloeobacter violaceus* ligand gated ion channel
IMAC	Immobilized metal ion affinity chromatography
PLGIC	Pentameric ligand gated ion channel
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholines
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
TMD	Transmembrane domain

1 INTRODUCTION

The nervous system is responsible for coordinating and controlling most of the vital mechanisms in us humans. It controls the rate of breathing, the blood pressure and the registration of new experiences among many other essential mechanisms. Pentameric ligand-gated ion channels (pLGICs) are one of the key components in our human nervous system, as they mediate signal transduction[1]. By conformational changes, pLGICs convert the chemical signals into electrical responses[2], which is crucial for a functional nervous system. The malfunction of pLGICs is thus believed to be involved in a variety of neurological diseases, such as depression and epilepsy. Because they are important players in the nervous system, pentameric ligand-gated ion channels also serve as important drug targets for general anaesthetics and alcohols[3]. In order to learn more about how the underlying mechanisms in the nervous system are regulated and enable better drug design in the future, we'll first need to learn more about how the pLGICs function.

The high resolution structures of human pLGICs available today provide valuable information about the linkage between structure and function but are limited in showing how the protein functions in its natural environment. The structural information that is available is mainly based on crystallography and cryo-microscopy, i.e. does not describe how protein function is affected by the membrane composition or how the ion channels function at normal temperatures. Thus, to fully understand how the pLGICs function it is crucial to study the protein complexes in a membrane system. Bacterial homologues are often used as a test-bed for method development and more advanced experimental characterization.

One of the bacterial homologs that is well known is the *Gloeobacter violaceus* ligand-gated ion channel (GLIC). GLIC's crystal structure has been determined in both an open and a closed state [4], [5], can be expressed in *Escherichia coli* (*E. coli*) and thus serves as an extremely good model system for studying the pLGICs. In this project, GLIC was extracted from *E. coli* cells and purified with chromatography methods. After insertion of the purified protein into proteoliposomes, the characteristics of the protein were confirmed using a port-a-patch electrophysiology method. This set-up allowed controlling and modifying the lipid composition and investigating the influence of the membrane on channel function, which is crucial for both developing future drugs as well as learning how the protein function in its natural environment.

1.1 Background

1.1.1 Membrane proteins

Membrane proteins interact with the cell membrane in various ways. The membrane itself consists of a phospholipid bilayer. The phospholipids have polar heads, facing both the extracellular and intracellular space, and hydrophobic lipid tails, creating a non-polar region in the centre of the bilayer[6].

Depending on how the membrane proteins interact with the membrane, they can be classified as intrinsic or extrinsic. The intrinsic membrane proteins are usually called transmembrane proteins and are, or have parts that are embedded, in the lipid bilayer. These proteins are known to interact with both the intracellular as well as the extracellular cell environment, including the hydrophobic core of the bilayer. The extrinsic membrane proteins interact with the polar heads of the bilayer or are bound to intrinsic proteins and do not enter the hydrophobic region at all. Most cell membranes include both types of membrane proteins in various compositions, depending on cell type and subcellular location (see fig 1)[6].

Because of the huge variety of membrane proteins and membrane protein interactions, the membrane proteins are involved in many different cell mechanisms. They regulate intracellular environment by controlling concentration gradients, interacting with other molecules in the extracellular space and converting chemical signals to electrical responses and thus enabling cell-to-cell communication[6].

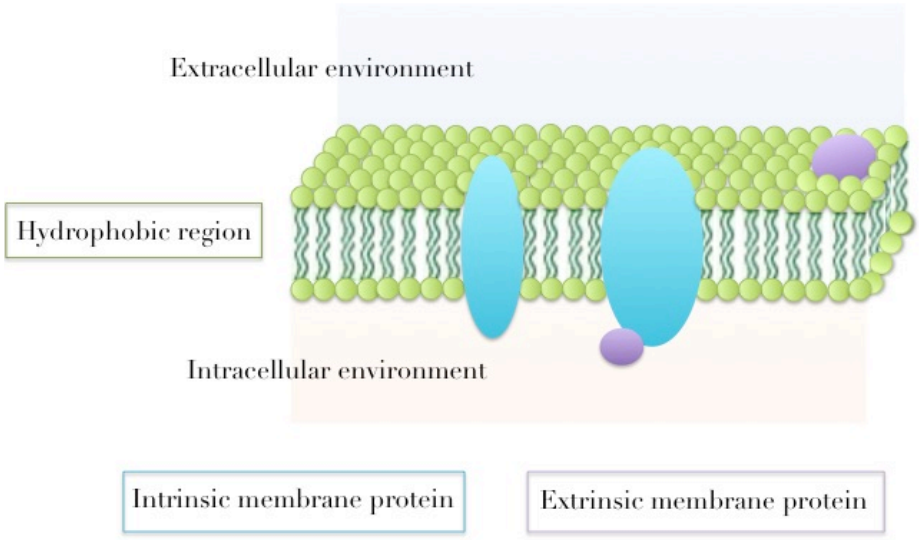

Figure 1: General overview of a biomembrane with membrane proteins. Intrinsic proteins are shown in blue while the extrinsic proteins are shown in purple.

1.1.2 Ligand-gated ion channels

The pentameric ligand-gated ion channels (pLGICs) are one type of intrinsic membrane proteins. The pLGICs consist of one extracellular domain (ECD) and one transmembrane domain (TMD). The agonists bind to the ECD, triggering a conformational change, which leads to an open-state conformation within the TMD, allowing the ion channel to mediate signal transduction[1].

Mammalian pLGICs include the $GABA_A$-, glycine-, nicotinic acetylcholine (nAChR)- and serotonin ($5\text{-}HT_3$)-receptors. These proteins are all part of the same superfamily of pLGICs, also referred to as "Cys-loop receptors" due to the conserved residue loop in the ECD.[2] The structural architecture of all pLGICs shows a quaternary similarity with five subunits, arranged in parallel to each other (see fig 2)[2].

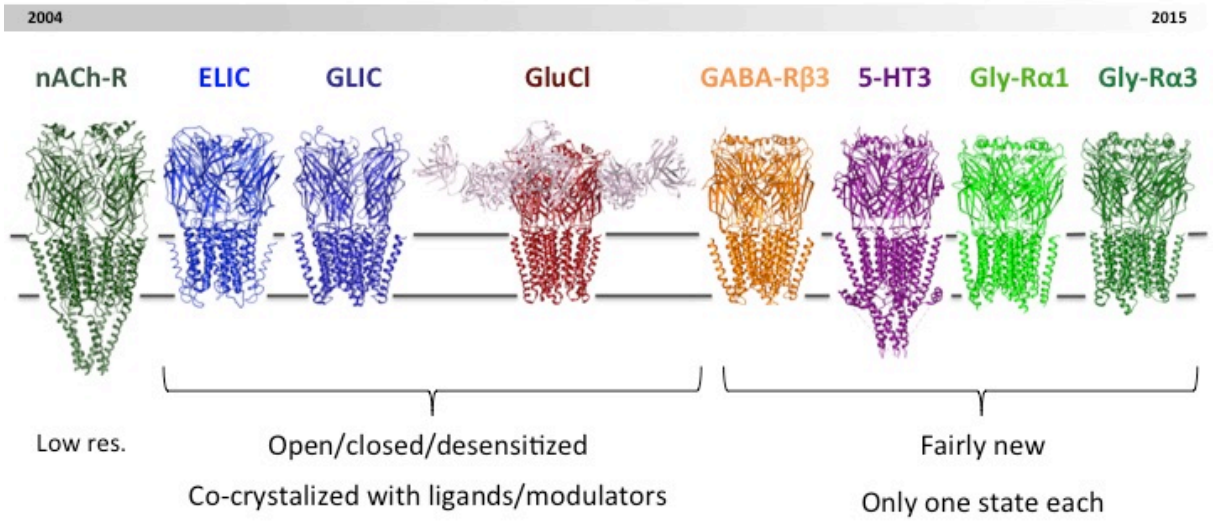

Figure 2: Structural overview of different pLGICs. The structures of ELIC, GLIC, GluCL have been determined in different states while the human receptors GABA, 5-HT3 and Gly just recently have been determined. (Figure courtesy of Stephanie Heusser, 2016)

Recently published studies have provided new insights regarding the full crystal structures of the human glycine-receptor as well as the mouse serotonin 5-HT3-receptor. These studies expand the previous knowledge about pLGICs greatly; the 5-HT3-receptor structure reveals a part of the intracellular domain[7] and the human glycine-receptor has been determined in the presence of strychnine, revealing inactivation-mechanisms previously unknown[8]. Du *et al.* have been able to create a template for constructing new pLGIC structures for data models, based on their work with the glycine-receptor from the zebrafish α1, by using electron cryo-microscopy to determine the structure bound to strychnine, glycine and ivermectin[9]. The knowledge of pLGICs is thus increasing every year but there are still questions to be answered, mostly based on the fact that all these brilliant

studies are based on crystal- and electron cryo-microscopy structures. These structures do not provide any information about the proteins in their natural environment due to that the structures are trapped in a crystal lattice at a cryogenic temperature. They do not show how surrounding lipids and other membrane components affect the pLGICs, which is crucial for understanding how the proteins really function. The mammalian pLGICs are more complicated structurally as well as they are more complicated to purify compared to the bacterial homologs. Most human pLGICs are heteropentamers and are not as stable as the bacterial homologs, which creates furthermore obstacles when performing experimental studies. To attain information about the proteins in the natural environment (i.e. inserted into membranes), it is still preferable to use the bacterial homologs.

Due to the difficulties of studying mammalian pLGICs in their membrane environment, water-soluble homologs of the protein agonist-binding site (ABD) have been used instead. The water-soluble homologs of the ABD of the acetylcholine-gated, cation-selective ion channel originated from the electric fish *Torpedo* (nAChR) are called AChBP and have provided useful information about conserved structures among several of the pLGICs as well as the protein behaviour both in presence and absence of agonists. It seems, however, as the conformation of ABD is highly affected by the TMD which makes the water-soluble AChBPs unable to provide definite observations regarding the gating of pLGICs. Hence, it was of great scientific significance when the identification of several prokaryotic pLGICs was published[2].

1.1.3 GLIC

The bacterial homolog *Gloeobacter violaceus* ligand-gated ion channel (GLIC) originates from the cyanobacteria *Gloeobacter violaceus*. Among other cyanobacteria, they lack thylakoids and instead use light-harvesting complexes on the inside of the plasma membrane. This creates a proton dependent photosynthetic apparatus over the plasma membrane[10].

GLIC is a cation-selective ion channel gated by protons. The protein complex is a homopentamer and has an approximately molecular weight of 195 kDa. Due to its five identical subunits (see fig. 3) and the fact that GLIC's crystal structure has been determined in both an open and a closed state [4], [5] as well as that GLIC show sensitivity to the same drugs that target several of the mammalian pLGICs, GLIC is a simple and good model system for both probing drug interactions as well as for understanding channel activation of pLGICs[2]. The overall sequence similarity to related proteins are quite low[11] but the structural identity is surprisingly high. Comparisons between GLIC and the human $GABA_A$-receptor show a structural

similarity of 83% (comparison made with TM-align, which uses dynamic programming iterations to superposition two structures and returns a TM-score value of how similar they are)[12]. Although GLIC lacks the intracellular domain found in other pLGICs, functionally important residues located both in the TMD and ECD as well as the pore region are conserved.[11]

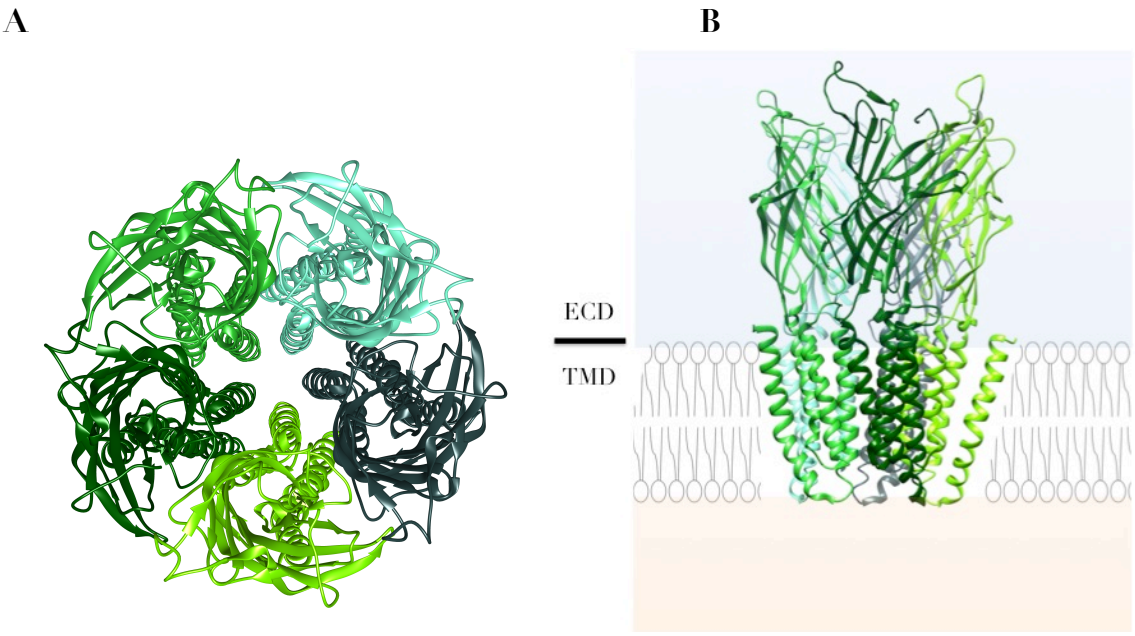

Figure 3: Structure of GLIC. A. GLIC viewed from the top. B. Displays an overview of the GLIC structure inserted into a membrane. Figures courtesy of Stephanie Heusser (2016).

GLIC is activated by a low pH. Some of the amino acid residues in the ECD become protonated when an agonist binds to the ECD, which allow the ion channel to change its conformation from a closed to an open state. When GLIC is in an open-state conformation, it transports positive Na+ ions throughout the membrane[13]. It has long been a question how cholesterol and other membrane components affect channels like GLIC and it is thus important to study GLIC in a membrane environment.

1.1.4 Port-a-patch electrophysiology

Patch clamp systems

In the late 19th century, Luigi Galvani proved that the legs of dead frogs responded in a twitching manner when electricity was applied. The specific effect of the muscle contraction after a stimulation of electricity was later called galvanism and is considered as the initial study of bioelectricity[14]. Since then, membrane potential has been measured long before ion channels were known. Hodgkin and Huxley (1952) performed a measurement of current-voltage relations in the membrane of a giant axon by using crude glass electrodes[15], which combined with the

development of the principle of voltage clamp by Cole (1949)[16] lead to the realization of how voltage clamp application can describe potential conductances. However, it was first in 1976 Neher and Sakmann could show the patch clamp technique that is still used today. By using a large-bore pipette, they were able to create a tight seal between the pipette and the cell instead of penetrating the cell. The tight seal that is formed is called a gigaseal due to its electric resistance in the order of 1 gΩ. The gigaseal is stronger than the membrane itself, which leaves the seal intact after removing the pipette from the membrane[16].

In 1981, Hamill *et al.* published an article regarding improved patch clamp-techniques that is considered to be the key article for patch clamp system[16]. The article summarizes and describes how two types of cell-free patch configurations can be performed. The two types are inside-out patch, whereas the cytoplasmic membrane is exposed on the outside of the pipette, versus outside-out patch, where the extracellular membrane is exposed. The article further describes how single channel recordings can be made under certain conditions (i.e. a cell diameter less than 20 µm)[17]. Since then, the techniques of patch clamping have been additionally improved and specialized throughout the years. [16]

Port-a-patch electrophysiology

The port-a-patch works as a miniature patch clamp system. The system consists of a borosilicate glass chip with an aperture big enough for one cell. By adding a solution of cells on the chip, one cell is automatically captured and sealed in a whole-cell configuration. The advantage of using a port-a-patch set-up is the ability of measuring single cells with a self-designed membrane system (see fig. 4). In that way, one can study one type of pLGICs directly, without other ion channels interfering, in an environment of own choice.

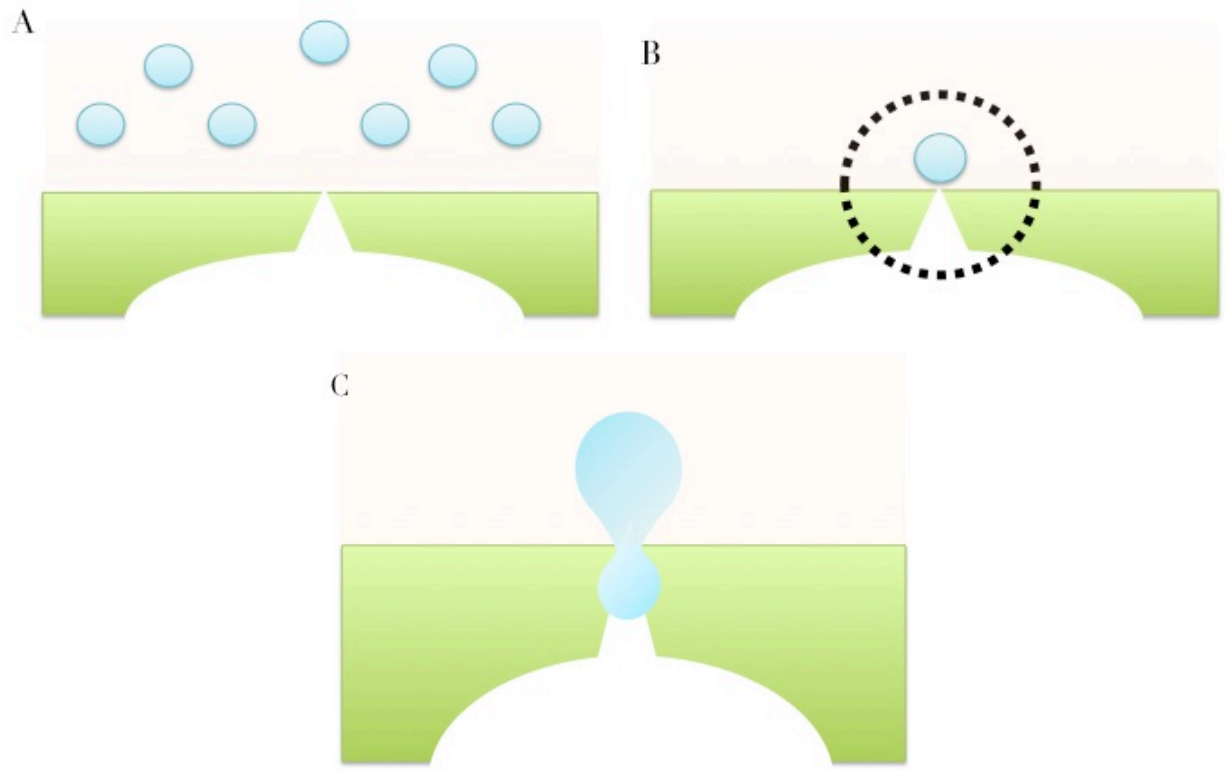

Figure 4: Schematic overview of planar patch clamp. A. Displays the set-up before cell contact while B. shows the cell contact. C. Display a zoomed in figure of the cell suction, i.e. the creating of the gigaseal.

The most commonly used lipids for experiments with bacteria are 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholines (POPC). They are broadly used to mimic cell membrane[18]. When the lipids have formed a planar lipid bilayer, the gigaseal is formed. The protein of interest is then added and incorporated to the artificial membrane. The advantage of using artificial membrane systems like the POPC is the possibility to design the membrane composition, by adding different components or changing the component concentration. When the protein complex (i.e. the ion channel) is incorporated, a measurement of conductance can be performed leading to further understanding of conducting properties as well as further knowledge about the effects of different membrane composition on ion channel function.

Membrane protein folding – how to insert the proteins?

The translocon is a protein complex that transports proteins. The translocon moderate how the polypeptides interact with the phospholipid bilayer, both throughout the membrane as well as laterally in the bilayer[19]. The translocon is thus responsible for integrating proteins into the membrane, i.e. insert membrane proteins in the membrane itself. Over a decade ago, a two stage model regarding membrane protein folding was proposed by Popot *et al.* The two stage model

describes how membrane proteins interact with each other to achieve the highest order of structure, even if they are inserted split wise in the membrane. The first stage describes how helices assemblies over the phospholipid bilayer while the following step further describes how the helices arrange themselves to interact with the surrounding helices. Popot *et al.* used bacteriorhodopsin (a protein that acts as a proton pump in different Archaea) to show how two different fragments of the membrane protein self-assemblies in the membrane, forming a globular apoprotein[20] (see fig. 5).

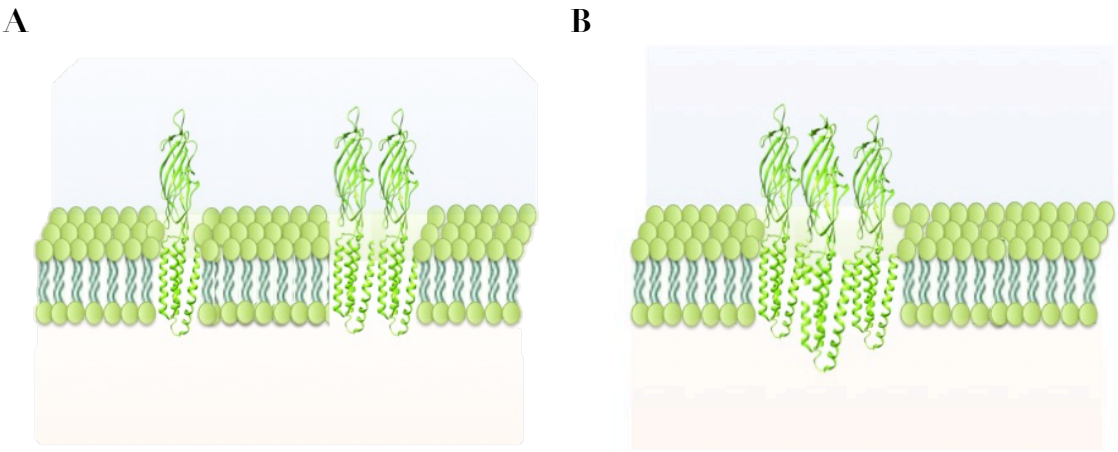

Figure 5: Schematic overview over a hypothetically arranged membrane protein in a membrane. A. Displays how three fragments of a membrane protein are inserted split wise into a membrane. B. Shows how they assembly according to the two stage model, proposed by Popot *et al*[21].

1.1.5 Purification of membrane proteins

Protein purification can be performed in a lot of different ways but the general idea is to perform:

- Cell lysis
- Protein extraction
- Chromatography methods

The first step is cell lysis where the cells are disrupted. This can be done in various ways: mechanical, chemical or both combined. To mention a few methods, freezing and thawing repeatedly, sonication and emulsification are commonly used. To ensure cell lysis, different chemical compounds can be added. In order to not allow proteases to start digesting the target protein, protease inhibitors can be added. Endonucleases (for example DNaseI, which cuts DNA non-specifically) are usually added as well; the lysate can have a high viscosity due to a high content of DNA. The DNAseI will digest the DNA which reduces the viscosity. After this, centrifugation often is performed to separate different parts from each other. The centrifugation forces the heavier parts to sediment in the bottom of the centrifuge

tubes and thus creates a separation. Depending on the choice of ultracentrifuge and on the properties of the protein of interest, time and g-force are set.

The following step is to extract the protein from the membrane pieces. Then a detergent has to be used. There are a lot of detergents available today and to be sure to have the most suitable detergent, a screening in advance is preferable. By adding a detergent, the membrane proteins most likely will leave the membrane pieces and aggregate with the detergent instead, due to that it is more energetically favourable (see fig. 6). After the solubilisation with the detergent, a centrifugation is often performed. This will remove the membrane pieces from the solution and leave the membrane proteins of interest attached to the detergent in the solution. Depending on the choice of detergent, the solution from now on needs to maintain a detergent concentration according to the detergents properties (i.e. the detergent critical micelle concentration, CMC).

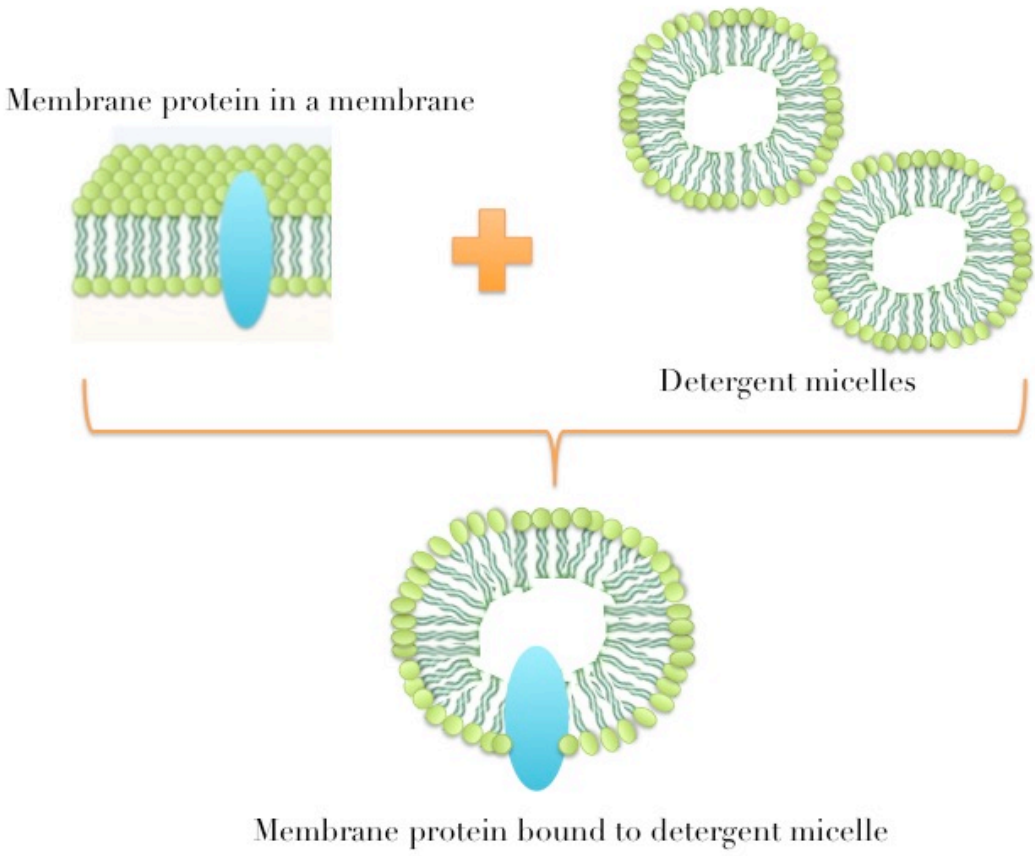

Figure 6: Schematic overview of how solubilisation with a detergent works.

To further purify the protein, different chromatography methods can be used based on the properties of the membrane protein of interest. Two commonly used chromatography column methods are affinity chromatography and gel filtration.

Affinity chromatography separates the proteins based on their affinity for a certain ligand. The most commonly used chromatography method for overexpressed proteins is affinity chromatography. The protein of interest has often been modified with a protein tag that functions as an anchor. When the solution runs through the column, only the proteins with an "anchor" will attach to the beads of the gel matrix. The proteins that do not have the affinity for the beads of the gel matrix will flow through the column straight away and the proteins of interest can then be eluted with a buffer, containing a substance with higher affinity to the gel matrix than the protein. For overexpressed proteins, a Histidine-tag (His-tag) is the most common "anchor". To enable separation based on the His-tag, an immobilised metal affinity chromatography (IMAC) is suitable. Ligands are charged with Ni^{2+} ions. The ligands are bound to the beads of the gel matrix. The Ni^{2+} ions effectively trap the proteins with a His-tag that later can be eluted with a buffer containing Imidazole, a histidine analogue. Imidazole is added in a high concentration and has higher specific interactions with the Ni^{2+} ions, leading to a replacement of the His-tagged proteins, i.e. "freeing" the His-tagged protein from the gel matrix.

Gel filtration (also called size exclusion chromatography, SEC) separates proteins based on their size. The gel matrix contains pores that force the molecules in the sample to go through them. Smaller molecules travel slower through the column because they have to spend more time in the pores compared to the larger molecules. The larger molecules can not fit as easily in the pores and are instead flushed out quicker. This creates a size separation where the larger compounds are eluted first and the smallest are eluted last.

Analytical methods

Polyacrylamide gel electrophoresis (page) with sodium dodecyl sulfate (SDS) is often used to analyse proteins and to determine their size. It gives an approximation of the molecular weight of a protein as well as it indicates how pure the sample is. The anionic detergent SDS linearizes the proteins and provides the proteins with a negative charge. When an electric source is connected to the gel, the linearized and charged proteins will start migrating through the gel according to their size. Smaller proteins will migrate faster. By using a ladder with known molecular weights, one can estimate the sizes of the proteins present in the samples. The bands that appear on the gel after staining (usually with Coomassie brilliant blue) also indicate how pure the sample is; fewer bands at different sizes indicate a more purified sample.

Western blot is often performed in order to identify proteins with certain known properties. The method uses antibodies with affinities for different compounds to display whether a compound, like a Histidine-tag, are present. Often two different antibodies are used: the first antibody is supposed to bind to the tag itself while the second antibody is supposed to bind to the first antibody. This ensures a visible detection as the second antibody carries an enzyme that convert a substrate to a detectable signal. There are two main types of detectable signals: either a colour-change of the bands on the membrane or by luminescence. A schematic overview of a western blot is shown in figure 7.

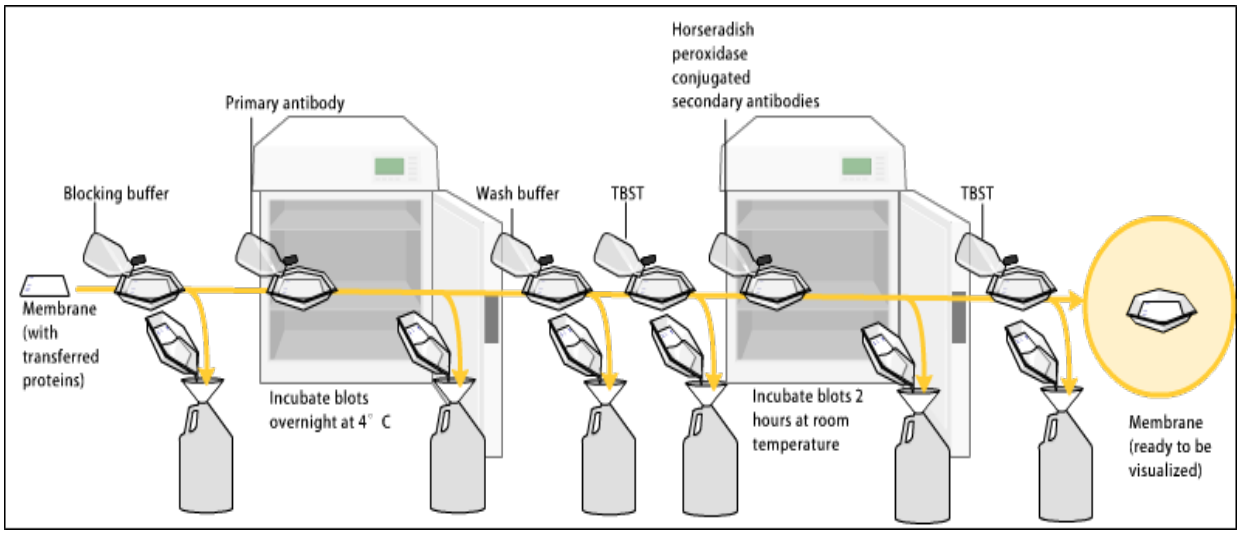

Figure 7: Schematic overview of a western blot. (Picture by Bensaccount at en.wikipedia)[22].

1.2 Aims and objectives

Aim with the project

- Purify recombinant overexpressed GLIC from *E.coli* cells
- Characterize GLIC's conducting properties

Objectives

- Extract GLIC from *E.coli* cells
- Use chromatography methods to purify GLIC
- Use identification methods (such as SDS-PAGE and Western Blot) to identify GLIC

- Create artificial membrane system
- Add purified GLIC to artificial membranes
- Use a port-a-patch electrophysiology set-up to characterize the conducting properties of GLIC

2 METHODS

2.1 Protein purification

2.1.1 Cell lysis and membrane preparation

The cell pellet was ordered from the Protein Science Facility (PSF) at SciLifeLab. The His-tagged monomeric GLIC was overexpressed in *E.coli* C43 cells and was delivered frozen (-80°C). All buffers and solutions used are further described in appendix 1.

Cells were re-suspended by gently pipetting up/down while adding buffer A (see appendix 1, table 8) until the solution was homogenous. Protease inhibitors (1 tablet of Roche protease inhibitor cocktail tablets) and DNaseI (20 µl of Amresco Ultra pure grade) were added to the solution. Cell lysis was performed by emulsification. After the emulsification, the samples were transferred from falcon tubes to centrifuge tubes (250 ml). The samples were centrifuged (20 000g, 45 min at 4°C). A Sorvall™ LYNX Superspeed Centrifuge was used with a Fiberlite™ F14-6x250y Fixed Angle Rotor (Thermo Scientific™). The supernatants were saved. The pellets were re-suspended (buffer A) and centrifuged again (5000g, 12 min at 4°C). The supernatants were saved while the pellets were discarded. All samples were kept on ice.

The supernatants were pooled and transferred to ultracentrifuge tubes (70 ml) and an ultracentrifugation was performed (64217g, 3h at 4°C). A Ti45-rotor from Beckman Coulter was used in a Beckman Coulter Optima™ L-90K ultracentrifuge. The pellets were saved and the weight of them measured carefully. The pellets were then pooled in a falcon tube (50 ml).

2.1.2 Membrane extraction and solubilisation

The pellet was re-suspended with buffer B (see appendix 1, table 8) to extract the membranes from the membrane pellet. The falcon tube containing the mix of cell pellet and buffer B was allowed to solubilize over night at 4°C (with shaker). The next day, the solution was transferred to ultracentrifugation tubes (70 ml) and ultracentrifugation was performed (64217g, 1h 10 min at 4°C) using a Ti45-rotor in a Beckman Coulter Optima™ L-90K ultracentrifuge. The supernatants were saved and transferred to viva-spin tubes (MWCO 100 kDa, 20 ml). The viva-spin tubes were centrifuged with an Eppendorf 5810 R centrifuge (2057g, 15 min at 4°C) in several repeated cycles until the volume of the sample was reduced to approximately 25 ml.

2.1.3 Affinity chromatography

A high-performance liquid chromatography (HPLC) system from Agilent technologies (1260 Infinity) was used together with a Histrap™ FF column (5x5ml, GE Healthcare) to perform an affinity chromatography at 4°C. The column was first washed 3x column volumes (CV) of dH2O and then equilibrated with buffer C (see appendix 1, table 8) 3-4xCV (until the baseline was flat). The flow rate was 0.6 ml/min.

The concentrated sample was injected in the column by using a Hamilton syringe (5 ml, Sigma-Aldrich), with an approximately flow rate of 1 ml/min. The column was left in a shaker for 30 min and then attached to the system again. Buffer C was used to elute fractions with non-binding proteins. These fractions were discarded. After 2xCVs, buffer C was changed to buffer E (see appendix 1, table 8). Buffer E was used step-wise, starting with 10%. The fractions were collected and carefully noted.

The fractions corresponding to the eluted peaks were pooled and stored in the fridge.

2.1.4 Gel filtration

A HPLC system from Agilent technologies (1260 Infinity) was used together with a Superose™ 6 10/300 GL-column (24 ml) from GE Healthcare to perform the gel filtration at 4°C.

The pooled sample from the affinity chromatography was concentrated, using viva-spin tubes (100 kDa, 6 ml) and Eppendorf 5810 R centrifuge, in cycles of 2057xg, 15 min at 4°C. 6 cycles were performed. The final volume of the pooled sample was 800 µl.

The Superose™ 6 10/300 GL-column was washed for 3xCV of dH_2O and then equilibrated with buffer C. The injection loop was used to inject the sample (800 µl) into the column. Buffer C was used during the whole run. Fractions were collected and the peaks were noted. Fractions corresponding to the peaks were not pooled but samples were loaded separately onto a SDS-PAGE.

2.1.5 SDS-PAGE

For the SDS-PAGE, pre-casted gels (Invitrogen Bolt™ 4-12% Bis-Tris Plus) and buffers and a pre-stained protein ladder (PageRuler Plus pre-stained protein ladder) from Thermo Fischer were used. The gels were loaded accordingly (unless stated differently):

Ladder - Sample 1 - Sample 2 - … - Sample N

The SDS-PAGE was performed at 4°C and the samples were prepared according to table 1.

Table 1: Describes how the samples were prepared before loaded on SDS-PAGE.

SDS-PAGE sample mix	**Volume**
Sample	20 µl
Sample Reducing Agent (10x)	5 µl
Bolt™ LOS Sample Buffer (4x)	12.5 µl
ddH_20	12.5 µl

Each of the wells were loaded with 50 µl of SDS-PAGE sample mix, while the well containing the pre-stained protein ladder was loaded with 4 µl. The SDS-PAGE was performed with 165 V for 35 min with SDS-PAGE running buffer (Thermo Fischer Scientific). The gel was then stained with Coomassie Brilliant Blue for 2h. After staining, the gel was washed with ddH_2O and de-stained with a de-staining solution (see appendix 1, table 9) over night.

2.1.6 Western Blot

Western blot was performed at BMC, Uppsala with the courtesy of professor Maria Selmer and post-doc Ana Laura Stern.

Two fractions from the peaks from the SEC chromatogram (corresponding to the first and second peak, i.e. vial 210 and vial 220) were added separately into two eppendorf tubes (300 µl). The tubes were marked as 1 and 2, respectively. Sample 1 and 2 were concentrated, using viva-spin 20 kDa (500 µl) in a VWR Mini-star microcentrifuge for 3 min. The samples, together with the ladder Precision Plus protein standard dual color (Bio-Rad), were loaded onto the pre-cast gel Mini-protean TGX Stain free (Bio-Rad). The wells were loaded according to table 2.

Table 2: Contents (with volumes) in each well of the SDS-PAGE performed at BMC. The samples in well 4 and 5 were not prepared according to standard SDS-procedure as they were not heated before they were loaded onto the gel (samples marked with *).

Well	Contents	Volume
1	Precision Plus protein standard dual colour	4 µl
2	Sample 1 (vial 210)	10 µl
3	Sample 2 (vial 220)	10 µl
4	Sample 1 (vial 210)*	10 µl
5	Sample 2 (vial 220)*	10 µl

The SDS-gel were then transferred onto membranes, using the Trans-Blot® Turbo™ Transfer System (Bio-Rad). The transferred blot was blocked with approximately 20 ml TBST and 5% nonfat dry milk (see appendix 1, table 10) for 10 min and then washed with TBST (20 ml) for 10 min. The first antibody Anti-His Mouse (Amersham™ ECL™ Prime Western Blotting) was added (2 µl) in TBST (15 ml) and was incubated for 2h. The transferred blot was then washed with TBST for 3x5 min, to remove unbound antibodies. The second antibody Anti-Mouse Peroxidase was then added (2 µl) in TBST (15 ml) and incubated for 2h. After this procedure, the blot was washed with TBS (see appendix 1, table 10) for 3x5 min. Detection was made with Bio-Rad Gel Doc™ XR+ System.

2.1.7 Absorbance measurements

NanoPhotometer® (NP80) from IMPLEN were used to determine the protein concentration in the fraction containing the target protein (i.e. vial 220). Buffer D (see appendix 1, table 8) was used as a blank. The absorbance was measured at 280 nanometer. To determine the concentration of GLIC, Beer-Lambert law was used together with the extinction coefficient for GLIC. The extinction factor for GLIC is

1.379. Beer-Lambert law (see equation 1) contains absorbance (A), the extinction coefficient (ε), the pathlength of the sample in cm (l) and the concentration of the protein in the solution in g/L (c).

$$A = \varepsilon \cdot c \cdot l \quad (1)$$

The IMPLEN NanoPhotometer® (NP80) measure the absorbance with a given pathlength of 1 cm. This results in the following equation:

$$c = A / \varepsilon \quad (2)$$

2.1.8 Yield calculations

To calculate the yield of how much target protein that was purified from the initial cell pellet, the following formulae was used:

$$Mass\ of\ end\ product = Concentration\ of\ end\ product \cdot Volume\ of\ end\ product \quad (3)$$

$$Yield = \frac{Mass\ of\ end\ product}{Mass\ of\ initial\ cell\ pellet} \quad (4)$$

2.2 Protein characterization

2.2.1 POPC

A glass vial (4 ml) was weighed and the weight carefully noted down. POPC (Avanti Lipids) was added (0.0190 g) with chloroform (100 µl) to create a POPC-mixture with a concentration of 20 mg/ml. The solution was let to evaporate overnight. The glass vial was then weighed and the weight carefully noted down. The dry film in the glass vial was re-hydrated with 0.9% NaCl-solution (1 ml). The suspension was gently pipetted up/down until the dry film was completely re-suspended.

A Mini-extruder (Avanti Lipids) was assembled and used for extrusion of the POPC-mixture. The Mini-extruder is constructed with a polycarbonate membrane filter in the centre. The POPC-solution was added to a Hamilton syringe (250 µl) and then connected to one side of the Mini-extruder. Another (empty) Hamilton syringe (250 µl) was connected to the other side of the extruder. By pushing the solution gently back and forth between the two syringes, the solution was pushed

through the polycarbonate membrane filter. This was repeated approximately 50 times.

2.2.2 Port-a-patch electrophysiology

The software that was used was PatchMaster provided by HEKA and Patch Control provided by Nanion. A Port-a-patch from Nanion connected to a HEKA patch clamp EPC 10 amplifier was used together with a NPC-1-chip (3-5 MOhm).

On the bottom side of the NPC-1 chip, a 5 µl droplet of intracellular solution (see appendix 1, table 11) was added. The chip was then mounted and the faraday top was screwed on. 5µl droplet of an external solution consisting of HEPES buffered Ringer solution was added to the chip (see appendix 1, table 11). The external electrode was connected to the external solution. The port-a-patch software was connected and an offset correction was made. Via *PressureControl* (a software tool), a negative pressure was generated. After these steps, the POPC-mixture was added to the chip (5 µl). To achieve a gigaseal, the pressure was adjusted with software tools.

3 RESULTS

3.1 Protein purification

The protein purification was performed several times with different setups (see table 3) for the affinity chromatography (see table 4) as well as the gel filtration (see table 5), and in some cases the protein sample preparation differs slightly. From now on, the first protein purification is referred to as A while the second optimized process is referred to as B. (The purification process described in method (p.15-20) refers to the optimized purification protocol of B.)

Table 3: Table of differences between two of the purification processes A an B. A refers to the first purification while B represents the optimized purification.

	A	**B**
Cell pellet	Delivered frozen (PSF)	Dissolved with buffer A, delivered frozen (PSF)
Cell pellet (g)	88	75 (dissolved in 2ml/g buffer A)
Membrane pellet weight (g)	8	4
DDM concentration (**solubilisation**)	0.2%	2%
Additional centrifugation	-	X
Additional ultracentrifugation	-	X

3.1.1 Affinity chromatography

Figure 8A and 8C had a flow rate of 0.3 ml/min and the protein sample preparation was prepared according to the method (see p. 15-20) with the exception for the detergent concentration. The detergent concentration in the solubilisation step was 0.2% instead of 2%. The sample volume loaded was 11 ml. In figure 8B, the setup was different compared to 8A. The protein sample preparation was prepared according to the method except for an additional centrifugation step after the first ultracentrifuge step, where the pellet from the first ultracentrifugation step was re-suspended with buffer A and then centrifuged with 5000g for 12 min at 4°C. An additional ultracentrifugation step was also performed with the pellet from the second ultracentrifugation, where the pellet was re-suspended with buffer B and then an ultracentrifugation was performed with 64217g for 1h 10 min at 4°C. The sample volume loaded was 27 ml and the flow rate was 0.7 ml/min.

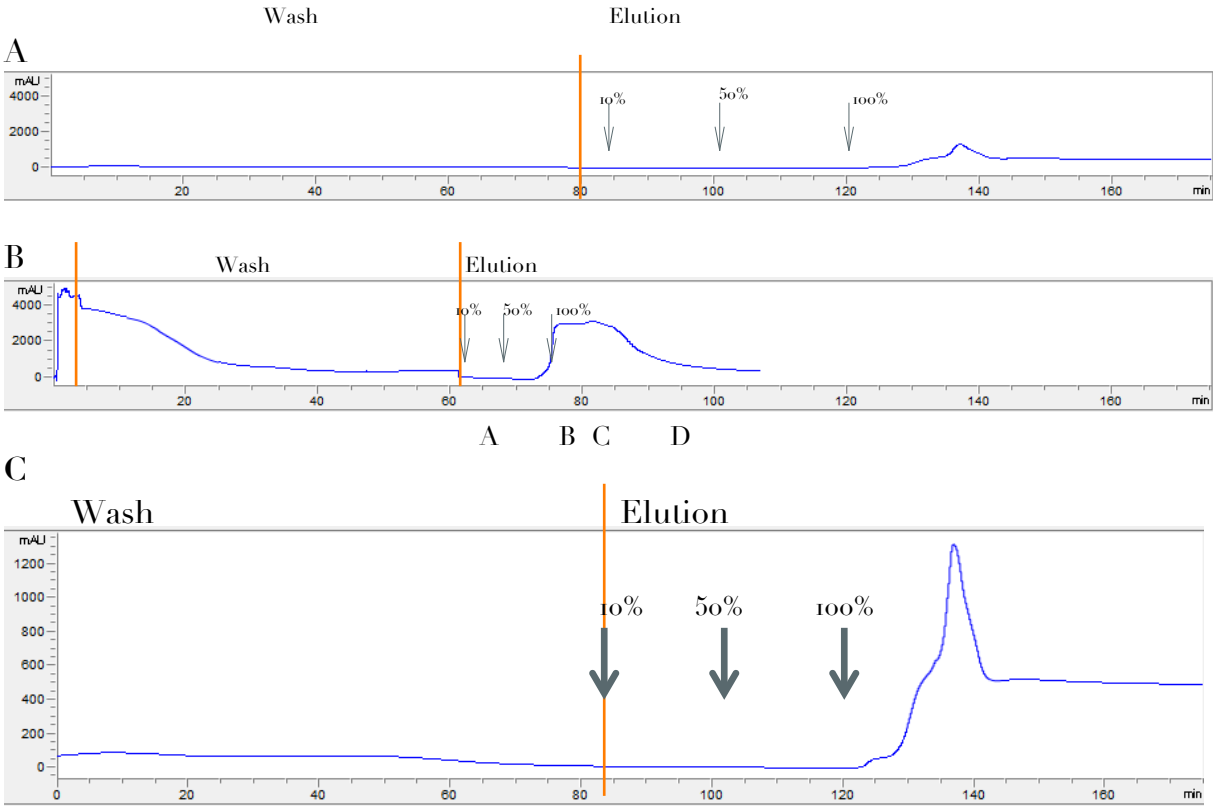

Figure 8: Chromatogram of performed affinity chromatography. Absorbance units (mAU) are on the y-axis and the time (min) is on the x-axis. Absorbance is measured at 280 nanometer. Wash and elution steps are marked. A. Affinity chromatography with a flow rate of 0.3 ml/min. A peak is visible between 120-140 min. The highest measured absorbance value is approximately 1300 mAU. **B.** Affinity chromatography with a flow rate of 0.7 ml/min. A wide peak is eluated from 75 min, 15 min (10.5 ml) after switching to elution buffer D. The highest measured absorbance value is approximately 3500 mAU. Fractions before, within and after the peak were saved (A-D). **C.** Shows the zoomed in chromatogram of 8A. In this chromatogram, the loading of sample is not visible due to a malfunction in the software used (Agilent OpenLab). The washing procedure and the start of the elution are visible and are marked as "Wash" and "Elution". The elution was made step-wise, with 10, 50 and 100% buffer D. A peak is eluated after 120 min, 35 min (10.5 ml) after switching to elution buffer D.

Table 4: Affinity chromatography setup and result for the purification processes A and B.

	A	B
Flow rate	0.3 ml/min	0.7 ml/min
Sample volume loaded	11 ml	27 ml
Final volume of pooled samples	6 ml	15 ml

The affinity chromatography (fig. 8A, 8C) resulted in a chromatogram with one peak with a shoulder, eluting after 120 min which is 35 min after switching to

elution buffer D (see fig. 8C). This corresponds to 10.5 ml. The highest measurement of the absorbance (A_{280}) was approximately 1300 mAU. The fractions corresponding to the peak and shoulder were noted and pooled together. The final volume of the pooled samples was 6 ml.

The affinity chromatography (fig. 8B) resulted in a wider peak that eluted faster, (after 75 min). The elution buffer E was added after 60 min, which gives 15 min retention time for the eluation of the peak (corresponding to 10.5 ml). From the fig 8B, a peak is visible right at the start, before the sample is injected in the column. The other peak, after eluting with buffer D, reached 3500 mAU. The fractions corresponding to the peak resulted in a pooled, final volume of 15 ml.

A closer look of the chromatogram shown in figure 8B shows that the broad peak in fact is divided into two peaks (data not shown). Fractions before and after the peaks, as well as fractions within the two peaks, were saved for SDS-PAGE (see fig. 11).

3.1.2 Gel filtration

The performed gel filtrations show a clear difference between A and B (displayed in figure 9). In figure 9A and 9C low peaks are visible after 45 min, which corresponds to a volume of 9 ml. In figure 9B peaks are visible after 30 min, which also corresponds to a volume of 9 ml. The fractions corresponding to the peaks in A were pooled but the fractions in B were not pooled.

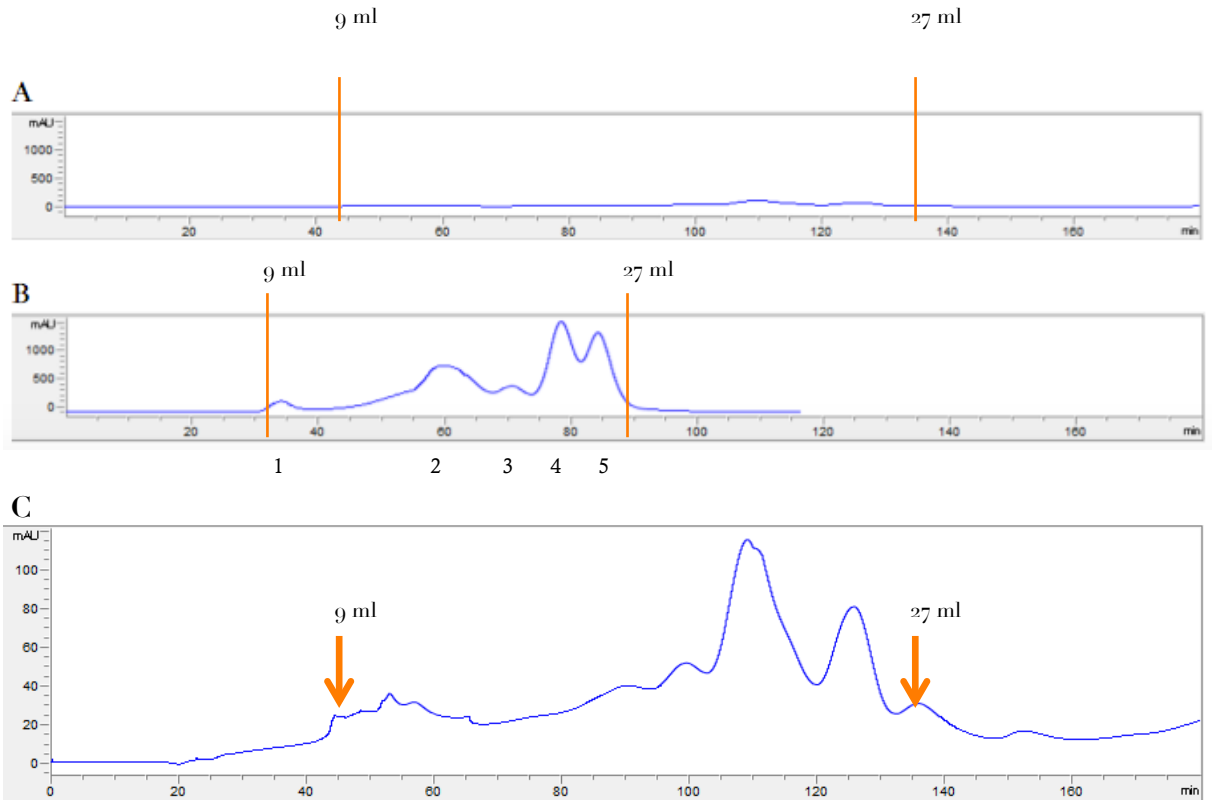

Figure 9: Figures of performed gel filtration. The absorbance units (mAU) are on the y-axis and the time (min) is on the x-axis. Absorbance is measured at 280 nanometer. The volume (ml) of the first eluted peak . A.Gel filtration performed with a flow rate of 0.2 ml/min. Low peaks are visible between approximately 45-135 min (corresponding 9-27 ml). The highest measured absorbance value is approximately 200 mAU. B. Gel filtration performed with a flow rate of 0.3 ml/min. The peaks are visible between approximately 30-90 min (corresponding to 9-27 ml). The fractions corresponding to peak 1-5 were saved. C. Shows the zoomed in chromatogram of 9A. The chromatogram shows a similar pattern with the chromatogram shown in 9B. Peaks with higher absorbance units of 20 mAU are eluted between ca 45-135 min, which corresponds to 9-27 ml. A lower peak is visible after 150 min, corresponding to 30 ml.

Table 5: Display the differences between purification process A and optimized purification process B in aspect to the gel filtrations performed. X describes the concentrated sample volume loaded onto the column, i.e. how many times the sample was concentrated.

	A	B
Concentrated (using Viva-spin)	6 ml → 6X	15 ml → 8X
Flow rate	0.2 ml/min	0.3 ml/min
Sample volume loaded	1 ml	0.8 ml

3.1.3 SDS-PAGE

Several SDS-PAGEs were performed on fractions from both purification process A and B. SDS-PAGE result from purification process A is not shown in this report, unless stated. Hence, the following results are based on the optimized purification process B.

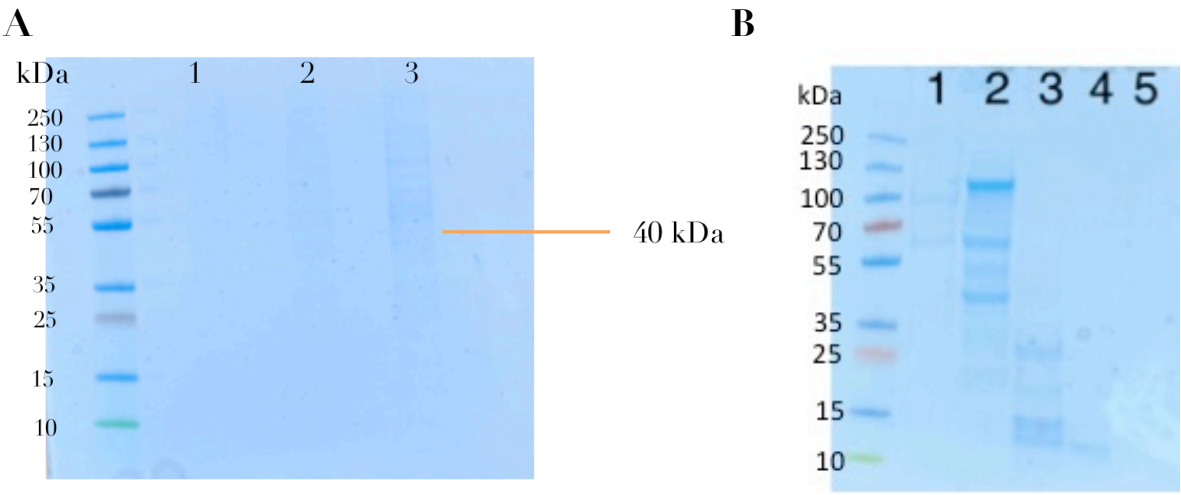

Figure 10: Two SDS-PAGE results from the first purification process (A) and the second optimized purification process (B). The samples were not concentrated. The size of the GLIC monomer is shown with an orange line A. Well 1-3 contains pooled samples from the gel filtration in fig. 9A. Well 1-3 contains 20, 30 and 40 ul of sample, respectively. B. Well 1-5 contains fractions from the gel filtration in fig. 9B. Well 1-5 represents fractions from peaks 1-5, seen from the right in fig. 9B.

In figure 10, two SDS-PAGE gels from purification process A and purification process B are shown. In figure 10A, the bands are vague and no band is clearer compared to another. The fractions corresponding to the peaks in the gel filtration (see fig. 9C) have been pooled for the SDS-PAGE (see fig. 10 A). The wells 1-3 contain different volumes of sample (20, 30 and 40 ul, respectively). In figure 10B, the fractions corresponding to the peaks (see fig. 9B) have been loaded separately. The fractions from the different peaks clearly show that the gel filtration have separated the protein sample based on size. Well 1-5 corresponds to fractions of peaks 1-5, respectively. In well 2, bands can be noted around 120, 60 and 40 kDa.

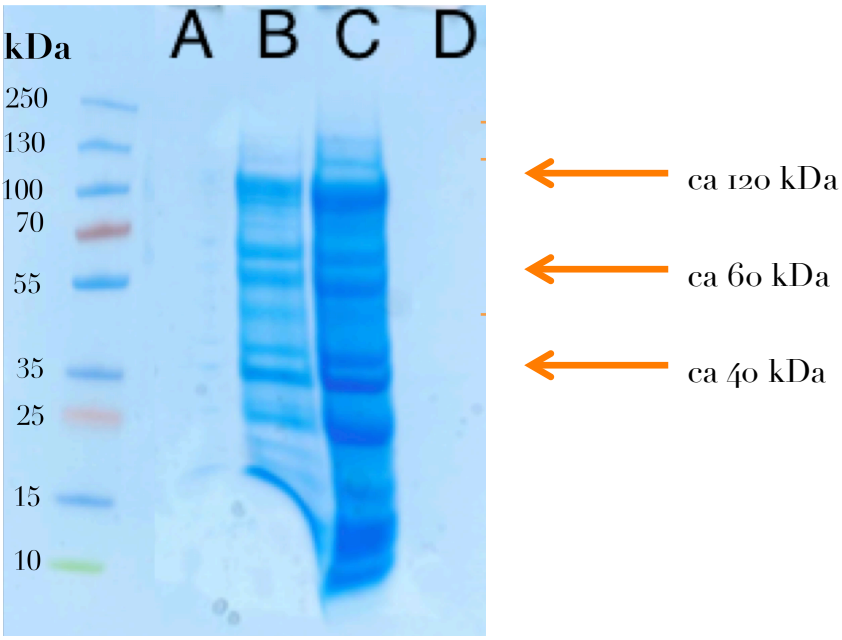

Figure 11: SDS-PAGE of fractions from the affinity chromatography (see fig. 8B). The wells A-D correspond to fractions collected before, in and after the peak. Well A corresponds to fractions before the peak, B-C correspond to fractions within the peak while D corresponds to fractions after the peak.

The SDS-PAGE results (see fig. 11) from the fractions corresponding to the peaks from the affinity chromatography (see fig. 8B) display multiple bands at different sizes (well A-D). The well B and C show bands at all sizes while A and D does not show any bands at all.

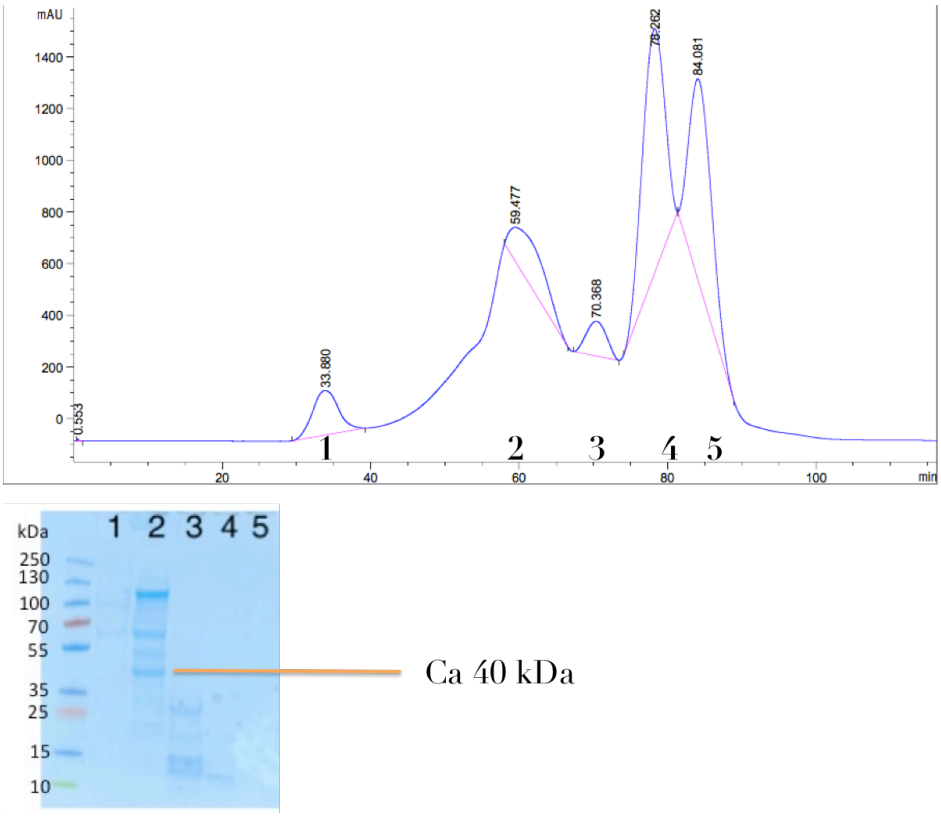

Figure 12: Gel filtration result from protein purification B with following SDS-PAGE results. The gel filtration was performed as described in fig. 9B. The wells 1-5 correspond to peaks 1-5, respectively. The orange band highlights a protein band in the middle of 55-35 kDa in well 2, corresponding to peak 2.

The SDS-PAGE results from the fractions of the gel filtration in fig. 9B (well 1-5) show that the fractions have been further purified (see fig. 11 compared to fig. 12). Well 2 shows visible bands at 120, 60 and between 55 and 35 kDa while well 1 shows bands at 60, 100 and 120 kDa. Wells 3-5 show bands at lower MW or no bands at all. The fractions from well 1 and 2 were selected to be analysed further with a western blot.

3.1.4 Western Blot

The two fractions corresponding to peak 1 and 2 in the gel chromatography (see fig. 12) were analysed further with a western blot. The result showed a clear band in well B and D, representing the fraction from peak 2 (vial 220) from the gel chromatography. The band is about 37 kDa (see fig. 14). The fraction from peak 1 did not show any visible bands (A and C).

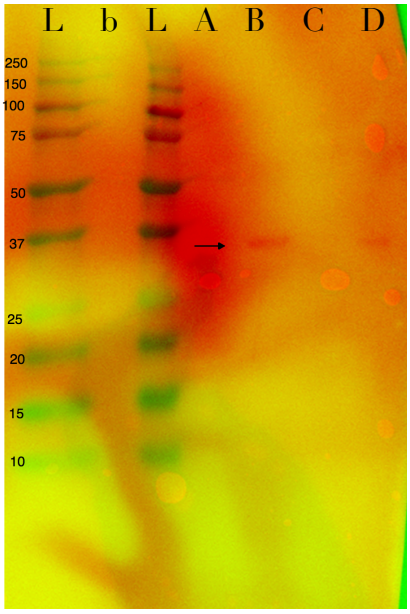

Figure 13: Picture from the western blot. L stands for ladder, b stands for blank well. The wells A, B, C, D contain samples after gel filtration. Well A and C contain the fraction from vial 210 and well B and C contain the fraction from vial 220. Fractions in well A and B are prepared according to regular SDS-protocol but fractions in wells C and D are prepared without heating. The results indicate that only well B and D contain a fraction with a his-tagged protein, at approximately 37 kDa.

From the western blot (see fig. 13) fractions from peak 2 seemed to contain a His-tagged protein. To be able to further analyse which of the two fractions that contained the protein of interest, the fractions were run on a SDS-gel separately. The result (see fig. 14) displayed that B, i.e. vial 220, shows a band at 40 kDa.

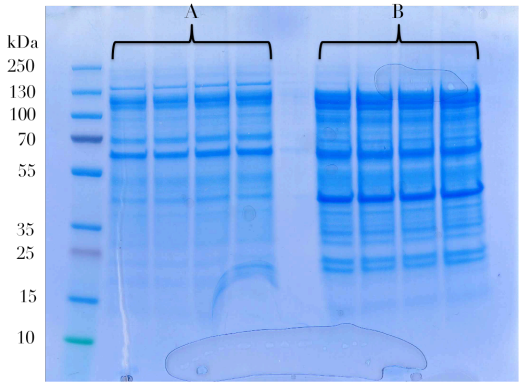

Figure 14: SDS-PAGE of the fractions corresponding to peak 2 (gel chromatography, see fig. 9). A represents fractions from vial 210 while B represents fractions from vial 220. Clearly, B (vial 220) contains an overexpressed protein with a molecular weight of 40 kDa.

3.1.5 Absorbance measurements

The sample from the gel filtration was diluted 1:9 with buffer A. The final concentration was determined by using the Beer-Lambert law (see equation 1 and 2). The results from the absorbance measurements are shown in table 6.

Table 6: Absorbance measurement of the fraction with target protein. The sample was diluted 1:9 with buffer A (see appendix 1, table 8).

Sample from	Containing	Dilution factor	A_{280}-value	Concentration
Gel filtration (vial 220)	GLIC monomer	1:9	0.063	0.46 mg/ml

3.1.6 Yield

The yield was determined by equations 3 and 4. The optimized purification protocol B resulted in 0.000018 mg protein per mg wet cell pellet (0.0018 %), which corresponds to 1.38 mg GLIC of 12% (purity per g wet cell pellet weight (see table 7). The purification factor of 12% was determined by the area beneath the peak of interest (data not shown).

Table 7: Table with result from yield calculations of cell pellet and end product. The yield was determined using equations 3 and 4 (see method, "Yield calculations").

	Mass (mg)	**Concentration** (mg/ml)	**Yield** (mg protein/mg cell pellet)	**Purification factor**
Cell pellet	75000	-	-	1
End product	1.38	0.46 mg/ml	0.000018	0.12

3.2 Protein characterization

3.2.1 Port-a-patch electrophysiology

The initial port-a-patch experiment was setup with only HEPES buffered Ringer solution added to the chip. No suction was applied and the measured resistance was 4.4 MOhm (see fig. 15A). To be able to study how a gigaseal would look like, the outer electrode was disconnected from the external droplet. The resistance measured was 1.37 GOhm and formed a "false" gigaseal (see fig. 15B). The POPC-mixture was added to the chip and suction (-45 bar) was applied in order to create a gigaseal. The resistance was measured to 25 MOhm (see fig. 15C).

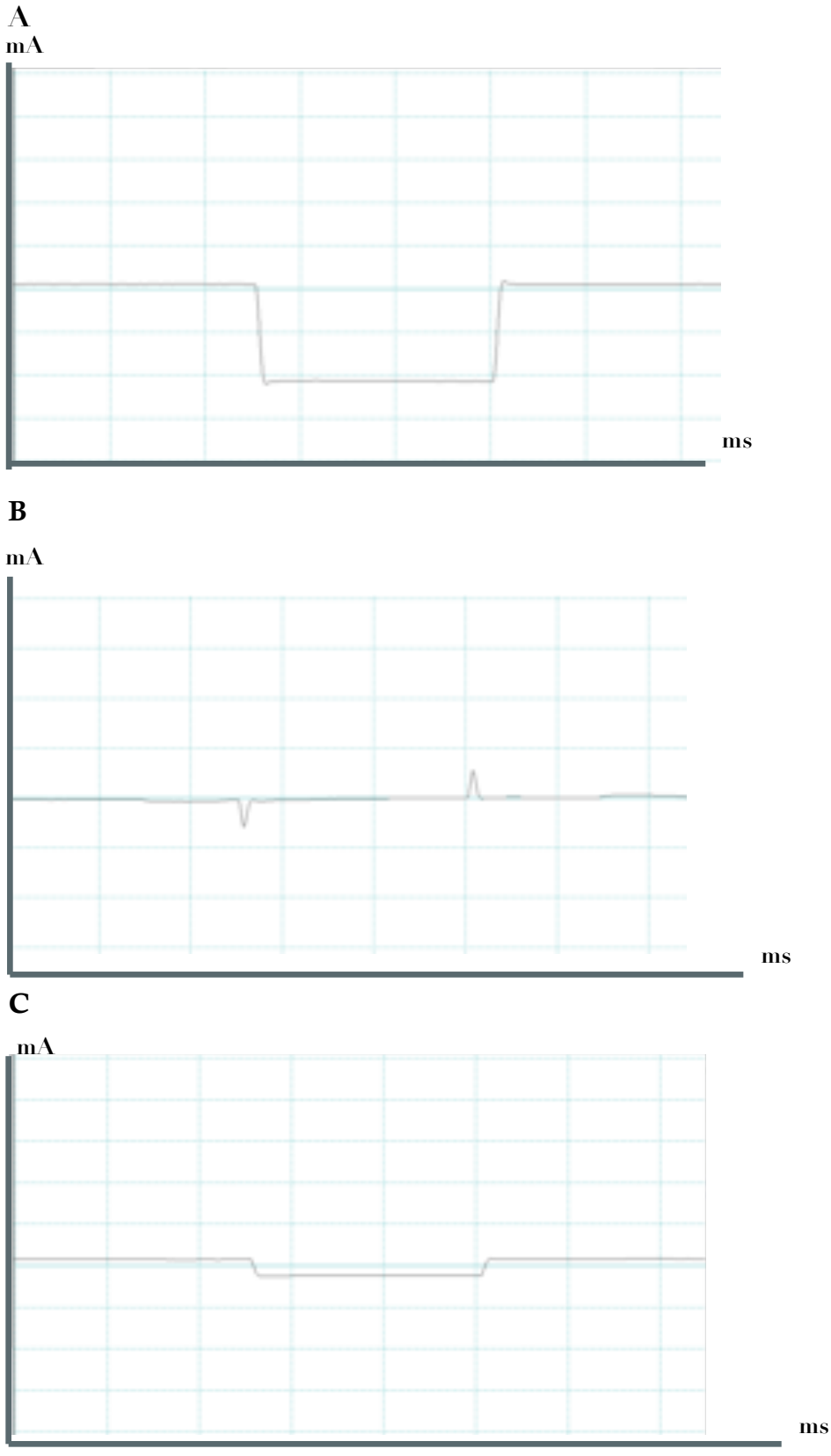

Figure 15: Port-a-patch experiment. On the Y-axis: The ion flow measured in mA. On the X-axis: time in ms. A. Only HEPES buffered Ringer solution buffer added on the chip. A resistance of 4.4 MOhm was measured. **B.** How a gigaseal should look like. The resistance was measured to 1.37 GOhm. **C.** POPC-mixture added. The resistance was measured to 25 MOhmn.

4 DISCUSSION AND CONCLUSION

4.1 Discussion

The struggles with purifying membrane proteins are many. Membrane proteins are often more complex to handle than other proteins, due to that they depend heavily on the membrane composition that surrounds them. The composition varies among different species and cells and makes it hard to estimate how the proteins will behave in a different environment, even if the environment is similar to the natural one. The first steps of purification (i.e. cell lysis) is performed quite straight forward but the level of difficulty gets significantly higher when it is time for the protein extraction. To be able to purify membrane proteins, one must use detergents in order to get the proteins to separate from the membrane pieces. The choice of detergent can be crucial; to be absolutely sure of a successful purification the right detergent has to be chosen. The choice itself depends on deep knowledge about the protein properties and hence it is important to do a screening of suitable detergents in advance. However, a screening is both time-consuming and expensive and is in most cases neglected due to this. Instead, previously published studies with detergents serve as a finger pointing in choosing detergent. In this project, the choice of detergent landed on DDM, that has been known to work with GLIC variants in previously performed studies[23]. However, the GLIC monomer that was used in this project differs from the GLIC monomer in other studies in one aspect: it is His-tagged instead of fused with a maltose binding protein (MBP) and it has a size around 40 kDa. The results from the analytical methods clearly shows that I have purified a His-tagged protein with a size of 37 kDa, indicating that I have purified GLIC which was my first goal in this project. The question is rather how much more optimized the process could have been.

A purification protocol for purifying His-tagged GLIC was not available and thus I had to develop one. The purification protocol that I drafted was optimized during the project. Results from SDS-PAGE (see fig. 8-10) clearly show a significant difference between the two purification processes A and B. The results from purification process A are vague compared to the results from the optimized protocol B. By adjusting the protocol (adding extra centrifugation and ultracentrifugation as well as changing into a higher detergent concentration in the solubilisation step), it seems like I was able to purify a higher concentration of protein. However, the concentration could not be measured for the protein sample saved from A due to lack of stable absorbance measurements and due to that both the affinity chromatography and gel filtration display higher mAU-value for the fractions purified from B than A, which could indicate that I indeed have purified

more protein in general. The chromatograms from both purification processes show a similarity both in pattern as well as in retention volumes for the peaks. The peak of interest (assumed to contain GLIC, see fig. 12) elutes after 16.5 ml (55 min, fig. 9B) and 16 ml (80 min, fig. 9C) in the gel filtration. The chromatograms from the affinity chromatography shows that the peaks are eluted after 10.5 ml in both purification processes (fig. 8).

The SDS-PAGE performed of samples from A was not visible unless pooled and concentrated. A vague band was noted at approximately 40 kDa, indicating that a protein with the same size as the GLIC monomer was purified in A. However, from the optimized purification protocol the band around 40 kDa is visible even without concentrating the sample (see fig. 10 B). Further analyse with western blot showed that one band in the sample was His-tagged. The band seemed to be about 37 kDa (see fig. 13), which is a bit smaller than the estimated 40 kDa band from the SDS-PAGE (see fig. 10, 11, 14). However, due to the use of different gels as well as different protein ladders, it is possible that these bands represent the same protein, i.e. GLIC. The protein ladder that was used in the SDS-PAGE has markers for 55 and 35 kDa, while the western blot has markers for 50 and 37 kDa. From the results of the western blot (see fig. 14), the markers of the protein ladder together with the location of the visible band enables a more specific estimation of the protein size and it is clearer that the His-tagged protein band is about 37 kDa. However, the markers of the protein ladder that was used in the SDS-PAGE are located above and beneath 37 kDa, which makes it harder to make the assumption that the band visible in the middle is ca 37 kDa. Hence, the assumption of 40 kDa was made. Although the difference of 37 and 40 kDa could indicate two different proteins with different sizes, the band seen in the SDS-PAGE at 40 kDa seems to be overexpressed because of its characteristic thick band which is consistent with the fact that GLIC was overexpressed in *E. coli*. The overexpressed GLIC was His-tagged, which indicates that the band visible at 37 kDa in the western blot (see fig. 14) represents GLIC. Together, this information and the result from the western blot indicates that the band that was assumed to be ca 40 kDa in the SDS-PAGE in fact is a band at 37 kDa. This indicates that the purification process was successful. Even though the vague band at 40 kDa in A were not analysed with western blot and no confirmation that the protein band represent a His-tag is available, it is likely that purification process A also was successful. However, to determine this and to confirm even further that the His-tagged band at 37 kDa represent the GLIC monomer, a mass spectroscopy should be preferred.

The concentration of GLIC in the sample from the optimized purification process B has been hard to measure due to both the presence of a detergent as well as the

impurities in the sample. The value of 0.46 mg/ml do not represent the correct concentration of GLIC in the sample but, together with the purification factor, it gives a hint of the true concentration of GLIC in the sample (see table 7). One reason to why it was so difficult to measure the absorbance can be the presence of detergent in the blank as well as in the sample. The detergent molecules form aggregates with the proteins and with other detergent molecules as well, which makes the solution non-homogenous. This makes it difficult to produce a steady baseline, i.e. the blank does not work well.

Another interesting aspect of purifying membrane proteins is their ability to form self-assemblies in the membrane. GLIC was overexpressed as a monomer in *E.coli* but should have formed a pentamer by itself, according to how membrane proteins usually fold when integrated in a membrane. When performing analytical methods such as SDS-PAGE and western blot, the pentameric structure should be torn apart and the proteins should be linearized. However, groups with experience of GLIC have stated that GLIC seems to aggregate even in denaturing conditions. This could be a possible explanation to the bands that are visible at 120 kDa (representing a possible trimer of GLIC) and 200 kDa (representing a possible pentamer of GLIC) (see fig. 11). One contradiction to this hypothesis is the western blot. The western blot did only display one band (at 37 kDa, see fig. 13), indicating that there is only a monomeric form of GLIC. However, the reason for the lack of band on the western blot at 120 and 200 kDa could be due to that the His-tag, located on the monomers C-terminal domain, could be blocked in the multimeric state. A blocked His-tag would not be able to bind to the first antibody, leading to the belief that there only is a monomeric form of GLIC present. A major concern with this theory is the affinity chromatography. If the His-tag is blocked, the multimeric forms of GLIC should not be able to attach to the gel column in the first place! This could explain the low yield (see table 7). For future work, the flow through from the affinity chromatography should be analysed with SDS-PAGE in order to see if this step is responsible for a significant loss of GLIC that could strengthen the theory about a blocked His-tag. Another possibility is of course that the bands of 120 and 200 kDa (see fig. 11) do not represent multimeric forms of GLIC at all but instead represent proteins with sizes of 120 and 200 kDa.

After all the optimized purification steps it still seems like there are multiple bands (see fig. 14) on different protein sizes that indicates that the GLIC monomer is present but other proteins are as well. To be able to purify it even further, multiple gel filtrations could have been performed (i.e. run all the fractions corresponding to the peaks in fig. 9B individually on a gel column). However, a better way would have been to change the properties of the gel filtration column. For example, a

longer column could have provided with a better separation. Another major concern is the affinity chromatography. This chromatographic method relies on the fact that the His-tag of GLIC can attach to the gel matrix. If the His-tag in fact is blocked, this would result in a significant loss of protein and an alternative affinity chromatographic method needs to be considered. However, even if the His-tag is available for binding, the affinity chromatography step needs to be optimized in other ways. In this project, a step-wise elution with Imidazole was used (see fig. 8). A major flaw in the procedure was that the step-wise elution was performed to fast. In figure 8C, the flow rate was 0.3 ml/min and the column size was 5 ml. The step-wise elution was initiated with 10% buffer D and after 20 minutes, a switch to 50% buffer D was made. Another switch (from 50 to 100%) was made after an extra 20 minutes. This might have been a too tight time interval and might have disallowed a good purification from other proteins that also had bound to the column. This could explain the shoulder on the peaks of the chromatograms (fig. 8). For future work, an Imidazole-gradient could be used in order to determine in which concentration of Imidazole the target protein elutes. After the determination of this, a step-wise elution could be used in a better way and hence optimize the purification process. If this would not work, another possibility would be to change the column entirely. In this project, the beads of the gel matrix were charged with Ni^{2+}-ions due to that it is standard when performing IMAC and working with His-tagged protein. However, there are other metal ions that could be suitable to use (ex. Cu^{2+} or Co^{2+}). For this project, tough, there is another major question to be answered: how pure must the sample be in order to insert it into the proteoliposomes and characterize it with the port-a-patch setup? This question is hard to answer due to the lack of guiding information from other studies and thus it must be determined through trial and error-experiments, by simply adding different concentrations of proteins as well as different purity grades of the sample and see what work and what does not. In theory, it would be enough with one functional GLIC pentamer and the purity of the sample should not have to be so high because the other proteins, making the assumption that they are not membrane proteins and thus do not affect GLICs functionality, would not interfere. However, this is hard to estimate due to the lack of proper studies with the port-a-patch setup.

The characterization part of the project required a successful purification of GLIC. The purification was successful but as the time ran out, the characterization part was neglected to a certain extent. The first step was to create the proteoliposomes by using POPC. The next step was to create a gigaseal with the proteoliposomes and then insert the purified GLIC in order to study the conducting properties. This, however, was never achieved. The gigaseal never formed with the POPC,

which disallowed the insertion of GLIC. The gigaseal only reached 25 MOhm when the lipids were added (see fig. 17). When the suction was increased, the gigaseal broke. This indicates that the proteoliposomes were not properly constructed; they probably formed aggregates instead of a planar lipid bilayer. A proper planar bilayer is required to form the gigaseal; the proteoliposomes should cover the aperture in the NPC1-chip and the suction should then create the wanted gigaseal. For future work, I would have preferred to spend more time on creating the proteoliposomes in order to create a more stable bilayer structure. This could have been achieved by working out new protocols for the preparation of proteoliposomes as well as changing the Mini-extruder setup (i.e. trying different configurations: heat, membrane filter size, different concentrations of POPC-mixture etc.) I do feel confident that proper proteoliposomes would have been stable and strong enough to create the gigaseal and I do believe that the insertion of GLIC would have worked.

4.2 Future aspects

Although the bacterial X-ray structures helps us a great deal regarding coupling the pLGICs structures to function, it is always best to study proteins in their natural environment to be able to fully understand their true function and characteristics. This work has provided new information about purifying a His-tagged variant of GLIC but the information about the characteristics of the conducting properties remain unknown. For future work, the characteristics need to be determined as well as the necessity of purity and concentration for conducting these types of experiments. Seen in a long distance, the research of pLGICs must develop into studying mammalian ion channels for even better knowledge. When using bacterial homologs, post-translational modifications and other biologically important components are different or even absent. Hence, it is of great importance to study mammalian pLGICs using mammalian cell lines. It will be a greater challenge though; both mammalian pLGICs and mammalian cell lines are more complicated to work with. This approach also gives rise to an ethical issue, as it always do when using mammalian cell lines.

4.3 Conclusion

The project aim was to purify and characterize the bacterial homolog GLIC from *E.coli* cells and study its conducting properties using a port-a-patch setup. The characterization with the port-a-patch setup was not achieved due to the lack of proper proteoliposomes. The proteoliposomes that were prepared were not as stable as they were supposed to, disallowing a gigaseal to be formed and thus were

GLIC not inserted into the proteoliposomes. However, the purification process was successful. A His-tagged protein at approximately 40 kDa (37 kDa) was identified with both SDS-PAGE and western blot after the purification process, indicating that GLIC was indeed purified using different chromatography methods. The protein sample purity can be debated, as there were several bands present on the performed SDS-PAGEs. However, one can easily see that the level of purity increases among the steps, indicating suitable choices of purification methods. The question regarding the necessity of a 100% pure sample is also under debate; for continued work with GLIC, it is not sure how pure the sample needs to be or how high the concentration must be in order to characterize its conducting properties. This is a future question to be answered and can only be determined by a trial and error setup

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7 APPENDIX - Buffers and solutions

Table 8: Buffers for purification of GLIC were made accordingly:

Buffer	Contents	pH
A	300 mM NaCl, 20 mM Tris	7.4
B	Buffer A + 2% DDM	
C	Buffer A + 0.1% DDM	
D	500 mM Imidazole + 0.1% DDM	

Table 9: De-staining solution recipe

Substance	Volume
99.9% Methanol	40 ml
99.9% Acetic Acid	10 ml
ddH_2O	50 ml

Table 10: Buffers for Western Blot were made accordingly:

Buffer	Contents	Total volume
TBS	20 mM Tris, 500 mM NaCl	1 L
TBST	TBS, Tween 20%	250 ml
TBST + milk	TBST, non-fat dry milk (semper)	50 ml

TBS: 20 ml 1M Tris was added with 250 ml 4M NaCl in a 1L flask. Distilled water was added to a final volume of 1L.
TBST: 125 µl Tween were added into a 250 ml flask with 250 ml TBS.
TBST + milk: 2.6 g of Semper non-fat dry milk was added to 50 ml of TBST.

Table 11: Description of solution used for port-a-patch experiments.

Solution	Contains	pH
Intracellular solution	130 mM CsCl (cesium chloride), 1 mM $MgCl_2$, 10 mM HEPES, 10 mM BAPTA-Na_4 (1,2-Bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrasodium salt	7.2
HEPES buffered Ringer solution	123 mM NaCl, 2 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, and 10 mM HEPES.	7.5

Both solutions were filtered through a 0.22 µm filt