

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**IMPROVING THE SALT REJECTION POTENTIAL OF
MEMBRANES BY USING CHANNEL PROTEIN AQUAPORIN OF
HALOPHILIC BACTERIUM, HALOMONAS ELONGATA**

GAZEL ÖYKÜM ÖZDEMİR
**A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE**
DEPARTMENT OF ENVIRONMENTAL ENGINEERING

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THESIS SUPERVISOR
PROF. DR. MELEK ÖZKAN

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

HALOFİLİK *HALOMONAS ELONGATA*
BAKTERİSİNİN SU TRANSFER PROTEİNİ
KULLANILARAK MEMBRANIN TUZ İTME
POTANSİYELİNİN İYİLEŞTİRİLMESİ

GAZEL ÖYKÜM ÖZDEMİR
YÜKSEK LİSANS TEZİ
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SUMMARY

Aquaporins are membrane proteins that allow the passage of water molecules and reject all other solutes. In most of the living creatures, aquaporin channel protein in the biological membranes provides high water transport capacity. Like other biological mechanisms aquaporin can be used as mimicking material for developing more efficient products in separation systems in order to be used in the fields of medicine, biochemistry, engineering etc.

In this thesis, a biomimetic filtration membrane was developed by using *Halomonas elongata* aquaporin based on the idea that it might provide more effective water transfer and salt rejection due to its strength of surviving in high concentrations of salt. *H. elongata*'s aquaporin gene was cloned in *E. coli* for the first time and the expressed aquaporin protein was purified from cell membrane. Proteoliposomes with *H. elongata* aquaporins were prepared in order to measure the water transport rate of the aquaporins. Water transport capacity of aquaporin containing liposomes was found to be higher than that of empty (control) liposomes up to 15 times indicating that aquaporin protein was successfully produced in its functional form.

A biomimetic membrane was prepared by incorporation of *H. elongata* aquaporin on polysulphone membrane. Water flux through this biomimetic membrane was shown to be higher as compared to the control membrane. Salt rejection capacity of the membrane can be improved by modification of the method used for aquaporin immobilization on the membrane surface. *H. elongata* aquaporin was shown to be an alternative to *E. coli* aquaporin for development of biomimetic filtration membranes.

Key Words: Aquaporin, desalination, *Halomonas elongata*, biomimetic membranes.

ÖZET

Aquaporinler su moleküllerinin geçişine izin veren ve diğer tüm çözünmüş maddeleri iten membran proteinleridir. Çoğu canlının hücre zarlarında bulunan bu aquaporinler yüksek su geçiş kapasitesi sağlamaktadırlar. Diğer biyolojik mekanizmalar gibi aquaporinlerde ilaç, biyokimya ve mühendislik benzeri alanlarda ayırma proseslerinde kullanılmak üzere daha etkili ve hızlı ürünler geliştirmek için bir mimik malzeme olarak kullanılabilir.

Bu tez kapsamında, *Halomonas elongata*'nın yüksek tuz konsantrasyonlarına yaşayabilmesine bağlı olarak aquaporinlerinin su transfer ve tuz itme özelliklerinin daha efektif olduğu düşünülmüş ve biyomimetik bir membran geliştirilmiştir. *H. elongata*'nın aquaporin geni ilk defa *E. coli*'de klonlanmış ve hücre zarından izole edilmiştir. *H. elongata* aquaporinlerinin su geçirme kapasitelerinin ölçülmesi için proteolipozomlar hazırlanmıştır. Fonksiyonel formunda elde edilen aquaporinleri içeren lipozomların su geçirme kapasitesi boş (kontrol) lipozomlarınkinden 15 kata kadar daha yüksek bulunmuştur.

H. elongata'nın aquaporinlerini içeren polysulfon bir membrane hazırlanmıştır. Hazırlanan biyomimetik membranın su akısı kontrol membranına kıyasla daha yüksek çıkmıştır. İmmobilizasyon metodunun geliştirilmesiyle tuz itme kapasitesinin daha fazla artırılması mümkündür. Biyomimetik filtrasyon membranlar geliştirilmesi için *H. elongata* aquaporininin *E. coli* aquaporinine alternatif olarak kullanılabileceği gösterilmiştir.

Anahtar Kelimeler: Aquaporin, tuzsuzlaştırma, *Halomonas elongata*, biyomimetik, membran.

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Explanations

Acronyms

AQP	:	Aquaporin gene
Aqp gene	:	Aquaporin gene
BSA	:	Bovine serum albumin
CA	:	Cellulose acetate
DI water	:	De-ionized water
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide triphosphate
DOPC	:	1,2-dioleoyl-sn-glycero-3-phosphocholine
GLUT	:	Glucose transistor
GS	:	Gas separation
GST	:	Glutathione S-transferase
HEAQF	:	<i>Halomonas elongata</i> aquaporin gene forward primer
HEAQR	:	<i>Halomonas elongata</i> aquaporin gene reverse primer
HopP	:	Halophilic outer membrane protein
IPTG	:	Isopropyl β -D-1-thiogalactopyranoside
LDH	:	Lactate dehydrogenase
LGIC	:	Ligand gated ion channel
LMH	:	L/m ² .h
MF	:	Microfiltration
MPD	:	m-phenylene-diamine
NF	:	Nanofiltration
NPA	:	asparagine-proline-alanine amino acids
OG	:	Octyl-beta-glucoside
ompC	:	Outer membrane protein C precursor
PBS	:	Phosphate-buffered saline
PC	:	Phosphatidylcholine
PCR	:	Polymerase chain reaction
PDI	:	Polydispersity index

PropP	:	proline/betaine transporter
PropU	:	glycine betaine and L-proline transporter
PS	:	Phosphatidylserine
RO	:	Reverse osmosis
SDS	:	Sodium dodecyl sulfate
SEM	:	Scanning electron microscope
TMC	:	Trimesoyl chloride
TMV	:	Tobacco mosaic virus
UF	:	Ultrafiltration
UV	:	Ultraviolet
β ME	:	β -mercaptoethanol

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1. INTRODUCTION

1.1. Aim, Contribution and Content of the Thesis

Water scarcity is both a natural and a human-made phenomenon. There is enough freshwater on the planet for seven billion people but it is distributed unevenly and too much of it is wasted, polluted and unsustainably managed. Increased water scarcity and stringent water quality legislation are fueling the expansion of the membrane-based water treatment market.

The membrane is a specific filter that will let water flow through, while it catches suspended solids and other substances. At the present time membrane processes are used in various fields including waste water treatment, distilled water production, fresh water production, food-drug industry separation operations, gas separation in petro chemistry industry etc. [1]. Membrane processes are designed to behave semipermeable layer in two different phases. This permeability occurs according to differences in pressure, concentration, temperature, electric potential, etc. [2].

The membrane processes are regarded as one of the most promising technologies for solving this problem. Converting the sea water into drinking water is a heavily studied subject in this area. During the separation process, pressure is required and it consumes high amount of energy which increases the cost of the process. If the present desalination methods cannot be improved in terms of process cost and efficiency, they will only be applicable in countries which has a wealthy economy. Fabrication of membranes which are more efficient in water filtration as well as salt rejection can be a solution for reducing high cost of desalination. Agre *et al.* discovered a unique protein that called aquaporinZ which is the main structure in membrane for water transport mechanism [3]. This discovery led him to won Nobel Prize in 2008 [4].

Studies on producing aquaporin incorporated filtration membranes for desalination are increasing day by day, but still there are few studies in literature. In most of these studies *E. coli* aquaporins are used. For example, Kumar *et. al* (2007) and Li *et.al* (2014) has developed aquaporin immobilized biomimetic membranes [5,

6] using *E. coli* AqpZ, and their results are quite satisfying that *E. coli* aquaporin embedded membranes has high capacity of water permeability and salt rejection capacity.

Halomonas elongata is an extremely salt-tolerant gram-negative bacterium. There are not many studies on porins of this specie. Only OmpC (outer membrane protein C) protein [7] and HopP (halophilic outer membrane protein) porin [8] were studied. Living in a salty environment might affect structure of membrane proteins since more strict control is necessary for protecting the cell from high salt concentration. In this thesis, it was hypothesized that *H. elongata* aquaporin may be more efficient in salt rejection due to its unique features. In this respect, this protein is open for further studies.

Every day new ideas are being propounded on membranes that they can be utilized in lots of different areas. By adding new properties to the membranes, they become more efficient in use. Bio-mimicking is an imitation of the models, systems, and elements of nature for the purpose of solving complex problems. Also, it is a way to bring multiple features into materials by direct use or modifying biological structures. Living cells, DNA, proteins are some of the biological materials that can be integrated into a receiving structure. Aquaporin protein, which is studied in this thesis, can be given as a specific example to these biological structures. Diffusion was thought to be the only way to transport water from membranes until the complete discovery of aquaporins. It is well understood that, in the result of Benga's and Agre's scientific contributions, most of the water demand in the cells eliminated by these proteins and they indicated that aquaporins increase the transport capacity up to 100 times [9-11].

In the first instance aquaporin1 protein was isolated from human red cells, then researches moved to a next level and aquaporins were extracted from a variety of living tissues. For instance, human kidney [12], mammalian optic lenses[13], hepatic bile ducts and water-resorptive epithelium of gallbladder [14] are some of the aquaporin rich biological membranes. Apart from that, aquaporin's high capacity of retaining the soluble substances like urea, glycerol, glucose allows high volumes of water pass through the membranes [15, 16]. Aquaporins block even the protons by means of its chemical and physical structure. It has six bilayer-spanning domains and in other respects the hourglass model essential for the formation of the water pore [5, 17]. This excessive water-selective protein is present in all kind of membranes which

need high water molecule transition. Aquaporins can be found also in bacteria, like plants and animals, and the mechanism of them now further known [11].

This thesis aims to create a functional membrane by using aquaporin proteins of a halophilic bacteria, *Halomonas elongata*. This study consists of several steps which are; cloning aquaporin genes, purification of the proteins and incorporation of these water channel proteins to the synthetic membranes. Liposomes carrying this specie of bacterial aquaporins, which is distinguished by its tolerance to high concentrations of salt, is expected to have high capacity of water transport and salt rejection. The membranes going be obtained in this study will be used with the aim of water filtration.

The results obtained within the scope of this thesis would be a helpful source for development of new materials which involves aquaporins and other pore proteins. This study will provide useful basis for new researches that aim to develop biomimetic filtration membranes.

2. LITERATURE REVIEW

2.1. Biomimetic approach

Biomimetic or biomimicry is to copy or transfer natural features with the aim of developing efficient products in order to be used in human necessity.

Biomimetic also means the use and implementation of concepts and principles from nature to creating new materials, devices and systems. There are various applications in different areas. It is classified as imitation and transfer. The imitation can be explained by how planes were modelled by considering birds physiological structure. Using the advantages of the functions of DNA, protein, bacteria or viruses provides new beneficial properties to biomimetic materials. Modifications needs to be applied to these biological molecules with respect to their specific structures. It is obtained by handling the specific features in design[18]. Use of Tobacco Mosaic Virus (TMV), a high aspect ratio cylindrical plant virus, for increasing the active electrode area in fabricated batteries is a successful example to biomimetic approach. Their structure increased the battery life up to 6 times due to self-connectivity and the shell's capability to bind nickel and cobalt [19]. Recent technologies allow immobilization of cell or their molecules into the different surfaces to bring similar features of living cells to the non-living structures. Aquaporin incorporation to the filtration membranes is one example to these applications[20]. Another type of study showed that, peritoneal transport studies using AQP1 knockout mice demonstrated that the osmotic water flux across the peritoneal membrane is mediated by AQP1[21]. These AQP channels were used for developing sensor devices. [22]. Biological cell membranes provide solid molecular evidence for fast trans-membrane water transport with high salt rejection. This specialty has spotlighted for incorporating aquaporin into biomimetic membranes for water reuse. Most aquaporin proteins appear to be exclusive water channels that will not allow the transport of ions or other small molecules because of narrow channels and unique charge characteristics. Therefore, this lead to a tempting to integrate the mentioned exclusive water channel proteins in water purification applications [23].

2.2. Aquaporin

2.2.1. Aquaporin and Its Applications on Filtration Membranes

2.2.1.1. Cell membranes

Cell membranes of living organisms have excellent water transport system, and some can generate this transport capacity in a wide range. By 1920, it has been understood that how a cell can maintain optimum conditions for its endurance in environments of high and low pH, salt and other solutes. By means of exploration of ion channels and co-transporters, transmembrane movements of solutes have been understood. Until this information scientist supposed that only diffusion is the mechanism of transportation for water in and out of membrane.

Transport through cell membrane is achieved by different mechanisms including diffusion, osmosis and active transport.

Diffusion: Articles move from a region where they are in high concentration to lower concentration. For diffusion, materials should be mobile and two environments should have different densities. There no energy consumption and no need for enzymes for this process. Some of the molecules which uses diffusion in living organisms are; galactose, glucose, alcohol, vitamins A-D-E-K, Cl, Na etc.

Osmosis: Diffusion of water from a semi-permeable membrane, in other words, transition of water molecules from a high concentrated stage to lower one.

Active transport: This type of transport occurs if the solute cannot enter into cell by diffusion. Only living cells can manage this system by using enzymes and energy.

All transport protein in the cell use a kind of system which has stated above. As an example GLUT (glucose transitor) coded proteins responsible from glucose transition [24], and LGIC (ligand gated ion channel) coded protein is liable from positive charged ions transition like potassium, calcium, etc. [25]. Furthermore, aquaporins control water transport system in the cells [26].

In 1992, AQP1 which is a membrane protein for water transport was discovered. Even though diffusion can hold the water transport need, aquaporins can increase the capacity up to 10 times, and even protons like H_3O^+ are rejected by them. In in-vivo conditions movement of water through the aquaporins are determined according to

osmotic or hydrolytic gradient [3]. Aquaporin which is the functional building blocks of cell membranes have been used due to its unique combination of offering high water permeability and high solute rejection for fabrication of synthetic biomimetic filtration membrane for water desalination and reuse [27].

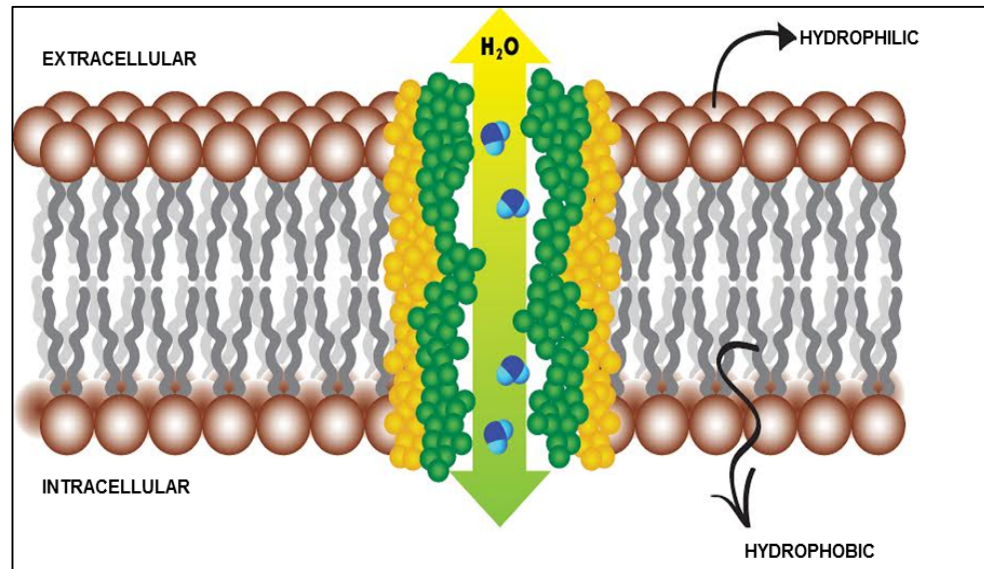


Figure 2.1: Aquaporin channel in cell membrane.

2.2.1.2. Aquaporin structure and function

Some members of aquaporin have excellent rejection capacity of solutes like urea, glycerol and glucose even in high water transport velocity [15]. These features are based on aquaporins water selective specific structure. Aquaporins have six domains that surrounds the membrane, and has an hourglass structure that formed from charged amino acids that created pores which allows it to transport water and reject solutes (Figure 2.2). Their configuration is always open, and water flows in both direction according to concentration [17].

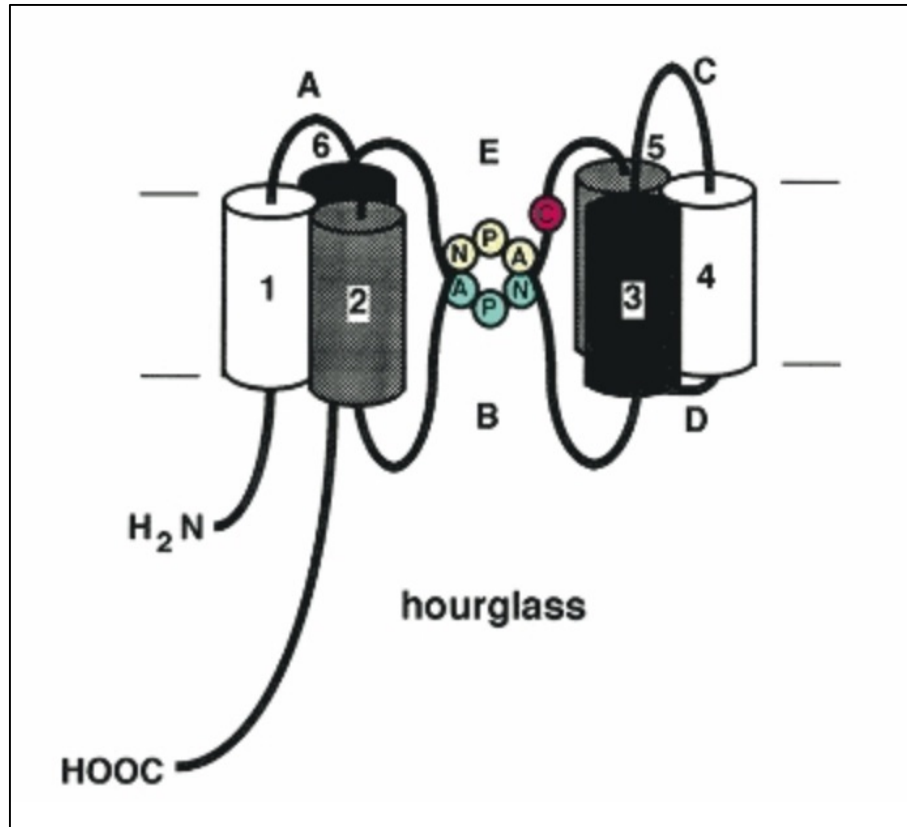


Figure 2.2: Aquaporin structure.

Aquaporin modified membranes permeability are much higher than phospholipid membranes [15]. Its structure and function provides this significant characteristic.

4 monomers merge together and create a tetramer, and transportation occurs in the center of these 4 individual monomers. 6 helix structure and A-B-C-D-E loops designed to allow only water molecule (H_2O) dimensions, even if there is a third H^+ ion they all rejected to the extracellular by the aquaporin. Histidine 180 and Arginine 195 creates the narrowest space which only allows water molecules to pass through. Since Arginine 195 is positively charged, it rejects other solutes like K^+ , Na^{++} . Water molecules moves sequentially in or out of the cell with changing hydrogen bond location by means of the aquaporins Arginine 195 and NPA motifs [28].

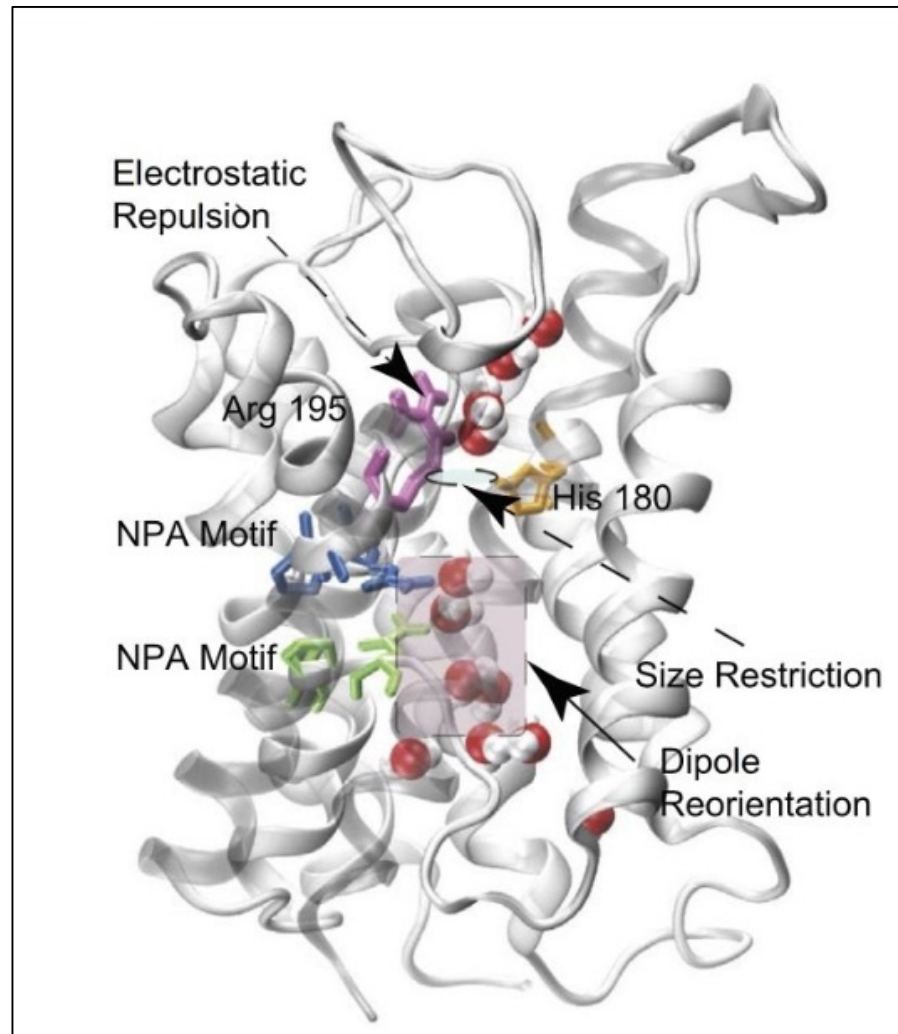


Figure 2.3: Aquaporin water transport system.

Water movement across biological membranes is not fully explained by simple diffusion through the lipid bilayer [15]. A great deal of research has been performed on discovery of aquaporins from different cell membranes. Existence of a set of proteins in the lipid bilayer for water transport was first reported by Peter Agre who won the Nobel Prize in 2003 for the discovery of aquaporins.

The most studied aquaporins are the mammalian and vascular plant water channels. There are 13 different aquaporins in mammals, from AQP0 to AQP12 [26] and more than 30 water channels have been found in plant genomes [29].

AQPs are not widespread in bacteria and archaea, in which water fluxes must then occur through the membrane by passive diffusion or unspecific pores [30, 31]. Several aquaporins have been discovered from different microorganisms including Gram negative bacteria *E. coli* [32] and *Rhodobacter sphaeroides* [33], gram positive

bacteria *Lactococcus lactis* [34], yeast *Saccharomyces cerevisiae* [35], and finally archaea *Methanothermobacter marburgensis*.

E. coli aquaporin, AqpZ was first cloned and characterized by Calamita et. al. (1995). Further characterization was carried out by Borgnia et. al. (1999) who showed that it is a highly stable tetramer with negligible permeability to glycerol.

In this thesis, we aimed to develop a biomimetic membrane with the aquaporin of *H. elongata*, a halophilic bacterium. It was thought that aquaporin of a halophilic might have higher solute rejection and water transport capacity.

2.3. Membrane Processes

Membranes are selective barriers that reject some of the solutes (molecules, ions, small particles etc.) but allow some components to pass through. Influent of a synthetic membranes called as feed, and liquid or gas that passes through named as permeate as shown in Figure 2.4 [36]. Membranes structures might be homo/heterogeneous, symmetric/asymmetric or solid/liquid. Also, they can be negative, positive, neutral and bipolar charged.

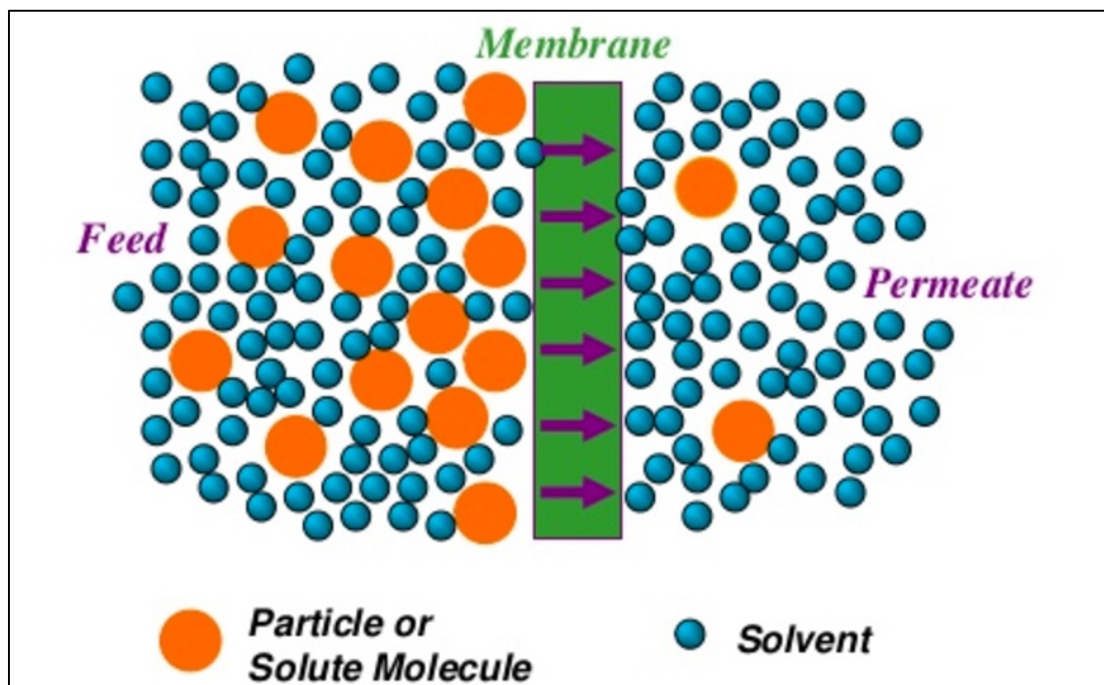


Figure 2.4: Membrane separation principle.

First membrane experiments were performed by using materials like cattle and pig skins. In the early 20thies nitro-cellulosic membranes with different pore size were developed. By the year of 1960, modern membrane applications were discovered and laboratory studies started. Later on, membrane technologies like surface polymerization, multi layered composite casting and covering processes developed, and these membranes pore size reached minimum to 0.1 micrometer which allows to select small sized particles. After 1980, new features added to existing technologies, and membranes became a useful tool in variety of areas like engineering, medicine etc. Different techniques like microfiltration, ultrafiltration, reverse osmosis, and electro-dialysis developed in order to satisfy variety of needs especially fresh water production [37].

Membrane processes were firstly used for separation of sea and well water. By the developments in the industry membranes were started to be used in different applications. Beside water separation processes, the membrane processes are used for different filtration purposes including separation of waste water, soft water production, food and drug industry and for gas separation in petro chemistry [1, 38].

2.3.1. Membrane Application Fields

Membrane separation is a technology which selectively separates applicable in variety of areas such as:

- Water and Pharmaceutical Purification
- Waste Water Treatment
- Seawater Desalination
- Food & Beverage (Dairy processing, clarification, de-bittering, bottled water, de-alcoholization of wine etc.)
- Solvent Purification and Recovery
- Biofuel Separation (Bioethanol, biodiesel, etc.)
- Petroleum & Petrochemical (Catalyst recovery, NPG purification, petroleum & petrochemical)
- Gas Separation & Purification (CO₂/H₂, NPG & LPG purification)

2.3.2. Membrane Processes Advantages and Disadvantages

The advantages include the following subjects.

- Because membrane processes can separate at the molecular scale up to a scale at which particles can actually be seen, this implies that a very large number of separation needs might actually be met by membrane processes.
- Membrane processes generally do not require a phase change to make a separation (with the exception of pervaporation). As a result, energy requirements will be low unless a great deal of energy needs to be expended to increase the pressure of a feed stream in order to drive the permeating component(s) across the membrane.
- Membrane processes present basically a very simple flowsheet. There are no moving parts (except for pumps or compressors), no complex control schemes, and little ancillary equipment compared to many other processes. As such, they can offer a simple, easy-to-operate, low maintenance process option.
- Membranes can be produced with extremely high selectivities for the components to be separated. In general, the values of these selectivities are much higher than typical values for relative volatility for distillation operations.
- Because of the fact that a very large number of polymers and inorganic media can be used as membranes, there can be a great deal of control over separation selectivities.
- Membrane processes are able to recover minor but valuable components from a main stream without substantial energy costs.
- Membrane processes are potentially better for the environment since the membrane approach require the use of relatively simple and non-harmful materials.

The disadvantages include the following subjects.

- Membrane processes seldom produce 2 pure products, that is, one of the 2 streams are almost always contaminated with a minor amount of a second component. In some cases, a product can only be concentrated as a retentate because of osmotic pressure problems. In other cases, the permeate stream can

contain significant amount of materials which one is trying to concentrate in the retentate because the membrane selectivity is not infinite.

- Membrane processes cannot be easily staged compared to processes such as distillation, and most often membrane processes have only one or sometimes two or three stages. This means that the membrane being used for a given separation must have much higher selectivities than would be necessary for relative volatilities in distillation. Thus, the trade-off is often high selectivity/few stages for membrane processes versus low selectivity/many stages for other processes.
- Membranes can have chemical incompatibilities with process solutions. This is especially the case in typical chemical industry solutions which can contain high concentrations of various organic compounds. Against such solutions, many polymer-based membranes (which comprise the majority of membrane materials used today), can dissolve, or swell, or weaken to the extent that their lifetimes become unacceptably short or their selectivities become unacceptably low.
- Membrane modules often cannot operate at much above room temperature. This is again related to the fact that most membranes are polymer-based, and that a large fraction of these polymers do not maintain their physical integrity at much above 100°C. This temperature limitation means that membrane processes in a number of cases cannot be made compatible with chemical processes conditions very easily.
- Membrane processes often do not scale up very well to accept massive stream sizes. Membrane processes typically consist of a number of membrane modules in parallel, which must be replicated over and over to scale to larger feed rates.
- Membrane processes can be saddled with major problems of fouling of the membranes while processing some type of feed streams. This fouling, especially if it is difficult to remove, can greatly restrict the permeation rate through the membranes and make them essentially unsuitable for such applications[39].

2.3.3. Pressure Driven Membrane Separation Systems

Membranes achieve separation by the influence of different driven forces such as temperature, electrical potential difference, pressure, concentration etc.[2]. Porous membranes separate with respect to size, shape and charge difference, and nonporous

membranes control separation by sorption and diffusion model. Membrane performance is determined with the parameters of selectivity and flux [40].

Pressure driven systems have different capture abilities with respect to their different pore sizes as shown in Figure 2.5 [41].

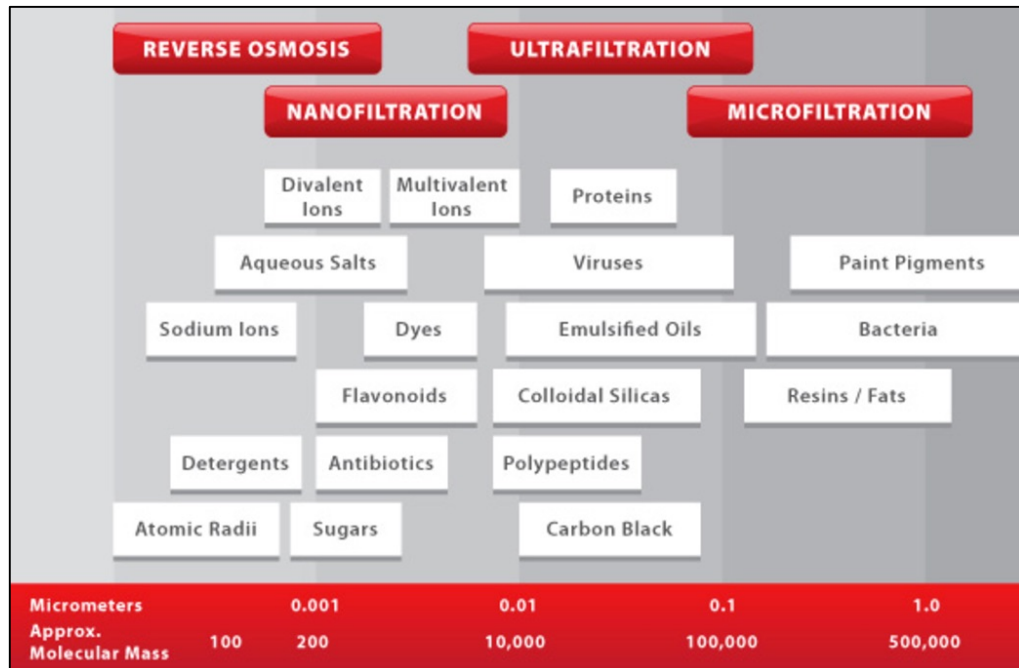


Figure 2.5: Membrane separation techniques by size.

Commonly used membranes in industries are pressure system ones. These are reverse osmosis [19], nanofiltration [42], ultrafiltration (UF) and microfiltration (MF) with respect to their pore sizes. Separation from these membranes occurs with the help of driving forces depending on the size and mass of the molecules size [43].

Table 2.1 gives the operation conditions of pressure driven membranes by means of operation pressure and pore size.

Table 2.1: Operation conditions of pressure driven membranes.

Filtration Type	MF	UF	NF	RO
Operation Pressure (Bar)	1-4	2-7	10-40	15-100
Pore size (µm)	0.1-1.5	0.01-0.05	0.001-0.01	<0.0002

2.3.4. Microfiltration Membranes (MF)

Microfiltration is a type of physical filtration process where a contaminated fluid is passed through a special pore-sized membrane to separate microorganisms and suspended particles from process liquid. It is commonly used in conjunction with various other separation processes such as ultrafiltration and reverse osmosis to provide a product stream which is free of undesired contaminants. Materials like bacteria, fat, yeast, algae etc. are treated with MF process [44].

2.3.5. Ultrafiltration (UF)

Ultrafiltration (UF) membranes, remove all fine silt, turbidity, bacteria and viruses, as well as chlorine-resistant organisms such as *Cryptosporidium* and *Giardia*. Ultrafiltration membrane productivity is high, while pressure differences remain low. UF membrane technology is used to produce potable water and process water, and is used in the pre-treatment process of large seawater reverse osmosis plants.

Ultrafiltration membrane technology has proven to be an excellent large-scale treatment method for surface water, the effluent of wastewater treatment plants, and groundwater projects with suspended solids levels of up to 100 to 200 mg/L [44].

2.3.6. Reverse Osmosis (RO)

Reverse osmosis is liquid filtering process in which a more concentrated liquid is forced to pass through a semi-permeable membrane by using pressure that block most of the dissolved or suspended contaminants.

Population growth, industrialization, active agricultural activity, unconscious consumption of limited ground and surface water recourses led to water scarcity. But these kinds of hitches enabled the discoveries of new technologies. By taking into consideration that almost 70% of the world is surrounded with salty waters, it is not surprising that researchers and industries focus on conversion of salty water to potable water.

The reverse osmosis (RO) processes were used to separate sea water and well water at the begging, however with the technological and industrial developments they have been involved in various different applications. The applied areas of

reverse osmosis processes are as follows: color removal process in waste water in textile industry, regaining metals in metal covering industry, regaining in food industry, treatment in waste water, color removal in paper production industry, iron, steel and mining industry, reusing agricultural drainage water etc. [45]. Reverse osmosis system is shown in Figure 2.6 [46].

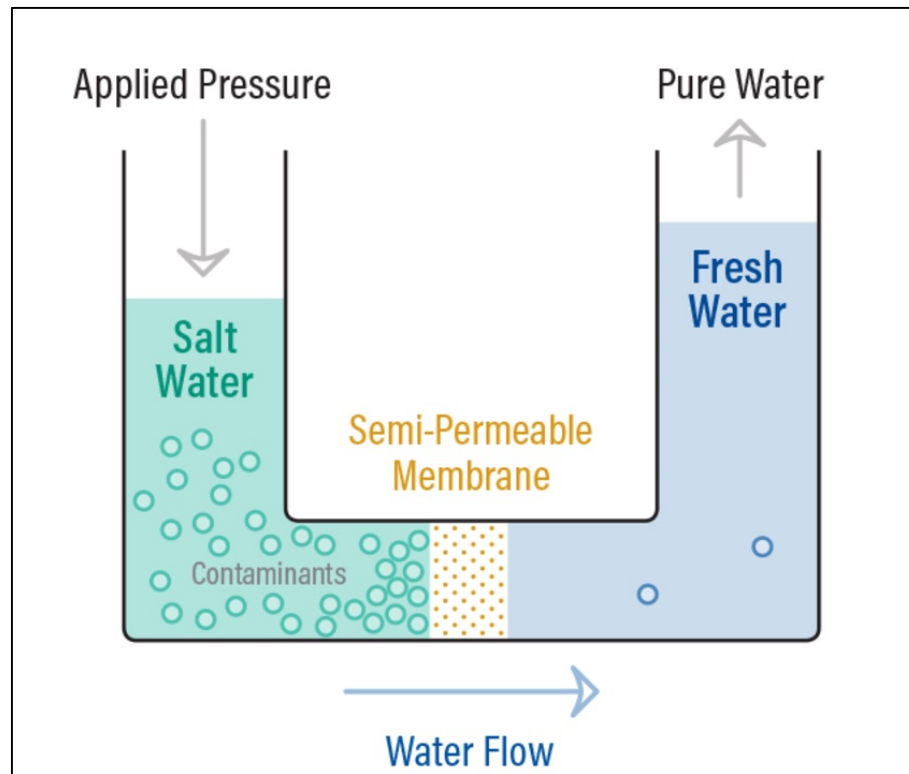


Figure 2.6: Reverse osmosis principle.

Reverse osmosis membrane is used for separation of all organic and inorganic materials from solutions that has so small molecular weight. This technique can reject particles sized between $0,0001$ and $0,001 \mu$ ($1-10^0 \text{ \AA}$) [47].

Even though reverse osmosis systems are highly developed at present, there have considerable difficulties on their management and design. Some of the problems are;

- Degradation and abrasion in membranes
- High energy cost
- Problems with salt penetration from membrane
- Feed water ratio and low flux

- Feed water temperature
- Bio growth in the system

2.3.7. Nano-filtration (NF)

Nanofiltration technique is used for separating the particles that are salt or maximum 10 times bigger molecules than salt. Treatment is performed by passing the solution through membrane by centrifugation.

Nanofiltration membranes (NF) have applications in several areas. One of the main applications is water treatment for drinking water production as well as wastewater treatment. NF can either be used to treat all kinds of water including ground, surface, and wastewater or used as a pretreatment for desalination. The introduction of NF as a pretreatment is considered a breakthrough for the desalination process. NF membranes have been shown to be able to remove turbidity, microorganisms and hardness, as well as a fraction of the dissolved salts. This results in a significantly lower operating pressure and thus provides a much more energy-efficient process [48]. Figure 2.7 shows the working principle of nanofiltration [41].

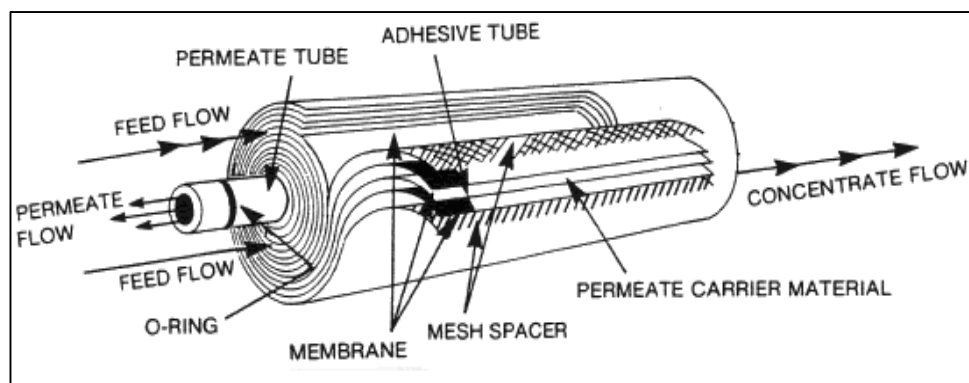


Figure 2.7: Nanofiltration working principle.

2.3.8. Desalination

The chemical process of changing seawater into potable or fresh water is called desalination. Thermal distillation and membrane processes are the two main approaches used around the world to desalinise water. Desalination processes may be used in municipal, industrial, or commercial applications. With improvements in

technology, desalination processes are becoming cost-competitive with other methods of producing usable water to respond to a growing demand. Stand-alone desalination plants can use renewable energy to operate. The pure water that is obtained after desalination must be re-mineralised to be adequate for human consumption. The concentrated brine produced in desalination processes needs to be disposed of properly [49].

The costs of desalination vary significantly depending on the size and type of the desalination plant, the source and quality of incoming feed water, the plant location, site conditions, qualified labor, energy costs and plant lifetime. Lower feed water salinity requires less power consumption and dosing of chemicals to prevent scaling. Larger plant capacity reduces the unit cost of water due to economies of scale. Lower energy costs and a longer plant running period reduce unit product water cost. The primary elements of desalination costs are capital cost and annual running cost. The capital cost includes the purchase cost of major equipment, auxiliary equipment, land, construction, management overheads, and contingency costs. The capital costs for seawater desalination plants have decreased over the years due to the on-going development of processes, components and materials. Annual running costs consist of costs for energy, labor, chemicals, consumables and spare parts. The energy costs strongly affect the running costs for thermal processes. Thus, distillation costs fluctuate more than reverse osmosis as they depend on changing energy costs. In regions where energy is fairly expensive, reverse osmosis is a favorable choice compared to any other thermal processes due to its lower energy consumption.

Aquaporin concentration polymerization is another point of consider for efficient water flux. Kumar *et al.* showed that permeability increased followed by a decrease as the concentration of protein is increased. They obtained the optimum permeability peaks at a protein-to-polymer ratio of 1:50 where the vesicle permeability was $\approx 3,000$ times greater than the pure polymer [5].

2.4. Organic Membranes

Different materials used for biomimetic membrane production such as polyacrylonitrile, cellulose acetate, polysulfone, Poly(amide-imide), etc. [28]. Ultrafiltration and Microfiltration organic membranes made by polyethersulfone,

polyvinylidenedifluoride, polyacrylonitrile. Membrane selection depends on a variety of factors, including the composition of the feed solution, operating parameters, application type, and separation goals. While organic and inorganic membranes have their own advantages and disadvantages, it is important to determine what type of membrane or polymer is most suitable for the application.

A majority of industrial membranes consist of synthetic or natural polymers; membranes with both types of polymers are known as organic membranes. Examples of synthetic polymers include polytetrafluoroethylene, polyamide-imide, and polyvinylidenedifluoride while natural polymers include rubber, wool, and cellulose. Artificial polymers are synthesized by the polymerization of a monomer or copolymerization of 2 monomers [39].

2.4.1. Cellulose Acetate Membranes

Cellulose Acetate is a natural plastic, which is manufactured from purified natural cellulose. Cellulose acetate has good chemical resistance to diluted aqueous solutions with acidic base and inorganic salts, parafinic hydrocarbons, superior alcohols and solutions with fatty matter. They can't be operated in high temperatures and their operation pH range is between 3-8. Its chemical formula shown in Figure 2.8 [39].

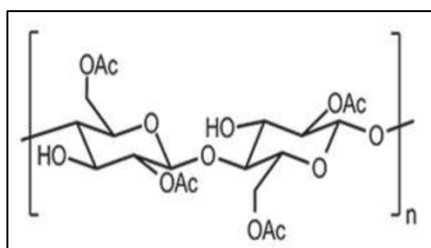


Figure 2.8: Cellulose acetate chemical formula.

2.4.2. Polysulphone Membranes

One of the most used type of membrane is polysulfone membrane. They are preferred for easy manufacturing, resistance to corrosive chemicals and stability and resistance to temperatures up to 75°C. Chemical formula of polysulphone is shown in Figure 2.9 [39].

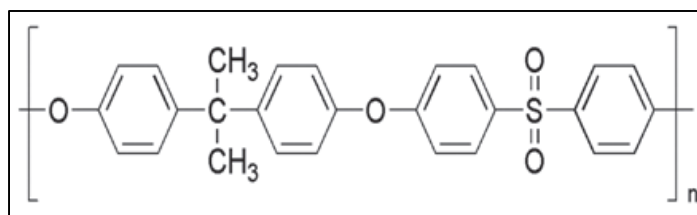


Figure 2.9 : Polysulfone chemical formula.

2.4.3. Polyamide Membranes

Polyamide membranes are the most commonly used membrane construction materials because of their lower pressure requirements and more flexible operating conditions. Polyamide membranes are oxidant (chlorine, chloramine, bromine, ozone etc.) intolerant and they carry an anionic charge. This type of membrane used for substrat material in membrane production, their operation pH range is between 2-10. Its chemical formula was shown in Figure 2.10 [39].

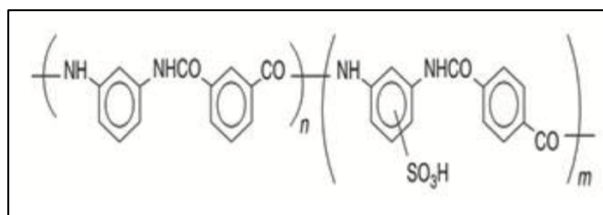


Figure 2.10 : Polyamide chemical formula.

2.5. Membrane Surface Modification

The major aim of surface modification is improving the performance of the membranes with a view to altering a wide range of characteristics of the surface, such as roughness, surface energy, surface charge, hydrophilicity, biocompatibility, and functionality. Although each method has its own advantages and limitations, interesting results can be obtained for the modified membranes in the form of improved properties [50].

Different methods can be used for modifications. Basic techniques are listed below;

Surfactants; treatment with water insoluble surfactant to enhance hydrophilicity.

Coatings; thin film application of different polymer or monomer-system to form a new surface via composite formation.

Chemical grafting; process of attachment of a low molecular weight active group (monomer) to a parent polymer or membrane.

Polymer blends; mixture of two or more different polymers can improve chemical properties of membranes and separation capacity.

Plasma; this technique of plasma modification is similar to chemical grafting in that a chemical modification is performed on the membrane polymer material.

The development of high-performance membranes involves the selection of a suitable membrane material and the formation of this material into a desired membrane structure. However, it is often necessary to modify the membrane material or the structure to enhance the overall performance of the membrane. Generally, the objectives for modification of pre-formed membranes are increasing flux and/or selectivity and increasing chemical resistance (solvent resistance, swelling, or fouling resistance) [51].

2.5.1. *Halomonas elongata*

H. elongata is a mesophilic (23-37°C), halophilic bacterium that can live in environments contain up to 32% salt concentration. For this bacterium's vitality salt concentration should be 0.5 M and for its optimum reproduction it should be 1.5-2 M [52]. There are several studies in the literature about type of *Halomonas elongata*'s osmoregulation due to its endurance in high NaCl concentrations [53]. Not only halophilic microorganism, but also non-halophilic ones have various mechanisms for survival. As an example; *Halomonadaceae* family is one of the moderate halophilic prokaryote, can adapt to the environments with wide range of salt concentration. One mechanisms for adapting high salt concentration that this strain has developed is storing potassium into the cells, as second mechanism; production of coherent solubles called osmoregulators such as amino acids, sugars and polyols or transferring those solubles from outside to inside of the cell. Systems which plays role on the transfer of these osmoregulators also exist in other kind of bacteria. ProP

(proline/betaine transporter) and ProU (glycine betaine and L-proline transporter) in *E. coli* and *Salmonella typhimurium* can be given as examples to these systems. Up to the date the only study about membrane proteins of *Halomonas elongata* is the discovery of a porin in *Halomonas* sp. 40 which is similar to *E. coli* ompC (outer membrane protein C precursor) porin, this porin named as HopP (Monosaccharide porin precursor) was cloned into *E. coli*, then expressed. While OmpC porin allows hydrophilic molecules to pass through, hydrophobics cannot [52]. Tokunaga et. al prepared proteo-liposomes in order to measure salt transfer potential of OmpC, and they had successful result. Because only aquaporins are specific to water, they have different responsibilities then porins like HopP and OmpC whose function is taking solutes into cell. Primary duty of aquaporins is to accelerate the water transition through the cell membrane [7].

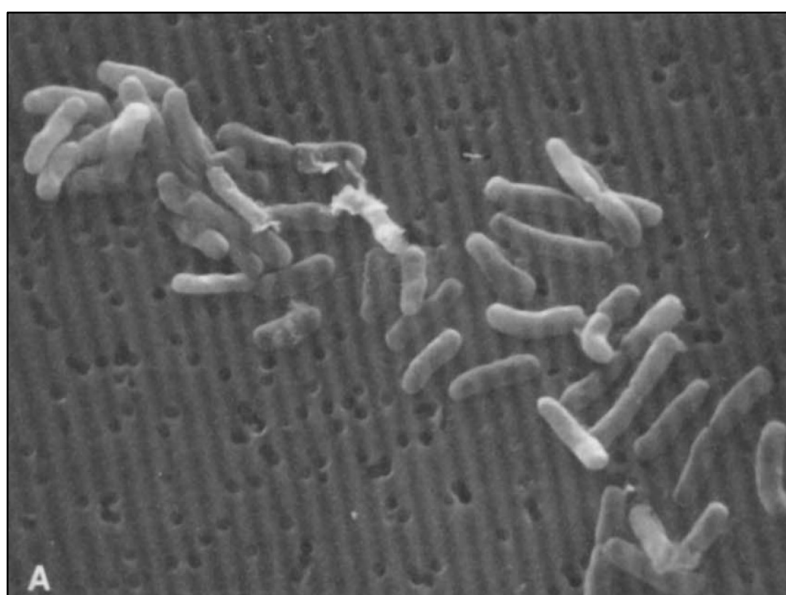


Figure 2.11: *Halomonas elongata* bacteria.

2.5.2. Surface Imprinting and Immobilization

Surface imprinting is the operation of integrated polymer synthesis that allows to bound the target molecule onto surface. Besides, surface immobilization is used for binding the target material onto activated surface. If modifications are performed with chemical bonds like covalent, ionic, dipole dipole etc. materials become more resistant to different conditions.

There are different methods for surface imprinting and immobilization procedures. Two of them which are mostly used for aquaporin incorporation listed and explained below.

Xie *et al.* have designed and fabricated a stable and functional polymeric membrane embedded with nano-sized aquaporin-vesicles through an innovative yet simple and easy to-implement method based on an AQPz-incorporated biomimetic membrane and surface imprinting technology. They proved that AQPz was functionally incorporated into polymer vesicles while retaining their water selectivity and transport capability. Also, AQPz-incorporated vesicles were successfully imprinted onto an ultrafiltration substrate membrane. They showed that AQPz has significant contribution to the membrane performance in terms of high water permeability and high salt rejection rate in either forward osmosis or the nanofiltration process. Figure 2.12 represents the aquaporin containing vesicle embedded membrane [54].

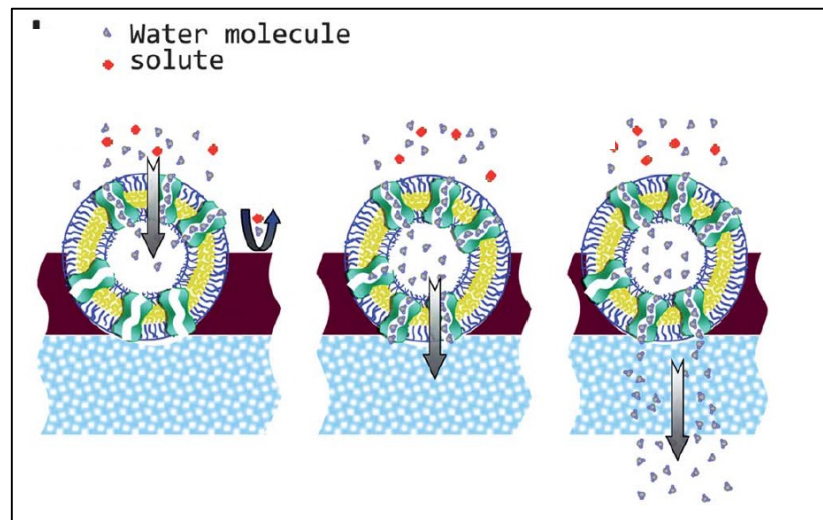


Figure 2.12: Aquaporin immobilized membrane representation.

Zhao *et al.* fabricated an aquaporin-based biomimetic membrane using the interfacial polymerization method. The resulting membrane showed good mechanical stability when tested up to 10 bars under RO conditions. High water permeability was observed at an applied pressure of 5 bar, and rejection was further improved with different applied pressures in the study [20].

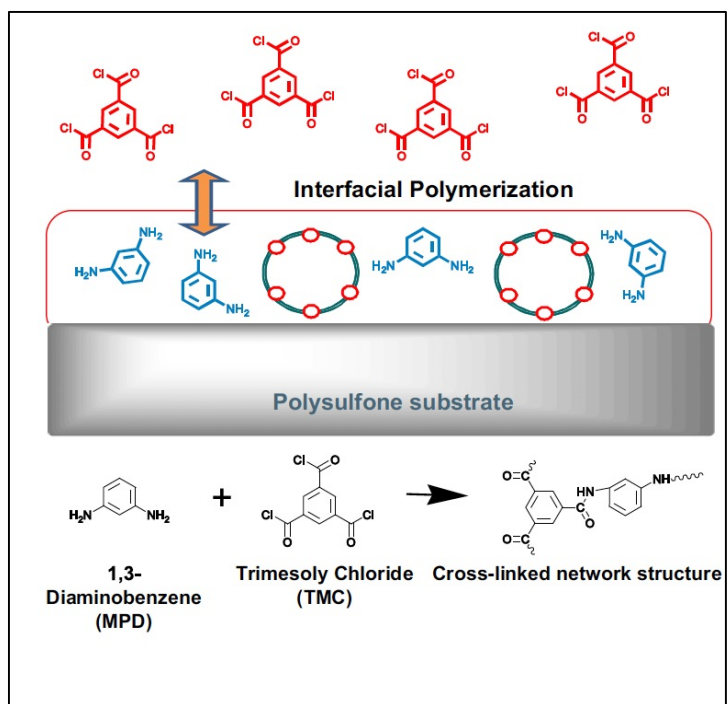


Figure 2.13: Interfacial polymerization of aquaporin-based biomimetic membranes.

3. MATERIALS and METHODS

3.1. Cloning and Protein Synthesis

3.1.1. Host Strains, Vectors, and Enzymes

E. coli Top10 and DH5 α competent cells were used as the host gene manipulation, while BL21 was chosen as recombinant protein expression host based upon its high efficiency expression of proteins. pGEM-T Easy Vector (Figure 3.1) was used as cloning vector and pGEX-4T (Figure 3.2) was used to construct expression vector.

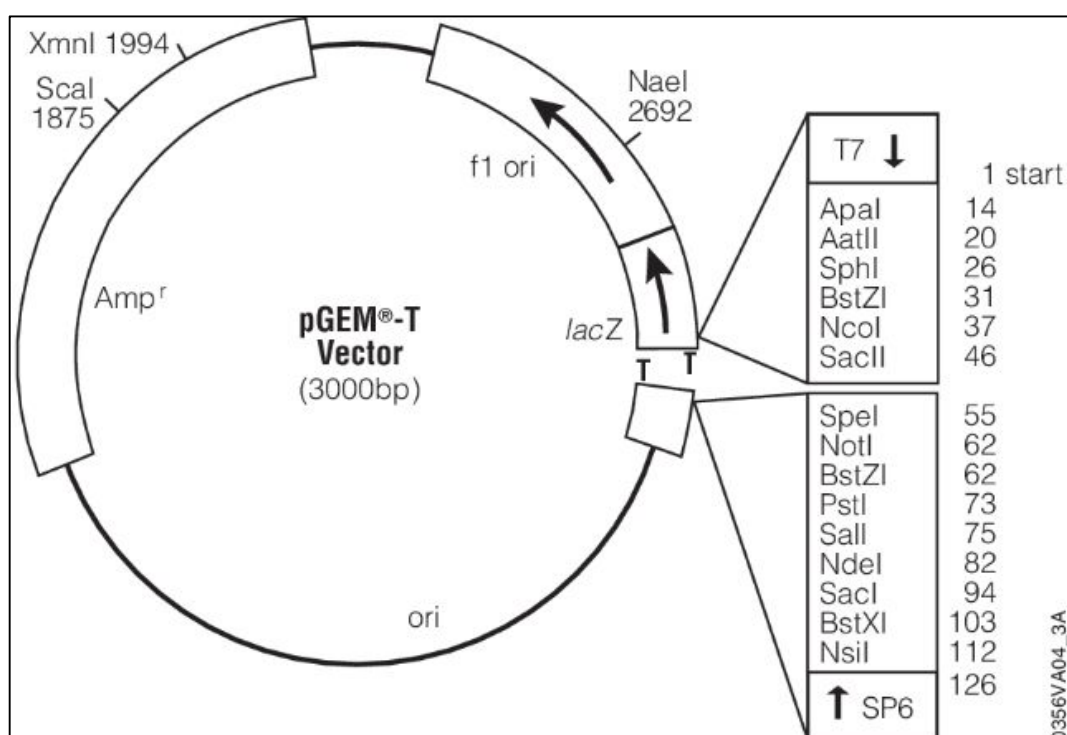


Figure 3.1: pGEM-T Easy vector map.

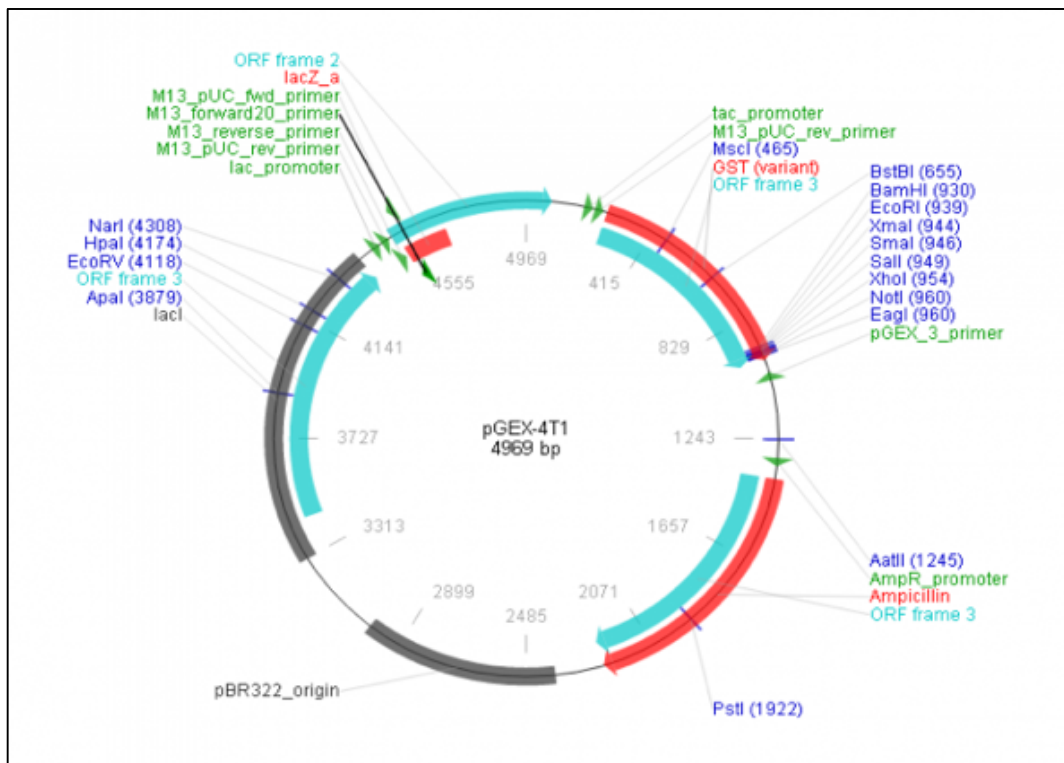


Figure 3.2: pGEX-4T vector map.

The restriction enzymes Sall and NotI (New England Biolabs, Beverly, MA), Tag DNA Polymerase and DNA ligase enzymes (Thermo Scientific, Waltham, MA) were used for cloning.

3.1.2. Component Cell Preparation

E. coli cells (DH5 α and BL21) harvested when the culture turbidity reached to OD₆₀₀: 0.5 absorbance value. The cells were treated with RbCl divalent cations for competency. The cell pellets were washed with the solution 1 and 2, then freeze with nitrogen and stored at -70° C.

3.1.3. Amplification of DNA Fragments

Genomic DNAs of *H. elongata* was extracted from living cells by using GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA). Forward and reverse primers were designed to have restriction enzyme sites and stop codons

(Iontek, Istanbul). PCR cloning kit (Thermo Scientific, Waltham, MA) was used for amplification processes. Reaction mixture contained 28 µl water, 50 ng template DNA, 5 µl of 10X PCR Mg²⁺ buffer, 50 pmol of each primer, 5µl of 2 mM dNTP, and 2 U of Taq polymerase. The PCR conditions were as follows; initial denaturation at 94°C for 10 minutes followed by 35 cycles of denaturation step (at 94°C for 1 minute), annealing step (at 55°C for 1 minute) and an extension step (at 72°C for 1 minute). These cycles were then followed by a final extension step at 72°C for 10 minutes. The expected product size was 747 bp.

Table 3.1: *H. elongata* primers.

Primer (Sall, NotI)	Sequence
HEAQF	<u>GGTCGAC</u> GCACAAGTACATCGCC (Sall)
HEAQR	GG <u>CGGCC</u> GCTCAGGCTGCATGGCTG (NotI)

3.1.4. Cloning and Expression of Vectors

The amplified DNA fragments of *H. elongata* were cloned into pGEM-T Easy Vector, and then transformed to *E. coli* DH5α. After blue-white colony selection on X-Gal/IPTG/ampicillin containing agar plates the plasmid isolation was performed from over-night agar culture (NaCl 10 g/L, yeast extract 5 g/L, peptone 10 g/L, agar 14 g/L) with Pure Yield Plasmid Mini-prep System (Promega, Madison, WI). Nucleotide sequence of the target DNA fragment in the cloning vector were confirmed by DNA sequencing (Iontek, Istanbul). Enzyme digestion (NotI, Sall) was performed for both target DNA fragment in pGEM-T and PGEX-4T2 cloning vector, and then recovered from gel by using Gene-JET Gel Extraction Kit. Target DNA and PGEX-4T2 vector which were cut with Sall and NotI, were ligated by T4 DNA ligase (Thermo Scientific, Waltham, MA) to construct the fusion expression vector pGEX-4T-HEAQP (ratio 1:4) in 16°C for 18 hours. Then reconstructed pGEX-4T-HEAQP was transformed into competent *E. coli* Top10cells. Transformants were selected on 100 µg/mL ampicillin containing agar plates, and then plasmids were purified from selected transformants. After verification of cloning by double digestion of the plasmids with Sall and NotI enzymes, the true plasmid was transferred into BL21 cells for the following protein expression steps.

3.1.5. Expression of AQP Proteins and purification from cell membrane

pGEX-4T-HEAQP in BL21 cells were grown overnight in 10 mL LB media containing 100 mg/mL ampicillin. This culture was used to inoculate a 750-mL culture in the same medium in a 1 L bottle. After on incubation the cells were induced with 1 mM IPTG for 3 hrs. Then the culture was centrifuged at 8000 rpm for 30 min and frozen at least one hour at -80°C prior to cell lysis. Cell lysis was performed in a buffer containing 100 mM K₂HPO₄, 1 mM MgSO₄, 1 mM PMSF, 0.02 mg/ml RNase, 1 mg/ml lysozyme at pH = 7.0 by sonication 30 times at 4°C in pulsed mode for 1 min at 50% duty cycle using an ultrasonic processor (Hielsher). Extraction of cell membrane proteins was performed according to [33]. Cell debris and unbroken cells were pelleted at 18,000 x g, 4°C for 30 min and the supernatant was centrifuged at 100,000 x g for 1 hr to pellet the membrane fraction. The supernatant was removed and membrane fraction was solubilized overnight in a buffer containing 5% Triton-X, 100 mM K₂HPO₄ (pH = 7.0), 200 mM NaCl, 10% glycerol and 2 mM β-mercaptoethanol (βME). Supernatant was filtered by 0,45 μm sterile filter. Supernatant was incubated with glutathione sepharose 4B (GE Healthcare) for 1 hr with gentle agitation. The resin was washed with up to 30 bed volumes of buffer containing 100 mM K₂HPO₄ (pH = 7.0), 1% Triton-X, 200 mM NaCl, 10% glycerol and 2 mM βME. The protein was eluted with L-glutathione reduced, 10% glycerol. Sample purity was estimated by running eluted fractions on a SDS-PAGE gel.

3.1.6. SDS-PAGE Analysis

Purified proteins analyzed with SDS-PAGE (SDS-Polyacrylamide Gel Electrophoresis) procedure

SDS-PAGE was performed using 12% separating gel and 5% stacking gel. Gels were firstly run at 100 V and then at 200 V once the dye entered the separating gel. The protein bands were visualized by Coomassie brilliant blue R250.

Stacking and separating gel contents were given in the Appendix.

3.1.7. Measurement of Protein concentration by Bradford method

1 mg/mL standard bovine serum albumin (BSA) was solubilized in %0.9 NaCl solution. Then 25 mg Coomassie brilliant blue G-250 was solubilized in 12.5 mL %95 ethanol, and 25 mL %85 phosphoric acid (H_3PO_4) added. Bradford solution was prepared by completing this dye solution to 250 mL with de-ionized water. This solution was diluted 5 times when its used and filtered from Whatman No.1 filter, then stored in room temperature in a glass bottle. Different concentrations of BSA were prepared as shown in the table below for standard preparation.

Table 3.2: BSA Standard Preparation Quantities.

Compounds	Tube No							
	1	2	3	4	5	6	7	8
1 mg/mL BSA(μL)	00	2	4	6	8	10	12	14
% 0.9 NaCl(μL)	100	00	00	00	00	00	00	00
Bradford Solution(μL)	900	998	996	994	992	990	988	986
Total volume(mL)	1	1	1	1	1	1	1	1

Prepared standards were hold for 10 min for fixation. Wavelength of UV Spectrophotometers Cintra 20 (GBC Scientific Equipment Pty Ltd., Australia) was adjusted to 595 nm. 2, 3, 4, 5, 6, 7, 8 numbered samples absorbance values were measured against numbered 1 clear (water loaded) tube. BSA solutions was prepared by taking 2, 4, 6, 8, 10, 12, 14 μL from solutions given in the Table 3.2. Standard graph was plotted using this data. Then, by using the standard curve unknown protein concentrations were calculated.

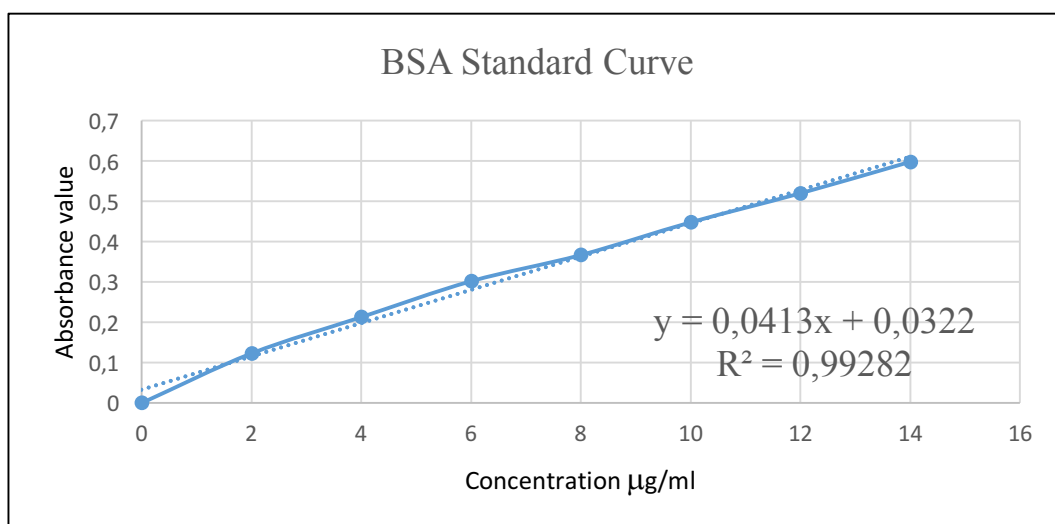


Figure 3.3: BSA Standard Graph.

3.2. Preparation of Vesicles Containing Aquaporin

3.2.1. Preparation of Vesicles

Lipid vesicles (i.e. liposomes) were formed through the film rehydration method[20]. Briefly, Phosphatidylserine (PS) (20 mg/mL), phosphatidylcholine (PC) (20 mg/mL), and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids INC, Alabama, USA) were prepared by dissolving lipids in a specific volume of chloroform. PS and PC were mixed in a ratio of 1:4 and DOPC was used alone in a round-bottomed flask. The chloroform was dried under N_2 gas to form lipid thin film. The film was kept in $4^\circ C$ for at least 1-2 hour to completely evaporate the chloroform. The dried polymer film was hydrated in 1 mL 1X phosphate buffered saline (PBS) to bring the final concentration to 10 mg/mL. The film was dissolved by using vortex for 15 min. The final solution was extruded 21 times through a polycarbonate membrane which has 100 nm pore size to get a liposome solution with a low polydispersity index (PDI). The preparation of AQP(aquaporin)-incorporated liposomes is also carried out by a similar procedure. An amount of AQP solution and %1 OG (Sigma-Aldrich, USA) was added into 10 mg/mL PS - PC liposomes phosphate buffer with a protein-to-lipid molar ratio of 1:200 during rehydration. The mixture was incubated for 1 hour with stirring at room temperature. Bio Beads (Bio-Rad Laboratories, Inc., USA) were used to remove the detergent. 0.05 g Bio Beads

were added to the mixture and stirred for 1 hour and this step was repeated by adding 0.15 g Bio Beads. After the formation of proteo-liposomes the mixture was extruded using the same method above.

3.2.2. Characterization of Vesicles

Nano Zetasizer (NanoZS, Malvern Instruments Limited, UK) was used to determine the size of liposomes and proteo-liposomes. The experiment was carried out at room temperature (22°C).

3.2.3. Vesicle permeability measurements

22SX20 Stopped-Flow Spectrometer (Applied Photophysics, UK) was used to detect the water permeability of lipid vesicles. For the stopped-flow measurements, fluorescence kinetic mode and a light source with a wavelength of 590 nm were selected. Vesicles were rapidly mixed with an osmotic draw solution (NaCl) driven by 8 atm pressurized nitrogen gas. Liposomes and draw solution mixing cause water efflux from the vesicle under the osmolality gradient. The rate constant k was established by curve fitting of the result data with single order exponential and used for water permeability (P_f) calculation using the following expression:

$$Y = A \exp(-kt) \quad (3.1)$$

$$P_f = \frac{k}{\left(\frac{S}{V_0}\right)V_w\Delta_{osm}} \quad (3.2)$$

where S is the initial surface area of the vesicles, V_0 is the initial volume of the vesicles, V_w is the partial molar volume of water (18 cm³/mole), Δ_{osm} is the difference in osmolality between the intra vesicular and extra vesicular aqueous solutions.

3.3. Aquaporin Incorporated Nanofiltration Membrane Preparation

3.3.1. Preparation of thin film composite polyamide membrane substrate

The polymer dope was prepared according to thin-film composite membrane procedure [55](16 wt.% PS, 77 wt% NMP, 5 wt% PEG, and 2 wt% LiCl was stirred at 70°C, degassed, and cooled down to room temperature (23°C). A 20 ml sample of dope was cast on a clean glass plate with a thickness of 200 μm using a film applicator (Paul N. Gardner Company, Inc., Pompano Beach, FL). The glass plate with the substrate film was immersed into tap water at room temperature to form PS substrate.



Figure 3.4: Preparation of polysulfone membrane substrate.

3.3.2. Preparation of AQP Vesicle Immobilized Membrane

The PS substrate first soaked in a MPD solution containing %1 MPD, 0,1% SDS, and 1 mg/ml proteo-liposomes for 10 min (MPD solution used for coating blank membrane and control membrane does not contain liposome and proteo-liposome, respectively). The soaked substrate was held horizontally at room temperature for 30 min, and excess solution was removed by nitrogen gas. The TMC solution (0.1% TMC in n-Hexane) was poured onto substrate surface to react with residue MPD to form rejection layer. Then membranes were kept in flowing tap water overnight to remove excess monomers and stored in Milli-Q water.

3.3.3. SEM analysis

The morphologies of prepared and dried membranes were observed by SEM analysis (Philips XL30 SFEG, Amsterdam, Netherlands). For cross-section images, dried samples were immersed in liquid nitrogen and fractured. Before imaging a layer of golden was coated on the membrane samples.

3.3.4. Dead-end measurements

In the study of Xie *et al.* (2013) and Li *et al.* (2014) cellulose acetate (CA) and poly(amide-imide) membranes were used as supporting materials, respectively. Then dead-end stirred cell and batch stirred cell filtration systems were used for flux measurements [6, 54]. In the filtration experiments, Sterlitech Corporation dead-end membrane system (HP4750, USA) (Figure 3.5) was used. Membrane area is 14,6 cm² and feed solution was 250 mL. Membrane was placed onto dead-end porous disk, then equipment was merged together. 5 bar pressure was applied to filter water or saline solution, and during filtration sample was stirred at 300 rpm. Filtrate was measured by precision scale.

The flux was calculated by using the equation given below.

$$J = \frac{V}{A} \times \Delta t \quad (3.3)$$

V shows total volume of filtrate in a specific period of time (L), Δt shows time, A shows membrane area (m^2), and J shows flux ($\text{L}/\text{m}^2 \cdot \text{h}$).

$$J_{SR} = (C_f - C_p) \times J_v \quad (3.4)$$

where J_{SR} shows salt rejection rate, J_v membrane flux, C_p and C_f are solute concentration in permeate and feed solutions respectively.



Figure 3.5: Dead-end reactor system.

NaCl rejections (as percentage) were calculated using the equation below:

$$R = \left(1 - \frac{C_p}{C_f}\right) \times 100\% \quad (3.5)$$

where R is rejection, C_p and C_f are solute concentration in permeate and feed solutions respectively.

4. RESULTS AND DISCUSSION

4.1. *Halomonas elongata* Aquaporin Gene Cloning

Halomonas elongata aquaporin gene was amplified by using specific primer duplex which has NotI and SalI restriction enzyme sites. DNA product belonging to 747 bp aquaporin gene was observed in agarose gel (Figure 4.1).

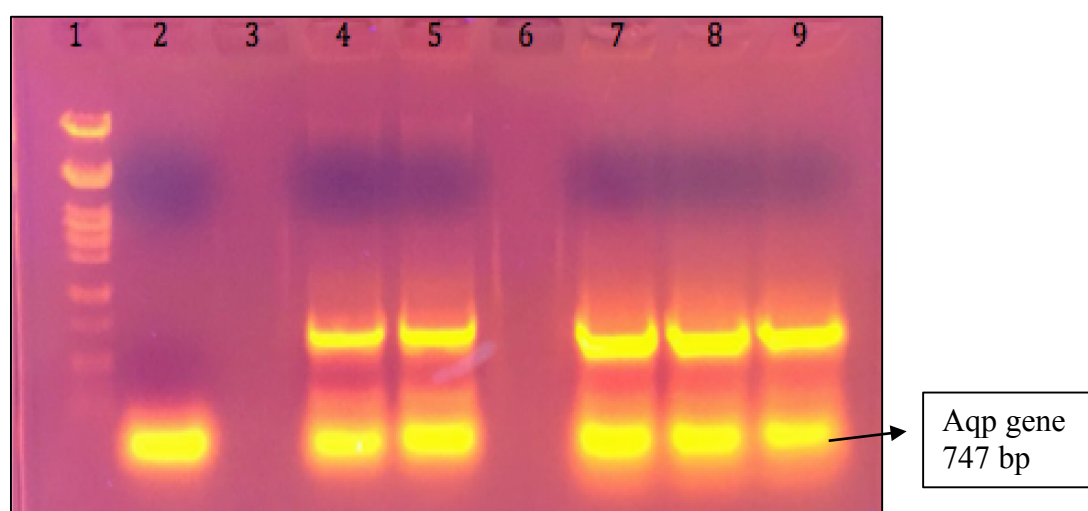


Figure 4.1: Amplification of *Halomonas elongata* aquaporin.

In the Figure 4.1 wells from 1 to 9 represent; 1) λ PstI Marker, 2) negative control 3) blank 4-5) Amplified aquaporin gene. DNA marker for reference points showed in Appendix.

DNA fragment belonging to *H. elongata* aquaporin gene was cut from the gel and purified by using gel purification kit. Then this eluted DNA fragment was ligated to pGEM-T Easy Vector according to the user's manual and ligation product was transferred into competent DH5 α host cells and then incubated at 37°C overnight. The next day blue and white colonies appeared on the agar plate containing X-Gal/IPTG and ampicillin. White colonies carrying the gene of interest were selected and cultured for further cloning experiment (Figure 4.2).

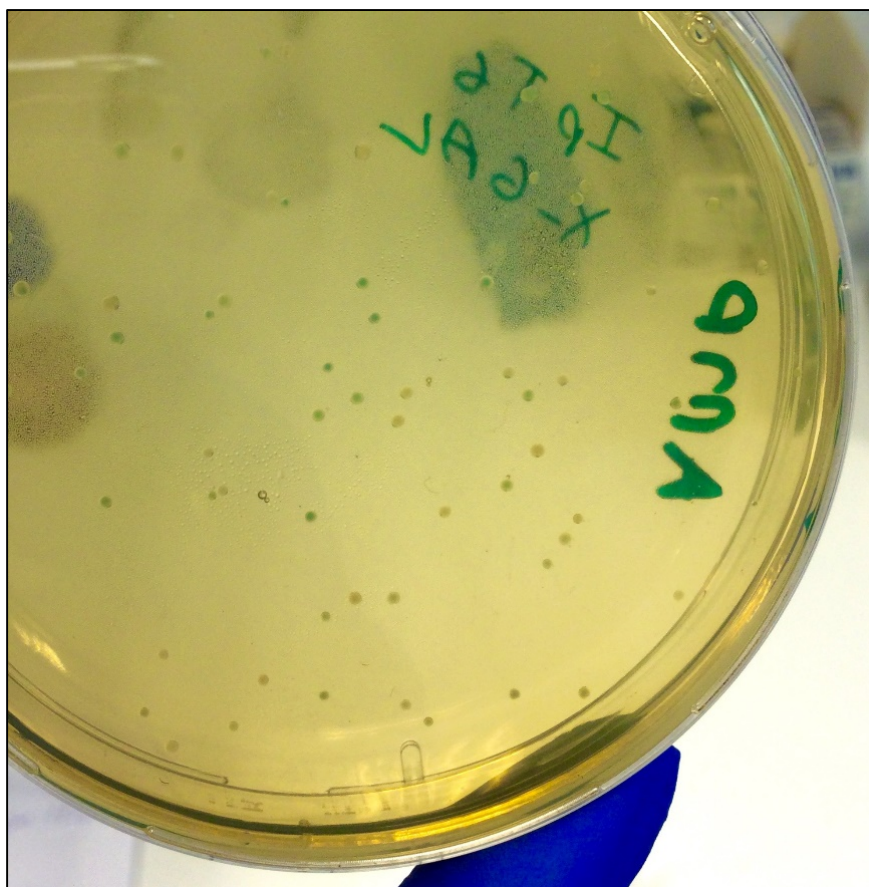


Figure 4.2: Blue and white colonies after transformation of HEAQP cloned pGEM-T vector.

White colonies were picked for screening positive clones that contains aquaporin encoding gene. Plasmids obtained from white colonies were extracted and PCR amplified by *H. elongata* aquaporin gene specific primers. As seen in Figure 4.3, 747 bp DNA fragment was obtained after PCR amplification confirming the PCR cloning was successful. The cells having gene product seen on well 8 (Figure 4.3) was chosen for further cloning experiments and named as pGEM-T-HEAQP.

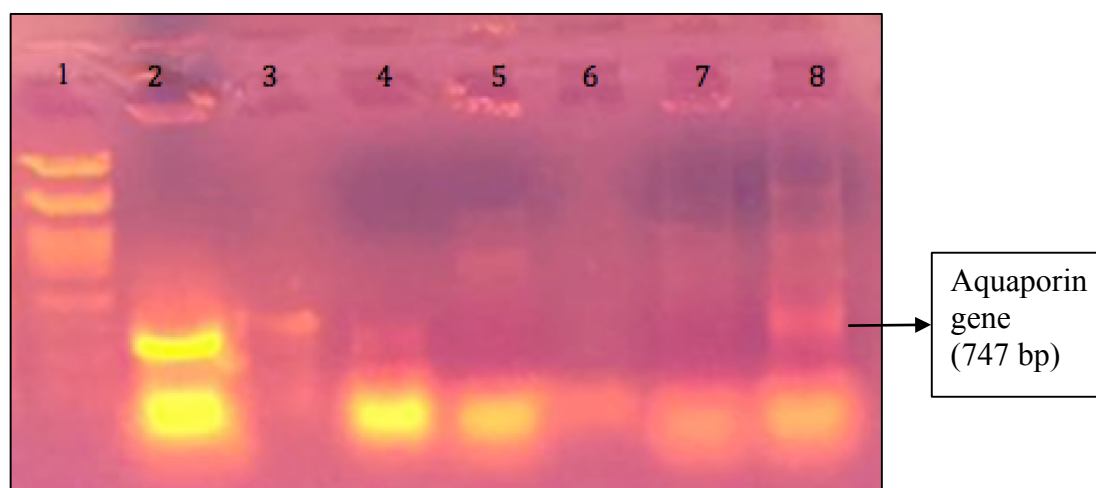


Figure 4.3: pGEM-T-HEAQP PCR verification.

In the Figure 4.3 wells from 1 to 8 represent; 1) λ Pst marker, 2) Genomic DNA, 3) Blank, 4) Control, 5) 1st transformant, 6) Blank, 7) 2nd transformant, 8) 8th transformant.

H. elongata aquaporin gene in pGEM-T-HEAQP was obtained by double digestion with Sall and NotI enzymes (

Figure 4.4), extracted from the gel and then ligated into double digested pGEX-4T2- LDH. (This vector contains previously cloned gene, LDH) vector as explained in materials and methods section.

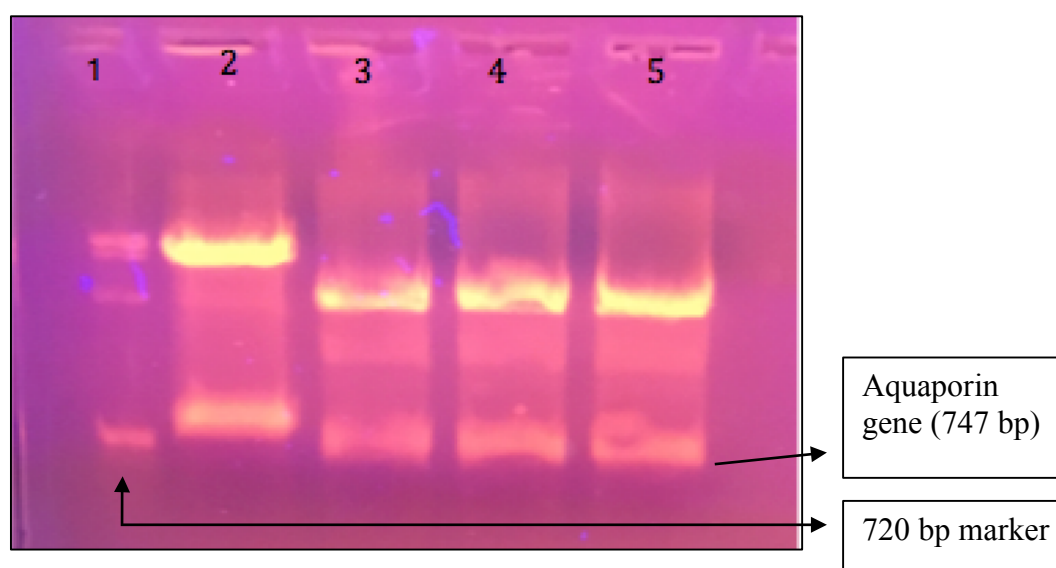


Figure 4.4: pGEX4T-LDH and pGEM-T-HEAQP NotI-Sall enzyme digestion.

In the Figure 4.4 wells from 1 to 5 represent; 1) Lab-made marker, 2) pGEX-4T-LDH, 3-5) pGEM-T-HEAQP.

For cloning *H. elongata* aquaporin gene into pGEX-4T2 vector approximately 100 ng/μL aquaporin gene inserts were ligated with pGEX-4T2 vector in the ratios given in Table 4.1. At first trial, transformation experiments were performed with *E. coli* BL21 and DH5α competent cells and no transformant was obtained after ligation and transformation (Table 4.1).

Table 4.1: pGEX-4T and HEAQP ligation ratio.

pGEX4T-2 (μL)	HEAQP (μL)	HEAQP (μL)	HEAQP (μL)	Result
4,0	4,0	1,0	1,0	0
2,0	6,0	1,0	1,0	0
1*	1,5*	1,0	1,0	0
1,5*	14,0	1,0	2,0	0
2,0	6,0	1,0	1,0	0
2*	3.5*	1,0	1,0	0
3,0	4,0	2,0	1,0	0
* sign refers that concentrations increased up to 400 ng/μL. (Concentration of vector is 200 ng/μl and insert concentration is 100 ng/μl)				

Different *E. coli* strains including K12, Top10, 8739 were prepared as competent cells for new setup.

Table 4.2: Ligation ratio and transformation results.

	pGEX4 T-2 (μL)	HEAQ P (μL)	Ligase (μL)	Buffer (μL)	Transformant numbers for each strain		
					Top10	K12	8739
Ligation 1	3	10	1,5	1,5	10	3	0
Ligation 2	8	6	1,5	1,5	0	0	0
Ligation 3	4	7	1,5	1,5	0	1	0

In Table 4.2 ligation ratios and number of transformant colonies obtained after ligation and transformation were listed. In total, 14 colonies from Top10 an K12 strains collected and 4 of them were selected for PCR verification.

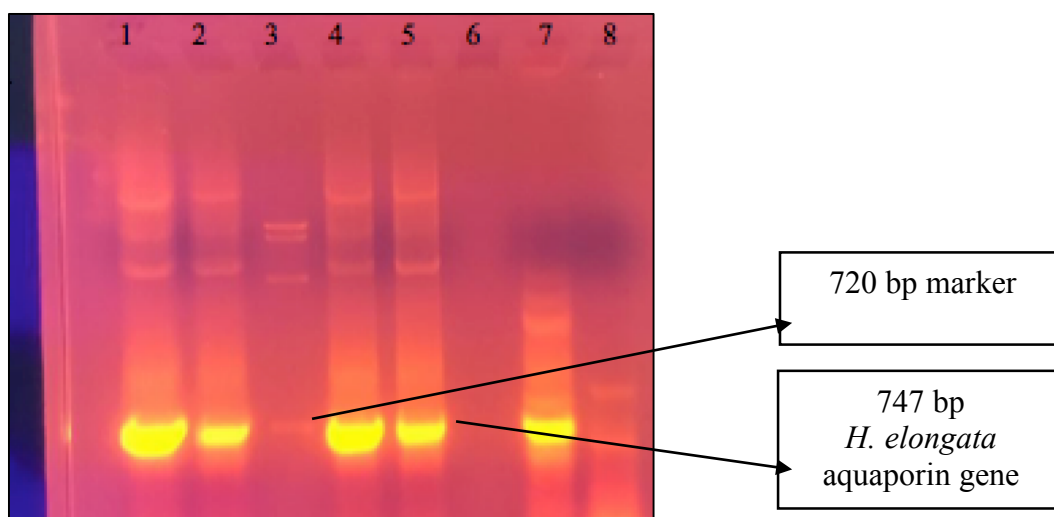


Figure 4.5: PCR verification for pGEX-4T-HEAQP.

In the Figure 4.5 wells from 1 to 8 represent; 1) 5th colony, 2) 6th colony, 3) Lab-made Marker, 4) 12th colony, 5) 13th colony 6) Blank, 7) Positive control, 8) Negative control.

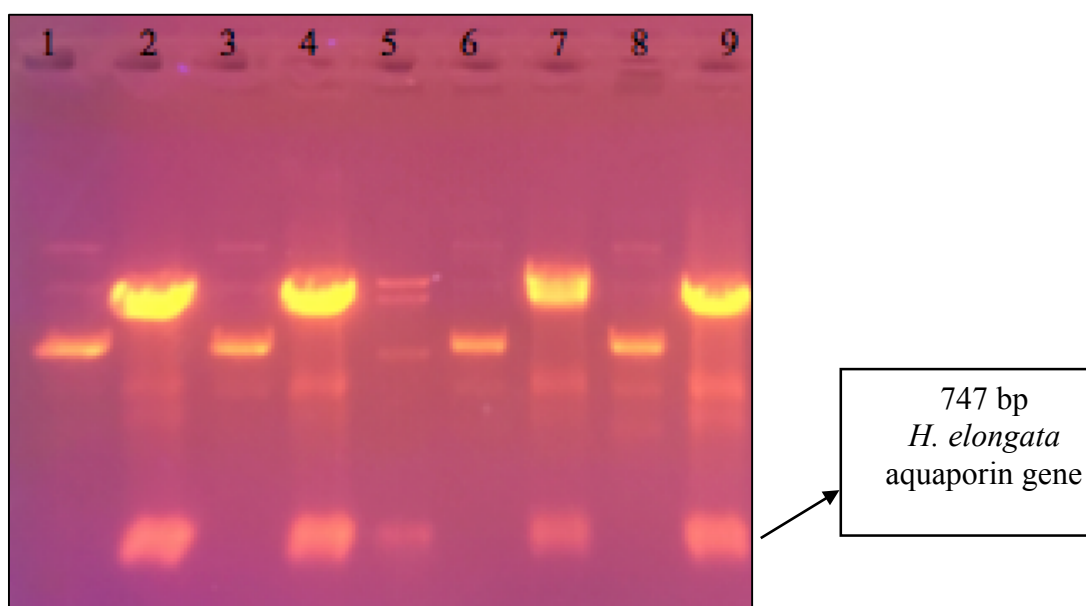


Figure 4.6: Restriction enzyme digestion verification of HEAQP gene containing pGEX-4T.

In the Figure 4.6 wells from 1 to 9 represent; 1) Undigested plasmid from colony 5, 2) Digested plasmid from colony 5, 3) Undigested plasmid from colony 6, 4) Digested plasmid from colony 6, 5) Marker, 6) Undigested plasmid from colony

12, 7) Digested plasmid from colony 12, 8) Undigested plasmid from colony 13, 9) Digested plasmid from colony 13.

As we can see in the

Figure 4.5 PCR results confirmed that the selected transformants contains HEAQP gene insert. To make sure about the cloning, restriction enzyme digestion was performed with NotI and SalI enzymes.

In the

Figure 4.6 around 750 bp insert is clearly seen after NotI and SalI digestion. After this verification, the insert in the vector was sequenced (Iontek, Turkey) and sequencing result further confirmed that the cloned gene is *H. elongata* aquaporin gene.

4.2. Protein Expression and Isolation

After verification of cloning of the recombinant vector pGEX-4T-HEAQP was transformed into BL21 cells for higher amount of protein production. Channel and soluble proteins which are GST tagged fusion proteins harvested by using Sepharose 4B beads and cleaved by thrombin from GST fraction. Cloned *E. coli* aquaporin protein from previous studies [56] and this new derived *H. elongata* aquaporin proteins are compared in s SDS-Gel (

Figure 4.7).

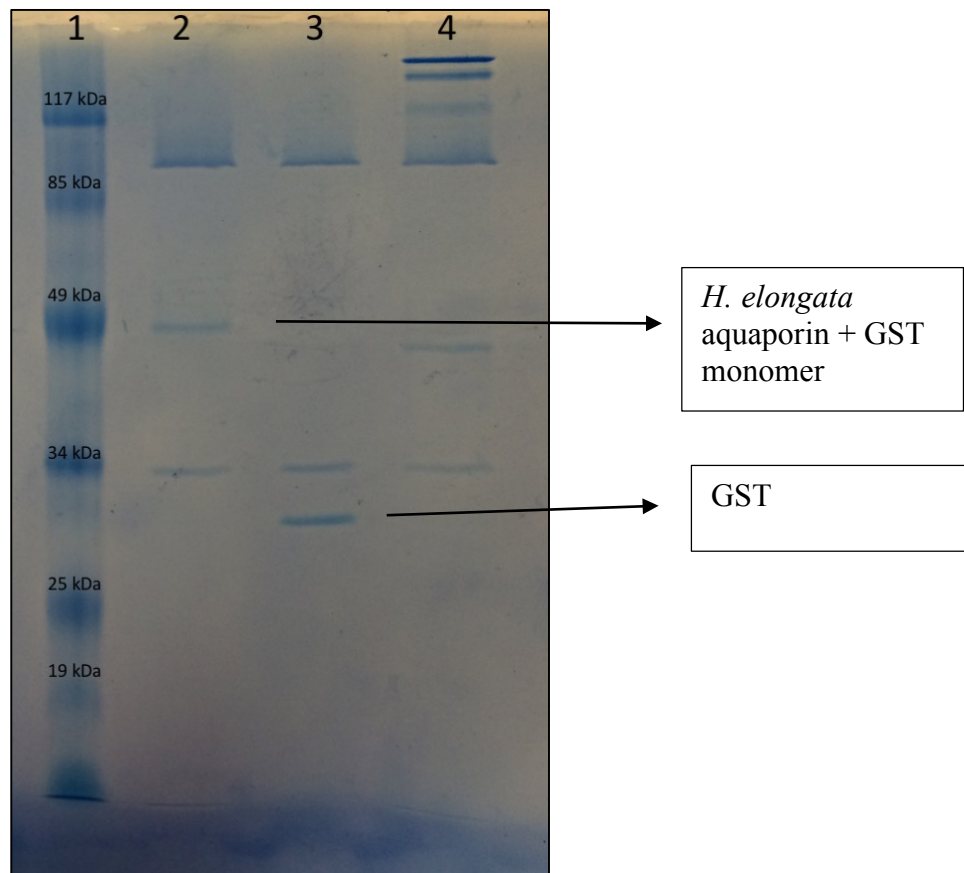


Figure 4.7: Cloned *H. elongata* aquaporin protein purification by Sepharose 4B beads.

In the Figure 4.7 wells from 1 to 4 represent; 1) Marker, 2) pGEX-4T-HEAQP, 3) pGEX-4T, 4) pGEX-4T-ECAQP. The 3rd well of the gel contains control (membrane proteins from cells containing empty vector pGEX-4T-2). It is clearly seen that there is no band belonging to *H. elongata* aquaporin and GST fractions can be seen around 26 kDa position. In the 4th well, *H. elongata* aquaporin proteins are seen in 2nd well as monomers (about 50 kDa together with GST). We can see that *E. coli* aquaporin proteins are tetrameric and has very high molecular weight. Cloning into pGEX-4T vectors results in fusion protein formation with GST (Glutathione S transferase) because the genes are cloned next to the GST gene in the vector. This GST protein is used as affinity agent for purification of protein of interest.

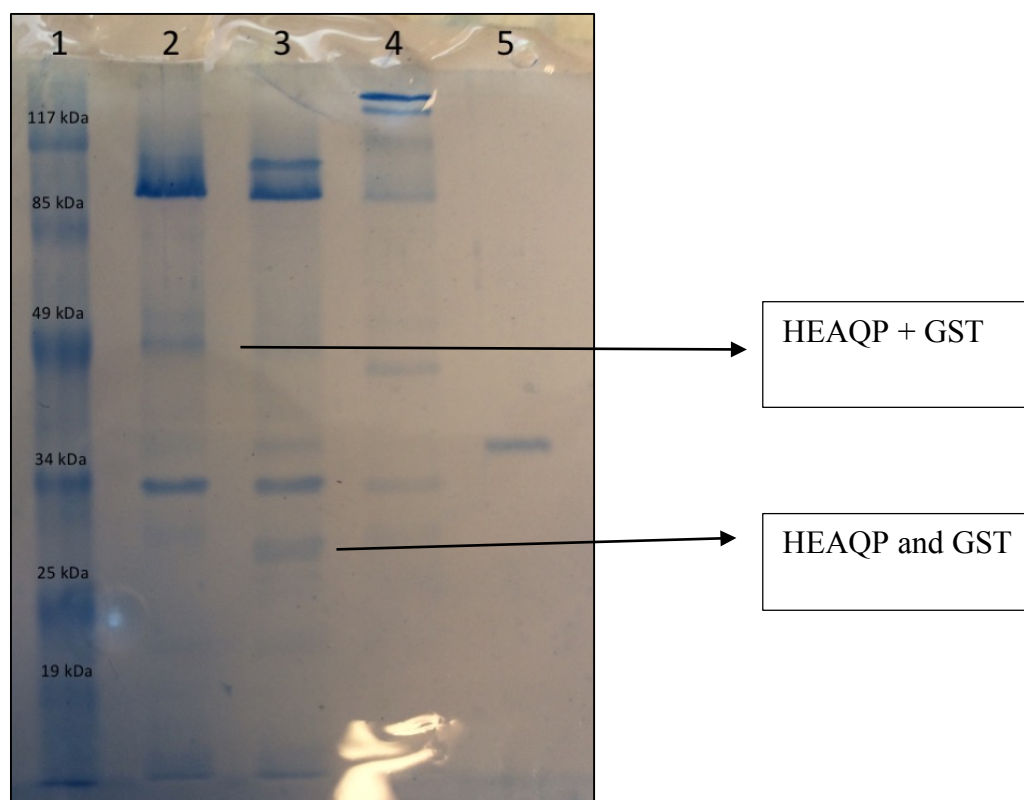


Figure 4.8: *H. elongata* aquaporin protein purification and thrombin cleaving.

In the Figure 4.8 wells from 1 to 5 represent; 1) Marker, 2) HEAQP, 3) HEAQP thrombin cleaving, 4) ECAQP, 5) Thrombin (3uL).

When purified HEAQP-GST was digested with thrombin, 50kDa protein fraction disappeared. Around 25 kDa protein bands (over each other) appeared proving that the fusion protein was successfully digested by thrombin enzyme. Because of similar size of HEAQP and GST, bands overlapped on the gel (

Figure 4.8).

For analyzing the resistance of *H. elongata* aquaporin protein (HEAQP) to denaturing conditions, the protein was incubated in different concentrations of β -mercaptoethanol.

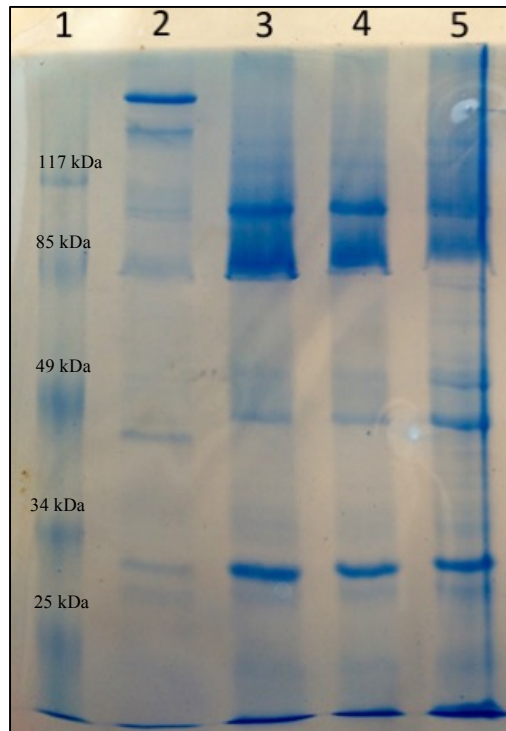


Figure 4.9: Effect of β -mercaptoethanol to protein denaturation.

In the Figure 4.9 wells from 1 to 5 represent; 1) *Marker* 2) *ECAQP* 3) *HEAQP(control)*, 4) 125 mM β -mercaptoethanol, 5) 250 mM β -mercaptoethanol.

When the β -mercaptoethanol increased to 250 mM monomer fraction concentration also increased, and fractions in the upper side of the gel thought to be dimer forms of the proteins.

4.3. Liposome Preparation

According to the method stated in 3.2.1, blank, tampon containing and protein containing liposomes were prepared and they were observed under Accu-Scope 3025 light microscope (Accu-Scope INC, US).

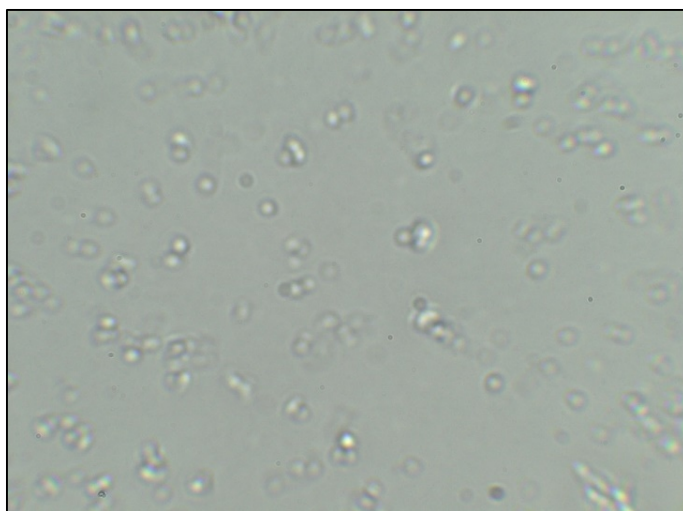


Figure 4.10: Light microscope image of liposomes(100X).

With light microscopy analysis, it was seen that the liposomes are uniform in size and they are intact as transparent round vesicles.

4.4. Vesicle Size Analysis

Sizes of PS: PC liposomes prepared by filtering from 100 nm filter and DOPC liposomes prepared by filtering from 200 nm filter. Both were determined by using Nano Zetasizer (NanoZS, Malvern Instruments Limited, UK) at room temperature (22°C). Blank liposomes (without AqpZ) and aquaporin containing liposomes prepared at the concentration of 0.5 mg/ml were used for all measurements. Sizes of PS: PC and DOPC liposomes given at Table 4.3.

Table 4.3: Nano Zetasizer results of liposomes.

Lipid type (filter size)	Blank liposome	AQP containing liposome
PS:PC (100 nm)	119.7 nm	101.2 nm
DOPC (200 nm)	174.8 nm	152.7 nm

Aquaporin containing liposomes sizes was found to be smaller than blank liposomes. The reason of this is explained as shrinking due to water loss from aquaporin containing liposomes. This result also supports that the aquaporins were

successfully placed in liposomes. Low PDI (polydispersity index) results shows that the results are confidential and the sizes of the liposomes are uniform.

4.5. Water Transfer Velocity Measurements of *Halomonas elongata* Aquaporins

Liposomes (blank and with HEAQP) were prepared by using two different method which are stated at heading 3.2.1. Different concentrations of NaCl solution mixed with prepared liposomes and their water transfer rates were measured by using Stopped-Flow Light Scattering Spectrometer. Water transfer rates of the liposomes are directly proportional with k (water transfer factor) values driven from light scattering data. It can be said that, as k value (water transfer factor) increases water transfer rate also increases. Water transport rate of *H. elongata* aquaporin was measured in different liposomes which has different pore sizes (100-200 nm) and different lipids compositions (PS-PC-DOPC). The liposome and salt concentrations were optimized for obtaining proper measurements in Stopped-flow light scattering spectrometer. 0.85 M, 1.5 M, 2 M, and 3 M NaCl containing solutions and 10 mg/ml liposomes were used for water transfer rate measurements.

Water permeability rates were calculated only for 0.85 M concentration feed measurements, and Δosm was found between 1X PBS and 0.85 M NaCl. For this calculation, other than NaCl molecules were neglected due to their low concentration in the solution. 1X PBS and 0.85 M NaCl solutions conductivity values were monitored and obtained $\mu\text{S/cm}$ values were converted to ppm. Then, ppm converted to g/L and this value determined as osmol. Osmolarity difference of these two solutions were found 1.2 osm. P_f values were calculated by using this Δosm value. Liposome radiuses obtained from Nano Zetasizer measurements. P_f values for 100 nm PS:PC and 200 nm DOPC liposomes were calculated.

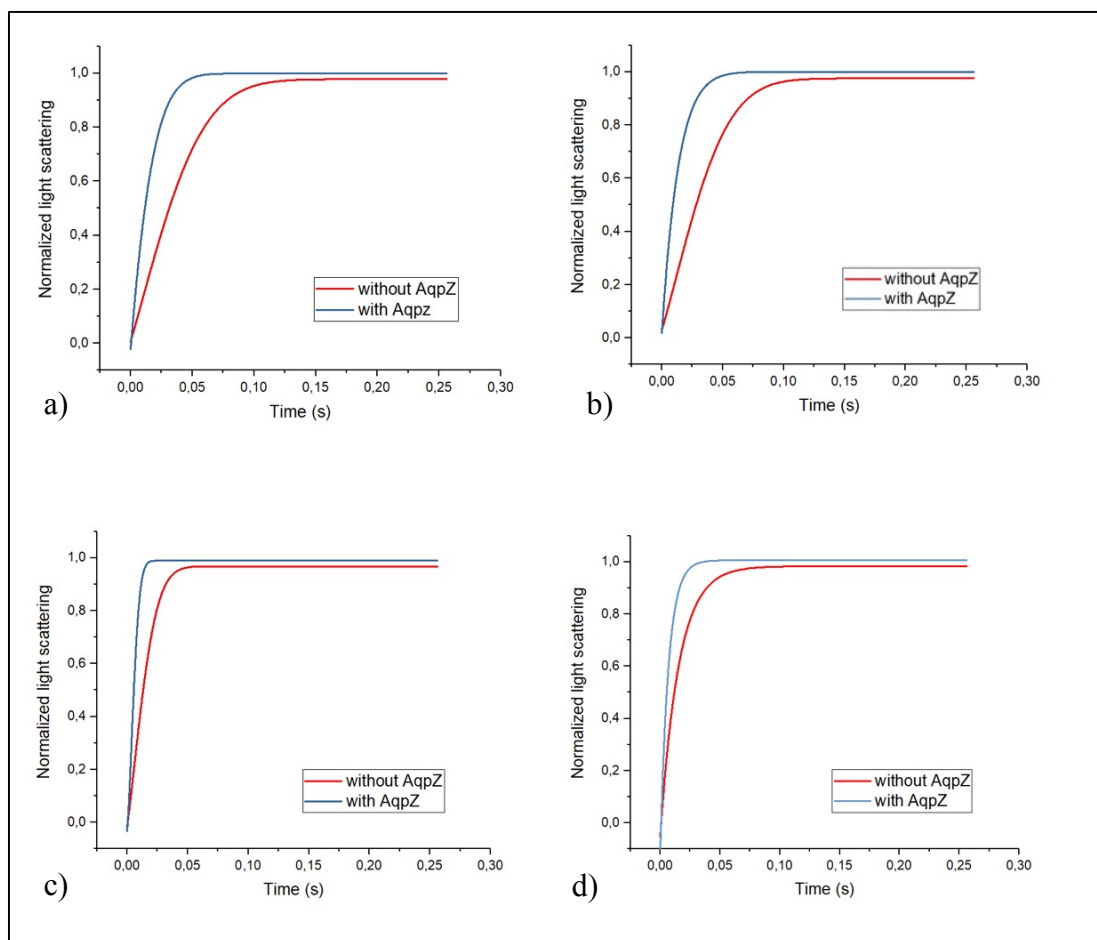


Figure 4.11: Normalized light scattering measurement results of 100 nm PS-PC liposomes. a) 0.85 M NaCl solution, b) 1.5 M NaCl solution, c) 2 M NaCl solution, d) 3 M NaCl solution.

The water permeability of the blank and aquaporin protein containing PS:PC liposomes for 0,85 M NaCl feed was found to be 2.16×10^{-3} and 4.54×10^{-3} , respectively (Table 4.4). k values (water transfer factor) obtained from the lines in Figure 4.15 are given in the table below.

Table 4.4: k and P_f values of PS-PC liposomes.

	k (1/s)				P_f (cm/s)
NaCl concentration	0.85 M	1.5 M	2 M	3 M	0,85 M
Blank liposomes	23.40	25.88	46.25	61.35	2.16×10^{-3}
Aqp containing liposomes	57.94	86.77	123.95	149.32	4.54×10^{-3}

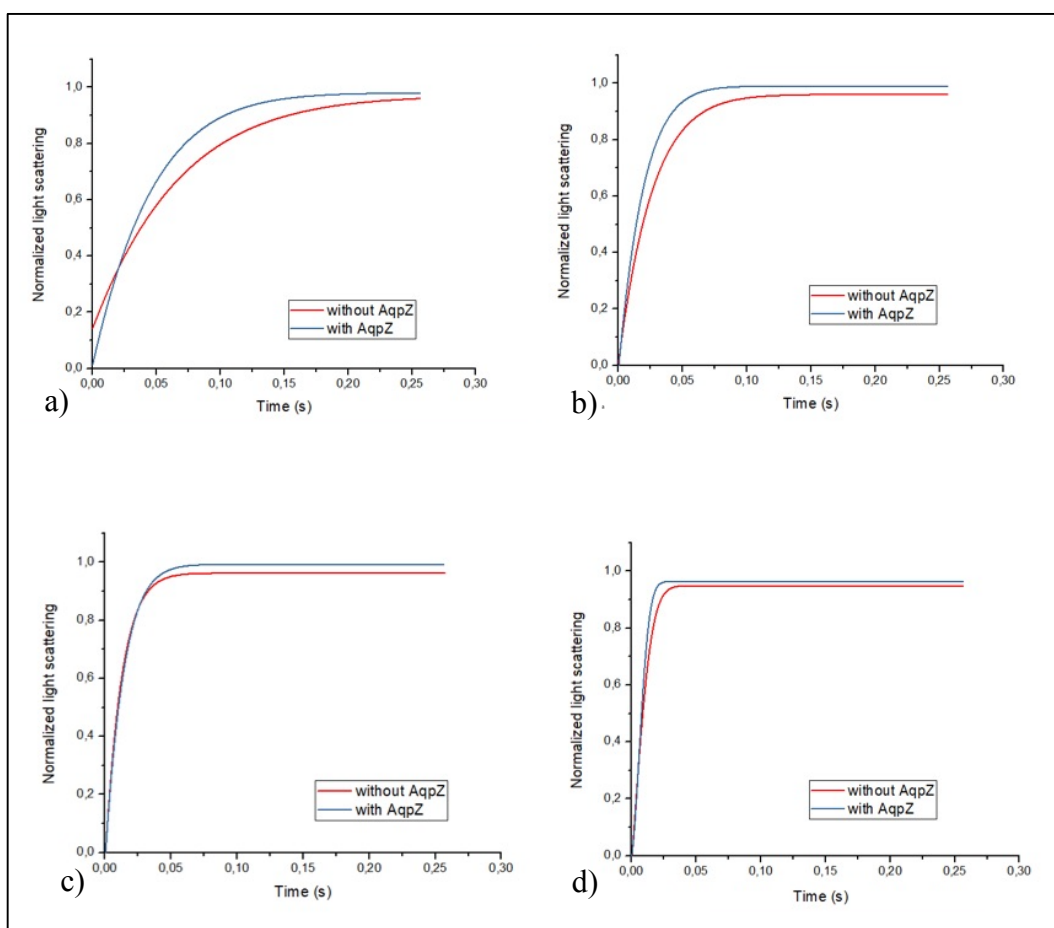


Figure 4.12: Normalized light scattering measurement results of DOPC liposomes filtered from 200 nm membrane. a) 0.85 M NaCl solution, b) 1.5 M NaCl solution, c) 2 M NaCl solution, d) 3 M NaCl solution.

The water permeability of the blank and aquaporin protein containing DOPC liposomes for 0.85 M NaCl feed was found to be 2.21×10^{-3} and 2.62×10^{-3} , respectively (Table 4.5). k values (water transfer factor) obtained from of lines in Figure 4.16 are given in the table below.

Table 4.5: k and P_f values of DOPC liposomes.

	k (1/s)				P_f (cm/s)
NaCl concentration	0.85 M	1.5 M	2 M	3 M	0,85 M
Blank liposomes	16.24	33.59	64.72	70.25	2.21×10^{-3}
Aqp containing liposomes	22.13	48.70	70.97	82.00	2.62×10^{-3}

All graphs show that aquaporin containing liposomes have higher water transfer capacity according to blank liposomes.

4.6. SEM Analysis of Membranes

As detailed in heading 3.3.1 and 3.3.2 different membranes were prepared according to characteristics below:

- PS membrane substrate (no interfacial polymerization)
- Blank coated (without liposome) membrane
- Blank liposome (without aquaporin) coated membrane
- Aquaporin containing liposome (with aquaporin) coated membrane



Figure 4.13: Coated membranes.

For Scanning electron microscope analysis firstly, membranes were coated with gold nanoparticles to get efficient display. Then SEM images of surface and cross section of membranes were obtained.

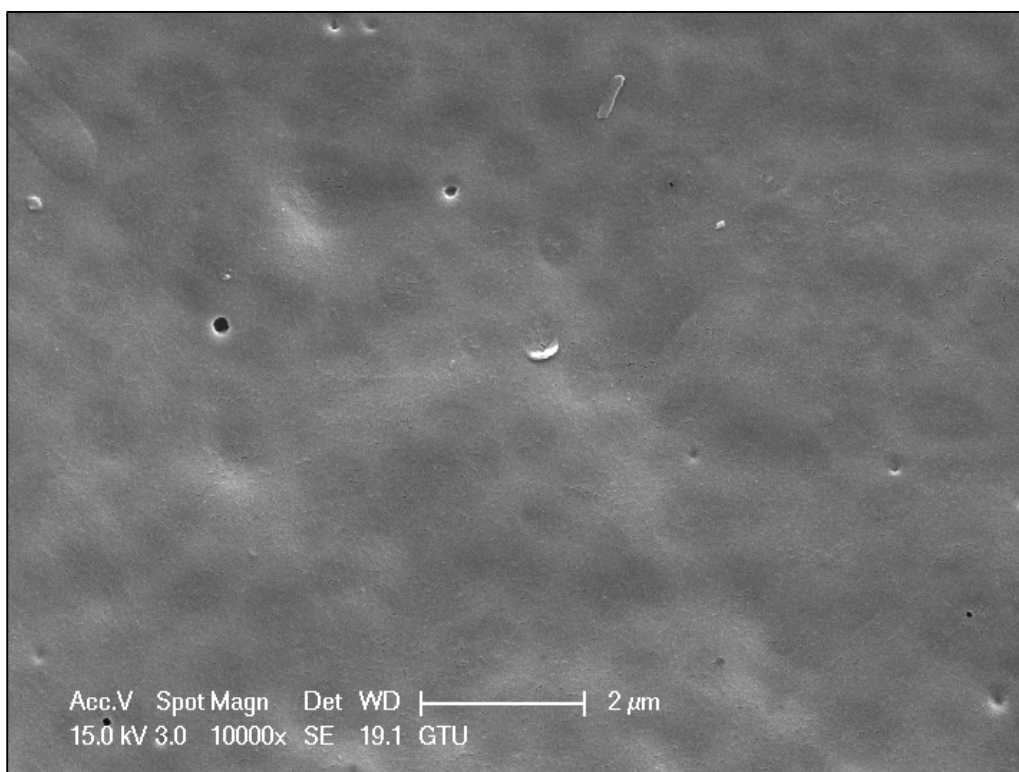


Figure 4.14: PS membrane surface.

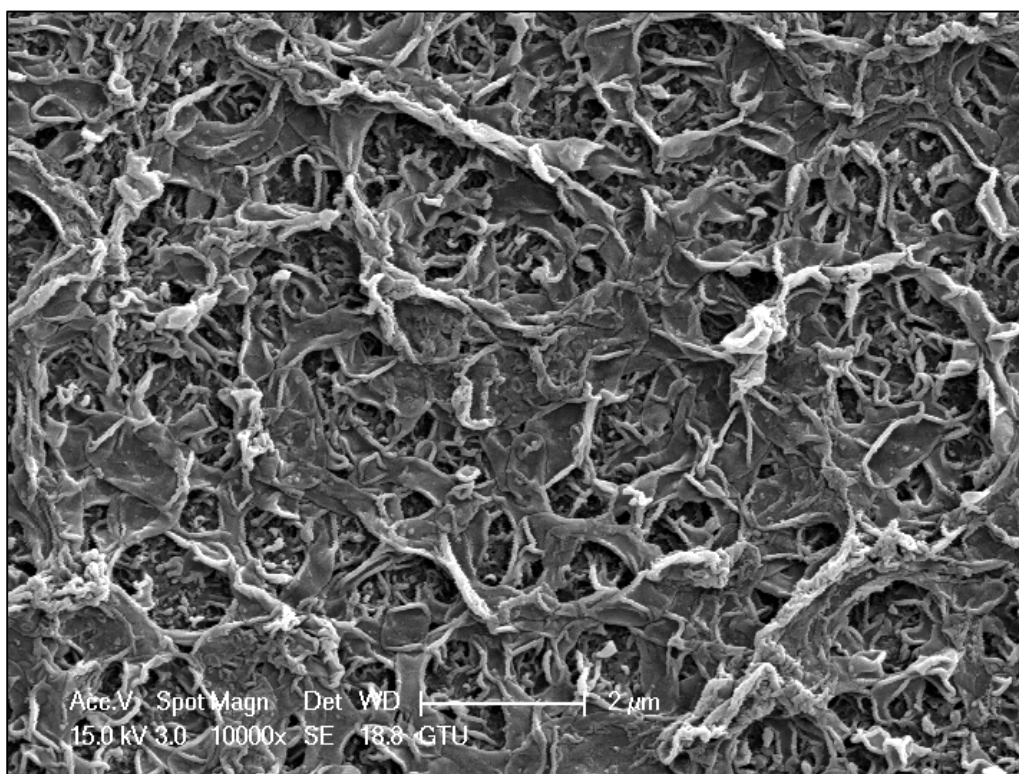


Figure 4.15: Blank (without liposome) coated membrane surface.

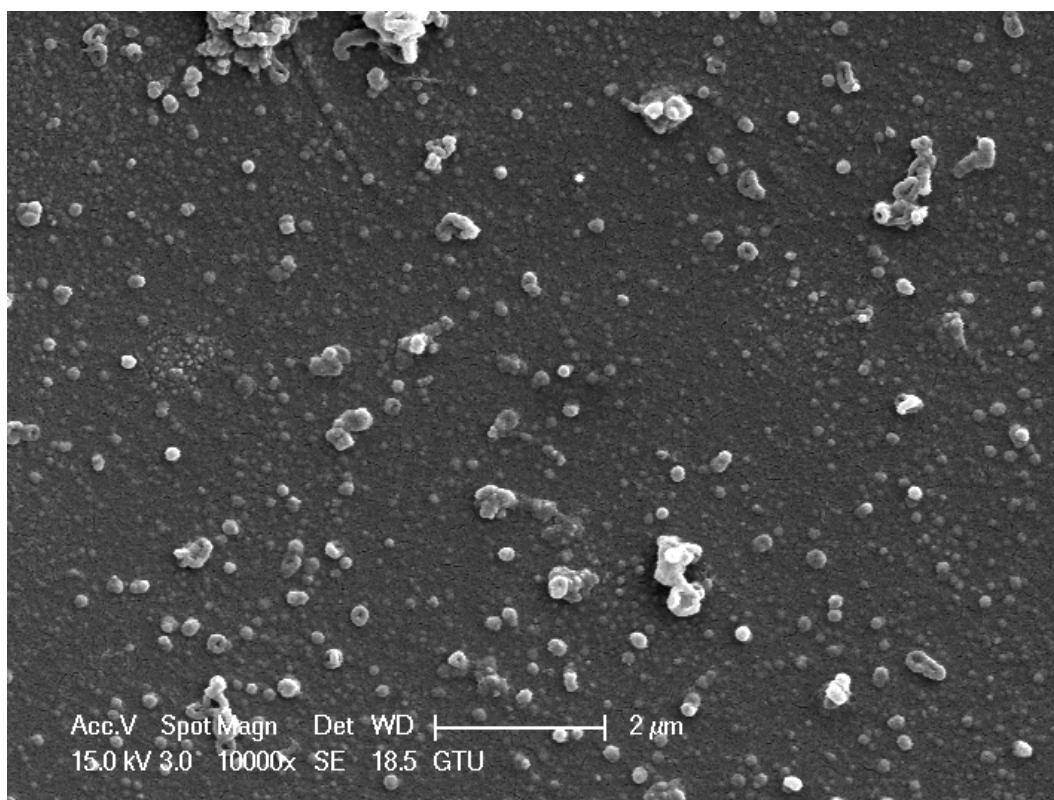


Figure 4.16: Liposome coated membrane surface.

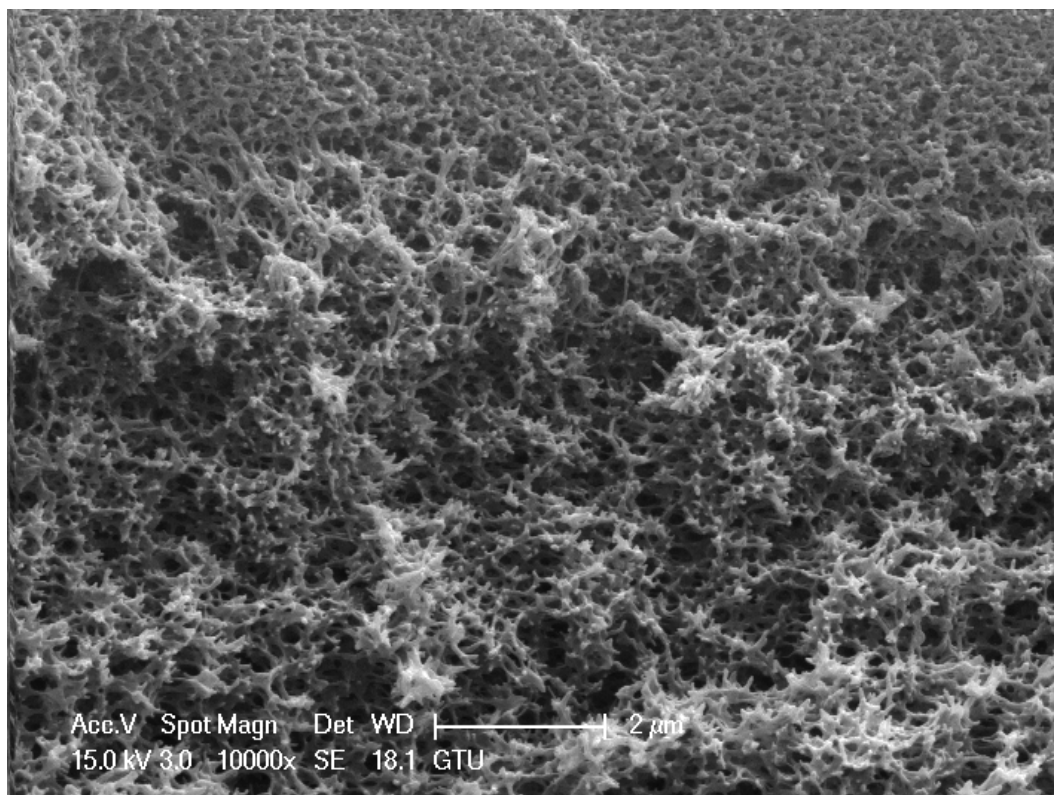


Figure 4.17: Blank (without liposome) coated membrane cross-section.

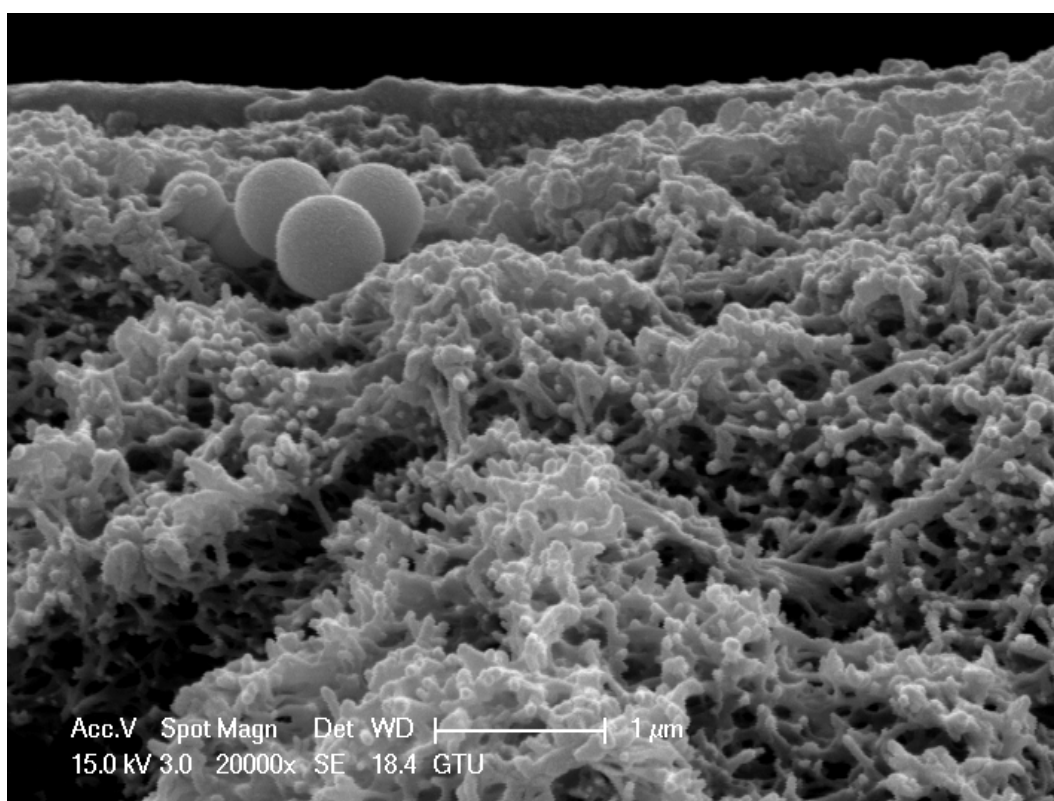


Figure 4.18: Liposome coated membrane cross-section.

As it can be seen in Figure 4.18 the proteo-liposomes were embedded inside the polyamide matrix. This is confirmed by SEM cross-sectional images.

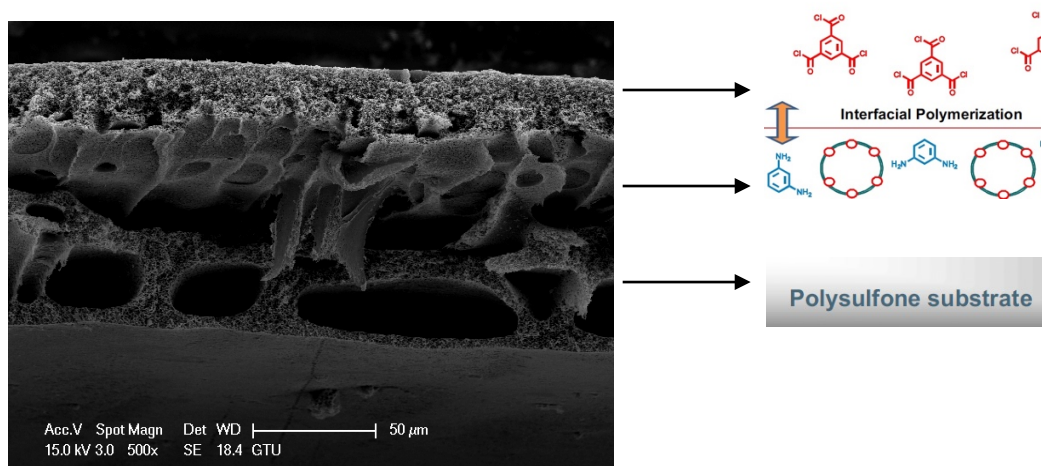


Figure 4.19: Blank coated membrane layers.

Figure 4.19 shows SEM image of polymerization layers of blank coated membrane.

4.7. Water Transport and Salt Rejection Capacity Measurements of *Halomonas elongata* Aquaporin Incorporated Membranes with Dead-end System

In this part, efficiency of the proteo-liposome incorporated membranes were measured using dead end reactor Figure 3.5. For this aim flux of empty liposome-embedded (control) and aquaporin containing liposome- embedded membranes were obtained and results can be seen in Figure 4.20. The experiment was performed twice with freshly prepared membranes in each experiment. Average constant fluxes for control membrane were calculated as 4.10 LMH and 2.05 LMH for DI water and 10 mM NaCl solution, respectively. Average constant fluxes for aquaporin liposome immobilized membranes are measured to be 34.24 LMH and 31.5 LMH for DI water and 10 mM NaCl solution, respectively. For the second trial with new membranes, Average constant fluxes for control membrane were calculated as 2.05 LMH and 1.36 LMH for DI water and 10 mM NaCl solution, respectively. Average constant fluxes for aquaporin liposome immobilized membranes are measured to be 6.84 LMH and 5.47 LMH for DI water and 10 mM NaCl solution, respectively.

Results showed that for DI water incorporation of aquaporins to the membranes provided about from 4 to 8 times increase in the DI water flux and, from 4 to 15 times increase for NaCl solution, respectively.

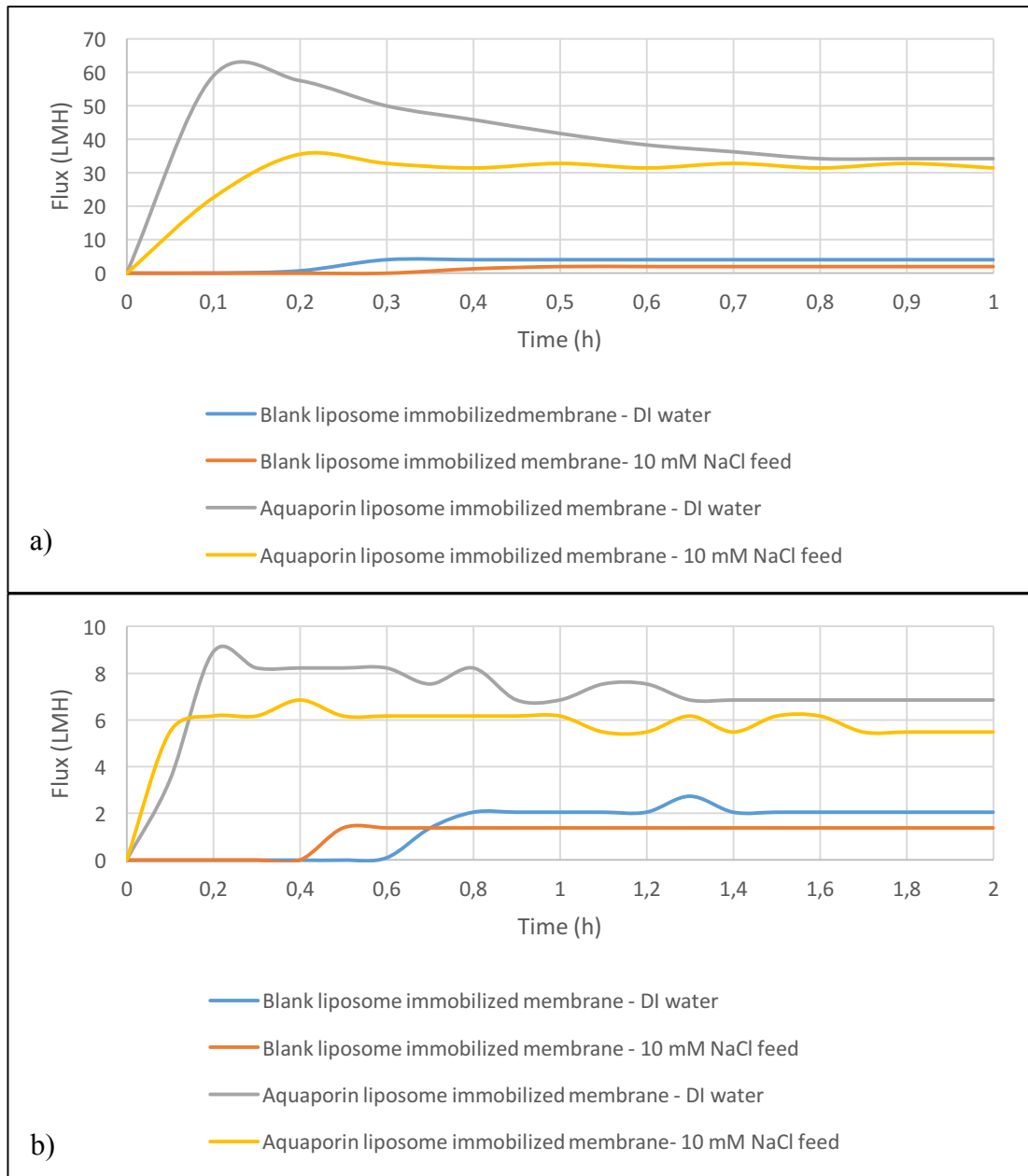


Figure 4.20: a) Membrane fluxes of the first experiment b) Membrane fluxes of the first experiment.

NaCl rejections of the membranes were calculated according to equation 3.5. For both first and second trial salt rejection of control membranes were found to be higher than that of aquaporin embedded membranes. Chemical coating of membrane surface results in very low water transfer, in other words, membrane is almost impermeable to water. Because of this, diffusion of NaCl ions through the membrane is difficult. And empty liposomes are lipids and they do not have any water channel. This lipid layer is also more resistant to both water and salt transfer as compared to

the channel containing liposomes. So that the results cannot be evaluated irrespectively of water flux. Results shows that aquaporin incorporation into the nanofiltration membranes improved water flux remarkably. Because the flux of aquaporin-embedded membranes are much higher than the control membranes, salt rejection rates of aquaporin containing membranes found to be 2.5-3 times higher than the control. Table 4.7 shows salt rejection rates depending on the flux values of the membranes.

Table 4.6: Salt rejections of the membranes.

Sample	Conductivity ($\mu\text{S}/\text{cm}^2$) and salt concentration (mg/L)		Rejections (%)	
	1 st experiment	2 nd experiment	1 st experiment	2 nd experiment
10 mM NaCl feed	1198 (730)	1198 (730)	-	-
Control membrane effluent	590 (360)	874 (533)	51	27.1
Aquaporin-embedded membrane effluent	992.7 (605)	982 (599)	17	18.1
*Control membrane refers for blank liposome immobilized membrane.				

Table 4.7: Salt rejection rates of the membranes.

Sample	J_{sr} (mg/m ² .h)	
	1 st experiment	2 nd experiment
Control membrane	269	1494
Aquaporin-embedded membrane	720	3945

5. CONCLUSION and RECOMMENDATIONS

In the present study, aquaporin of a halophilic bacterium *Halomonas elongata* was firstly cloned into *E. coli* cells and purified from cell membrane using GST purification system. In contrast to tetrameric *E. coli* AqpZ, *H. elongata* aquaporin was found to be present mostly as dimer or monomer form. Increasing β -mercaptoethanol concentration resulted in dissociation of dimers to monomers at 250 mM.

Water transfer rate of *H. elongata* aquaporins were measured by using Stop flow light scattering spectrometer. Two types of lipids (PS:PC and DOPC) were used for preparation of proteo-liposomes containing *H. elongata* aquaporin. Results showed that *H. elongata* aquaporin is functional and water transfer rate of aquaporins increased with increasing salt concentration of the medium because of the osmolarity difference.

For membrane fabrication, aquaporins were embedded in DOPC and immobilized with m-phenylene-diamine (MPD) and trimesoyl chloride (TMC) on polysulphone membrane. DOPC was preferred because it is a synthetic lipid and more stable than PC. Zhao *et al.* (2012) used same immobilization method for proteo-liposome embedding. This method is simple and cost effective, because of that large membrane areas can be prepared. Besides, the membrane prepared with carbodi-imide chemistry method was reported to have high mechanical stability [57]. Different from our study, they investigated performance of *E. coli* aquaporin by using cross-flow filtration system. Similar to the result of Zhao *et al.* we found that flux through aquaporin containing membranes was higher than that of empty liposome containing membranes suggesting that the aquaporins are transferring water efficiently when embedded in MPD.

In our study, we also used dead-end reactor, which is rarely seen in the literature for flux measurements of aquaporin incorporated membranes. Similar to the result of Zhao *et al.* we found that flux through aquaporin containing membranes was higher than that of empty liposome containing membranes suggesting that the aquaporins are transferring water efficiently when embedded in MPD. Different from our study, they investigated performance of *E. coli* aquaporin by using cross-flow filtration system.

Although, water permeability of *H. elongata* incorporated membrane was measured to be quite high (5.47 LMH), salt rejection capacity was 18.1% which is not very satisfactory. However, salt rejection capacity of the membrane cannot be evaluated independently of the flux values. Since increasing water flux value results in increase amount of rejected salt due to the high volume of desalinated water. Zhao et. al. also mentioned the effect of flux on salt rejection in their study [20]. Salt rejection rate (mg/m².h) of *H. elongata* aquaporin embedded membrane is higher than the control membrane in our study. Nevertheless, this interfacial immobilization method used in the present study should be modified or improved for increasing salt rejection capacity of aquaporin containing membranes. Applied pressure in dead end reactor system may also influence the performance of aquaporins. However, Wagh *et al.* also used dead-end filtration system for filtration of *E. coli* aquaporin containing membranes and they found quite high rejection values. But they used different immobilization method including gum arabic and amphiphilic polyvinyl alcohol with alkyl side chains (PVA-alkyl) [57].

In conclusion, *H. elongata* aquaporin based biomimetic membrane was successfully fabricated. SEM micrographs and water flux experiments shows that the resulting membrane has high mechanical strength. Likewise, this polymerization technique provides protection to extrinsic factors. Salt rejection capacity of the membrane is remained to be improved more by optimization of the preparation protocols (such as temperature, proteo-liposome size, concentration of aquaporins and vesicle loading on the membrane). Concentration polarization which is concentration of aquaporin might be the reason of decrease in the salt rejection values. This study reveals the capacity of new material developments with biomimetic approach in the field of environmental technologies. Moreover, this study is a great source for enlightening biomimetic product improving and, inspiring new researches about mimic materials.

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BIOGRAPHY

Gazel Öyküm Özdemir was born on 30th of January 1993 in İstanbul, Turkey. She started his bachelor's degree in Environmental Engineering of Sakarya University in 2011. After her graduation from BSc in 2015, she started Master of Science education in Environmental Engineering program of Gebze Technical University. In the summer of 2017, she graduated from MSc with this thesis.

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APPENDICES

Appendix A: Markers and and SDS-Gel Recipes

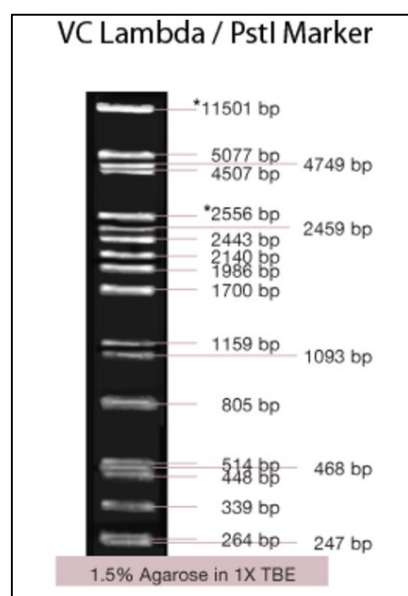


Figure A1.1: DNA marker.

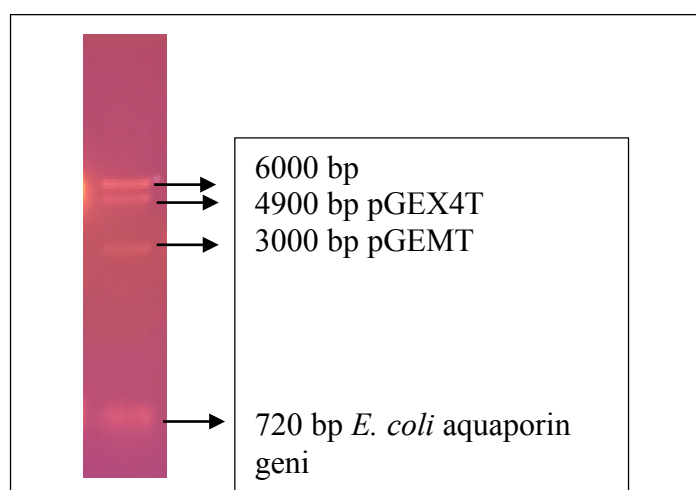


Figure A1.2: Lab made DNA marker.

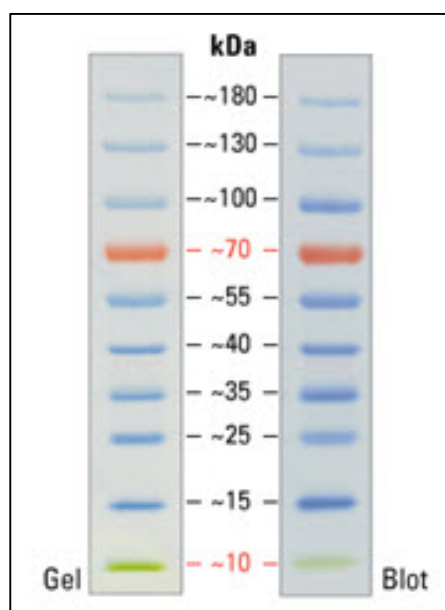


Figure A1.3: Thermofischer page ruler prestained protein ladder.

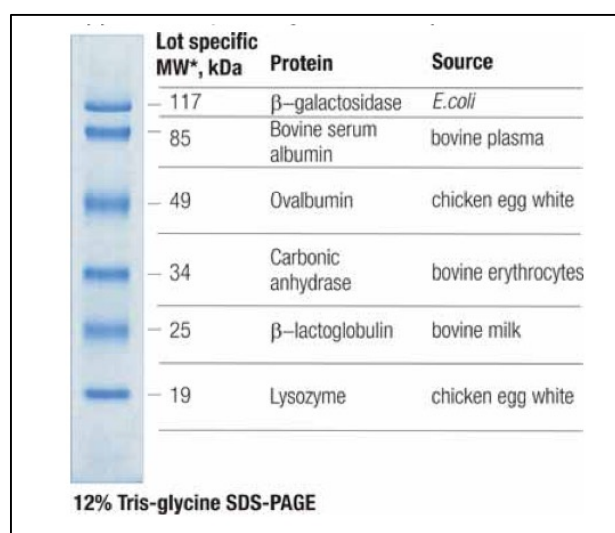


Figure A1.4: Fermentas Prestained Protein MW Marker.

Table A1.1: Separation gel recipe (for 10 ml solution).

Chemical	6%	8%	10%	12%	15%
DI water	5.2 ml	4.6 ml	3.8 ml	3.2 ml	2.2 ml
Acrylamide 30% Bisacrylamide 0.8%	2 ml	2.6 ml	3.4 ml	4 ml	5 ml
1.5 M Tris pH:8.8	2.6 ml	2.6 ml	2.6 ml	2.6 ml	2.6 ml
10% SDS	100 μ l	100 μ l	100 μ l	100 μ l	100 μ l
10% APS	200 μ l	200 μ l	200 μ l	200 μ l	200 μ l
TEMED	20 μ l	20 μ l	20 μ l	20 μ l	20 μ l

Table A1.2: Stacking gel recipe (for 5 ml solution).

Chemical	5%
DI water	3 ml
Acrylamide 30% Bisacrylamide 0.8%	670 μ l
1.5 M Tris pH:8.8	1.25 ml
10% SDS	50 μ l
10% APS	200 μ l
TEMED	15 μ l