Photoelectric Characterization of Bacteriorhodopsin Reconstituted in Lipid Bilayer Membrane

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Photoelectric Characterization of Bacteriorhodopsin Reconstituted in Lipid Bilayer Membrane
Photoelectric Characterization of Bacteriorhodopsin Reconstituted in Lipid Bilayer Membrane

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Microelectronics-Photonics

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Abstract

The objective of this work was to conduct basic research in biologically inspired solar energy conversion solutions. A photosynthetic protein (Bacteriorhodopsin) was reconstituted in a bi-layer membrane. Then, when a laser beam was shined on the membrane, the photon energy was used by the protein to pump protons across the membrane. The translocation of protons across the membrane was measured as photocurrent. For this purpose, a system was built to characterize the lipid bilayer membranes and to measure the photocurrent. The lipid bilayer membrane was characterized by its capacitance and resistance. A picoampere photocurrent was observed when Bacteriorhodopsin protein was present in the bilayer membrane and a 532 nm laser was used as light source. The obtained values were consistent with the reports from literature.

An introduction to the generation of photocurrent by photosynthetic proteins in lipid membranes was reviewed and their applications are discussed in this thesis.
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I finally thank my wife, Larisse, my son, Jayden, and my families and friends all over the world for the unconditional emotional support.
Dedication

This work is dedicated to the two women in my life: my wonderful wife, Larisse Laure Tantchou-Kamwa, and my lovely mother, Julienne Kamwa; my little one, Jayden Kamwa may follow the path.....
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Chapter 1: Introduction

Motivation of this Work

In order to meet the huge energy demand of our time, one of the most promising alternatives to conventional energy sources is the conversion of sunlight into electrical power. Research into new and transformative biological means of solar energy conversion might reveal alternative processes to common solar harvesting methods.

The aim of this work was to develop a biologically inspired solution to solar energy conversion. A protein (Bacteriorhodopsin) was used because of its light-driven proton pump capability. The protein was reconstituted in a bi-layer membrane, and then a laser light beam was shined on the membrane inducing a measurable proton translocation also called photocurrent.
Chapter 2 : Bacteriorhodopsin

2.1 Definition

Bacteriorhodopsin (BR) is a small and robust membrane protein with light-driven proton pumping capability as shown in Figure 2.1 [1]. The protein has a molecular weight of approximately 26 KDa, with a total of 248 amino acids. The name Bacteriorhodopsin derives from Bacteri, which means bacterial mutation, and Rhodopsin, which is the visual chromoprotein of higher animals. In fact the BR structure is very similar to the Rhodopsin found in higher animal retinal. Bacteriorhodopsin provides cells with energy to live, by generating a proton gradient around them [2, 3, and 4].

![Diagram of Bacteriorhodopsin](image)

Figure 2.1 Bacteriorhodopsin in its environment [1].

Bacteriorhodopsin is found in Halobacterium halobium, a microorganism with a high affinity for salty environments [3]. Halobacterium halobium can be grown in high saline
environments under light and low oxygen pressure. Halobacterium halobium acquires its energy through its light response system also called photophosphorylating system, the important part of which is called purple membrane [4]. The purple membrane, a round / oval sheet, can be isolated from a mixture of membrane fragments, obtained through a hypotonic disruption of a Halobacterium halobium bacterial cell. The analysis of a purple membrane shows that it is made of lipids (ether of phosphoglycerol and dihydrophytol) and a single protein called Bacteriorhodopsin [2].

Compared to other available photosynthesis proteins, Bacteriorhodopsin (BR) is more robust and stable. It exhibits the ability to stay active for many years while maintaining its original structure at up to 140 °C. When captured inside a membrane and illuminated, BR is able to act as a proton pump; it translocates protons from one side of the membrane to the other (vectorial charge transport). This pumping activity is increased when BR fragments are all oriented in the same direction, with respect to the lipid membrane. Having the BR fragments unvaryingly oriented, ensures that protons flow only in one direction, and creates the proton gradient only on one side of the membrane. The produced photovoltage is characterized by its bipolarity which is expressed in a first fast (ps) negative signal followed by a positive slower one (in µs or ms). The longer signal indicates the proton transfer by the BR fragment [5].

### 2.2 History & Theory (working principle)

Bacteriorhodopsin (BR) is, with Halorhodopsin (HR), the simplest known light energy converting systems under biological species. These proteins are able, when reconstituted in lipid membranes, to induce an electrochemical gradient upon illumination [6]. The focus of this research was to investigate the different properties of the Bacteriorhodopsin. More than 30 years after its discovery, techniques in biophysics, biochemistry, and structural biology have advanced
scientific understanding of how BR works. The interest in Bacteriorhodopsin was not first driven by its ability to live in high concentrated salt, with light as unique energy source. Instead, BR appeared to be a very simple model protein, useful to investigate cell-membrane receptors. In addition, BR was identified as a model protein that could be used to replicate and analyze the transmission / communication through membranes. Similarly to BR, the membrane-receptors, also known as G-protein, are made of seven helices. In human bodies, these helices incorporate the majority of the known drug targets [3].

In the 1930, although minimal, recognition was given to the proton conduction in biological energy transduction systems for the first time. While Halobacterium salinarum (HS), the archaeon of Bacteriorhodopsin, was discovered in 1966, initial data were collected only between 1970 and 1978. The similarity between BR and Halorhodopsin was established in 1970. Two years later, the BR ability to translocate protons across membranes upon illumination, as well as its role in the light-induced proton pumping, was established. Around 1977, the understanding of BR structure and its function was advanced. Bacteriorhodopsin was identified as an intrinsic membrane protein with around 26 kDa = 26 kg/mol. The membrane protein appeared to be located in the cell membrane, where it forms a purple membrane (PM) comprised of crystalline patches.

In 1975, observing the structure of a purple membrane under low resolution, investigators were able to identify a cluster of seven alpha-helical segments that cross the thick purple membrane forming a quasi right angle. Even though the retinal (which is a molecule of a linear pigment) was not clearly identified, it was supposed to be located in the hydrophobic interior of the cluster. It was suggested that this seven-alpha-helical structure acts as retinal isomerase (the enzyme that catalyzes the chemical reaction). This structure was also suspected to be responsible
for the retinal large red shift and also for the increases of the chromophore acid dissociation constant (pK) [6].

In 1976, Michel and Oesterhelt [7], presenting the purple membrane structure for the first time, proved that Bacteriorhodopsin from Halobacterium salinarum / halobium can generate a voltage up to 280 mV. The protein was identified as a channel-like bundle of seven transmembrane helices, which were branded with the letters A, B, C, D, E, F, and G; the channel consisting of all but the A, C, and E helices. The cell was supposed to be made of both a cytoplasmic (CP) and extracellular (EC) half. Both half cells were supposed to be oriented by the chromophore retinal, with the retinal bound to the lysine 216 in helix G via the so-called Schiff base [8]. In 1979, the BR protein was sequenced for the first time by two laboratories. An attempt to establish a relationship between spectroscopic data and protein structure was made. Seven hydrophobic segments were acknowledged in the investigated sequence. The seven segments identified in the sequence seemed to be the “helices” that were previously investigated within the transmembrane [6]. The spectroscopy observation of the BR photoreaction allowed researchers to identify five thermal states during the photocycle. The state lifetimes varied from less than a few picoseconds to a few milliseconds. These states were identified as K, L, M, N, and O following the transitions: \( \text{BR} \rightarrow \text{r K590} \rightarrow \text{r L550} \rightarrow \text{r M410} \rightarrow \text{r N~520} \rightarrow \text{r O640} \rightarrow \text{BR} \); as indicated in Figure 2.2 [9]. Only the first transition of the cycle requires light, the energy needed to translocate protons is stored inside the protein. The light absorption through the intermediate states K, L, and M helps short-circuit the cycle and release protons. When a proton is released from the outer membrane surface (LrM transition), it appears on the outside medium after less than one millisecond. A few milliseconds later, the proton is taken up from the inner medium during the so-called N-decay as presented in Figure 2.2.a. During these early research
stages, changes in the amino acid residues inside the protein as well as the Schiff base pK (acid dissociation constant) appeared to be the driving force for the protons [6].

From 1975 on, visible light and time-resolved absorption spectroscopy at low temperature provided the basis data for modeling photocycles in order to understand proton transport. A central part of the investigations was dedicated to the verification of earlier assumptions. The later development of new software made it possible to apply global analyses to extensive new data sets. The main advantage of the data model was the coverage of a wide range of temperature and pH values. In addition, it has been possible since then to analyze characteristic effects like photo selection and light scattering, and to investigate the possible existence of Bacteriorhodopsin types with different photocycles. Back reactions and diverging pathways have also been defined [6].

Since its publication, the cycle process proposed originally remains the most established among researchers [6]. Investigations proved that the charge displacement toward the cytoplasm detected in the BR → rK590 transition is very fast and small; a charge motion toward the exterior follows it in the rL550 transition as shown in Figure 2.2 [9]. Combined, both steps complete around 20% of the charge translocation; the transfer between M and BR accounts for the remaining 80%. In 1990, electron crystallography allowed the exploration of three-dimensional BR. The protein plane resolution was found to be around 3.5 angstroms and the normal plane around 10 angstroms [6]. Further investigations confirmed that the natural BR photocycle determines the proton translocation. The retinal being the first light-excited component inside BR, it isomerizes from its original state “all-trans” to the 13-cis state (A=1 → M=13) forming a product identified as M. Between the all-trans state and M, the three intermediate states J (10-cis), K(11-cis), and L(12-cis) are formed (Figure 2.2.b [9]).
Figure 2.2 (a) Intermediate states translocation time sequences. (b) BR intermediate states translocation sequences flow [9].

The M (or Mcis) formation sees a release of the protons from the Schiff base to the extracellular side via amino acids called “Aspartate 85” (D85). Next, the Schiff base acid dissociation constant (pK) is shifted from pK 13 to pK 4.0, later confirmed by Sheves et al. [10]. A second pK shift occurs while still in Mcis state from pK 4 to pK 10. Then, the intracellular side (CP) takes up the protons via the next amino acid Aspartate 96 (D96). And Oesterhelt and Hess [11]; and several other authors called this shift “Molecular Switch”. The M state is subdivided into M1 corresponding to the proton’s accessibility to the extracellular side and M2 which represents its accessibility to the cytoplasmic side (Figure 2.2.b [9]). The spectroscopic identification of M1 and M2 showed that the switch between M1 and M2 is driven by a change in the protein, which is preceded by a change of the retinal configuration. The irreversibility of the M1→M2 transition was established by Sass et al. in 1997 [12]. The transition step from M1 to M2 is the key molecular switch of ion transport inside natural occurring type BR. In 1983, Dubrovskii et al. [13] found out that the M decay can be held up to a factor of 10, by
preilluminating Bacteriorhodopsin, in whole cells, or in proteins containing phospholipid (proteoliposomes). They suggested that the preillumination is able to reprotonate the Schiff base from the outside, inducing the slow decay of the M phase. Experiments conducted with BR as dried purple membrane samples – BR proteoliposomes or BR in planar lipid membranes – confirmed that the photocurrent is reduced when an electrical potential is applied against the proton pumping direction [8].

Underlying mechanisms which drive the transition step from M1 to M2 were investigated by Luecke et al. [14]. Their research confirmed that BR is made of seven membranes forming a helical structure, connected to either the extracellular or the cytoplasmic side of the cell membrane via short loops. Normally, the cells enveloped by the membrane are impermeable to ions and life depending nutrients. In fact, each Bacteriorhodopsin particle contains a retinal (Figure 2.3.a [9]). While one end of the retinal is compressed deep into the protein, the top end is attached to a nitrogen atom of a lysine residue in helix G, one of the seven helices. The researchers explained that the presence of visible light changes the retinal structure. This structure change allows the retinal to capture light energy. Next, the polypeptide inside Bacteriorhodopsin uses the captured energy to push a single proton through the seven-helix bundle outside of the cell. During this process, the whole molecule exhibits several intermediate states which have been characterized through spectroscopy. These states have different colors and were identified as already mentioned above as K, L, M, N, and O: Figure 2.2 [9] shows the L, M, and N states as well as the ground state [3]. Figure 2.3 [9] shows the charge transfer inside Bacteriorhodopsin; the observation of Bacteriorhodopsin from the helix C is depicted in (a). The S193 (bottom) area is the location of the F-G interaction on the extracellular side of the membrane. The yellow dotted lines represent the hydrogen bonds, the green dots the water
Figure 2.3 (a) Charge transfer inside bacteriorhodopsin. (b) Structure of the hydrogen bonds [9].

molecules, and the purple color the retinal. In (b), the structure of the hydrogen bonds starting from the Schiff base is represented. The proton is first freed from the inside / cytoplasmic side of the membrane and then passed to the extracellular surface (S193) via the E204 protonation [3].

More recently, electron cryo-microscopy applied on two-dimensional crystals inside cell membranes was used to determine the structure of the Bacteriorhodopsin ground state. Subsequently Luecke et al. [14], and Edman et al. [15] determined the structure of the K and M intermediate state using microcrystals X-ray crystallography. Shortly thereafter, the N intermediate was identified using electron microscopy of two-dimensional crystals [2]. Bacteriorhodopsin mutants with slow proton-pumping cycles were used to determine the M and N intermediate. The spectral characterization of the proton transport intermediate processes BR→v K → L → M1 → M2 → N → O → BR [16] confirmed that the proton translocation by the BR protein, involving amino acid residues and internal water molecules, usually follows the isomerization of the BR retinal. It particulary showed, according to Lórenz-Fonfría and Kandori [17], that the deprotonation, which is in fact the proton release from BR, occurs in the M sub-
state and not as sometime stated in the L→M transition. In fact, the proton release is delayed relative to the protonation of Aspartate 85. In addition, and according to Zimányi et al. [18], the proton release in the transition of the M substrate is followed by the proton reuptake, which normally occurs in the transition N→O when pH > pKa. Normally, when pH < pKa, the proton uptake occurs during the N→O transition prior to the proton release during the O→BR transition. Under illumination of BR, an electric response from the proton transport can be observed. In general, the BR photoelectric response of BR-based devices is investigated through a BR film structure [19]

2.3 Applications

2.3.1 Imaging device

Vsevolodov and Dyukova [20] were the first to launch the use of Bacteriorhodopsin as advanced functional material in the 1980s. The group modified Bacteriorhodopsin samples and was able to use them to create the “Biochrom film,” the first BR-based imaging device.

2.3.2 RAM & Hologram

H.T. Tien [21], using spinach leaf extract to form artificial black lipid membranes, was among the firsts to demonstrate the photoelectric ability of BR. The resulting photochromic effects were subsequently used in the development of hologram and high-speed random access memory (RAM) (Figure 2.4) [22] [23].
2.3.3 Motion detectors

Bacteriorhodopsin membranes which exhibit fast photoelectric signal (with time rising in picoseconds), are solid candidate for the design of high-speed photodiodes and memories.

Further applications in the manufacturing of motion detectors and color discriminators are shown in Figure 2.5 [24]. The principle of both devices is based on ‘differential responsivity’ [22].

Figure 2.4 BR based molecular memory system [22].

Figure 2.5 BR based ‘micromouse’ robot with two biophotosensors (dotted circle) [24].
2.3.4 Alternative solar energy conversion

Figure 2.6 [25] shows the diagrams of the top and cross-section views of a photocell based on BR. Due to its proton pumping ability, BR has been in focus for alternative solar energy conversion methods. In general, the observed photovoltage is in the range 0.2-15 mV per BR layer. The stationary photocurrent can reach a maximum of 40 pA/cm$^2$ per BR monolayer. Indium-tin oxide (ITO) is used as an optical window, and is most of the times also an electrode. The substrate is a thin film of PolyEthylene Terephthalate (PET), with deposited ITO as shown in Figure 2.6 [25]. Only a half cell made of a purple membrane suspension is used. The electrode polarity is not defined when the external bias is applied [19]. Photo devices using BR as light driven proton pumps can be fabricated by placing three droplets incorporating a BR suspension on electrodes. The electrodes are then placed around a fourth droplet.

![Figure 2.6 Bacteriorhodopsin-based photocells: design (a) and finished product (B) [25].](image)

On the other electrode, a fifth droplet containing a different species ($\alpha$HL, for example) is placed. The electrical circuit is completed by incorporating the pore into the final Droplet
Interface Bilayer (DIB) [19]. Figure 2.7 [26] shows three Bacteriorhodopsin-containing droplets (mauve) arranged around the fourth one. The three droplets are connected to the same electrode. The fifth droplet (green) contains a protein-based ion channel which connects it to the central droplet. Through the current amplitude and back-calculation, it is suspected that a huge amount of BR copies are in service in each membrane.

Powering a droplet network can be achieved, if necessary, by using BR with white light as the source of power. This is a means of constant energy generation compared to bio-batteries in which energy production leans on pre-existing ion gradient and will, thus, automatically run down. Functional systems can be created based on the high stability of DIB networks. This occurs when biological processes are mimicked using DIBs. For this purpose, incorporating / engineering membrane protein networks (ion channel, pore, and pumps) in DIBs needs to be completed. A bio-battery, using three coupled droplets and working electrochemical gradient, was already designed and tested [27].

Figure 2.7 BR based droplets network [26].
Chapter 3: Bilayer Lipid Membranes

3.1 Definition

Lipid bilayers are one of the most important structures in nature; they are responsible for protecting the inside of cells and for communication with other entities. It is widely believed that lipid bilayers, as a basic element of cell membranes, are responsible for many of the cells properties. It is extremely difficult to access and/or manipulate the inside of a cell. Equivalent systems made of solid supported lipid bilayers are excellent candidates to investigate cells properties [28]. Figure 3.1 [29] shows the detailed diagram of a cell lipid membrane.

![Lipid membrane diagram](image)

Figure 3.1 Lipid membrane diagram [29].

3.2 Background

The development of artificial bilayer systems was driven by the desire to understand the complex nature of two-dimensional fluid plasma membranes as shown in Figure 3.1 [29]. Mueller et al. [30] were the first to develop a reliable artificial system for the investigation of planar phospholipid bilayers. The system, known as black lipid membrane, was made of brain
lipid extracts. The name Black Lipid Membrane (BLM) was derived from the fact that intrusions, noticed inside the membrane, decayed while thinning the lipid mass. The system was made of phospholipid solution painted across a 1 mm septum. This septum was used to connect two chambers filled with conductive aqueous solution.

Later, Tamm and McConnell [31], found a way to form and deposit the membrane on solid substrate. Boxer et al. [32] thereafter developed a process to fabricate lithographically patterned corals out of lipid bilayers. Spinke and Yang [33] found that it is possible to use thin polymer films to couple lipid bilayers with different substrate materials such as metals, oxides, and semiconductor electrodes. Their results pioneered the formation of polymer supported phospholipid bilayers on solid substrate.

### 3.3 Diphytanoyl phosphatidylcholine (DPhyPC)

Diphytanoyl (3,7,11,15-tetramethylhexadecanoic) phosphatidylcholine (DPhyPC) is an artificial lipid. The DPhyPC structure exhibits connected carbon chains that are specific to archaebacterial and non-mammalian membranes. The DPhyPC structure is shown in Figure 3.2 [9]. Figure 3.2.a shows a bilayer membrane while Figure 3.2.d and Figure 3.2.e are showing the single components of the bilayer structure. The hydrophobic tails are connected to the hydrophilic heads through glycerol. Since the hydrocarbon chains are saturated, DPhyPC is more robust against membrane degradation compared to lipids with unsaturated carbon chains. The broad usage of DPhyPC in investigative experiments leans on two main properties. First, bilayer membranes formed with DPhyPC are stable in most of the biologically relevant fluids. Second, the DPhyPC based membranes exhibit a very low transition temperature (≤120 °C). The area per lipid rate of DPhyPC membranes (A = 80.5 Å²) is by far the largest among commonly used lipid
species. Aside from DPhPC, biological species used to form lipid membranes include, beta-bodipy assolectin, agarose type 7, alamethicin and gramicidin D.

Tristam-Nagle and Kim [34] proved that the water permeability in DPhyPC membrane is strongly related to the membrane area, while the thickness of the hydrocarbon has only a secondary effect. The group suggested that DPhyPC membranes are made of two interfacial headgroup layers and a central hydrocarbon core layer.

Figure 3.2 (a) DPhPC based membrane consisting of (b) DPhPC 3D model (c) Kekule structure of the different various groups, (d) corresponding cartoon lipid molecule, and (e) membrane [9].

Based on these suggestions, Shinoda et al. [35] found that a smaller diffusion rate is the most relevant factor in the water permeability of saturated hydrocarbon.

The saturated / branched hydrocarbon chains do not significantly affect interactions between DPhyPC bilayers. Compared to unsaturated / linear bilayers, the water permeability of DPhyPC membranes is higher and depends on the area. This means that unsaturated hydrocarbon
membranes are able to reduce the water flow through the hydrocarbon region. Therefore, using DPhyPC to investigate the peptide / lipid, ion channels in complex organisms does not produce accurate results. However, it does provide investigators with sufficient information to suggest theories which can later be tested through other means [34].

The carbon chains in mammalian cell membranes are unsaturated, contrary to DPhyPC-based membranes. Therefore, using DPhyPC membranes to simulate mammalian in vivo environments must be done very carefully. Adjustments considering the difference between the two environments have to be identified and addressed; for example, the influence of chain branching on bilayer structures on the material properties (bending, robustness), function, and permeability.

DPhyPC formed membranes are influenced by temperature variation. In fact, the DPhyPC packing structure is temperature dependent. The packing structure also depends on the presence of water. Under a ratio of 16 water molecules per lipid, the DPhyPC headgroup starts changing its orientation and, thus, the lipid gound structure. In the presence of water molecules, DPhyPC dehydrates in a cubic like structure. Finally, at a ratio of six water molecules per lipid, DPhyPC dehydrates in a hexagonal structure [36].

3.4 Applications

DPhyPC is a good candidate to be used in artificial system while exploring processes such as: peptide–lipid interactions; model ion channels; electrophysiological measurements; and, basic lipid mixtures.

The bilayer membranes formed with DPhyPC are stable in most biologically relevant fluids. DPhyPC might be useful for the investigation of the connection between the membranes intrinsic curvature and its packing structure [34].
Chapter 4: Overview of Experimental Devices

In this chapter the devices and methods used in this work are introduced. The main advantage of the setup used was that it required a computer, an AC/DC converter, a patch clamp amplifier, and a digitizer, all affordable and, commercially available devices. However, the calibration of the system with the right protocol to implement measurements was very complex.

4.1 Faraday cage

In order to reduce the noise during the recording of photocurrents, the entire experiment was protected from electrical and vibrational interference. Moreover, it was necessary for the Bacteriorhodopsin photoactivity to be activated by the laser light source only, once the lipid-BR membrane was formed. Thus, it was important to shield the lipid-BR solution / membrane from surrounding light sources. For these reasons, the whole experiment was mounted and performed in a Faraday cage as depicted in Figure 4.1.

![Figure 4.1 Faraday dark chamber.](image-url)
4.2 Chambers

4.2.1 Description

The two-chamber system used for the experiment was purchased from Warner Instruments. The system was made of one large chamber in which a second chamber (a cup) was inserted (Figure 4.2 & Figure 4.3). The bilayer aperture (diameter = 0.147mm) was incorporated in the cup wall.

![Diagram of a two-chamber system with labels for electrodes, agar solutions, bilayer membrane, tightening screw, front chamber, back/cup chamber, and salt bridges.]

Figure 4.2 Bilayer cups and chambers design setup.

The large chamber was made of black Polyoxymethylene (POM) and the cup was made of white POM. POM is a thermo-plastic with high stiffness, low friction, and excellent dimensional stability. These attributes fulfilled the precision required from the used parts. A screw on the back of the large chamber held the cup and the septum in the defined position. It was important to avoid over-tightening the screw since this could crack the cup and shortenend its lifetime.
In order to avoid over-tightening, a rubber plug (tightening bolt) was inserted between the screw and the cup. A glass window on the large chamber allowed a live observation of what was happening inside the cell during the experiment. The plexiglass was glued into place with a solid adhesive. Up to four additional screws were used to secure the glass (Figure 4.2 & Figure 4.3).

4.2.2 Using the chamber

The bilayer membranes were formed across the aperture located on the cup-wall (septum). The space left over after inserting the cup represents the front chamber, while the interior of the cup was the second chamber. This configuration is the most common one in the field.

Agar salt bridges were used to ensure the electrical connections between the headstage and each chamber.
4.2.3 Polydimethylsiloxane “PDMS” chamber

4.2.3.1 Description

Alternatively to the cup chambers, a chamber system made of Polydimethylsiloxane (PDMS), a silicone rubber state substrate, was used to form and test bilayer membranes. The system was composed of two identical PDMS chambers and a silicon substrate which incorporated the window as shown in Figure 4.4.

The chambers were designed and fabricated as part of this research. Figure 4.4 shows a sketch of the PDMS chamber system. Each of the two walls #1 incorporated a cave-like-chamber #2. A drop on each wall surface #3 served as location for the silicon chip / substrate #4. The substrate was previously fabricated by photolithography, creating a micro-sized window in its center #5.

![Figure 4.4 Sketch of the Polydimethylsiloxane “PDMS” chamber.](image)

Three mini channels were incorporated inside each wall #6. The channels were the connections between the outside and the chambers. Syringes and wires were connected to the channels to control the experiment. In order to close and seal the system, the substrate was
placed in its location on one of the walls, and then the second wall was placed on top of it. Next, the system was placed inside a metal form which was then closed with screws. The screws provided the pressure needed to seal the system.

4.2.3.2 Using the chamber

The PDMS chamber was designed as an alternative system to form bilayer via the folding method. As mentioned above, the silicon chip was placed between the two PDMS compartments. The system was sealed using metal forms to press both compartments against each other. The chambers were, in fact, holes enclosed inside the PDMS parts. The chambers were accessible via mini channels. Up to three mini channels were integrated in each PDMS part. The first channels #6 on the bottom were used to inject the buffer solution inside the chamber. The second channels #6 on the rear top were used as ventilation and, when necessary, excess solution could be sucked out from there. The third and last channels #7 were located on the top front of each PDMS wall; these channels #7 remained closed, as indicated in Figure 4.4, until the buffer solution was raised in the right position below the window. Only then, the third holes #7 were punched and used to inject the BR-DPhyPC-n-decane solution in one chamber and the DPhyPC-n-decane solution in the other chamber. Then, as will be described in 5.1.2.1 the solution was raised above the window and the bilayer was formed.

The PDMS chamber and the cup-chamber (POM) system operated according to the same principle. The main advantage of using a PDMS chamber system was the use of a silicon substrate. As described earlier, a solid state substrate onto which a micropore was drilled hosted the formed membrane. The substrate chip presented the advantages of being easily transportable and fixable into other devices for additional investigations of the formed membrane. Although the membranes fabricated with the PDMS system exhibited the same average resistance and
capacitance as the membranes fabricated with the system purchased from Warner Instruments (Chapters: 4.2.1, 6.1.2, 6.1.3, and 6.1.4), it was, unfortunately, to this point, not possible to generate defensible photocurrent with it.

4.3 Electrical Signal Measurement Setup

The clamp system was used to measure the capacitance and the resistance of the formed membranes as well as the produced photocurrent. The clamp system was made of electrode salt-bridges, a digitizer, an amplifier, a headstage, the connection cables, and a computer.

4.3.1 Voltage clamp

The voltage clamp was the main technique used to record the membrane’s capacitance and the photocurrent. This method included a voltage (function-) generator with two electrodes: a "cathode electrode" and an "anode electrode." The voltage clamp used a negative feedback to keep the cell at a defined voltage (holding voltage).

4.3.2 Patch clamp

Generally used in electrophysiology, the patch clamp is a refinement of the voltage clamp technique. The patch clamp technique allowed accurate examination of single or multiple ion channels in cells. This technique was particularly useful for the investigation of excitable cells and bacterial ion channels.

4.4 Salt-bridges

Agar salt bridges are commonly used for the electrical connection of bath solutions in order to control and reduce the ion transfer from the electrical environment. In this work two salt-bridges were used in the electrical connection with the amplifier. Salt-bridges were composed of 2-5% agar in a 1 M KCl or NaCl solution. The agar was inserted into glass tubings
which act as the bridge between the solution and the electrical circuit. Figure 4.5 [37], shows the principle of a salt-bridge application. The bridge can be stored for up to one month.

![Salt-bridge principle](image)

Figure 4.5 Salt-bridge principle [37].

### 4.5 Digitizer

The Digidata 1440A from Molecular Devices is a low-noise data acquisition system designed for ease-of-use and fast results [38]. This device is best suited for continuous data acquisition from an operating system undertaking multiple tasks. The connection to the controlling computer / laptop was established via a USB 2.0 interface. This allowed an easy installation as well as an extremely rapid signal transmission due to the 16-bit data acquisition of the Digidata 1440A. This high bit range allowed measurements up to a maximum sampling rate of 250 kHz per channel with a total data throughput rate of 4 megasamples per second. The best advantages of using the Digidata 1440 were the extremely low inherent digitizer and channel noises. The Digidata 1440A is, with sixteen inputs, four analog output channels, and eight digital outputs especially well organized. An additional digital output is dedicated to trigger external devices. Four additional analog inputs for remote instruments and the DB-25 connector for the digital outputs are located in the back.
The Digidata 1440A works with the pClamp 10 software from AxoScope designed for electrophysiology under Microsoft Windows (Microsoft Windows 7, Appendix G, was used in this work). After installation, the Digidata 1440A was ready to perform and gather data immediately. No extra programming was required.

4.6 Amplifier

The Axon Axopatch 200B (AA200B) from Molecular Devices used in the experiments in this work is a well established amplifier system used to capture capacitor feedback [39]. The AA200B is excellent for basic investigations of ion channels. This device is best suited for ultra-low-noise recordings.

4.7 Headstage

The electrodes were connected to a pre-amplifier, also called headstage, on one end. The headstage gathered the membrane potential and forwarded the signal to the main amplifier. The headstage also supplied the voltage electrode with power. Figure 4.6 [40], shows the internal circuit of the headstage used, the Axon Instruments CV 203BU [41]. The power level was defined by the operator, produced by an external (function) generator, and transferred to the headstage via the main amplifier.

Figure 4.6 Decoupled headstage amplifier with gain [40].
It is recommended to use agar salt bridges (Chapter 4.4) to connect the headstage to the bath, since the response of membrane channels can be affected by the presence of silver electrodes in the bath solution. The chamber system offers wells (Figure 4.2 & Figure 4.3) which were filled with 0.1M NaCl. The wells are adjacent to the baths; this means that the agar bridges used to close the circuit from well to bath were as short as possible. The wells were connected on one side to the bath via a salt-bridge and on the other side to the headstage via an electrode.

4.8 Computer and software

4.8.1 pClamp 10 Analysis Software (Clampex)

The main software used in this work was the Clampex 10, a suite of the widely used pClamp 10 from AxoScope [39]. pClamp 10 is a data acquisition and analysis software used in micro analysis. It is best suited for the control and recording of voltage-clamp, current clamp, and patch-clamp experiments. The pClamp 10 program collections include the possibility to synchronize simulation, to detect events, and to perform online analysis. Clampex 10 as part of the collection was particulary suited for expanding the range and the quality of the data acquisition. This was very important in the experiments since the basic research performed here involved the application of several experimental protocols.

4.8.2 Clampfit

The Clampfit 10 from AxoScope [39] was the second main software used in this work. Clampfit 10 is most appropriate for the analysis of layout and graphs produced with Clampex. This software allows data filtering after recording and routine fittings. The widely used I-V curve and voltage spectrum are included in its functionality. Moreover, Clampfit 10 allowed event detection and analysis under template and single-channel mode. It was further possible to link the threshold analysis observation to the data acquisition.
Chapter 5 : Experiments

In this chapter, the experiment steps, as well as the correlation between them, are presented from the mix of biological solutions to the generation of photocurrent.

5.1 Membranes

5.1.1 Solutions

Pure 1,2-diphytanylsn-sn-glycero-3-phosphocholine (DPhyPC over 99%) with the formula \( \text{C}_{48}\text{H}_{96}\text{NO}_{8}\text{P} \) (846.252 g/mol) was purchased from Avanti Lipids [42] in powder form. Bacteriorhodopsin: BR (27052 g/mol) was acquired from Sigma Aldrich [43]. Hexadecane n-decane with the formula \( \text{CH}_3(\text{CH}_2)_8\text{CH}_3 \) (142.28 g/mol), was purchased from TCI America [44], and the natriumchloride NaCl (58.44 g/mol) from J.T.Baker [45]. Except from the n-decane, all species were purchased in powder form.

The lipid solution used in the experiments was made of a combination of the selected lipids (DPhyPC) suspended in n-decane. Visible turbidity in the solution was identified as a potential sign of contamination, which would make the lipid solutions unusable for the experiment. Therefore, the solution setups and storage were performed with high accuracy and care. Two different mixes were completed; the first was a 1% weight per volume (w/v) solution made of 2.5 ml of n-decane added to 25 mg of the DPhyPC. The obtained DPhyPC-n-decane solution was partitioned in multiple tubes and stored in a freezer at -80 °C. The second solution combined BR and n-decane in a two micromolar (2 µM) concentration. This solution was mixed and also stored in the same freezer. The third, and last, solution was a mixture of 1 µl of the DPhyPC-n-decane solution and 10 µl of the previously mixed 2 µM BR-n-decane solution.

This mixture was prepared at the start of every experiment and immediately used. The goal was to achieve a ratio of 1 BR molecule per \( 3.2\times10^4 \) DPhyPC molecules [46]. Ultimately,
the front (Cis) chamber (negative electrode) contained the membrane solution 0.01 mg/ml BR plus 1% w/v DPhyPC and the back (Trans) chamber (positive electrode) contained the 1% w/v DPhyPC solution.

Because of its conductivity, a one molar sodium chloride solution (NaCl) was mixed and used as a buffer. For this purpose, 58.44 g NaCl powder was mixed to 1 L deionized water. Since high conductivity might compromise the measurement, the salt solution was diluted to 0.1 mol/l.

5.1.2 Membrane forming

The procedure of forming bilayers included first cleaning the chambers. It is important to keep the pH of cleaning solutions over 4 and below 9 to avoid the degradation of the chambers. Next, the chambers were dried with argon and placed on a wipe on the lab-bench. The 1% w/v DPhyPC-n-decane solution was taken out of the freezer and shaken with a vortex for about one minute. Then, in order to ease the adhesion of the lipids on the aperture, the cup-septum was pre-coated with the lipid solution. For this purpose, the window was pre-painted with the lipid cocktail using a paint brush. First, the brush was dipped into the lipid solution then it was revolved on both sides of the window until a uniform coating was achieved.

Takagi et al [48] noticed that pre-painting the septum with the lipid solution renders the region around the septum hydrophobic and under these circumstances, the contact with the lipid-solvent chains is better established and, the membrane is easier formed.

5.1.2.1 Folding Methods

The long chains of fatty acids, which have been synthesized under abiotic conditions, suggest that the folding of two monolayers to form a bilayer is a process that might have played a role in prebiotic evolution. Montal and Mueller [46] already developed a technique to form lipid bilayers from monolayers. The main advantage of this process is in the possibility to form
asymmetric bilayers. In order for the bilayer to remain on the substrate, it is important that each monolayer exerts the same opposite pressure at equilibrium. By looking through the microscope, a meniscus can be observed over the aperture while raising the solution in the back-chamber. This is due to a capillary action since the monolayer-coated aqueous phase wets the substrate [47].

As shown in Figure 5.1 [9] the window was initially pre-painted with a bit of the DPhyPC-n-Decane solution. Subsequently, the chambers were filled up with the sodium chloride buffer solution to a level under the aperture (Figure 5.1.c). Next, the membrane solutions were injected on the top of the buffer solution. The BR free solution was injected in the positive and the BR-containing one in the negative chamber (Figure 5.1.d). Finally, the NaCl levels were raised above the window level, first in the back, and then in the front-chamber, as indicated in Figure 5.1.e. This was done by injecting more sodium chloride in each compartment. According to the DPhyPC property, a bi-layer membrane, made of the folding of two monolayers, formed itself at the window, isolating both chambers electrically from each other (Figure 5.1.f).

![BLM Formation Steps – Folding Bilayer Method](image)

**Figure 5.1** Folding method Bilayer membrane formation steps [9].
5.1.2.2 Painting Methods

Although in less use than the folding methods, painting a membrane is still one of the most used membrane formation techniques because, this method is easy to test. First, the DPhyPC-n-Decane solution was obtained from the freezer and used to pre-paint both sides of the window (Figure 5.2.b). Subsequently, the chambers were also filled up with the buffer solution. This time, however, the buffer solution was raised to a level over the window (Figure 5.2.c). After connecting the system as described in Chapter 5.2, and inserting the electrode, the Clampex program was opened on the computer and the amplifier was turned on. A paint brush was modified (all bristles removed but one or two) and its tip was used to paint a bit of the solution on both sides of the window (Figure 5.2.e). The painting was performed until a reading appeared on Clampex. Then, the 100 µV “seal test” was turned on. Next, the paint brush was dipped into the lipid solution again and the painting was resumed. This procedure was continued until the capacitive current rose on the Clampex-screen (Figure 5.2.f). As mentioned before, when the membrane broke (signal disappears), it was easy to reform it by repainting.

Another variation of the painting method included first of all the pre-painting of the hole with the DPhyPC-n-Decane solution using the functionalized brush. Subsequently, the chambers were also filled up with the buffer solution to a level above the window. Next, the BR-containing solution was absorbed into a syringe. The syringe was used to form a lipid bubble which was then used to paint the window until the membrane was formed, similar to what was done with the paint brush.

5.2 Patch Clamp Circuit

5.2.1 Assembly & Design

The devices described in Chapter 4 were connected and tested. As depicted in Figure 5.3,
the voltage signal was first sent to the membrane via the amplifier over the headstage to the electrodes. This was achieved by connecting the headstage with the bath (line C1) and by connecting the (function) generator output to the “rear switched external command” located in the back of the amplifier (line C2). Next, the generated voltage was sent to the computer for monitoring by the operator. This was done by using a “T-joint” to connect the generator output with the channel 3 of the digitizer (Chapter 4.5) which was connected to the computer via the USB2 port. Next, the voltage signal generated by the amplifier for the measurement of the resistance was sent to the computer via the digitizer by connecting the scaled output of the amplifier to channel 0 of the digitizer (line C3). The amplified or reduced response signal provided by the membrane through the amplifier for the capacitance measurement was sent to the computer by connecting the 10 mV output to the channel 1 of the digitizer analog input (line

Figure 5.2 Painting Techniques BLM Formation [9].
C4). Last, the generated current signal was sent to the computer via the amplifier and the
digitizer by connecting the 10 kHz (I) output of the amplifier with channel 2 input of the digitizer
(line C5). The voltage signal used to evaluate the membrane resistance was transmitted to the
cell by connecting the digitizer analog output to the second external command of the amplifier
(line C6). It was further required to connect the Gain, Frequenz, and Cell capacitance
(“telegraph outputs”) of the amplifier, respectively, to channels 0, 1 and, 3 of the digitizer
telegraph input. This allowed a direct measurement (and display) of the membrane or cell
characteristics (line C 7, 8 & 9). All connections were identified and set up for analysis using the
Clampex program before starting the experiment.

5.2.2 Measurements and recordings

Planar bilayer lipid membranes and natural unmodified membranes are quite similar in
their capacitance and resistance. This was reported by M. Montal and P. Mueller [47]. It was
necessary to make sure that the formed structure is really a lipid bilayer and not an obstruction of
the aperture. For this purpose, the capacitance of the formed membrane was tested by turning the
“electrode fast” knob on the amplifier clockwise (10 pF/rotation) until the capacitance reached at
least 60 pF. This induced a baseline on the monitor. Then, through additional rotation and
pushing the plus or minus button, a 200 mV AC signal was induced. At this value the membrane
broke and the current signal from the membrane disappeared; otherwise it was not a real
membrane [48].

Alternatively, other methods used to measure cellular dynamic changes included: the regulation
of the capacitance compensation from the patch clamp amplifier electrical circuit; the careful
investigation of the time constant of a cellular step change; and, the usage of a sequence voltage
to calculate the capacitance of the input impedance coming from a power spectral analysis [9].
Figure 5.3 Patch clamp electrical circuit.
5.2.2.1 Measuring Membrane Capacitance

As presented above, the black lipid membrane electrical capacitance is usually derived from a voltage measurement. The capacitance of non-solvent-bilayers and monolayers tends to be higher compared to the capacitance of solvents containing bilayers. It was supposed that the solvent (like the n-decane that was used) obstructed the charges from gathering in the region near the BR group. This could be the reason why solvents containing bilayers exhibited only low resistance. Another explanation might be the thicker membrane resulting from the added solvent [49, 50].

The total membrane capacitance could be calculated through the integration of the transient capacitive current produced during voltage-clamp steps. This method is used to acquire capacitance data in most electrophysiological systems (e.g., pClamp, Molecular Devices). The total membrane capacitance could also be measured using the phase-shift generated through sine-wave current input or using voltage-clamp and applying a voltage ramp [51].

The recording quality played a crucial role in the accuracy of the total capacitance calculation. Even though noise could be reduced to a very low level, the obtained total capacitance could be affected by the transient signal, drifts and other unstable events. For example, the accuracy of the slowest time constant was central in measurements. Fast signal changes by low sampling rates significantly affected the results as well. It was important to set a high sampling rate on the gain [51].

The function generator was set to provide the system with 10 mV at 100 Hz peak-to-peak with a -5 mV to +5 mV amplitude. The supplied voltage was adapted to the bath condition inside the amplifier. With the system under 10 mV, the capacitance formula was used to calculate the membrane capacitance by measuring the resulting current:
\[ C = \frac{\Delta Q}{\Delta V} = I \times \frac{\Delta t}{\Delta V} \] (Equation 5.1)

Where \( C \) is the capacitance in faraday (F), \( Q \) is the charge in coulomb (C), \( V \) is the voltage in millivolt (mV), \( I \) is the current in Picoampere (pA), and \( t \) is the time in second (s).

The membrane square-wave response to the applied triangular-wave voltage is illustrated in Figure 5.4.

Figure 5.4 Measurement setup.

5.2.2.2 Measuring Membrane Resistance

Before starting the resistance measurement, a low pass filtering was required on the patch clamp amplifier output in order to limit noise; then, a high gain was required to limit noise resulting from digitization uncertainty [9].
The function generator was turned off, and the amplifier was used to measure the resistance. A steady 10 mV was set to supply the cell. Subsequently, Ohm’s-law was used to calculate the membrane resistance by plotting the resulting current against the applied voltage; the slope of the plotted line was inverted to obtain the resistance value [49, 50].

\[ R = \frac{V}{I} \]  

(Equation 5.2)

where \( R \) is the resistance in gigaohm (GΩ), \( V \) is the voltage in millivolts (mV) and, \( I \) is the current in picoampere (pA).

### 5.3 Photocurrent Generation

The generation of a photocurrent was achieved by shining a laser beam on the membrane. The light induced proton pumping in cells is always directed outward. This fact proves that Bacteriorhodopsin molecules have a defined orientation inside the membrane [48]. In order to orient the BR particles, an alternate current (AC) was applied to the chambers while forming the membrane.

The voltage-free system first showed a straight line indicating zero voltage and no current activity from the measurement devices. Shortly after shining the laser beam on the membrane, however, the BR confined in it was excited by the energy (photons) from the laser beam and started translocating protons from one chamber to the other. The proton-pumping induced a difference in the proton concentration between the chambers which caused a change in the electrical potential of the system. The change of the electrical potential was measured as photocurrent.
Chapter 6 :  Results & Discussion

6.1 Results

This chapter presents the results obtained from selected experiments. In general, experiments were performed using the painting or the folding method. However, as mentioned in Chapter 5, using the painting method included some inaccuracy due to the reliance on the operator qualification. In fact, in the painting method the operator forms / paints the membrane by hand as opposed to the folding method where the membrane forms itself upon buffer injection. The folding method was therefore selected for the photocurrent generation.

6.1.1 Resistance of membrane formed with painting method

Figure 6.1 shows the plotted data collected from a membrane made with DPhyPC and n-Decane only. The membrane was formed through the painting method (Chapter 5.1.2.1). The obtained I-V curve was fitted in order to calculate its slope which, as mentioned in Chapter 5.2.2.2, represents the membrane resistance.

\[
R = 34.3 \pm 5 \, \text{G}\Omega \\
= 2.5 \times 10^{+05} \, \text{G}\Omega/\text{cm}^2
\]

Figure 6.1  BR free membrane I-V curve: Painting Method.
The resistance of the plotted membrane was calculated to be \( R = 34.3 \pm 5 \, \text{G}\Omega \). This value is in line with literature which states membrane resistances should be greater than 0.1 G\( \Omega \) and can even go over 80 G\( \Omega \) [46].

### 6.1.2 Resistance of membrane formed with folding method

Figure 6.2 also presents the plotted I-V curve of the data collected from a membrane made of DPhyPC and n-decane. This time, the folding method was used to form the membrane. The calculated resistance here was \( R = 27.7 \pm 5 \, \text{G}\Omega \), which is also inline with the reference data [46].

![Graph showing the I-V curve for the folding method](image)

Figure 6.2 BR free I-V curve: Folding method

In general, it was noticeable that membranes formed with the folding method exhibited a straighter I-V curve than membranes formed by painting. This characteristic is due to the fact that the folding method produces more homogenous membranes since the membrane forms itself compared to the painting method where the membrane is formed by an operator. A relationship between the method used and the membrane resistance value could not be determined.
6.1.3 Resistance of membrane containing bR formed with folding method

Figure 6.3 and Figure 6.4 below illustrate the data gathered from a membrane made of the DPhyPC + n-Decane solution and the DPhyPC + n-Decane + BR solution. Figure 6.3 exhibits two distinct regions for the real signals (red) and the noise (black). These two regions were present in all previous experiments. Figure 6.3 shows a clear separation between the signal (red) and noise (black) trace before 510 ms. After 510 ms, both signal and noise had approximately the same amplitude and were constant. At 510 ms the applied voltage switches to positive values, generating less noises and an overall constant membrane signal.

![Figure 6.3 Raw data of a BR containing membrane fabricated with the Folding Method.](image)

As before, Figure 6.4 shows the plotted I-V curve of the collected data. The calculated resistance was \( R = 20.8 \pm 5 \, \text{G}\Omega \). This type membranes exhibited lower resistance compared to BR-free membranes. In fact, due to the presence of BR, the membrane is no longer impenetrable since the bonds between BR and DPhPC are not as compact as between DPhPC and DPhPC.
The fact that the I-V curve does not go through the origin (point zero) in Figure 6.2 and Figure 6.4 is due to the noises generated by the system, in fact as also noticed in Figure 6.3 the current signal (red) is always accompanied by a noise signal (black). The “no-voltage measurement” (Figure 6.6), shows that even without applied voltage the system produces a (noise) signal. This feature was not identified as an obstacle to the membrane characterization since the noise data were filtered out before calculations.

The experiments described above revealed three main findings:

1) the trace of the collected data cannot be used to conclude that the membrane has a high or low resistance;

2) the folding method exhibits more stable membrane which is verified by the straight current-voltage plot (I-V curve) trace; and,

3) only the slope of the IV-curve is relevant for the calculation. The position of the curve in the orthogonal system can be adjusted as needed.

Figure 6.4  I-V curve of a BR containing membrane: Folding method

R = 20.8 +/- 5 GΩ
= 1.5x10^4 GΩ/cm^2
6.1.4 Capacitance of membrane formed with folding method

The membrane capacitance was measured and calculated as indicated in 5.2.2.1. Figure 6.5 illustrates magnified data between 0 and 240 milliseconds. Figure 6.5 is used to verify that the triangle waves from the function generator will exit as square waves after interacting with the membrane. Multiple capacitance values were calculated. It was noticed that membranes formed with the painting method exhibited a lower capacitance than membranes formed through the folding method. This can be explained by the fact that most membranes formed through painting were monolayers since it was not possible to properly paint the interior of the back-chamber. The bilayer formed through the folding method, however, exhibited capacitance values between 0.3 and 0.5 +/- 0.2 pF/cm²; these values were in line with literature [27, 52, 53] which reports membranes formed under the same circumstances have a capacitance of \( C = 0.3 +/- 0.2 \text{ pF/cm}^2 \) to \( 0.5 +/- 0.3 \text{ pF/cm}^2 \).

![Figure 6.5 Capacitance measurement raw data magnified. \( C = 0.5 +/- 2 \text{ pF/cm}^2 \).](image)

6.1.5 No-voltage data acquisition of membrane formed with folding method

Figure 6.6 shows the no-voltage control data. This experiment was performed to capture any signal from the setup before exciting the membrane with laser light. For this purpose, the
function generator and all other input sources were switched off. Then, outcoming signals were recorded. As one can see, except the constant noise, no other signal coming from the system was noticed. Since photocurrent would disrupt the trace of the noise, and would thus still be measurable by setting the noise signal as constant, it was not necessary to focus on reducing outcoming noise.

Figure 6.6 No-voltage control data.

Figure 6.7 shows the control experiment which was performed to confirm that the photocurrent signal was associated with the presence of BR in the membrane.

For this purpose, the laser light was shined on a membrane made of the DPhyPC + n-Decane solution only and formed through the folding method. As can be seen, the laser light has no effect on the BR-free membrane.
6.1.6 Photocurrent of membrane formed with folding method

Figure 6.8 illustrates the activated BR membrane. The 532 nm (green) laser was shined intermittently on the formed membrane. The “on” and “off” states are depicted by peak signals in the graph. The system was still operated without voltage. It may be noticed that the basis line is a little higher than zero picoampere. This is due to the fact that a remanent current is kept between two excitations. A longer time between the excitations would have resulted in the recorded signal going to zero before the next excitation. For protocol purposes, the “on” and “off” sequences were set up for aproximatively 10-20 seconds each. Nevertheless, the stable signal until the first exitation can be regarded as the reference line from which the generated current is read. As shown in Figure 6.8, the generated current was much smaller (~3 +/- 2 pA) than the reference indication of 5.2 to over 100 pA (up to 300 nA/cm²) [27; 52; 53].
As previously mentionend, BR molecules will pump protons only in one direction. This means that if the BR fragments are oriented against each other, the resulting photocurrent will be influenced by the fraction of current that is cancelled out due to different orientations. BR exhibits dipole behavior and can, therefore, be oriented with applied voltage during the membrane formation. Figure 6.9 shows the BR orientation process. A 10 mV potential is applied during the membrane formation. This voltage forces the BR into the voltage direction.

Figure 6.10 shows the generated photocurrent after BR orientation. The photocurrent can clearly be identified, here each time the laser light is switched on, the constant noise increases to approximately 40 +/- 5 picoampere. Compared to the reference, this photocurrent is defendable. Further experiments provided similar results as well. As presented in the introduction, the BR fragments inside the membrane are responsible for pumping protons across the membrane.
The pumping direction is not influenced by the direction of the light source, but only by the BR orientation. Thus, without harmonized direction, BR fragments will pump protons as per their individual orientation. This means that part of the generated photocurrent will be cancelled in noise. It is thus crucial to force the BR in the same orientation by applying a defined voltage during the formation of the membrane.

Figure 6.9 BR Orientation process.

Figure 6.10 Photocurrent generated with a Br containing membrane with orientation
6.2 Discussion

The results obtained in this work attest to the capability of Bacteriorhodopsin to translocate protons upon illumination. The parameters, although interchangeable, must be set accurately. In this case the relationship between the DPhyPC and the BR concentrations must be carefully followed. Further, the concentration of the buffer solution should be around 1M KCl or 1M NaCl [54].

The observed variation of the generated current with the light intensity was explained by Herrmann and Rayfield [54]. The researchers suggested that initially filled states are rapidly emptied when the light is switched on. They further observed that using lipid membrane systems allows a direct measurement of electrical properties of the light-dependent proton pumping.

Usually, at time zero when the light is switched on, the photocurrent is allowed a few seconds to stabilize and then measured. Since both sides of the membrane are filled with the same buffer, the observed peak can only be attributed to a charge translocation induced by the BR captured inside the lipid membrane upon illumination. The peaks decay rapidly and settle down at a steady value indicating that proton pumping has been regulated. The proton release at the end of the photocycle is related to the initial step in the photoreaction cycle.
Chapter 7: Summary and Conclusions

The Photoelectric Characterization of Bacteriorhodopsin Reconstituted in Lipid Bilayer Membrane was realized through: the fabrication and testing of an experimental setup to form bilayer membranes; the use and evaluation of the Folding and Painting methods; the characterization of membrane capacitance and resistance; and, the successful generation of photocurrent.

This work supports the understanding of the theory and principle of forming and characterizing membranes, and producing bio-photocurrent with Bacteriorhodopsin as a biological species.

The results obtained in this work confirmed that the setup and species used are suitable for the generation of photocurrent. Since the produced photocurrent is proportional to the amount of BR molecules used, it is conceivable to vary the experimental parameters in order to optimize the harvested current. Further, the robustness of the substrate supporting the membrane makes it possible to transport the formed membrane into a structure imaging device such as a transmission electrons microscope in order to investigate its structure.

Moreover, biological fields such as cell-communication could be positively impacted. In vitro biological ion channels and transport systems (so far inaccessible by electrical analysis) could be investigated through the artificial double layer system presented in this work. However, new methods for screening ion channels are desirable in order to support the gathering of information about intentional and unintentional channel blockers. The realization of such an artificial system will lead to the conception of devices that will be able to target tissues and deliver specific drugs. Such devices will be required to sense, compute and transmit signals [19].
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43. Sigma-Aldrich 3050 Spruce St. St. Louis, MO 63103
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66. P. Galajda and P. Ormos. “Copyright 2003 from The Bacteriorhodopsin, by Reproduced by permission of Peter Galajda.”

Appendix A: Supplementary figures used in oral thesis defense

Figure 1 BR absorption spectrum [2].
Figure 2 a-m Occurrence and working principle [66 and 67]

Figure 2a BR occurrence in high saline environments, here the salt ponds in San Francisco bay.

Figure 2b Swimming Halobacterium Salinarum the archaeon of BR (purple stains).
Figure 2c The three main structures of the Halocbacterium Salinarum: the Flagel for navigation; the ADP↔ATP conversion center; and, the light activated BR for protons translocation.

Figure 2d BR patches (green) and Retinal (purple).
Figure 2e BR patches (gray) and Retinal (green-purple).

Figure 2f BR patches (dark gray) and Retinal bonds (white, green and blue).
Figure 2g BR retinal in ground states (no translocation).

Figure 2h BR retinal in exited states after receiving a photon.
Figure 2i After being exited by the laser, the retinal transfers one proton from its Schiff base to the near located Aspartate 85.

Figure 2j The protons is then transferred via water molecules and added to the BR-free-chamber buffer solution.
In order to compensate the translocate protons, the retinal seizes a proton from Aspartate 96.

The retinal goes back into its ground state after taking a proton from the Aspartate 96; the Aspartate 96 compensates the loss by seizing a proton from the BR-containing-chamber buffer solution.
Figure 2m Aspartate 96 goes back to its ground state, and a new cycle can start.
Appendix B: Description of Research for Popular Publication

Bacteriorhodopsin: a viable alternative to solar energy conversion.

Have you ever wish to live in a purple world? If not, then start thinking about it because purple might soon be one of the most encountered colours. Researchers have demonstrated that Bactheriorhodopsin a purple, small, and robust membrane protein, with light-driven proton pumping capability can be used to generate current. When captured inside a membrane and illuminated, Bactheriorhodopsin (BR) is able to act as a proton pump; it translocates protons from one side of the membrane to the other (vectorial charge transport). This pumping activity is increased when BR fragments are all oriented in the same direction, with respect to the lipid membrane. Having the BR fragments unvaryingly oriented, ensures that protons flow only in one direction, and creates the proton gradient only on one side of the membrane. In its natural living environment Bacteriorhodopsin, provides cells with energy to live, by generating a proton gradient around them. Investigators at the University of Arkansas were able to generate up to $2.6 \times 10^5$ pA/cm$^2$ using Bacteriorhodopsin (BR); Sodium Chloride (NaCl); N-decane (alcohol), and Diphitanoyl Phosphatidylcholine (DPhPC), as sole biological species. This implies that up to 2600 Ampere could be generated by covering around 1000 m$^2 \approx (1/6$ of an American football field) with light responsive Bacrtheriorhodopsin. This energy could be used to cover needs such as: motor vehicles (200-300 A at the start, then 80 A); supply typical in North American (USA, Canada, Mexico) needs of continuous current around 200 A / household; and much more. However, for this vision to become reality, researchers are expected to stabilize the current generation process and to ensure a low cost and less complicated exploitation. Most importantly we must all agree to live in a more purple world.
Appendix C: Executive Summary of Newly Created Intellectual Property

The newly designed and fabricated chamber, made of Polydimethylsiloxane (PDMS chapter 4.2.3) represents the main intellectual property that was created in the course of this research. This chamber could be considered from a patent perspective. Beside the PDMS chamber, the whole experimental process is unique in its simplicity. It includes an easy fabricated and tested experimental setup to form, and characterize bilayer membranes, as well as the use of: Bacteriorhodopsin (BR); Sodium Chloride (NaCl); N-decane (alcohol), and DiphitanoylPhosphatidylcholine (DPhPC), as sole biological species to generate photocurrent. This process can, however, not be considered as an intellectual property since it is a simplified version of an already existing one.
Appendix D: Potential Patent and Commercialization Aspects of listed Intellectual Property Items

D.1 Patentability of Intellectual Property (Could Each Item be Patented)

Because they are fairly known among researchers, methods and processes used in this work (Painting, Folding, and photocurrent generation upon illumination) cannot be claimed as intellectual property.

D.2 Commercialization Prospects (Should Each Item Be Patented)

The simplicity of the experiments in this work allows them to be performed in most rudimentary research rooms, including schools and hobby laboratories. This aspect can be used to commercialize “kits” that teaches the formation of bilayer membranes and the generation of photocurrent. This could be beneficiary in teaching the multiple methods of converting solar energy.
Appendix E: Broader Impact of Research

E.1 Applicability of Research Methods to Other Problems

Results from this work might be extended to the imaging of Bacteriorhodopsin, captured inside a bilayer membrane. This would provide additional data about the Bacteriorhodopsin working principle, and eventually the very similar structured ATPase (brain transport protein). Another area of interest where this work would serve as foundation would be the investigation of the dependency of the generated photocurrent on applied voltage. It is further conceivable to harvest, or forward, the produced photocurrent as an energy source for other processes.

E.2 Impact of Research Results on U.S. and Global Society

This work provides an additional alternative to conventional energy production process. Even though the generated current cannot be used to cover the needs at a considerable range, the presented mean of producing current is a welcomed technology to face the global warming issues of our time.

E.3 Impact of Research Results on the Environment

Apart from the measurement and handling instruments used in this work, all involved materials are biocompatible, from the PDMS chambers up to the used biological species (BR; DPhPC; N-Decane; NaCl; DI water). This feature conveys the presented experiments a high environmental friendliness.
Appendix G: Identification of All Software Used in Research and Thesis Generation

1- Computer #1: DELL
   Model Number: Dell Optiplex 390
   Serial Number: 7215609313
   Location: PHYS119
   Owner: Prof. Jiali Li

2- Computer #1: DELL
   Model Number: Dell Optiplex 390
   Serial Number: JHKMRH1
   Location: PHYS125
   Owner: Prof. Jiali Li

3- The Digidata 1440A from Molecular Devices
   Model Number: Digidata 1440A
   Serial Number: 813085
   Location: PHYS125
   Owner: Prof. Jiali Li

4- The Axon Axopatch 200B (AA200B) from Molecular Devices
   Model Number: Axopatch 200B (AA200B)
   Serial Number: 821103
   Location: PHYS125
   Owner: Prof. Jiali Li

5- The Headstage from Axon Instruments CV 203BU
   Model Number: CV 203BU
   Serial Number: 851172
   Location: PHYS125
Owner: Prof. Jiali Li

6- Polyoxymethylene (POM) two-chamber system from Warner Instrument
   Model Number: BCHM13
   Serial Number: 64-0451
   Location: PHYS125
   Owner: Prof. Jiali Li

7- Software #1:
   Name: Microsoft Office 2007
   Purchased by: UA Physics Dept.

8- Software #2:
   Name: pClamp 10 from AxoScope
   Purchased by: Prof. Jiali Li

9- Software #3:
   Name: Clampfit 10 from AxoScope
   Purchased by: Prof. Jiali Li

10- Software #3:
    Name: Adobe Acrobat Professional 10.0
    Purchased by: University of Arkansas Site License
Appendix H: All Publications Published, Submitted and Planned

None