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Electrochemical Modulation of the Optical Properties of Bacteriorhodopsin

By
Philip H. Danks

A thesis submitted to
The faculty of Graduate Studies and Research
In partial fulfilment of
The requirements for the degree of

Master of Science

The Department of Chemistry
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Ottawa, Ontario
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Electrochemical Modulation of the Optical Properties of Bacteriorhodopsin

Submitted by

Philip H. Danks

In partial fulfilment of the requirements for
The degree of Master of Science

Prof. Z.Y. Wang, Thesis Supervisor

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Abstract

The objective of this thesis was to attempt the modulation of the M state of bacteriorhodopsin via electrochemistry. This required the identification and synthesis of electroactive materials whose redox chemistry was compatible with the environment in which the M state of the protein could readily accessed.

Several materials that could serve as a proton sink/source were investigated. During this investigation, the synthesis of 2,3-dicarboxyaniline from a novel monomer, *o*-aminophthaloyl hydrazide was performed. Dyes of the phenothiazine class were also investigated and proved promising, however the stability of electroactive layers of these dyes was poor.

The modulation of the M state of the protein was achieved in an electrochemical cell using tungsten oxide as a proton source/sink layer. This modulation was rapid but the magnitude was small due to absence of a suitable counter-electrode and the small pathlength of the device.

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Symbols and Abbreviations

bR	Bacteriorhodopsin
CV	Cyclic Voltammogram
DMF	dimethylformamide
D₈₅	Aparagine 85
E₂₀₄	Glutamic Acid 204
GuaHCl	Guanidinium Hydrochloride
HeNe	Helium-Neon laser
ISFET	Ion Selective Field Effect Transistor
ITO	Indium-Tin Oxide
SHE	Standard Hydrogen Electrode
O.D.	Optical Density
PEDOT	Polyethylenedioxythiophene
TEMED	N,N,N',N'-tetramethylethylenediamine
WO₃	Tungsten Oxide
λ_{max}	wavelength of maximum absorption

The photocycle of bacteriorhodopsin has been exhaustively studied and yet its exact mechanism still remains unclear. What has been gleaned from this work is that the photocycle of bacteriorhodopsin is populated with discrete intermediates whose spectral characteristics correspond to an alteration of the chromophore and the environment that surrounds it. What is noteworthy is that the spectra of two of these intermediates, the ground state and what is termed the M state differ greatly and that the lifetime of several of these intermediates can be controlled by external factors². This is one of the reasons for the interest in this material for optoelectronics.

Fig. 2: Ground and M state of bR. Note the large hypsochromic shift in the absorption maximum of the M state

Another important property of the protein is that the chromophore in its ground state has a large extinction coefficient ($63\,000\text{ mol L}^{-1}\text{cm}^{-1}$) and a large cyclicity. This latter term refers to the number of times that the protein can enter the photocycle productively before the protein denatures and therefore no longer functions. These properties taken together mean that the material is quite sensitive to light and has a good operational lifetime. This arises from the fact that the protein's function *in vivo* is coupled to the survival of the organism and thus the proteins efficiency is subject to selection pressures¹.

Several other properties make this material attractive for optoelectronic applications. Most of these properties arise from the fact that the natural environment of *Halobacterium halobium* is a salt marsh and as a result the bacteria has evolved to withstand a wide variety of harsh conditions. In turn, bacteriorhodopsin has several properties that are uncommon amongst proteins such as an excellent stability to extremes of pH and a high tolerance to thermal and mechanical stresses. This greatly simplifies the manipulation of the protein and allows a wide variety of processing steps that would otherwise result in damage to the protein.

1.2 Current Applications

There is a wealth of proposed optoelectronic applications for bacteriorhodopsin. Unfortunately, many of these devices have not progressed beyond the prototype phase. Most of these applications can be separated into two classes. The first class of applications are those that employ the proton gradient created by the illumination of bacteriorhodopsin. Several different means to detect this proton gradient have been proposed ranging from the response of an ion selective field effect transistor² (see Fig. 3) to processes that involve the detection of a transient changes in illumination by the charging of an indium tin oxide (ITO) electrode in solution. Some of the proposed applications range from utilitarian (alarms) to fanciful (artificial retinas) but to date none of these applications have progressed beyond the prototype stage.

Fig. 3: Proposed photodetector based on bR. Upon the illumination of the bR layer, vectorial transport of protons into the region of the ISFET lowers the resistance of the gate and causes an increase in current across the transistor.

The second class of applications is centred on exploiting several of the intermediates of the photocycle for applications such as holography⁴ and flat panel display applications. These applications centre on one particular intermediate in the photocycle of bacteriorhodopsin, the M state.

1.3 M state of bR

The chromophore of bacteriorhodopsin is introduced into the protein via the reaction of retinal with an amino acid residue found midway between the alpha helices that span the plasma membrane of the bacteria. The product of this reaction is a protonated Schiff base with some unusual properties. Firstly, the absorption maximum is shifted dramatically from that of the free chromophore. This change in the absorption maxima is referred to as the opsin shift and is responsible for the purple colour of bacteriorhodopsin. This shift arises from at least two factors that arise from the special environment provided by the protein. Firstly, the counter ion to the protonated Schiff base is especially weak. This serves to destabilise the ground state of the chromophore relative to the excited state, which, in turn lowers the energy required to achieve the excited state. The second factor arises from the fact that in the protein the chromophore is found in its all-*trans* form rather than the preferred 6-*cis* form which reduces the steric clash between the methyl groups of the ring and the polyene system⁵.

Fig. 4: Retinalbutylimine. In the absence of opsin shift the absorption maximum is 440 nm

One noteworthy consequence of the opsin shift is that upon the deprotonation of the Schiff base, the integral step in the formation of the M state, this shift is reduced and this results in a dramatic change in the absorption maxima of the protein.

From the perspective of optoelectronic devices this intermediate is a useful one and considerable effort has gone into understanding the nature of the M state. From this work, site directed mutagenesis has provided mutants displaying anomalously long half lives for this intermediate and these mutants have been used in holographic and “e-ink”⁶ applications.

Several other means are available to alter the M state lifetime of bacteriorhodopsin. Alterations in humidity, encapsulation in media with lowered proton mobility, chemical additives and an increase in pH can all affect the formation and half-life of the M state³. One of the best ways to alter the lifetime of the M state is with the use of guanidine hydrochloride (GuaHCl) in conjunction with solution pH⁷. Controlling these two conditions allows the alteration of the M state lifetime by several orders of magnitude (ms to hours) without losing the functionality of the protein. This allows one to envisage an application in which at a fixed GuaHCl the modulation of the pH would provide a direct means to modulate the lifetime of this useful intermediate.

1.4 Electrochemistry and Modulation of the M State

Many electrochemical processes involving organic molecules involve the release or uptake of protons. A typical example is the redox couple of benzoquinone shown below:

The half wave potential for this particular reaction is subject to the relationship⁸;

$$E_{1/2} = E^{\circ} - (0.05915 / n) \log Q$$

$$\text{Where } Q = [\text{Hydroquinone}]/[\text{Quinone}] [\text{H}^+]^2$$

This relationship predicts that with an order of magnitude increase in Q the halfwave potential will shift to a more negative value.

Based on the preceding relationships two observations can be drawn. The first observation is that an increase in pH will occur next to an electrode at which a species that behaves in this manner is being reduced. Furthermore, this change in pH can be readily reversed by placing the cell potential below the cell's half wave potential. This serves as the basis by which the M state of bacteriorhodopsin could be rapidly and conveniently modulated. The second observation is that the half-wave potential of a compound for which a proton participates in its redox couple will move to more negative values upon an increase in pH and that with a change of one pH unit the halfwave potential will shift by 59.16/n mV . This latter point allows the rapid identification of materials that would be suitable for this modulation and the range in which the material exhibits a Nernstian response to changing solution pH.

1.5 Candidate Materials: Electroactive Polymers

One potential means to electrochemically modulate the M state of bacteriorhodopsin is through the use of electrochemically active polymers.

Electrochemically active polymers have been the focus of intensive study in the hope that these materials can be used for displays, so-called "smart windows" and a variety of other cheap consumer electronics. The cited benefits are a reduction in cost owing to the ease of synthesis and processibility of these materials.

One useful synthetic method of preparing these polymers is by the technique of electrodeposition, in which a polymer film is deposited onto one electrode of an electrochemical cell. This strategy has the distinct advantage combining the synthesis and the processing of the polymer into one step. Although the synthesis of each particular polymer differs somewhat, commonly the technique consists of sweeping the potential of the cell to a sufficiently positive potential to activate the monomer in solution by the creation of radical cations. These cations initiate the polymerisation reaction and the resulting polymer is drawn from solution onto the surface of the electrode. The cell is then swept to a negative potential and the process is repeated until a film of a desired thickness is produced⁹. The accumulation of the electroactive polymer during this process is seen as a stepwise increase in the current of the cell and serves as a useful measure of the films' activity.

Fig. 5: Film generated by electropolymerization and the corresponding cyclic voltammogram

In order to create an electrochemical cell suitable for the modulation of bR at least two electroactive materials are required. The first material should serve as a source/sink of protons in order to modulate the M state and can be either p- or n-doping. The second material required is one that serves as a source/sink of electrons in order to complete the electrochemical cell and should be opposite in doping characteristics to the proton source/sink.

1.6 Rationale and Objectives

Current devices employing bR exploit the properties of this protein in static environment. In other words, once the device is prepared, the properties of the protein cannot be modulated and therefore limits the applications that this protein can be employed in. The modulation of the properties of bacteriorhodopsin would add functionality to current devices and raises the possibility of several of constructing other new devices.

A proposed device is illustrated in Fig. 6. For display purposes this device is intended to operate under constant illumination. Light serves to place the bR predominantly in the M state and thus the protein appears yellow. Upon the oxidation of the proton source/sink, protons are released into the electrolyte and acts upon the bR to shift the population of the protein in the M state to the ground, or purple state. The electrons liberated by the oxidation then flow to the other electrode and serve to reduce the material on the other electrode.

Fig. 6: Proposed device for the electrochemical modulation of bacteriorhodopsin

The objectives of this thesis are as follows;

1. To identify electroactive materials that can serve as a proton sink/source for modulation of the M state of bR.
2. To identify complementary electroactive materials to serve as a companion material to the proton source/sink.
3. To find a matrix in which bR can be placed that allows access to the M state upon illumination and allows its modulation to take place.
4. To attempt the construction of the proposed device and to characterise its properties.

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Chapter 2: Design and Synthesis of Bacteriorhodopsin Matrix

2.1 Design Parameters

The matrix of the proposed device illustrated in Fig. 6 must serve two functions. The first function is to supply counterions to the charges generated upon the oxidation and reduction of the electroactive material of the device, or in other words, it must serve as a reservoir of electrolyte for the electrochemical cell. Its second, and far more important function, is to serve as a matrix for bR in which the M state is readily accessible. Furthermore, this matrix must allow its pH to be altered readily, and finally, the matrix should impart stability to the bR in order to increase the useful lifetime of the device.

Guanidine HCl is a charged polar amine that significantly increases the lifetime of the M state¹. The exact mechanism of its function is unknown but it is speculated that it binds with the residue responsible for the releasing the proton to the solution upon the conclusion of the photocycle. This binding interferes with the hydrogen bonding network that links the amino acid residues from the site of proton uptake to the site of its release. This hydrogen bonding network ensures that the proton pumping occurs in a vectorial manner since it regulates the pKa's of the residues that deprotonate the Schiff base and release it on the other side of the membrane². Interference in this network does not allow this regulation to take place and therefore alters the photocycle.

Fig. 7: Structure of GuaHCl and the postulated effect on M state

The effect of GuaHCl on the lifetime of the M state is greatly increased by an increase in pH. In fact, the increase in the lifetime of the M state depends logarithmically on the solution pH. This can be seen by examining the graphs adapted from Yoshida et.al.¹.

Fig. 8: Increase in M state lifetime at 2 M GuaHCl concentration

Fig. 9: Logarithmic Dependence of M state lifetime on pH

Several important design parameters arise from an examination of the graphs.

The first noteworthy feature is the change in slope in Fig. 8 corresponds to a half-life of the M state in the order of 50 ms and occurs at a GuaHCL concentration of 2M. One should note that the half-life at the inflection point (~50 ms) is significantly longer than the natural length of the photocycle at this pH (~15 ms). This inflection point therefore corresponds to the onset of a photostationary state, wherein the M state accumulates since its lifetime is significantly longer than the lifetime of the states required for it to reach this state. From a device standpoint, this is an important finding for two reasons. Firstly, an accumulation of the M state means that to an observer, a film of bacteriorhodopsin under these conditions appears yellow, in contrast to the purple of the ground state. Second, it is desirable to retain the ability to rapidly switch between a photostationary state in which the M state is the predominant intermediate and one in which the ground state is the predominant state. This allows a rapid conversion from a yellow film to a purple one.

There are two ways of achieving this; the first is trivial and results from simply changing the irradiation intensity. The second means is by changing the pH to effect a change in the photostationary state population.

This dependence of the lifetime of the M state on pH can be easily seen in Fig. 9. One can see from this graph that from pH 6 onward the impact of changing the pH on the lifetime of the M state is maximised and that the relationship is a logarithmic one. The region in which this dependence is strong is quite useful since the number of protons required to change the pH within the fixed volume of an electrochemical cell in this region is relatively small compared to if this region fell, for example, at pH 5. From the standpoint of a device this is an important finding since;

- i. the logarithmic alteration of the M state lifetime may allow the colour of the film between the electrodes to be changed provided the required pH change can be made**
- ii. the amount of electroactive material required to effect this change is at a minimum owing to the location of the onset of the logarithmic dependence of the M state on solution pH**

From the standpoint of the matrix these findings result in three design parameters.

- 1. The GuaHCl concentration of the matrix should be no more than 3M in order to preserve the ability to rapidly switch between the yellow and purple states.**
- 2. The pH of this matrix to access the M state (yellow) should be 8 whereas the pH of this matrix should be 6 to access the ground (purple) state.**
- 3. The matrix should have little or no buffering capability in order to facilitate the desired change in pH.**

2.2 Immobilisation of bR in Polyacrylamide

Polyacrylamide is an excellent matrix for the immobilisation of proteins due to its ease of synthesis and, in contrast to many polymer preparations, the protein can be present during the polymerisation reaction without suffering damage. It is primarily used as an electrophoresis medium for the separation of proteins and therefore it is a natural choice for a matrix.

When polyacrylamide is used as a matrix for electrophoresis it is synthesised as a crosslinked polymer gel from an aqueous solution of acrylamide and N,N-bisacrylamide, a crosslinking agent. As this solution is aqueous, bR can be suspended in this solution, and once polymerisation is complete, the protein is immobilised in this matrix. This approach was adopted for the synthesis of a bacteriorhodopsin matrix suitable for the application outlined in Chapter 1.

Fig. 10: Structure of acrylamide and N,N-methylene bis acrylamide

The initiation of polymerization is typically carried by two means, chemically via the use of an oxidant or photochemically. In the latter case, a photosensitiser is used to transfer the chemical energy of an excited state generated by irradiation to a species capable of initiating the polymerisation reaction³.

The method chosen to initiate polymerisation for the work performed was the use of a photosensitiser, riboflavin. This method was chosen because it allows greater flexibility in the preparation and manipulation of films of this material. This greater flexibility stems from the fact that once a chemical initiator is added the solution must be mixed rapidly and placed into its mold quickly or inhomogeneities in the matrix are created. This is particularly true of solutions with a very high viscosity since rapid mixing is difficult and the onset of gelation is rapid. The use of riboflavin allows the solution to be mixed in its final form and introduced to its mold and then irradiated to yield a high quality film.

The mechanism of riboflavin initiated polymerisation involves the interaction of the triplet state generated by irradiation of riboflavin with visible light and a tertiary amine to generate a radical species. This highly reactive species then initiates the polymerisation reaction³.

Fig. 11: Mechanism of riboflavin initiated polymerisation

Another advantage of this method of polymerisation stems from the fact that these conditions are not oxidative. This has the benefit of not exposing the protein to a harsh oxidant, which could possibly denature the protein.

2.3 Preparation of Matrices for Bacteriorhodopsin

As previously mentioned, one advantage of the photochemical initiation of acrylamide polymerisation is that very viscous solutions can be prepared in advance of the initiation of polymerisation. This feature became quite important as the convenient preparation of bR matrices requires the use of glycerol.

The reason for this is a consequence of the kinetics of the photocycle of bR. As one can see from Fig. 12 the transition from the O state to the ground state is the rate-limiting step in the photocycle of the protein⁴. This intermediate, unlike the M state of bacteriorhodopsin cannot be easily modulated. One additive that increases the lifetime of the O state is glycerol⁵. The use of this additive was believed to be necessary based on the following considerations.

Fig. 12: Actual Photocycle and Simplification for Kinetic Model⁵

A kinetic description of the photocycle of bacteriorhodopsin is very difficult unless significant simplifications are made. One such simplified scheme is depicted in Fig. 12. The qualitative analysis of this model, assumes that the interconversion between these two intermediates is a first order process, and that there is no delay between the arrival of the protein to the ground state and it's subsequent excitation. This latter point is achieved in practise by high illumination intensities. With these assumptions in mind the photostationary states of the protein are given by⁶;

$$A_{\infty} = \frac{k_2}{k_1 + k_2} x[A]_0 \quad B_{\infty} = \frac{k_1}{k_1 + k_2} x[B]_0$$

where A_{∞} = Concentration of A at equilibrium
 A_0 = Initial Concentration of A
 k_1 = rate constant of $A \rightarrow B$
 k_2 = rate constant of $B \rightarrow A$

In this scheme, to achieve a film with a high contrast, ideally one would wish to be able to modulate the pH such that k_1 was greater than k_2 at one pH, and the reverse was true at a new pH value. This would result in a change in the dominant photostationary state and thus the colour of the film would reflect the absorption of this intermediate exclusively. Unfortunately this cannot be done since none of the intermediates, other than the M state, exhibits a strong dependence on pH. This means that one must set the value of the M state lifetime equal to that of the lifetimes of the other intermediates in order to approximate the ideal condition. From a practical standpoint, this is very difficult to achieve since the lifetime of all the other intermediates is small (~10 ms) and fine control over the M state lifetime is difficult since the relationship between its lifetime and the pH of the media is a logarithmic one.

One solution to this is the introduction of glycerol to the media. This additive increases the lifetime of the O state and therefore relaxes the need for extremely fine pH control. This additive is very viscous and therefore requires that the polymerisation of the matrix be initiated via irradiation with visible light.

Fig. 13 illustrates a successful proof of principle. Here two identical polyacrylamide gels were prepared with the exception of the pH. One can see that under strong illumination, which places the protein in a photostationary state, one cuvette appears purple as a result of the dominance of the ground state. The other cuvette, at the higher pH, appears yellow reflecting the dominance of the M state under the same illumination intensity.

Fig. 13: Bacteriorhodopsin containing gels at two illumination levels.

The presence of optical contrast solely by the modification of pH illustrates that if this pH change can be effected electrochemically, then access to the two states of bacteriorhodopsin under constant illumination is possible.

2.4 Experimental

Preparation of gel cuvettes

A mixture of 5.0 g of acrylamide and 0.1 g of N,N-methylene bisacrylamide was added to an 80% v/v solution of glycerol. This liquid was stirred for approximately two hours with very gentle heating (30°C). Once the solids had dissolved the solution was cooled with continual stirring.

Several other solutions are required for the preparation of the polyacrylamide matrix. A catalysis solution containing 1.2% v/v TEMED and the initiation solution prepared by placing 4 mg of riboflavin into 50 mL of ddH₂O.

To prepare a gel, 2.65 mL of the acrylamide containing glycerol solution was mixed with 0.21 mL of the TEMED solution and 0.42 mL of a bR solution (~13 mg/mL). The liquids were thoroughly mixed and 0.8359 g of GuaHCL was dissolved in the resulting mixture. To this mixture, 0.21 mL of the riboflavin solution was added and mixed in thoroughly. From this point onward in the preparation care was taken to avoid exposing the gels to strong light sources. The pH was then adjusted to a desired value with KOH or HCl solution using a 10 µL microsyringe and a pH meter. Once the desired pH value was reached, the gels were heated to ~50°C in a water bath and placed under a fluorescent light for 30 minutes. Gelation occurred within 7 minutes to yield a rigid hydrogel.

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Chapter 3: Electroactive Polymers for the Modulation of the M State

3.1 Introduction

The potential applications of electroactive polymers have been the focus of interest for many years since, in theory, these polymers are cheap and easily processed materials with novel properties. Of these polymers, many are unsuitable for modulation of the M state of bacteriorhodopsin since there is no proton involved in the redox couple of this material. The work of the following chapter outlines the materials investigated for the modulation of the M state of bacteriorhodopsin.

3.2 Evaluation of Polymethacroyl-graft-dopamine

Dopamine is a biologically active compound whose redox chemistry involves protons and is therefore potentially useful for the modulation of the M state of bR.

Fig. 14: Redox couple of dopamine

This molecule is of interest to researchers since the o-quinone form of this molecule reduces the overvoltage required to oxidize nicotinamide adenine dinucleotide (NADH), a hydride donor in biological systems. In the absence of this compound, the oxidation of this compound occurs at 1.1 volts vs. SHE and therefore the oxidation of the solvent (water) interferes with the electrochemical determination of this compound. The modification of an electrode with an o-quinone moiety reduces this overvoltage by 450 mV in certain cases¹.

The prevalence of this compound in biological systems makes its detection in trace amounts very important and therefore numerous attempts have been made to fabricate electrodes bearing the o-quinone moiety.

Dopamine is an attractive compound from the standpoint of electrode modification because it can be readily grafted onto polymers bearing a functional group that is reactive towards amines. One such polymer is a commercially available acid chloride polymer, poly(methacryloyl chloride)¹.

Fig. 15: Synthesis of poly(methacryloyl chloride)-graft-dopamine

Despite the reported stability of this compound to repeated oxidation and reduction the observed stability of the compound was poor. From the CV below one can see that a rapid loss of electroactivity occurred in the first three cycles.

Fig. 16: Cyclic Voltammogram illustrating rapid inactivation of polymer in basic media

The reason for this rapid inactivation is thought to be a result of the fact that the o-quinone moiety is susceptible to nucleophilic attack. Once this group is attacked by a nucleophile, the resulting product no longer has the desired electrochemical properties. This finding implies that this moiety is unsuitable for applications at basic pH since it will be readily inactivated by hydroxide ions. As a result this material and others bearing the o-quinone moiety were not pursued further.

3.3 Evaluation of Carboxylated Polyaniline

Polyaniline is a very well known electroactive polymer that can be synthesised either chemically or via electrochemical deposition. This polymer is also electrochromic in that the location of its absorption maxima is dependent on its oxidation state. This material possesses 3 distinct redox states, pernigraniline, emeraldine and leucoemeraldine whose colours are blue, green and yellow respectively².

One feature of polyaniline is that in order for the material to remain conductive it must be doped with protons. For this reason, this material loses its electroactive behaviour at pH values greater than 4 and is therefore not suitable for the electrochemical modulation of bacteriorhodopsin at near neutral pH.

To increase the useful pH range of this material substituents are added that are capable of donating charge and protons to the backbone of the polymer. The most common approach is to incorporate sulfonyl groups *ortho* to the polymer backbone³.

Fig. 17: Sulfonated Polyaniline

This material is referred to as self-doping since the species that donates the charge to the backbone in order to maintain its conductivity is part of the polymer itself. As a result of this property the material is no longer dependant on protons from the solution to maintain its conductivity and therefore the pH range of the material increases from four to six.

Recently a new self-doping material was synthesised from 2,3-dicarboxyaniline. Upon the polymerisation of this material the following polymer was obtained whose pH activity was reported to extend into the neutral range. Unfortunately, this material has a low degree of polymerisation due to two features of the monomer⁴.

Fig. 18: Structure of 2,3-dicarboxyaniline

The mechanism of polyaniline formation is seen below. An examination of this mechanism reveals that a nucleophilic monomer is required and that protons are released with the addition of each repeat unit. As a result, the polymerisation of 2,3-carboxyaniline is difficult since the monomer is deactivated initially by the presence of two withdrawing groups and once the pH dips to sufficiently acidic values intramolecular hydrogen bonding further deactivates the monomer. As a result of the last point this reaction requires that the reaction be performed in the presence of base.

Fig. 19: Mechanism of aniline polymerisation and inactivated carboxylated monomer

In an attempt to increase the degree of polymerisation the use of a new monomer, o-aminophthaloyl hydrazide, was investigated. This monomer is believed to be more reactive since its cyclic structure does not permit intramolecular hydrogen bonding and the hydrazide moiety is believed to be less withdrawing than the carboxylic acid substituents of the original monomer.

The polymerisation of o-aminophthaloyl hydrazide, resulted in the formation of the carboxylated polyaniline. This represents the formation of the polymer from a new monomer. The polymer was isolated as a brown solid that was readily soluble in pyridine and somewhat soluble in DMF. The molecular weight of the polymer was not significantly improved despite a variety of reaction conditions.

Upon the initiation of polymerisation by the rapid addition of an oxidant to a basic solution of the monomer, purple chemiluminescence was observed. This is a property of o-aminophthaloyl hydrazide, also called luminol. The origin of this chemiluminescence is uncertain, but it is thought to arise from the formation of an excited carboxylate dianion by the addition of oxygen to the dianion of hydrazide⁵. This excited state then decays to yield a photon.

Fig. 20: Origin of chemiluminescence of luminol

The inability to synthesise high molecular weight polymer from o-aminophthaloyl hydrazide is most likely due to the fact that the polymerising species is predominantly the carboxylate dianion generated from the hydrazide dianion by adding an oxidant to a basic solution. It is therefore subject to the previously mentioned processes that decrease its reactivity.

The material generated formed excellent brown films when spin coated onto an ITO substrate from a concentrated pyridine solution and these films were weakly electrochromic. GPC analysis revealed that the degree of polymerisation was quite low with an estimated degree of polymerisation of 8 for the largest oligomer. This data, combined with a comparison of the infrared spectrum to that of oligo(2,3-dicarboxyaniline) confirms the identity of the polymer.

Although the material was successfully synthesised it is not a suitable material for its intended application. The first drawback of this material its electrochromism was so weak it was not visible to the eye. As a result the electrochromic material would not complement the anticipated colour change of the bacteriorhodopsin. The second drawback is that the adhesion of thin films of the polymer was excellent but upon the deposition of more material the adhesion grew rapidly poorer. Thicker films are desirable in order to increase the charge capacity of the device but these films were quite fragile and their utility was limited. Lastly the major drawback of the material is that although the useful range of the film was increased by 2.5 pH units over the parent material polyaniline, the electroactive regime of the polymer did not extend enough into the basic range for the effective modulation of the pH of the cell.

3.4 Evaluation of Thiazine Dyes

3.4.1 Evaluation of Thionine

Several phenothiazine dyes are commercially available in high purity. Many of these dyes can be electropolymerized by repeated cycles of oxidation and reduction to yield smooth, uniform blue films. One such dye, available in very high purity (99+%) is thionine. The redox chemistry of this dye is as follows⁶;

Fig. 21: Redox Chemistry of Thionine

λ_{max} in H₂O = 598 nm

Polymerisation occurs via the creation of cation radicals that react to form a polymer in which the units are linked together at either of the two positions ortho to the amino groups of the monomer⁷.

Fig. 22: Proposed structure of polymer

Films of excellent quality can be readily prepared from a solution of the monomer, however the polymerisation is quite slow sometimes requiring up to 70 sweeps in order to yield a film with good optical density.

From the standpoint of the proposed device, thionine meets many of the outlined requirements. In its reduced form, the material serves as a source of protons and its colour is bleached. Upon oxidation, the material releases protons and darkens which is complementary to the anticipated change in the colour of the bacteriorhodopsin containing matrix between the electrodes. Furthermore, it retains redox activity to pH values as high as ten and therefore is active in the proposed pH regime of the device.

This retention of redox activity and its dependence on pH can be readily seen in the shifting of the redox couple upon a change in the electrolyte pH. This is illustrated by the cyclic voltammogram below in which the peaks migrate to more cathodic potentials upon an increase in the pH of the electrolyte.

Fig. 23: Cyclic Voltammogram of Polythionine film at two pH values.

The optimisation of the polymerisation conditions led to a significant improvement over the literature procedure for obtaining polythionine films. This improvement is believed to be simply a result of an increase in the concentration of the monomer. This was achieved by changing the polymerisation solvent from water to an acidic solution of water/ethanol. This optimisation is important in the context of the device since the amount of electroactive material determines the amount of protons released to the bacteriorhodopsin containing matrix.

Although this material was the best material identified to date several problems were identified with these films. The first problem with the films is the low contrast between the oxidised and reduced states. This problem is exacerbated by an increase in pH since the extent of the electrochemical reaction decreases with an increase in pH. This low contrast can be offset by improving the overall contrast of the proposed device. This can be achieved by using a polymer film whose absorption maximum is not shared by bacteriorhodopsin resulting in an absorption over a larger span of wavelengths and therefore appearing darker to the observer in the oxidised, low pH (bR ground state) condition of the proposed device. This can be achieved in practise by the use of the phenothiazine dyes Azure A and B which are N-methyl derivatives of thionine.

Fig. 24: Spectra of bR, thionine and Azure B. Note the greater separation of the absorption maximum of bR and Azure B

3.4.2 Evaluation of Azure A

Azure A, a commercially available phenothiazine dye, absorption maximum is red-shifted owing the conversion of a primary amino group of the structure of thionine to a tertiary one. This bathochromic shift is due to an increase in electron donating ability of the substituted amino moiety.

Fig. 25: Structure of Azure A

λ_{max} in H₂O = 633 nm

The efficient polymerisation of this moiety required a change in the conditions. It was found that a basic aqueous solution was resulted in the most efficient conditions for polymerisation.

The efficient polymerisation of Azure A in a basic solution is believed to occur due to the assembly of dimers in solution⁸. This dimerisation can be observed by a blue shift in the colour of the solution. With an increase in basicity the solution takes on a brown colour and precipitate formation occurs. This precipitate is believed to be the formation of higher order aggregates that are no longer soluble.

Fig. 26: Aggregation initiated by an increase in basicity and structure of dimer

The formation of these dimers is believed to be the cause of the increase in the amount of material deposited at high pH, since upon the formation of the radical cation, another dye molecule is present in an ideal orientation for the coupling reaction that occurs ortho to the amino moiety.

The films formed via electrodeposition share the optical properties of the free monomer in solution and therefore this dimerisation must be disrupted by association with the surface of the electrode. This illustrates that the approach to improving the contrast of the overall device is sound.

3.4.3 Evaluation of Azure B

Azure B is another methylated derivative of thionine. This derivative possesses the most red-shifted absorption maxima as a consequence of its three N-methyl substituents.

Fig. 27: Structure of Azure B

λ_{max} in H₂O = 648 nm

This dye formed the most optically dense films. The reason for this increase in reactivity may be a result of an increase in the forces that drive the association of the dye molecules to form a dimer since it is dependant with the cube of the absorption maxima⁸. For this reason films formed from this dye would represent an improvement over the polythionine films in that it would increase the overall contrast of the device. Furthermore, the capacity of the device should increase, as the deposition of more material should result in the release of more protons with the oxidation of the material. Unfortunately, this was not the case since the films redox couple became pH independent once the pH of the electrolyte increased beyond seven. This can readily be seen by the lack of movement of the cyclic voltammogram at these two pH's.

Fig. 28: Independence of Azure B redox couple to solution pH

This independence is observed during the sol-gel encapsulation of methylene blue, another closely related phenothiazine dye, and this effect is attributed to the dense structure of this film. It is therefore believed that the close packing of the dimers in thick film of Azure B prevents the diffusion of the protons present in low concentration at higher pH values and thus the charge is neutralised by other cations present in solution⁸.

3.4.4 General Observations

The largest problem associated with the phenothiazine dyes is that the reduced states of the polymer/dimer are soluble in the electrolyte and therefore when the cell is held at a negative bias the electrode delaminates. All of the dyes have this problem to some degree and therefore the electrode stability is poor. A common strategy to improve the adhesion of material to ITO electrodes is the pre-treatment of the surface with alkoxysilane however in this case this procedure had little or no effect.

3.5 Experimental

Synthesis of poly (methacroyl-graft-dopamine)

1.87 g of dopamine HCl was dissolved in 4 mL of DMF with warming to 60 °C. 1.2 mL of triethylamine was added to the stirring solution and a fine white powder precipitated over the course of ten minutes. The liquid was isolated by gravity filtration with the assistance of 10 mL of acetone. The acetone was removed in vacuo. A dried, two-necked round bottom flask was fitted with a reflux condensor capped with a drying tube and purged with argon. The DMF solution of dopamine was introduced to the round bottom through a column of anhydrous aluminum oxide followed by two aliquots of 10 mL of DMF via argon pressure. The column of alumina was removed and 1 mL of 25% poly(methacroyl chloride) in dioxane was added. The reaction mixture yellowed almost immediately and deepened in colour to a brown solution over the course of 18 hrs under reflux. 1 mL of anhydrous EtOH was added to the refluxing mixture and the flask was cooled. The reaction mixture was concentrated and precipitated in water. A tarry solid was isolated and the precipitation was repeated to yield a fine brown powder that was isolated by filtration. This material was readily soluble in pyridine and was spin coated onto an ITO electrode for electrochemical study.

Optimised Synthesis of Carboxylated Polyaniline

A saturated solution of o-aminophthaloyl hydrazide with 4 equivalents of tetrabutylammonium hydroxide in DMF was prepared and purged for 10 minutes with nitrogen. To the stirring solution 2 equivalents of benzoyl peroxide were added rapidly and the solution darkened immediately and gas was evolved. The solution was allowed to stir for 3 days under N₂ upon which it was acidified with conc. HCl and allowed to stand for 1 day. A brown solid precipitated and was isolated by filtration. GPC analysis of a DMF solution indicated that this material was oligomeric. It was also soluble in pyridine. This permitted spin coating of this material on ITO to yield films with excellent uniformity and good adhesion.

Several other syntheses were attempted using DMF/water solutions using NaOH as a base and ammonium persulfate as an oxidant. These preparation yielded lower molecular weight oligomers probably due to their poorer solubility in this solution.

Fig. 28: GPC of carboxylated polyaniline

Optimised Electropolymerisation of Thionine

10 mg of thionine and 150 μ L of 12M HCl was added to 25 mL of 4:1 EtOH/H₂O and degassed with nitrogen. An ITO electrode was placed in a three electrode cell and swept between 1400 mV and -400 mV at a rate of 50 mV/s. Polymerisation, as judged by an increase in current at the redox peaks, would persist for approximately 70 cycles. Upon the conclusion of the electropolymerisation, a mirror smooth, blue film was obtained. This film was washed with distilled water and then cycled for ca. 5 minutes in fresh electrolyte (600 to -400 mV).

Optimised Electropolymerisation of Azure A and B

4 mg of the dye was dissolved in 15 mL of water and 100 μL of 12M HCl was added while the solution was degassing. 1M KOH was slowly added until the solution changed colour (blue to purple). The solution was quickly stirred and electropolymerisation was started immediately (1400 to -600 mV, 50 mV/s). A blue film was rapidly deposited in approximately 16 sweeps. The film was washed with distilled water and cycled for ca. 5 minutes in fresh electrolyte.

Fig. 29: Electropolymerisation of Azure B

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Chapter 4: Electrochemical Modulation of the M State

4.1 Identification of Counter-electrode Material

Upon the identification of polythionine as a polymer that could effect the desired pH change it became necessary to identify a material that could serve as a suitable counter-electrode. The criteria for this counter-electrode were;

1. No acid/base chemistry. This was required since the release of protons from the polythionine electrode would result in an uptake of protons from the media at the counter-electrode and therefore the media would not change pH.
2. Stability to basic conditions
3. A complimentary colour change to assist the O.D. change of the device overall.

Once this material was identified, the preparation of prototype devices could be attempted in order to test the properties of the proposed device.

One suitable material is polyethylenedioxythiophene (PEDOT) prepared via electropolymerisation. This material must be prepared by electropolymerisation for the intended application since the chemical polymerisation of this material required the use of a polymeric sulfonic acid dopant. This dopant participates in the redox couple of the material and thus is a source of acid-base chemistry. This acid/base electrochemistry cannot, however, be used in the same fashion as polythionine since the colour changes upon oxidation/reduction are the opposite of the anticipated changes of bacteriorhodopsin. These properties render the commercially available material unsuitable for the proposed device.

4.2 Electropolymerisation of PEDOT

The formation of uniform films of PEDOT from an acetonitrile solution of its monomer is complicated by the fact that the deposition of the polymer onto the electrode is very sensitive to slight changes in concentration in front of the electrode. Furthermore, the ITO electrode must be extremely clean in order to obtain a uniform deposition. Without compensating for these effects the films obtained by electropolymerisation are of extremely low quality.

Fig. 30: Electrochemically generated films of PEDOT. Note the difference in the quality of the films

Changes in concentration in front of the electrode are brought about by the fact that during the electropolymerisation, the electrical field between the electrodes that assists in the deposition of the polymer is not uniform and oscillates in direction as a consequence sweeping the potential in a cyclical fashion. As a result, the location and shape of the counter-electrode is critical in obtaining uniform films of PEDOT.

Many different electrode configurations were tried and the best results were obtained with the configuration seen in Fig. 31. This configuration ensures that a uniform field exists between the electrodes at all times and therefore the deposition occurs in a uniform fashion.

Fig. 31: Parallel plate assembly

4.3 Cell Assembly

In order to assess whether or not the redox chemistry of the electrochemical cell was capable of changing the photostationary state population of bacteriorhodopsin an electrochemical cell with the smallest volume and highest O.D. must be constructed. This requires that the bacteriorhodopsin containing matrix be as thin as possible but also have an appreciable O.D. This requires the use of lyophilized protein in order to minimise the increase in volume upon adding the protein. Careful preparation allows polyacrylamide films of 100 μm with an O.D. as high as 1.2 to be constructed. These films were then soaked in a glycerol/GuaHCl solution whose pH had been adjusted to the appropriate value and used to prepare the device.

4.4 Electrochemical Modulation of Bacteriorhodopsin

4.4.1 Polythionine/PEDOT Solid Cell

To observe the electrochemical modulation of bacteriorhodopsin the following electrochemical cell with a polythionine electrode to serve as the proton source/sink layer and a PEDOT counter-electrode was constructed. The GuaHCl concentration was set such that the M-state lifetime was very long so that upon strong illumination the change in O.D. at 410 nm could be observed over the course of ~2 minutes. This configuration would seriously limit the practical application of the cell but serves as a means to test the hypothesis that the electrochemical change in pH can effect the M-state of the protein. This experiment differs from the intended application in that the change in pH effects a static population of bacteriorhodopsin in the M state.

The experiment was carried out by assaying the change in transmittance at 410 nm in the dark immediately after strong illumination of the cell. Prior to the illumination, the polythionine was oxidised or reduced as a means to alter the pH of the bacteriorhodopsin containing matrix. The oxidation of polythionine corresponds to the release of protons to the matrix and therefore the expectation is that upon the removal of illumination, the rise of transmittance at 410 nm will be faster than if these protons had not been released. This behaviour was observed and is seen in Fig. 32. The slower rise in the transmittance when the cell bias is set to place polythionine in its reduced form is evidence that the cell pH is in fact being altered, and that this alteration in pH affects the kinetics of the photocycle of bacteriorhodopsin.

Fig. 32: Decay of M state monitored by Change in Transmittance

The reproducibility of the effect was investigated since the polythionine layer was known to be unstable. The activity of the cell was checked before and after the collection of data corresponding to the two pH values by cyclic voltammetry. The effect, although small, could be observed between each trial and when the data was averaged the curve seen in Fig. 32 was obtained.

The short operational lifetime of the cell constructed for these investigations is the major reason that polythionine cannot be used for any practical device based on the modulation of bacteriorhodopsin. This problem was circumvented by the use of tungsten oxide as the proton source/sink.

4.4.2 Tungsten Oxide/PEDOT Solid Cell

Tungsten oxide undergoes a reduction with an associated cation insertion into the inorganic film. This results in the film taking on a deep blue colouration from an initially transparent state. This material is an excellent charge storage material since it can accommodate up to 20 mC/cm² of charge and thus can store many more protons than any electroactive polymer¹. Unfortunately, this material is similar to PEDOT in that the colour change is anti-complementary to the anticipated change for bacteriorhodopsin. This material is, however, an excellent material to further study the effect identified using polythionine since it is very stable and can deposit much more charge into the bR matrix.

The experimental setup is diagrammed in Fig. 33. Under this design the HeNe laser beam (632.4 nm) serves both as a means to excite the bR and to detect any response of this material to a change in cell bias. It is important to note that the electroactive material has been removed from the path of the laser beam. With this experimental design, a decrease in the photodetector current should stem only from the growth of the population of bR in the ground state upon the acidification of the media. Care was taken to ensure that the laser path was not occluded by any of the electroactive material on the electrodes.

Fig. 33: Experimental apparatus for the detection of the modulation of the M state

With this experimental design the modulation of a dynamic population of the M state is possible since the laser illuminating the cell ensures that the bR is constantly photocycling. For these experiments the additive concentration and pH of the bacteriorhodopsin matrix was tailored to allow a rapid change in the photostationary state population. The successful modulation of the population is seen in Fig. 34. The blue curve represents the current generated upon the application of voltage to the cell. The peaks of this curve represent the discharge of protons into the bR matrix. The purple curve represents a change in the photodetector current. Note that upon the release of protons to the media, the current from the photodetector drops as a result of the attenuation of the signal. This attenuation is consistent with an increase in the ground state photostationary state as a result of the acidification of the bR layer via the release of protons from the tungsten oxide.

This effect was reproducible and the modulation between the two photostationary populations was rapid as it could be achieved within one second. The amplitude of the effect was quite small and corresponds to a change in O.D. of less than 5%. This finding is most likely a result of a limit placed on the oxidation of the proton doped tungsten oxide since the PEDOT counter electrode can only accept $\sim 1 \text{ mC/cm}^2$ of charge. The amount of current generated by the cell suggests that the tungsten oxide electrode is releasing more than 1 mC/cm^2 upon oxidation and therefore another reduction process is occurring at the counter-electrode other than the reduction of PEDOT. This unidentified process is most likely the reduction of some species in the electrolyte directly at the ITO electrode. This process was indirectly observed as a permanent change in the colouration of ITO beneath

the PEDOT. The possibility that this was responsible for the observed effect was checked by a control cell in which the PEDOT and the bR was removed. The permanent change in the ITO was observed and a very small modulation in the signal was also observed but, importantly, this modulation was opposite in magnitude to the effect observed in the presence of bR. As a result of this observation, the magnitude of the modulation observed in Fig. 34 attributed to bacteriorhodopsin is in fact slightly larger since the attenuation of the laser is being offset by an increase in transparency of the counter-electrode.

4.5 General Remarks

The main problem with the device in its current configuration is the mismatch in the charge capacity of the inorganic layer and the organic layer. This requires that a suitable counter-electrode be found whose charge capacity is equal to that of the inorganic layer, tungsten oxide. A survey of the literature was conducted and no inorganic material that was capable of releasing the anions required to balance the charge of the electrolyte layer upon the release of protons from WO_3 could be found. This represents a barrier to the successful development of a practical device.

A second problem with the device is that the change in absorbance of the device stemming from the electrochromic layers significantly overshadows the modulation generated by the bR matrix. This suggests that the requirement of complementarity of the electrochromic activity of the cell must be abandoned if an effect solely due to bR is to be observed by the naked eye.

Optically passive materials with characteristics similar to tungsten oxide have been developed such as iridium and cerium/vanadium oxide films but the cost of using this

material is prohibitive. If these films could be made available, the lack of a suitable counter electrode remains.

Summary

The electrochemical modulation of the M state of bacteriorhodopsin was demonstrated with two configurations. The first configuration used an electrochemically generated polymer, polythionine, as a proton sink/source material to affect the kinetics of the photocycle of bacteriorhodopsin. A small change in the lifetime of the M state was observed and it was dependent on the redox state of polythionine, from which a proton is released upon oxidation. The second configuration employed tungsten oxide as a proton sink/source material and allowed the modulation of a dynamic population of the M state. The effect was small owing to a mismatch in capacity between the inorganic electrode and the organic counter-electrode and the small pathlength of the device.

Considerable effort was expended to optimise the polymerisation conditions for the phenothiazine dyes. For the N-methylated derivatives, a dramatic increase in the amount of material deposited was observed with an increase in solution pH. This effect was attributed to the formation of reactive dimers in a basic solution. For the non-methylated derivative, thionine, an increase in coverage occurred with a change in solvent and was believed to be a result of an increase in solubility. The main drawback of these systems was that with repeated cycling, the polymers delaminated from the electrode.

Several other redox active polymers were investigated. A dopamine derivatised polymer was synthesised but its stability was very poor in basic solvents as the electrochemically generated species was reactive.

A polyaniline derivative, 2,3-dicarboxyaniline, was synthesised from a novel monomer, *o*-aminophthaloyl hydrazide. The synthesis of this material from this monomer confirms that the chemiluminescent reaction associated with this material in fact generates

the dicarboxylate anion, and this is believed to be the reactive monomer that formed the polymer. This material was not suitable as a proton source/sink material for the modulation of bR since it was not electrochromic and could not be placed on an electrode in sufficient amounts.

A consideration of the kinetics of bR and the effect of various additives suggested the pH range and GuaHCl concentration required for the rapid modulation of the M state. These parameters were investigated with the protein entrapped in a polyacrylamide matrix and it was found that useful modulation could be achieved over 2 pH units (6 to 8). The convenient modulation of the M state was found to require the presence of glycerol and this in turn required the use of riboflavin to photoinitiate the polymerisation of acrylamide.

Contributions to Knowledge

- A photostationary population of bR in the M state can be altered by a change in the pH of the electrolyte of an electrochemical cell. This results in a change in the absorption at 570 nm of the bacteriorhodopsin containing film that can be used to modulate the intensity of a beam of light passing through the film.
- The synthesis of 2,3-dicarboxypolyaniline can be carried out from luminol. This is further evidence for the suggestion that the chemiluminescent reaction of luminol generates 2,3-dicarboxyaniline.
- The efficient electrodeposition of N-methyl derivatives of thionine is best carried out in basic media. This increase in reactivity is believed to stems from the formation of dimers in solution whose structure places the reactive sites of the dye in close proximity.