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Ionophore/Lipid Bilayer Assembly on Soft Organic Electrodes for Potentiometric Detection of K^+ Ions

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1. Introduction

The major goal on intrinsically conducting polymers (ICP) technology development has been to combine the electrical and optical properties of these new materials with the mechanical and processibility properties of commodity bulk polymers. New conductive materials that offer significant application potential as substitutes, and new products having properties difficult or impossible to achieve by existing materials, can now be produced.
The most common synthetic methods are the oxidative chemical polymerization of aniline using ammonium persulfate, electrochemical polymerization; and most recently enzyme catalyzed polymerization of aniline has also been reported (Jin 2001).The reactions of aniline with oxidants proceed in two fundamentally different ways. The oxidant can either donate oxygen to the aniline molecule or it can remove a hydrogen atom from its amino group, the latter is the prevalent method that leads to polyaniline (PANI) (Ćirić-Marjanović 2008). It was shown by Wan and co-workers that during oxidative chemical polymerization, the molar ratio of acid to aniline affected the probability of formation of fibrous nanostructures dictating the final product morphology (Zhang 2002).
In the oxidative chemical polymerization method aniline and ammonium persulfate are mixed at a predetermined stoichiometric ratio and reacted in acidic medium producing a green precipitate of PANI salt. For the electrochemical method an applied potential in lieu of an oxidant is used to oxidize and polymerize the aniline on the anodic electrode surface. In 2002 the International Union of Pure and Applied Chemistry (IUPAC) in an effort to standardize the process of oxidative polymerization of aniline using ammonium persulfate, issued a Technical Report with the goal of obtaining PANI with a defined conductivity (Stejskal 2002). This report constituted the first organized robustness study of the oxidative chemical polymerization of aniline, and it included eight chemists from five different institutions executing the same IUPAC preparation protocol. The electrochemical method was excluded from this report since its efficiency has been proved to be a function of the electrode area and therefore not suitable for large-scale production. An efficient polymerization of aniline is achieved only in an acidic medium where aniline exists as the anilinium cation. The reaction is exothermic with a defined and reproducible temperature profile that can be used to monitor the progress of the reaction (Beadle 1998). Generally

there is an induction period of about 4 minutes where the temperature stays constant at pH ≤ 2.5, followed by polymerization period where the temperature raises close to 40°C, then the temperature start falling during the post polymerization period.

The presence of residual aniline must be minimized to obtain the best yield of PANI and a stoichiometric ratio peroxydisulfate/aniline of 1.25 is recommended, the polymerization is complete within 10 minutes at room temperature, however it slows down to 1 hour when the reaction container is cooled between 0-2 °C (Sulimenko 2001). PANI has a nitrogen heteroatom incorporated between the phenyl rings along the polymer chain imparting flexibility and allowing the existence of the six different forms known (Naarman 1987) as seen in Figure 1 and Figure 2. The PANI powder synthesized by the chemical oxidative polymerization in acidic solution is the emeraldine salt (ES) form which when treated with alkali becomes the emeraldine base (EB) form. Oxidation and reduction of the ES form yields the pernigraniline salt (PNS) form and the leucoemeraldine salt (LES) form respectively, while same treatment of the EB form produces the pernigraniline base (PNB) and the leucoemeraldine base (LEB). The individual base and salt forms can convert into each other by protonation/deprotonation.

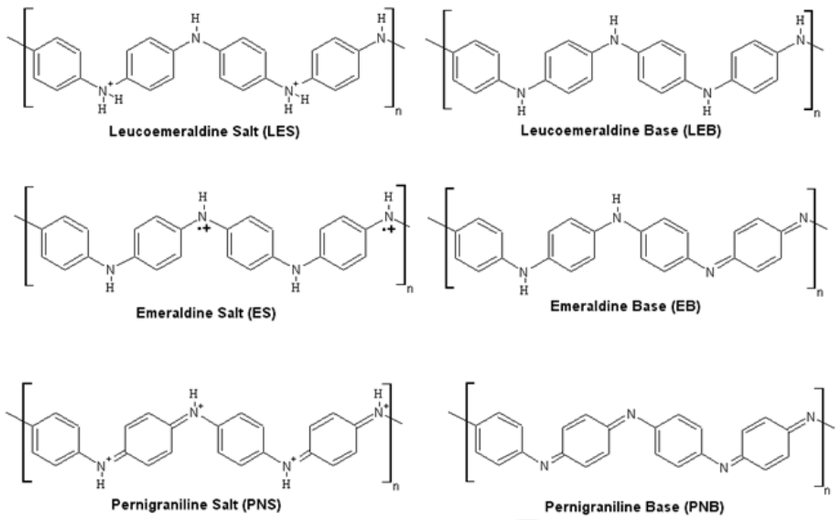

Fig. 1. Chemical structures of the six different polyaniline chemical forms

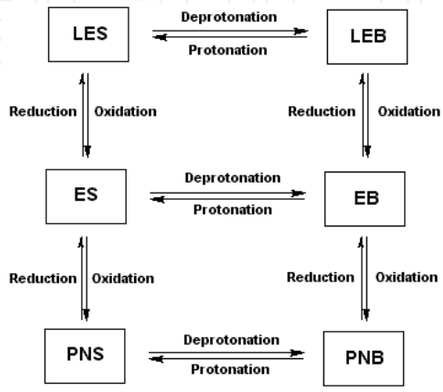

Fig. 2. Polyaniline interconversion chart for the six different chemical forms

Polyaniline has a conduction mechanism that is unique among conductive polymers in that its most highly conducting doped form can be reached by two different processes:

- Protonic acid doping of the EB form and
- Oxidative p-type doping of the LEB form.

The term doping was coined to refer to the introduction of impurities in an extremely pure semiconductor in order to change its electrical properties. However this terminology has also been used to refer to a process consisting in the introduction of charge carriers in ICPs. Charge carriers are created by removing or adding electrons to the delocalized π-electrons through a mechanism that is more precisely termed as a redox reaction (Zhang 2001).
Through the process of doping the number of electrons in the PANI chains are either decreased, or increased resulting in structural defects in the bond interaction and localized electronic states. Since the geometry of the chain between the radical and the ion must be distorted, and the energy of the distorted geometry is normally higher than that of the ground state, separation, and delocalization of the radical ion associated with a lattice distortion are called polarons and are the basis of the hopping conductivity typical of ICPs.

2. Functionalization of polyaniline

Several solutions to the intractability of PANI have been devised throughout the years, all these solutions aim to improve solubility and processability in organic solvents. Large organic anions such as dodecylbenzenesulfonate and camphorsulfonate are used as dopants, also sulfonation has also been used to improve solubility and processability in aqueous media. Zheng investigated the incorporation of flexible alkyl chains into polyaniline through N-alkylation of PANI LEB form to improve it tractability, and found that long alkyl side chains facilitate the formation of mesophases by functioning as flexible spacers to decouple individual mesogenic units for the entire polymer chain (Zheng 1994). Han et al through review and observation that many Lewis base solvents, e.g. pyrrolidine, piperidine and morpholine are effective solvents for the PANI EB form, proved that the class of compound provided concurrent reduction and substitution between PANI and nucleophiles, and therefore a facile approach for modifying a PANI backbone with various electron-donating groups, e.g. amino, alkylthio and alkoxy (Han 1997). The protonation of the imine nitrogen could possibly promote nucleophilic attacks at the meta position of the protonated quinoid ring, followed by a 1,3 proton shift of the meta-proton to the para-imine nitrogen and, ultimately, a conversion of the less stable quinoid ring to the more stable benzenoid. Highly conductive aniline copolymers containing butylthio substituents have been prepared from unsubstituted polyaniline confirming that a concurrent reduction and substitution route performed on the solid-state matrix of PANI is a better way for preparing aniline copolymers, than the conventional copolymerization (Han 2001). The butylthioaniline copolymers obtained by this process are highly soluble in common organic solvents, such as THF, dioxane, 2-methoxyethyl ether, and 2-methoxyethanol [47] which are nonsolvents for the parent PANI.

3. Electrochemical properties of polyaniline

The most useful electroanalytical technique for the characterization of PANI is cyclic voltammetry (CV), in this technique the potential applied across the electrode-solution interface is varied and the resulting current is measured. The versatility of CV allows a mechanistic study of redox systems, this is of great utility for the identification of peaks

associated with the PANI redox couples, further characterization can be done using the peaks individual potentials in the voltammogram, and by changing the scan rate.
A typical cyclic voltammogram of PANI in aqueous 0.5N sulfuric acid is shown on Figure 3, two well resolved peaks at half-potential ca. 0.2 and 0.7 V corresponding to two quasi-reversible redox processes, the first corresponds to the conversion of PANI protonated amine units (LES) to the semiquinone radical cations in ES, while the the second can be assigned to the conversion of ES to the fully oxidized and protonated quinoidal form PNS.
The shape of the cyclic voltammogram is independent of the nature of the aqueous acid electrolyte only when strong acids are used (Batich 1990), however, the redox potentials strongly depend on the film thickness, scan rate, and pH of the medium. As the pH is increased the redox couple peaks shift to a higher positive potential, this is due to differences between partial and complete protonations of the amine nitrogens (Lukachova 2003, Varela 1990). The composition of the electrolyte medium has also been found to influence the redox mechanism, since both the cation and anion present participate in the PANI redox mechanism Genies 1990). Electrogravimetric studies in aqueous media (DeSilvestra 1992, Kabumoto 1988) have shown that the mass of PANI polymer increases during oxidation (charging) and decreases during reduction (discharging) for the first redox couple. Mass changes depend upon the molecular weight of the anion, and there is not effect from the cation, this is an indication that the anions present in solution are combined into the polymer films to compensate for the positive charges resulting from the oxidation from LEB to ES, and then driven out during subsequent reduction.

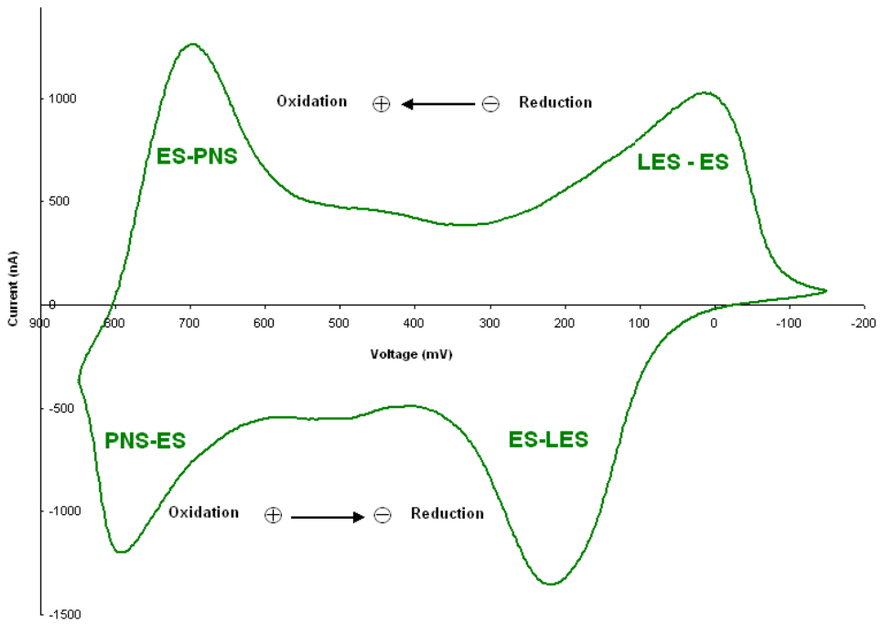

Fig. 3. Cyclic voltammogram of a polyaniline film on glassy carbon microelectrode (7 mm^2 disk in 0.5M H_2SO_4 from –0.150V to 0.850 V, scan rate = 100 mV/s.

4. Ion-Selective polyaniline electrodes

Today, conducting polymers have become one of the most important ion-to-electron transducers in solid-state ion selective electrodes (ISEs). They are based on the

potentiometric measurement technique which is very attractive for practical applications because it allows the use of small-size, portable, low-energy consumption and relatively low-cost instrumentation[57]. Furthermore, the development of solid-state ISEs without internal filling solution gives durable and maintenance-free ion sensors. The principle of ion-to-electron transduction is derived from the fact that ICPs are polymers with conjugated double bonds, and thus considered electroactive materials that have widespread use in the field of chemical sensors. Oxidation of the conjugated polymer backbone is accompanied by anion insertion or cation expulsion, as shown on equation 1 and 2.

$$P + A^- \rightleftarrows P^+A^- + e^- \quad (1)$$

$$PA^-M^+ \rightleftarrows P^+A^- + M^+ + e^- \quad (2)$$

Where P = neutral conducting polymer unit, P^+ = oxidized conducting polymer unit, A^- = anion, M^+ = cation and e^- = electron. Equations 1 and 2 describe two limiting cases where either anions or cations are mobile, respectively. The similarity between these two equations immediately suggests that conducting polymers can work as ion-to-electron transducers in ISEs as they are frequently used today.

5. Polymer supported biomimetic membranes

Lipid-bilayer membranes supported on solid substrates are widely used as cell-surface models that connect biological and artificial materials (Turner 1996). They can be placed either directly on solids or on ultrathin polymer supports that mimic the generic role of the extracellular matrix. Biological membranes consist largely of a lipid bilayer that imparts a fluid character, there are proteins embedded in it and carbohydrates attached to its surface which facilitates communication and transport across it.

The complexity of biological membranes and their interactions with intra and extracellular networks make direct investigation difficult and for this reason artificial membranes have played an important part in unraveling the physical and chemical characteristics of membranes and their contributions to membrane function. The most commonly used experimental cell-surface model for the past 20 years have consisted of phospholipid bilayers deposited onto solid substrate. Solid-supported membranes are prepared by directly depositing lipid monolayers and bilayers onto surfaces, which maintain excellent mechanical stability without losing their fluid nature (Tanaka 2005, Groves 2003 and Sackmann 1996).

The fluidity and stability of the solid-supported membranes planar surfaces have a distinct advantage over freestanding “black lipid” membranes or spherical lipid vesicles models because it makes it possible to carry out experiments and use analytical methods that are difficult or impossible to use with other model systems. Methods like total interference fluorescence, nuclear magnetic resonance (NMR), Fourier-transform infrared spectroscopy, Surface Plasmon resonance, and X-ray and neutron scattering can all be used to probe the structural and dynamic properties of solid supported membranes (Kalb 1992, Baruerl 1990, Tatulian 1995, Terretaz 1993, Kjaer 1987, Kalb 1990, Majeski 1998).

In solid-supported membranes the artificial membranes and their solid supports are close together. They typically approach each other to within 5-20Å. This small gap leaves a water reservoir that is usually not sufficient to prevent protein subunits from coming into

direct contact with the bare substrate. Such direct contact can be avoided by using polymer supports of typically less than 100 nm thickness that "cushion" or "tether" the membrane.

When using a polymer to 'cushion' a supported membrane, it usually acts as a lubricating layer between the membrane and the substrate. This will assist self-healing of local defects in the membrane over macroscopically large substrates (~cm2) and allow the incorporation of large scale transmembrane proteins without the risk of direct contact between protein subunits and the bare substrate surface. J. Majewski and J.N. Israelachvili prepared softly supported polymer-cushioned membranes which consisted of thin layer of branched, cationic polyethyleneimine (PEI), and the bilayers were formed by adsorption of small unilamellar dimyristoylphosphatidylcholine (DMPC) vesicles (Wong 1999). Recently glycolipid-containing bilayer have been shown to be an effective tethering system (Lipkowski 2010, Chen 2009, Brosseau 2008).

Our objective of this research is the derivatization of PANI with thiolated phospholipids, with the purpose of assembling covalently bonded phospholipid layers on this ICP. The EB form was the starting material, as its chemical structure presents one unique opportunity for derivatization though a concurrent substitution and reduction of its diiminoquinoid rings to diaminobenzenoid rings forming the PANI leucoemeraldine base (LEB) form. Alkylthiols and alkylamine nucleophiles have been used to derivatize EB, obtaining the LEB reduction product with an assembled hydrophobic layer on it; in a similar fashion as thiols self-assemble on gold surfaces. However as far as we know, this work has not yet been attempted with thiolated phospholipids. As part of this research some thiolated phospholipids were also synthesized by treating 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) with heterobifunctional cross-linkers also known as thiolation agents, which are commonly used for coupling -NH_2 containing compounds and –SH containing molecules together by a covalent bond. However this work required multiple synthesis/separation steps and exhaustive purification which were suspended once a thiolated phospholipid commercial product named 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol sodium salt (PTE) was found to be available.

Once PTE was substituted on the PANI films supported on carbon glassy (CG) electrodes, attempts were made to assemble K^+ ion specific electrodes, based on the immobilization of the ionophore valinomycin and the pentadecapeptide gramicidin forming biomimetic lipid membranes. Potentiometric titrations with K^+ ions were performed to determine the assembled membranes saturation curves, and to determine if the mechanism of adsorption was consistent common cellular facilitated transport mode.

Phospholipid, also known as phosphoglycerides, are triglyceride-derivatives in which one fatty acid has been replaced by a phosphate group and one of several nitrogen-containing molecules. The hydrocarbon chains are hydrophobic as in all fats. However, the charges on the phosphate and amino groups make that portion of the molecule hydrophilic resulting in an amphiphilic molecule.

As amphiphilic molecules, the phospholipids are major constituents of the cell membrane wherein with their hydrophilic polar heads face aqueous surroundings like the cytosol and their hydrophobic non-polar tails face each other. If suspended in water, these molecules can orient themselves at the air/water interface with their hydrophobic chains upwards in the air and their hydrophilic head groups downwards into the aqueous phase. The chemical structures of 1,2-dipalmitoyl-sn-glycero-3- phosphothioethanol sodium salt (PTE) and 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine (DPPE) are shown in Figure 4 as A and B,

respectively. The preparation of thiolated phospholipids requires a primary amine group as observed in DPPE.

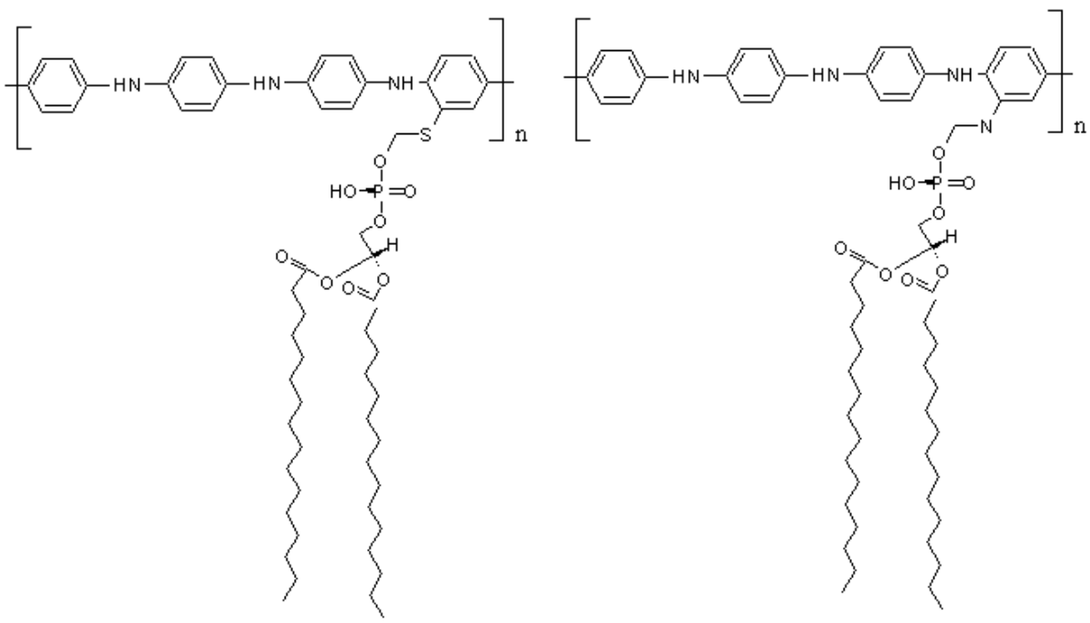

Fig. 4. 1,2-dipalmitoyl-sn-glycero-3- phosphothioethanol sodium salt (PTE) and 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine (DPPE)

6. Adsorption of amphiphilic molecules at electrode interfaces

A phenomenon that can markedly affect the results of electrochemical experiments, is the tendency of amphiphilic ions or molecules to adsorb on the electrode surface and their aggregation into supramolecular structures (Rusling 1991). Neutral molecules can adsorb on the electrode surface primarily because of the hydrophobicity in aqueous solution. Generally, the less soluble a molecule is, the stronger it adsorbs (Kissinger 1996). Bonding between the electrode surface and π electrons and nitrogen lone pairs electrons (as in –SH and $–NH_2$) of the molecule can also enhance adsorption.

The PANI coated Pt and GC electrodes used in this research has proven to be a more versatile approach to prepare a chemically modified electrode (CME) that just a simple polymer coat as initially shown by Miller and Bard (Miller 1978, Merz 1978). The diiminoquinoid rings in the PANI film backbone can form a covalently bonded densely packed layer of phospholipids, primarily because of increased hydrophobicity of the electrodes which facilitates the approach of the phospholipid and the eventual functionalization of the backbone by the –SH group.

When redox reagents are absorbed on an electrode surface or confined on a thin layer of solution adjacent to it, the reversible cyclic voltammograms are not governed by diffusion controlled processes and this phenomenon can markedly affect the results of any electrochemical experiment. Adsorption is responsible for unusual electrochemical behavior[2] and for this research it played an important role in enhancing the substitution of PTE on PANI.

Derivatization of electrochemically synthesized PANI films on GC electrode was performed by using DPPE phospholipid which contains $-NH_2$ nucleophile. The chemical structure of DPPE is shown on Figure 4. DPPE vesicles were made by followingly: The procedure consisted of weighing 5.2 mg of DPPE into a glass scintillation vial, dissolved in 2 mL of chloroform, then evaporated under a stream of nitrogen, and dried under vacuum for 60 minutes. The DPPE solid was then hydrated in 8 mL of 0.5M H_2SO_4 by intermittent agitation within a period of 60 minutes (shaking every 10 minutes), then the mixture was frozen and thawed five times using a salt-water ice bath and a water bath at 37°C, and extruded by filtering through an Acrodisc GHP 0.45µm syringe filter after each freeze/thaw cycle . The final solution contained DPPE small unilamellar vesicles (SUVs), which were transferred to an electrochemical cell. A PANI coated GC electrode was used as the working electrode, a platinum wire as the counter electrode, and an Ag/AgCl as reference electrode. The cell was cycled 50 times from –0.150V to +0.850V at scan rate of 100 mV/s. For comparison, the same procedure was repeated to prepare PTE SUVs which were then used, to treat an additional PANI GC electrode using the same procedure described above

CVs of conventional PANI in acidic solutions show two redox couples[11], as shown in Figure 5, the first pair of peaks located at low potentials correspond to the LE-ES/ES-LES redox couples, while the second couple located at higher potential is due to the ES-PNS/PNS-ES. A review of the data show that the LES-ES transition peaks corresponding to the fully benzenoid radical cation, and the ES-LES transition both experimented a blue shift. Contrarily the ES-PNS transition peak corresponding to the fully oxidized quinoidal form showed a red shift while the PNS-ES transition peak disappeared after an initial red shift. Overlay CVs are shown on Figure 6.

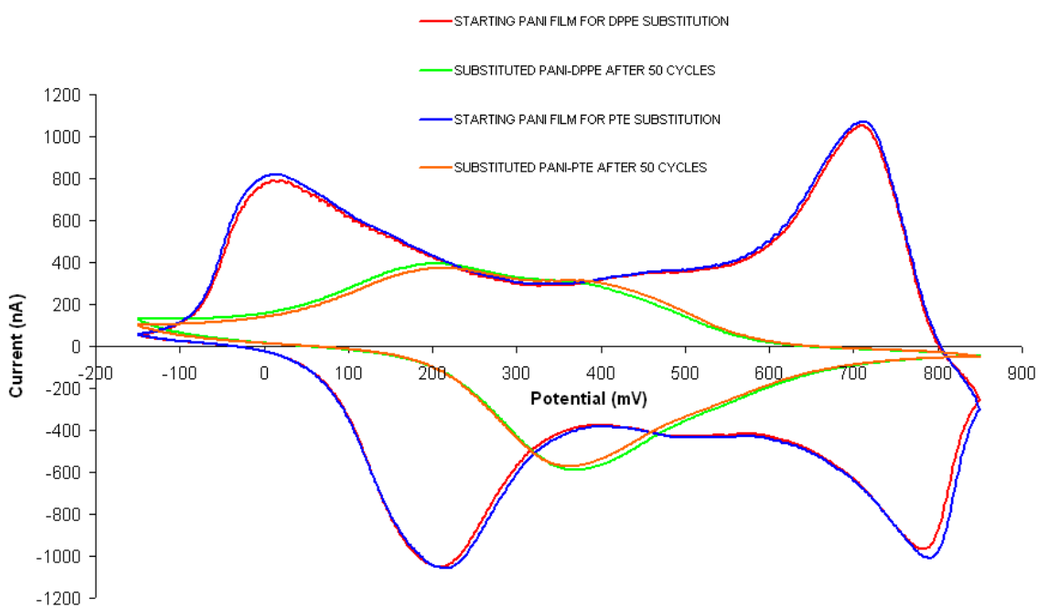

Fig. 5. Overlaid voltammograms for PANI films before and after the substitution with DPPE and PTE

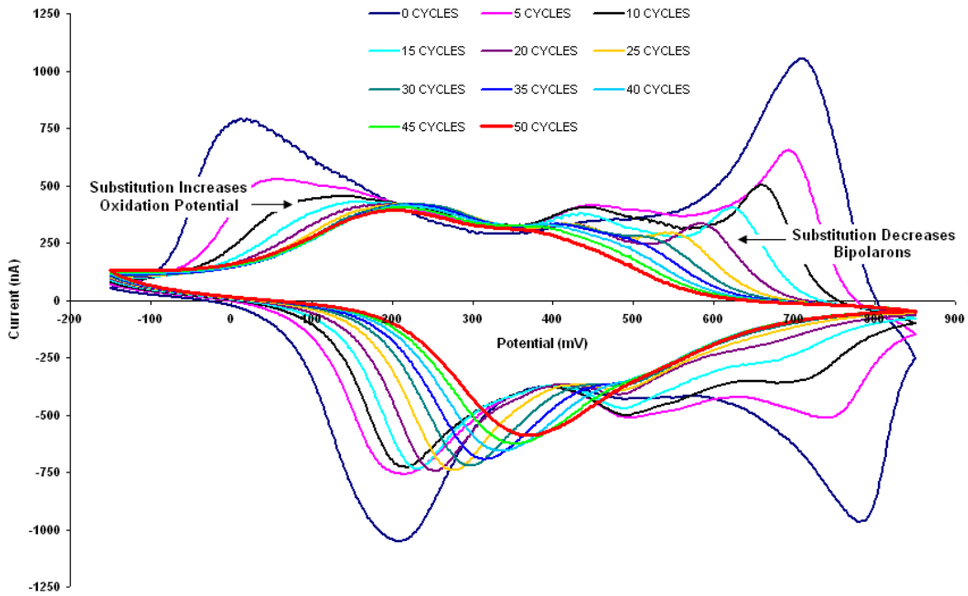

Fig. 6. Potentiodynamic substitution of PANI film with PTE

The overlaid evolution of the potentiodynamic substitution of PTE on a PANI film is displayed on Figure 6, it basically shows that the PNS-ES peak disappears after only 10 cycles, and then two new peaks on the anodic and cathodic planes appeared on the CV. It is believed that after the 10th cycle, any ES that is created by the potential cycling, thus reacted immediately with PTE to become the newly substituted LES product. The LES-ES shifts towards the center of the CV along with the remainder ES-PNS Peak which is continuously depleted by its transformation to ES and then to LES. At the end of the 50th cycle, only three peaks remain, the fully reduced and PTE substituted LES-ES transition, its corresponding ES-PNS radical cation and the blue shifted PNS-ES transition.

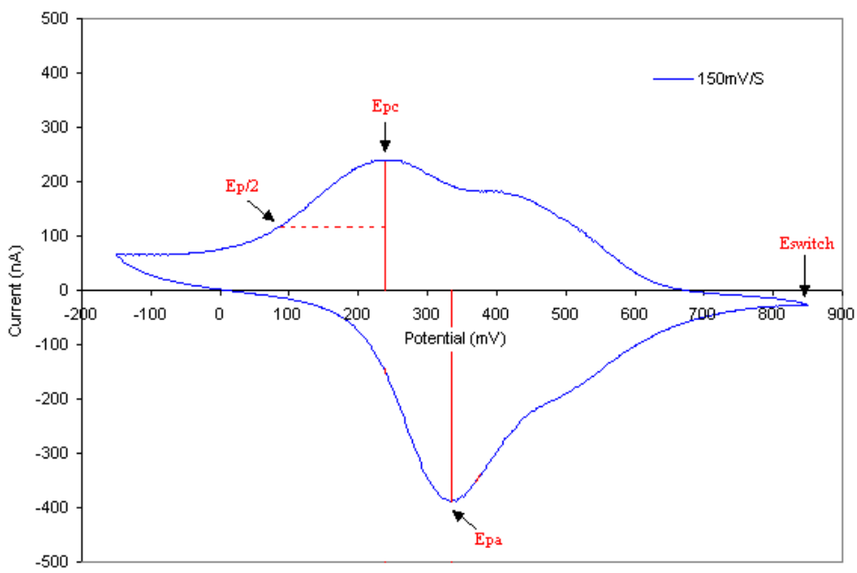

Fig. 7. Cyclic Voltammogram of PTE substituted PANI GC Electrode

The disk shaped GC electrode coated with derivatized PANI-PTE products showed a distinctive voltammogram, consistent with a gradual increase of the oxidation potential for the benzenoid forms and a decrease in redox potential for the diiminoquinoid forms, thus indicating a decrease on the bipolarons. The CV of the PTE substituted PANI is shown on Figure 7 using a scan rate of 150 mV/s

The CV on Figure 6 shows a gradual evolution of the substitution of PTE on the PANI film supported on the GC Electrode. At the end of 50th cycle one could wonder what could be the extent of the surface functionalization and how to measure it. Several techniques come to mind to make such measurements, some rather macroscopic like contact angle measurement could in fact measure the change in hydrophobicity on the PANI surface, other like ATRIR could measure the degree of substitution on the PANI film, and the use of a Crystal Quartz Microbalance (QCM) being an ultrasensity mass detector could measure the change in mass during the reaction progress. Finally electrochemical impedance spectroscopy (EIS) could also be used to measure the film capacitance. Film capacitance changes when lipids films attach to the electrode surface, it has been shown that lipid SAMS on gold electrodes can be modeled as flat plate capacitors in series (Rose 2006).

Due to the intrinsic geometry of the GC electrode, the GC disk is attached right at the bottom of a PTFE body. This makes it difficult to handle for placing on a conventional contact angle measurement apparatus. Same issue occurs with the ATRIR with requires a measurement tip to touch a horizontal section of the sample. To get a ATRIR spectrum the sample must fit under the ZnSe measurement tip and that could not be done with the GC electrode.

The synthesis of PANI on Pt and GC electrodes along with concurrent derivatization reactions, lead to the chemical and electrochemical substitution/bonding of phospholipids lipid layers on the conductive polymer substrate. The goal is to present the steps taken to assemble lipid membranes containing ion carrier proteins on electrodes coated with different conductive polymers, with aim of creating simulated natural systems. Once the membranes were assembled, their integrity and response was investigated by measuring their potentiometric responses by titration with K^+ ions.

7. Potentiometric detection

Potentiometry is one of the most frequently used analytical methods in chemical analysis[30]. This analytical technique is characterized by the measurement of the potential difference between two electrodes while maintaining the electric current under a nearly zero-current condition. In most common forms of potentiometry, the potential of the WE electrode varies depending on the concentration of the analyte, while the potential arising from a second RE electrode is ideally a constant. In practice the general principle of potentiometry deals with the electrochemical force (EMF) generated in a galvanic cell where a spontaneous chemical reaction takes place. The measurement of the electrochemical cell potential under zero-current conditions is used to determine the concentration of analytes in measuring samples.

PANI films have been used for the fabrication of ultramicroelectrodes sensitive to pH, showing some advantages over conventional glass electrodes (Slim 2008, Zhang, X 2002). Many other applications of PANI included potentiometric sensing of creatinine (Pandley 2004), dodecyl benzene sulfonate ISE (Karami 2004), nitrates ISE (Mazeikiene 1997), and quantitation of salinity (Diniz 1999). Films of other conductive polymers like PPy, poly(3,4-

ethylenedioxythiophene), and poly(3-octylthiophene) have been casted on solid supports to make potentiometric nanoelectrodes (Bakker 2008). PPy and PANI are considered today as the most promising conductive polymers for the development of sensors devices, owing to its good biocompatibility, conductivity, and stability.

The electrodes assembled during the experimental work fit in the category of all-solid state, as there was no need of using an internal reference solution. To avoid the drift problems observed on coated-wire electrodes (CWE), Cadogan et al. used an ICP as an intermediate layer between the ion selective membrane (ISM) and the substrate (Cadogan 1992), where the ICP was acting as an ion-to-electron transducer, thus eliminating the mismatch between the ionic conductivity in the membrane and the electronic conductivity in the substrate. This mismatch causes a high charge transfer resistance at the interface and the drift.

A drawing representation of the electrochemically derivatized PANI film is shown on Figure 8. The surface coverage was tested and showed that the thiolated phospholipid derivatized surface was hydrophophic, as indicated by the drift to negative potential. This also proved that the PANI-PTE surface did not exhibit ion exchange capabilities. At this point the surface became a platform for biomolecule immobilization as PANI could provide rapid electron transfer for use as a biosensor.

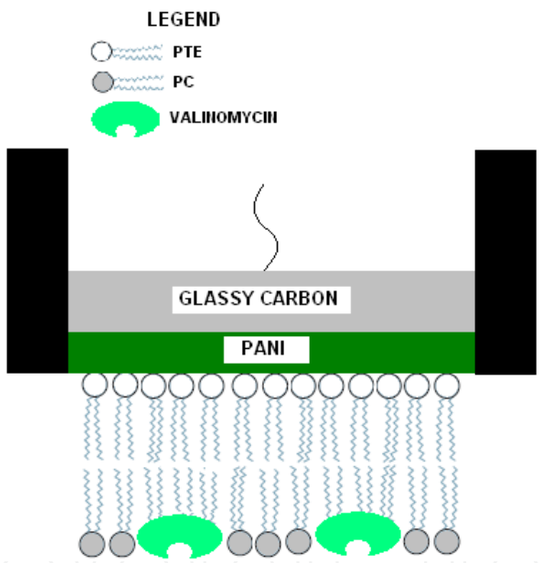

Fig. 8. Assembled bilayers with ionophore on GC electrode

In order to induce ion selectivity, ion-recognition sites as provided by ionophores must be immobilized in the derivatized PANI film, either covalently or non-covalently (Vasquez 2005). In solid contact as it was the case of the experiments performed on this research, PANI acts solely as an ion-to-electron transducer between the ISM and the electronically conducting substrate.

The cell membrane is lined with narrow protein-lined pores, known as ion channel proteins; these proteins are molecular entities that catalyze the flux of ions across bilayer lipid membranes. They exhibit three essential properties: first; an ability to catalyze high transport rates: second, an ability to select between closely related ions, e.g. Na^+ and K^+, and third, regulation by external stimuli such as ligand binding or a change in transmembrane voltage, that is the property of being gated. A classification of natural ion channels

according to the most common gating stimulus are: change in membrane potential (voltage-gated), drugs or chemical transmitters (ligand-gated), and mechanical deformation (stretch-gated). Gating is thought to involve conformational changes of the ion channel which alters selective permeability. We applied gramicidin and valinomycin as the ionophores in this work.

Gramicidin is an antibiotic obtained form the bacterial species Bacillus Brevis, it is a linear pentadecapeptide usually called, gramicidin D, which is a heterogeneous mixture of six antibiotic compounds called Gramicidins A, B, and C at the levels of 80%, 6% and 14% respectively. Gramicidin has been used in the fabrication of ion-channel based biosensors [3], because this hydrophobic linear polypeptide forms channels in phospholipid membranes that are specific for monovalent cations.

Valinomycin is a potassium selective ionophore obtained from the cells of several Streptomyces strains, capable of increasing the transport of potassium across cell membranes and thereby causing damage to bacteria cells.

Eisenberg[41] considered the behavior of ion channels as enzymes, pointing out that ion channels modify the flux of ions the same way enzymes modify the flux of reactants, by stabilizing the transition state between substrate and product. Ion channels also have substrate and products just as clearly defined as do enzymes. The substrate of a channel is just the permeable ion on one side of the membrane, and the product is just the permeable ion on the other side of the membrane. The transition state of one ion-channel consist basically of a step when the ion is in the pore, there the protein provides polarization charge to neutralize the permanent charge of the permeating ion. To continue describing the analogy, the substrate and product before passing through an ion channel, have different free energies, just as they do for enzymes. Substrates and products of enzymes are different chemical species with different free energies at the same location. Substrates before and after passing through an ion channel (product) are the same chemical species, at different locations. The spatial gradient of electrochemical potential drives diffusion just as the chemical gradient free energy drives a chemical reaction. Therefore an ion channel is a catalyst for diffusion through membranes and a channel is an enzyme, even if it does not facilitate ordinary chemical reactions. Naumann and Knoll have developed an ion channel – lipid bilayer assembly using His-tag connection to the electrode offering the possibility to follow electroactive membrane protein functions (Nauman 2003, Ataka 2004). Lipkowsky have used glycolipid conjugation tethering the bilayer with the electrode (Lipkowski 2010, Brosseau 2008). The cushion acts effectively as a soft support for the bilayer membrane allowing its dynamic function. The only example of assembling valinomycin ion channel on a conducting polymer-based organic electrode has been published by Bobacka et al who physically mixed valinomycin as the ionophore with poly(octyl thiophene) and used the system effectively as a selective potassium potentiometer. We have prepared the ion channel within the bilayer and then deposit the system on a conductive polymer, polyaniline.

Thiolated PANI GC electrodes prepared were coated at the physiological temperature of 37°C, with large unilamellar vesicles (LUVs) mixed with the ion carrier protein valinomycin (mw 1111.36 g mol^{-1}). Proteins have the advantage of being amphiphilic and thus anchor within a membrane and float within it. The procedure is shown below:

Pipetted 2 mL of egg L-α-phosphatidylcholine (concentration of 20 mg/mL in chloroform) to glass scintillation vials and evaporated under a stream of nitrogen, then kept under vacuum for 1-hour. Added 1.3 mg of valinomycin , followed by 20 mL of 0.5M H_2SO_4 and vortexed for 5 minutes to make a suspension. Transferred an aliquot of the suspension onto

a 10 x 75 mm glass centrifuge tube and carefully suspended one thiolated PANI GC electrode inside the tube, with the aid of Parafilm®, such that it did not touch the bottom of the tube. Placed in a temperature controlled bath for 60 minutes at 37°C. Removed gently from the tube, and slowly washed the PTFE electrode body with Milli-Q water, never touching the bottom of the electrode. Special care was taken to protect the assembled membrane by suspending the electrode on the electrochemical cell lid and equilibrating in 10 mL of Milli-Q water until a steady potential reading was reached.

8. Potentiometric titration using valinomycin PANI modified electrode

A glass electrochemical cell containing 10 mL of Milli-Q water was outfitted with a three holed PTFE lid. The valinomycin modified electrode, and the Ag/AgCl reference electrode used two holes, while the third hole was left open to add a KCl titrant solution. The two electrodes were connected with a BNC cable to a pHmeter operating in potential mode, and allowed to equilibrate until reaching a steady absolute potential reading (approximately 60 minutes). The pHmeter display was adjusted to relative potential mode, displaying a zero value before starting the addition of titrant. The titrant potassium chloride solution was prepared as follows:
Weighed 105.6 mg of potassium chloride and transferred to a 50 mL volumetric flask, dissolved and diluted to volume with Milli-Q water for a final concentration of 2.112 mg/mL. The titration results are summarized on Appendix C, the absolute potential (ABS) at equilibrium was –268.5mV. Measurements were taken at 2 minutes intervals between additions, stirring with a magnetic bar for the first 30 seconds, then stopping and taking the reading after the remainder 1.5 minutes. This time is usually enough for the potentiometer to display a stable reading. The titration curve is shown on Figure 9 and its characteristic Nernstian plot on Figure 10.

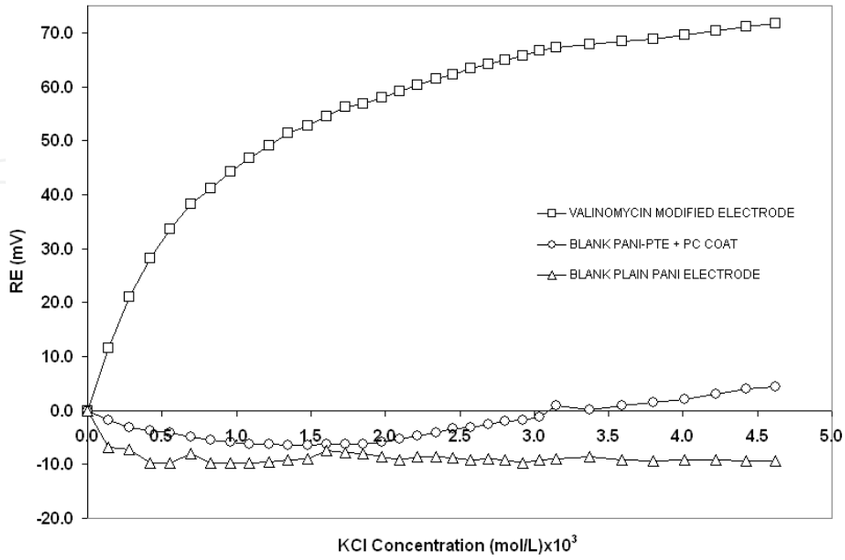

Fig. 9. Overlaid potentiometric titration curves for PANI-valinomycin modified electrode

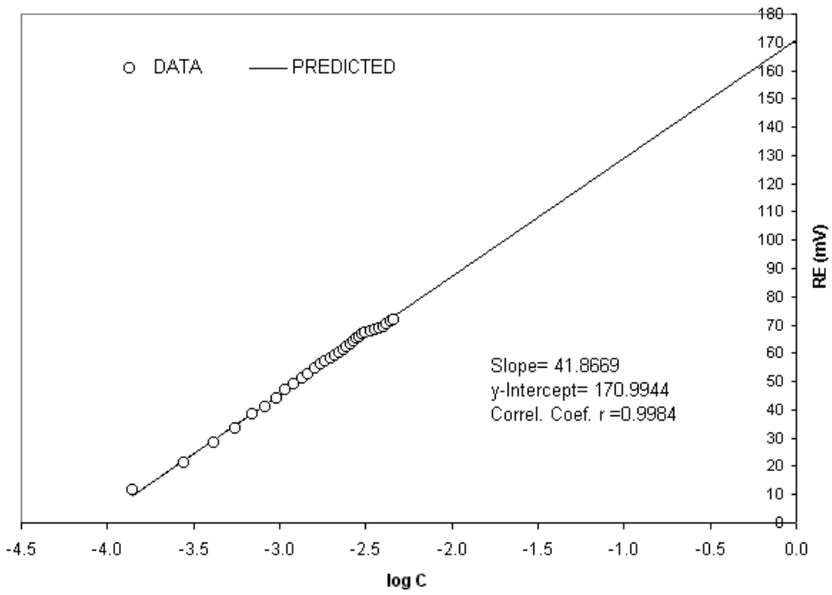

Fig. 10. Nernstian Plot for potentiometric Titration with PANI-valinomycin Modified Electrode

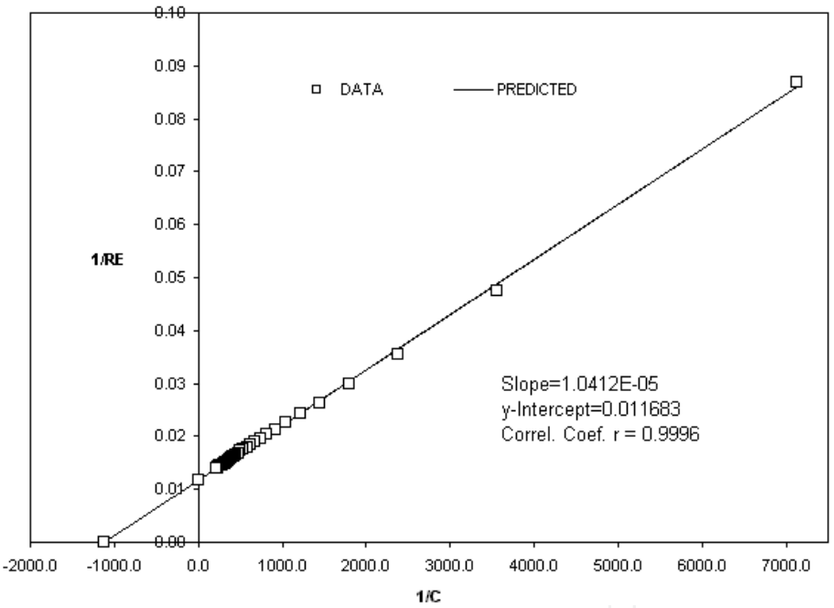

Fig. 11. Lineweaver-Burk plot for PANI-valinomycin modified electrode

The perfect fit of the titration data to the Michaelis-Menten model as shown on Figure 11, is a clear evidence of facilitated diffusion mechanism on the assembled biomimetic membrane on GC electrode.

9. Analogy to enzyme kinetics Michaelis-Menten equation

Ion carrier proteins exhibit Michaelis-Menten kinetics (Nelson 2000), and their affinity for specific ions can be measured in terms of two parameter, the Michaelis constant K_m, which

measures the affinity for the ion carrier as an analogy to enzyme-substrate interaction, and the V_{max} which measures the maximal velocity of the reaction at saturating ion concentration as represented by the rectangular hyperbolic curve.

In enzymatic reactions the slowest step is the conversion of [ES] to [E] + [P] , and in such cases the value of k_{cat} is much less than k_2, thus $K_m \cong k_2/k_1=K_d$ the dissociation constant for binding of S to E, thus the value of K_m describes the affinity of an enzyme for its substrate, and hence the stability of the enzyme-substrate complex. The smaller the value of K_m the more avidly the enzyme can bind the substrate from a dilute solution and the smaller the concentration of substrate needed to reach half-maximal velocity.

A Lineweaver-Burk plot is a graphical representation of enzyme kinetics based on the plotting of double reciprocals that facilitates the interpretation of the Michaelis-Menten plot.

The Lineweaver-Burk double reciprocal plot is notorious for distorting data, and it is unreliable for the calculation of enzyme kinetics parameters (Rose 2006), however it is very useful as a graphical representation of the different types of enzyme inhibitions, e.g. it can used to distinguish between competitive, noncompetitive and uncompetitive inhibitors. In competitive inhibition one can note that inhibitors have the same y-intercept ($1/V_{max}$) as uninhibited enzyme but different slopes (K_m/V_{max}) and x-intercepts ($-1/K_m$) are observed for the data sets.

For noncompetitive inhibition the curves have the same x-intercept but different slopes and y-intercepts. Finally in uncompetitive inhibition the curves have different intercepts on the y and x axes, however the slopes are the same.

Diffusion is a mean of passive transport; it results from the thermal, random movement of ions and molecules. There are three main types of diffusions; simple, channel, and facilitated diffusion. Simple diffusion does not involve a protein and occurs when a small, non-polar molecule passes through a lipid bilayer without being rejected. Channel diffusion involves channel proteins where material moves through an open aqueous pore. Facilitated diffusion is dependent on single ion moving along ionophores that operate on a bind, flip, and release mechanism. This type of diffusion mechanism allows hydrophilic molecules to move into the hydrophobic region of the membrane without being rejected.

The valinomycin modified lipid bilayer assembled on PANI works by selectively complexing the K^+ ions from their Cl^- counterions in the test sample solution, thereby causing a charge separation and a corresponding change in electrical conductivity in the membrane. This causes the generation of an EMF, which could be defined as the force with which positive and negative charges could be separated and it represents the potential value in the Nernst equation.

The electroconductive PANI film which is supporting the lipid membrane acts as a good ion-to-electron transducer. Because valinomycin is selective on its affinity for K^+ ions any measurable change in potential is due solely to the presence of this ion. In summary the ionophore valinomycin has an effect on the electrical properties of phospholipid bilayer membranes, such that it effects the solubilization of K^+ ions within it, thereby providing a carrier mechanism by which this ion can cross the insulating hydrophobic or hydrocarbon interior of the bilayer. When accumulated in the lipid membrane assembled on PANI electrode they cause a voltage differential which can be determined using an external reference electrode. Because only K^+ binds to the valinomycin in the membrane, the conductive path only appears for K^+. Therefore, the potential developed is attributable

solely to the K^+ concentration. The electrical neutrality of the lipid membrane is maintained by a reverse flow of H^+.

10. Potentiometric titration using gramicidin PANI modified electrode

A thiolated PANI GC electrode was coated with a suspension of the pentadecapeptide gramicidin (mw 1882.3 g mol^{-1}) in PC LUVs, and allowed to equilibrate at the physiological temperature of 37°C. The procedure is shown below:

Pipetted 2 mL of egg L-α-Phosphatidylcholine (with a concentration of 20 mg/mL in chloroform) into a glass scintillation vials and evaporated under a stream of nitrogen, then kept under vacuum for 1-hour. Added 2.8 mg of gramicidin, followed by 15 mL of 0.5M H_2SO_4 and vortexed for 5 minutes to make a suspension. Transferred an aliquot onto a 10x75mm glass centrifuge tube and carefully suspended one thiolated PANI GC electrode inside the tube with the aid of Parafilm® such that it did not touch the bottom of the tube. Placed in a controlled temperature bath for 60 minutes at 37°C, removed gently from the tube, and slowly washed the PTFE electrode body with Milli-Q water, never touching the bottom of the electrode. Special care was taken to protect the assembled membrane by suspending the electrode on the electrochemical cell lid and equilibrating in 10 mL of Milli-Q water until a steady potential reading was reached.

The titration experiment was conducted in a glass electrochemical cell containing 10 mL of Milli-Q water and outfitted with a three holed PTFE lid to support the gramicidin modified electrode, and the Ag/AgCl reference electrode, with the third hole left open to add the titrant solution. The two electrodes were connected with a BNC cable to a pHmeter operating in potential mode. The electrodes were allowed to equilibrate until reaching a steady absolute potential reading, and then switched to relative potential mode thus displaying a zero value before starting the addition of titrant.. The titration results are shown on Appendix D and are depicted graphically in Figure 12 and 13.

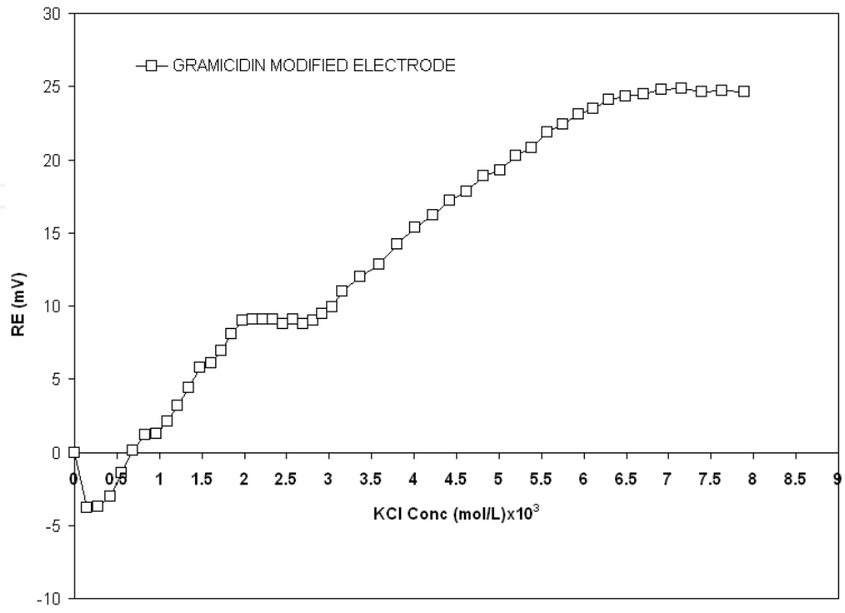

Fig. 12. Potentiometric titration curve for PANI-gramicidin modified electrode

The electrode showed a change in response at a concentration of about 0.2 mg/mL of KCl as shown on Figure 12. The trend line shown on Figure 13 includes all the data point, however a close examination of this attempted fit to a Nernstian plot, revealed what appeared to be two linear sections of different slopes separated by a horizontal segment, an a clear evidence of an apparent mixed bimodal titration mechanism as shown on Figure 14. The first segment has a smaller slope than the second segment, indicating than the binding of K^+ ions was initially slow, and then changed after reaching a concentration of approximately 0.2 mg/mL, increasing by a factor of approximately 4. The gramicidin molecule has physical dimensions[42] of 30Å-long by 15Å-wide, while the valinomycin molecule dimensions are 5.6Å-long by 15.6Å-wide. For this reason the gramicidin molecule spans about 5 times deeper into the lipid bilayer compared to valinomycin.

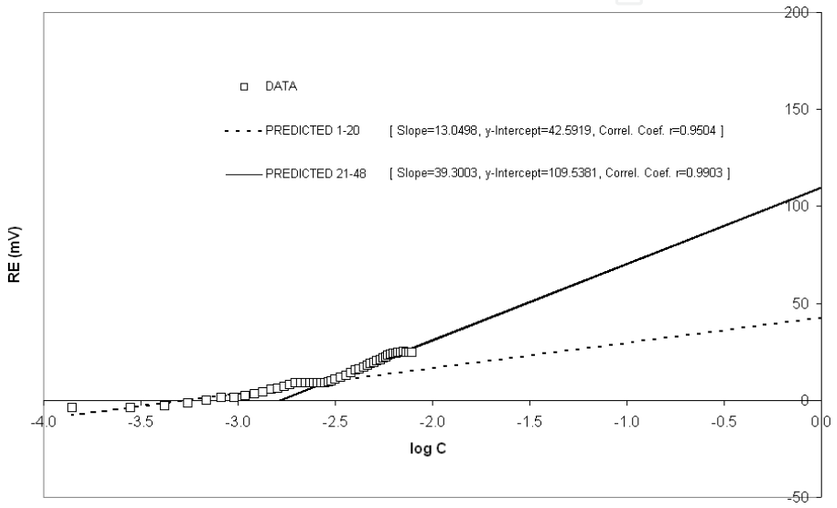

Fig. 13. Nernstian plot for potentiometric titration with PANI-gramicidin modified electrode

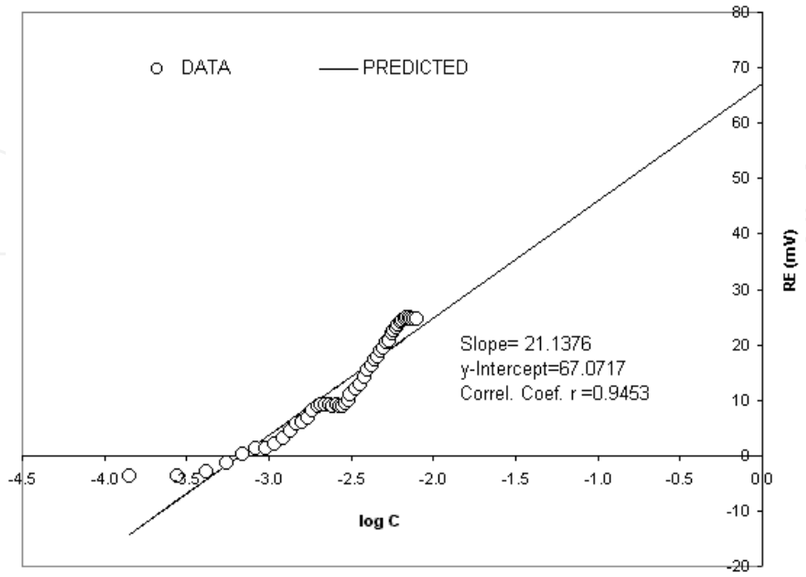

Fig. 14. PANI-gramicidin modified electrode mixed bimodal titration mechanism

The bimodal titration mechanism may be due to the fact that assembled simulated were not tethered. Tethered bilayer lipid membranes (tBLMs) offer a quasi-natural environment where membrane proteins can be embedded in and investigated. A tBLM consists of a lipid bilayer that is coupled covalently via a spacer group to a solid support on an electrode, offering homogeneity and fluidity that is important toward the biomimesis of biological membranes, and also helps accommodate larger proteins.

The suspension of gramicidin and valinomycin in PC allowed the assembly of the ionophores on the derivatized phospholipid layer on PANI GC electrodes. The ion carriers settled in the assembled phospholipid layers creating hydrophilic pores which can open and allow inorganic ions, to pass through. In general, ion carriers are quite specific for the type of solute they will transport and it occurs very fast by a mechanism called facilitated diffusion. Facilitated diffusion is a process of diffusion, and a form of passive transport that facilitates spontaneous passage of molecules or ions across a biological membrane passing through specific transmembrane transport proteins.

11. Conclusions

A successful assembly of biomimetic membranes on phospholipid derivatized PANI-GC electrode was achieved by using the ion carrier proteins gramicidin and valinomycin. The valinomycin assembled electrode showed a titration curve consistent with facilitated diffusion mechanism as this carrier has been shown to have a great affinity for the K^+ ion. Evidence of such affinity was provided by a perfect fit of the titration data to a Michaelis-Menten enzyme-substrate model. The electrode showed a great detection slope of approximately 42 mV/decade.

The gramicidin modified electrode showed a bimodal titration mechanism which was probably caused by its larger size as compared to valinomycin. No tethers were used to attach the simulated membrane to the electrode. The slope of the first segment of the titration curve was approximately 13 mV/decade, and after reaching a KCl concentration of approximately 0.2 mg/mL, the simulated membrane appeared to swell to the point of at which its fluidity improved, then the sensitivity of the electrode increased to a slope value of 39.3 mV/decade. It appeared that valinomycin is a better ion carrier to bind K^+ ions for this type of electrode, as it showed enough fluidity, and a good sensitivity without the need of tethers

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