DEVELOPING RESISTIVE-PULSE SENSORS USING ARTIFICIAL CONICAL NANOPORES IN TRACK-ETCHED POLYMER MEMBRANES

By
LLOYD PEYTON HORNE, JR.

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To my wife, Jodie, and my son, Zackary
ACKNOWLEDGMENTS

In late 2004, I left a job in the pharmaceutical industry that I had held for years to return to school full-time to pursue a Ph.D. At first, I was criticized by many and understood by few. Nevertheless, it was my strong interest in teaching and academic research that propelled me to make such a sacrifice. In simplest terms, the educational process by which one earns a doctorate boils down to just that, sacrifice. There are times of self-doubt, high times associated with experimental success, and many low times of frustration and exhaustion. There are also frequent periods of time one must go without family and friends to conduct research. To go through this process successfully and keep one’s spirit intact requires a tremendous amount of support and encouragement from others in your life. I am very fortunate to have had a great deal of support from my family, friends, and colleagues during my tenure at the University of Florida.

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The objective of this research is to develop sensors based on the resistive-pulse method using conical nanopores and investigate properties of such pores that impact their sensing capabilities. In the first section of this work, sensing of a model protein is demonstrated using a single, conical nanopore embedded in a track-etched polymer membrane. The pore surface was modified with a thin, conformal gold film and subsequently functionalized with thiol-modified poly(ethylene glycol) (PEG) to prevent non-specific protein adsorption. Single protein molecules were detected and counted as downward current-pulses as they were electrophoretically driven through the pore. The frequency of these current-pulses was found to vary exponentially with the applied transmembrane potential. Removal of the PEG and gold layers revealed current-pulses that went both upward and downward. Such a phenomenon had not been previously observed with resistive-pulse sensors constructed from track-etched conical nanopores. The impact of this effect on components of the current-pulse signature is discussed.

Previous resistive-pulse sensors derived from track-etched polymer membranes have been configured such that the net surface charge on the analyte, the surface charge of the pore wall, and electrode at the tip opening all have the same polarity (i.e., negatively-charged). The second part of this work presents an example of a resistive-pulse system where the net surface charge on
the analyte and electrode at the tip are both opposite in polarity of the pore surface (i.e., both are positively-charged). That is, the resistive-pulse sensing of a model cationic analyte, poly-L-lysine-conjugated gold nanoparticles using a single, conical nanopore in track-etched poly(ethylene terephthalate) (PET) is presented. Current-pulses were observed down to the femtomolar concentration level and exclusively upward. Such pulse direction reflects the ion current-enhancing effect of the counter-ions accompanying each nanoparticle into the nanopore sensing zone. A definition for the limit of detection in resistive-pulse sensing is presented and discussed.

The third part of this work focuses on developing resistive-pulse sensors in an alternative polymer material, polyimide. Progress towards a two step-etch method for tailoring the tip diameter of conical nanopores in polyimide is introduced. Controlling the tip opening diameter during fabrication is absolutely critical to constructing functional resistive-pulse sensors. The tip diameter was observed to scale linearly with the final value of the ion current during the two-step etch. Furthermore, the extent of ion current rectification was found to be inversely proportional to tip size at the tip sizes evaluated. An approach towards loading electrolyte into conical pores in polyimide is introduced involving the use of a wetting agent, vacuum degassing, and perfusion. Carboxylated, fluorescent nanoparticles were then detected using a single, conical nanopore in track-etched polyimide. Current-pulses were exclusively upward and detected using much lower applied transmembrane potentials than those typically used for resistive-pulse sensors housed in PET membranes. This was attributed to the large cone angle and correspondingly lower pore resistance characteristic of conical pores fabricated in polyimide.

The fourth part of this work introduces an alternative strategy to electroless gold deposition for functionalizing the surfaces of single, conical nanopores with PEG based on EDC/sulfo-NHS
coupling chemistry. Minimizing non-specific pore surface adsorption is absolutely critical in resistive-pulse sensing. X-ray photoelectron spectroscopy and ionic pore conductance were utilized to study the non-specific adsorption of three model proteins, BSA, fibrinogen, and lysozyme, to the surfaces of single, conical nanopores before and after functionalization with amine-modified PEG. The presence of the PEG was found to reduce the non-specific adsorption of each protein to varying extents. Thus, this represents progress towards producing more biocompatible nanopores for developing resistive-pulse applications for biological analytes.

The final part of this work presents cone angle studies on pores fabricated in PET membranes. The electric field strength distribution inside two single, conical-shaped nanopores having identical tip diameters but different base diameters (i.e., one large and one small) was evaluated via finite element simulations. These simulations show the electric field strength, which is directly proportional to current-pulse frequency, increases more with increasing cone angle than with increasing transmembrane potential. Thus, this provides the impetus for fabricating larger cone angle nanopores. However, before doing resistive-pulse sensing, methods for fabricating, controlling and reproducing large cone angle nanopores are needed. A high voltage etching approach in high track density PET membranes wasn’t successful due to increased resistive-heating. Thus, a non-aqueous approach to fabricating single, conical nanopores having a larger cone angle than pores typically produced via the aqueous two-step etch method is presented. Using this approach, the effect of increasing cone angle on ionic pore conductance and ion current rectification was evaluated. Increased ionic pore conductance and decreased ion current rectification were observed with the larger cone angle pores relative to those having a smaller cone angle.
CHAPTER 1
INTRODUCTION AND BACKGROUND

Introduction

Nanoscale materials have attracted tremendous interest in recent years. Such materials have been generally defined as structures comprised of one dimension of 100 nm or less in size. As the length scale of materials approaches that of single molecules, their inherent physical properties change and differ from that of their much larger, or bulk, counterparts. Consequently, such unique characteristics have found potential applications in a variety of areas including biotechnology, medicine, electronics, and energy.\(^1\)-\(^{16}\)

In particular, nanopore research constitutes one area of growing interest in nanoscience. This is due, in part, to the prevalence of highly selective and sensitive nanopores in the human body. Such biological nanopores, or ion channels, play a critical role in many key biological processes.\(^{17}\)-\(^{18}\) Therefore, biological ion channels provide an excellent paradigm for designing highly selective and sensitive chemical and biological sensors. However, there are several drawbacks from using biological nanopores which preclude their use in any practical sensing device (\textit{vide infra}). As a result, there has been significant efforts towards developing artificial nanopores as a feasible alternative.\(^{19}\)-\(^{64}\) Such synthetic analogues are attractive due to their mechanical robustness and chemical stability, controllable pore size, and easily tunable surface chemistry. Artificial nanopores also provide a vehicle in which the fundamental transport properties of biological ion channels can be investigated without dealing with their characteristic fragility.

In recent years, several technologies have been utilized for fabricating nanopores in artificial materials. Some of these approaches include focused ion beam (FIB) methods and electron beam lithography,\(^{23}\)-\(^{38}\) micromolding,\(^{39}\)-\(^{44}\) carbon nanotube-embedded polymers,\(^{45}\)-\(^{50}\)
femtosecond laser ablation, base etching of silicon wafers, and single ion-track etching. Nanopores have been constructed in a diverse array of materials including organic-inorganic hybrid materials, silicon-based inorganics, carbon nanotubes, and thin polymer membranes. There has been increasing interest in using nanopores as the sensing element for developing analytical devices based on the resistive-pulse method. This approach has been used to detect metal ions, small molecules, nucleic acids, proteins, viruses, and nanoparticles.

In the Martin group, we have been investigating the fabrication and application of conically-shaped nanopores in thin polymer membranes. These pores are prepared via the track-etch method. Track-etched membranes have been utilized to investigate fundamental transport properties, to perform bioseparations, and as templates for fabricating open tubular and solid nanomaterials (i.e., nanotubes and nanowires). Single, conical-shaped nanopores have also been used to construct resistive-pulse sensors.

The research presented herein discusses recent investigations on nanopore fabrication, resistive-pulse sensing, varying pore surface chemistry, and varying cone angle in asymmetric nanopores using thin polymer membranes. Chapter 1 presents the background information which supports the research. First, the resistive-pulse method is discussed along with examples of two different nanopore constructs: (1) those derived from biological transmembrane proteins and (2) pores fabricated in artificial materials. Then, the track-etch method is discussed along with pore characterization and surface functionalization.

The Resistive-Pulse Method

The resistive-pulse method has been around for decades. The Coulter Counter®, a commercially available instrument that counts and sizes biological cells and colloidal particles, operates on this principle. In fact, approximately 98% of all cell counters are of the Coulter®
This device was developed in the 1940s by W. H. Coulter and patented in 1953. The Coulter Counter® contains a small diameter aperture (15 μm – 2 mm) that is placed in between two electrolyte solutions and subsequently filled with electrolyte. An electrode is placed into each solution and used to pass an ionic current through this aperture. The solution of particles to be counted is added to one of the electrolyte solutions, and these particles are driven through the aperture by applying pressure or a potential difference (i.e., either a constant current or constant voltage).

When a particle enters the aperture, it displaces a volumetric fraction of electrolyte solution that is equivalent to the volume occupied by the particle. As a result, the ionic resistance of the aperture increases while the particle is present in the aperture. This is registered as a transient voltage- or current-pulse by the device. The magnitude of such pulses is proportional to the size (volume) of the particle. The frequency of these pulses is proportional to the particle concentration. Coulter Counters® can size particles that are 400 nm – 2 mm in diameter using apertures 15 μm – 2 mm. The operating particle sizing range is primarily determined and limited by the aperture diameter. Thus, to detect single molecules, nucleic acids, or proteins, this suggests that much smaller aperture diameters are required.

In fact, in the 1970s, DeBlois, et al., postulated that if they could fabricate smaller apertures, then smaller analytes (i.e., virus particles) could be measured. They used apertures, or pores, fabricated via the track-etch method (vide infra). Using a single, cylindrical pore diameter <500 nm, Deblois and coworkers were able to detect and accurately size Mason-Pfizer virus, Rauscher murine leukemia, and simian sarcoma virus particles of 140 ± 3 nm, 122 ± 2 nm, 110 ± 3 nm in diameter, respectively. Such values were found to be in good agreement with those obtained by other methods and reported in the literature. Their device could routinely
measure virus particle concentrations on the order of $10^9 - 10^{11}$ particles/mL in just a few minutes.

Although their approach was very creative and seemed promising, it was plagued with several problems: (1) they were only able to fabricate multipore membranes (pore density $>10^6$ pores cm$^{-2}$, (2) the cylindrical pore shape and large aspect ratio caused more than one particle to simultaneously reside in the pore during translocation, and (3) pore blockage due to non-specific adsorption of particles along the pore interior. Problems (1) and (2) have since been resolved using a single ion-etching technique$^{57,58}$ and conical pore geometry,$^{59,95,96}$ respectively. Problem (3) has been addressed indirectly in multiporous membranes$^{117}$ and directly with single, conical-shaped nanopores as part of the research presented herein. Nevertheless, reduction of pore diameter is a critical pre-requisite for detecting smaller size analytes.

The nanopore-based experiment is analogous to those early experiments based on the Coulter® principle. That is, a membrane containing a single, conical-shaped nanopore is immobilized between two halves of an electrolyte-filled, U-tube cell, and filled with electrolyte (Figure 1-1).$^{74,75,89,90,91,95}$ An electrode, typically Ag/AgCl, is immersed into each half-cell. A transmembrane potential difference is applied across the nanopore, thereby generating an ionic current which is measured as a function of time. When a charged analyte is driven via electrophoresis into the pore, it displaces a volumetric fraction of electrolyte that is related to analyte size (i.e., molecular volume) and increases the pore resistance. As a result, a transient decrease, or blockage, of the ionic current occurs. This phenomenon is generally observed as a downward current-pulse.$^{74,75,89,90,91,95}$ That is, the ionic current during a current-pulse is less than the baseline current. As mentioned previously, analyte concentration is directly proportional to the frequency of such current-pulses.$^{89,95}$ Analyte identity, or selectivity, is encoded in both the
magnitude and duration of the current-pulses. The duration of these current-pulses is also related to effective surface charge on the analyte.

Recent research efforts in resistive-pulse sensing have been focused on the detection and characterization of smaller size analytes such as ions, small molecules, nucleic acids, proteins, and small particles. As discussed in a later section, there are advantages for using conical-shaped nanopores derived from ion track-etched materials for developing such sensors. Such artificial nanopore systems have been used to detect small molecules, proteins, nanoparticles, and DNA. Biological nanopores have also been utilized as the sensing element in resistive-pulse devices.

**Biological Nanopores**

Biological nanopores, or ion channels, represent a broad class of highly selective and sensitive transmembrane proteins that play critical roles in many key biological processes. As a result, biological ion channels provide excellent archetypes for designing chemical and biological sensors. Ion channels are generally comprised of proteins and/or subunits thereof that assemble to form a selective passage barrier between one region and another (e.g., separating the extracellular environment from the intracellular space of cells). In simplest terms, these channels regulate the selective transport of ions and neutral molecules into and out of the cells. As a result, such channels mediate cellular communication.

Biological ion channels are generally categorized according to how they function. For example, ligand-gated ion channels alternate between “open” and “closed” states based upon the corresponding binding and release of a ligand, or chemical messenger. While in the “open” state, ion transport occurs; however, while in the “closed” state, ion transport is blocked. One of the most widely studied ligand-gated channels is the nicotinic acetylcholine receptor. These receptors selectively bind the neurotransmitter, acetylcholine. This recognition process causes a
conformational change in the receptor and subsequent opening of the ion channel.\textsuperscript{17,18} Voltage-gated ion channels represent another type of ion channel which function based on a cell membrane potential.\textsuperscript{127} Examples of such channels regulate both nerve signal transduction (i.e., action potentials of axons) and muscle contraction (i.e., cardiac and skeletal muscle).

Additionally, ion channels were the subject of the 2003 Nobel Prize in Chemistry which was jointly awarded to Peter Agre and Roderick MacKinnon for their discoveries on aquaporins and potassium ion channels, respectively. Aquaporins are transmembrane channels that regulate the passage of water molecules across the cell membrane.\textsuperscript{128-131} Such channels are present in most all organs including the gastrointestinal tract, kidneys, and nervous system. Thus, aquaporins play a key role in maintaining homeostasis and offer potential applications for addressing major medical problems. Potassium channels play a central role in many key biological processes as well. MacKinnon, et al. first explained the selectivity of such channels for potassium ions.\textsuperscript{132-138} That is, these channels only permit potassium ions to pass through. Other ions, such as sodium, are rejected due to their smaller size with a selectivity ratio \((K+/Na+)\) of 10,000/1.\textsuperscript{136}

Moreover, due to their widespread importance, biological transmembrane channels are one of the most widely studied platforms for developing resistive-pulse sensors. Research conducted on such channels has provided the impetus for developing molecular scale, resistive-pulse sensors. Sensors based on \(\alpha\)-hemolysin,\textsuperscript{66-70,78,79,82,83-88,93,94,118,119,121,126} maltoporin,\textsuperscript{139} and the outer membrane protein F (OmpF)\textsuperscript{140} have all been described, but \(\alpha\)-hemolysin is the most commonly utilized and remains the benchmark by which all other resistive-pulse sensors are compared.
Alpha-hemolysin (α-HL) is a transmembrane protein exotoxin comprised of 293 acids and produced by *Staphylococcus aureus*. When α-HL comes in contact with a lipid-bilayer membrane, it self-assembles into a nanoscale pore that penetrates the membrane (Figure 1-2). This channel provides a very narrow passageway between the cell interior and extracellular environment. For this reason, α-HL has been implicated in cell lysis. This nanopore also provides a highly sensitive and selective detection passageway that has been used to detect a wide variety of target analytes via the resistive-pulse method. Either native or engineered forms of α-HL have been utilized to detect enantiomers of drug molecules, DNA, nitroaromatic compounds, metal ions, small organic molecules, anions, proteins, and polymers.

The typical resistive-pulse experiment begins with α-HL embedded in a planar lipid bilayer support. An appropriate electrolyte solution is placed above and below a α-HL-embedded lipid bilayer membrane. Electrodes are inserted into each solution and an applied potential difference causes ionic current to flow through the interior of α-HL by the movement of cations and anions present in the electrolyte. This ionic current quickly reaches and maintains a steady-state as long as analytes are not present. When an analyte is introduced into the electrolyte, it is driven by electrophoresis into α-HL where it transiently increases the pore resistance as it enters and passes through the lumen.

There are several advantages for using α-HL to construct resistive-pulse sensors. First, α-HL is commercially available and its structure is known. Furthermore, the pore size is reproducible and can be achieved from one bilayer to the next. The key advantage is that highly selective molecular recognition capabilities can be imparted into the lumen of the α-HL protein via chemical and genetic engineering. For instance, nucleic acids have been selectively detected with an α-HL pore that was chemically modified with a covalently attached single
Similarly, arginine-modification of the α-HL lumen has provided a means to selectively detect phosphate ester anions. Metal ions have also been detected by introducing four histidine resides in the lumen.

Despite all of the sensitivity and selectivity that stem from these approaches with α-HL, several major drawbacks persist. As mentioned above, the fragility of the lipid bilayer membrane housing the α-HL pore precludes the use of this protein nanopore in any practical sensing technology. That is, the lipid bilayer membrane only lasts for a few hours before rupture, which is too short of time for any repeat-use analytical device. Furthermore, planar lipid bilayers are incapable of withstanding the broad range of applied potentials, temperatures, and pHs that artificial pores can endure. The use of α-HL in resistive-pulse sensing is applicable only to very small analytes because the inner constriction of α-HL is ~1.5 nm. Although α-HL-based devices have these disadvantages, α-HL remains the standard by which other resistive-pulse sensors are compared. Consequently, recent research efforts have focused on the development of resistive-pulse sensors based on nanopores derived from artificial materials.

**Artificial Nanopores**

By replacing the biological nanopore and lipid bilayer with an artificial nanopore, much greater chemical stability and mechanical robustness can be achieved. Nucleic acids, proteins, and nanoparticles have been detected with such artificial pores. For example, Crooks, et al. embedded a single carbon nanotube within an epoxy resin. This resin was microtomed and utilized as the sensing element in a resistive-pulse detector for nanoparticles of different surface charge densities. Their carbon nanotube device was also used to study DNA transport using fluorescence microscopy.

The most commonly utilized fabrication methods involve the application of either ion or electron beams to create nanopores in silicon oxide and silicon nitride membranes. Golovchenko
used a focused ion beam (FIB) method to fabricate nanopores in Si₃N₃ membranes,¹²,¹³ and Letant, et al. used FIB for making pores in silicon membranes.¹⁴ Bashir, et al., and other groups have utilized electron-beam approaches to fabricating pores in SiO₂.²⁸,³⁰,³⁵ Similarly, Timp, et al. employed a electron-beam method for creating pores in Si₃N₃.³¹ Such pores have been used to primarily study DNA but some protein studies have also been conducted.

Sohn, et al. reported another, rather unique method of artificial pore fabrication based on the micromolding of poly(dimethylsiloxane) (PDMS).⁴⁰-⁴⁴ Such pores have been utilized to detect λ-phage DNA and colloidal nanoparticles, as well as directly study antigen-antibody interactions on antibody-conjugated colloidal particles. Nanopores in PDMS have also been used in multianalyte immunoassays to detect human granulocyte and macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) antigens. Mayer, et al. developed a laser ablation fabrication technique in which a femtosecond-pulsed laser is utilized to drill a conical pore in glass.⁵¹-⁵³ Such pores have been used to detect viruses, virus-antibody complexes, and particles.

Additional methods of fabricating single nanopores include etching silicon wafers under alkaline conditions,⁵⁴ and track-etching of surfaces that have been tracked via swift heavy-ion irradiation.⁵⁵-⁶⁵ The Martin group has used such a track-etch approach to fabricating single, conical-shaped nanopores in a variety of different polymer membranes⁷⁴,⁷⁵,⁸⁹,⁹⁰,⁹⁵,⁹⁶ as well as muscovite mica.¹⁰⁶,¹¹³,¹⁴⁶ These conical pores have been utilized for the resistive-pulse detection of single-stranded phage and double-stranded plasmid DNA,⁸⁹ small double-stranded DNAs,⁹⁰ small molecules,⁷⁴,⁷⁵ proteins,⁹⁵ and nanoparticles.⁹⁶
Fabrication of Conical Nanopores in Polymer Membranes

Ion Track-Etch Methodology

The ion track-etch method has been practiced commercially for decades to make a wide variety of pore diameters that are available at many different pore densities. That is, membranes containing pore diameters ranging from 10 nm to as large as 10 μm, with pore densities on the order of $10^5$-$10^9$ pores cm$^{-2}$ are commercially available. These porous materials have been commonly utilized for filtration applications (e.g., laboratory, cell culture, and process filters), as templates for fabricating tubes and wires, and for investigating fundamental transport phenomena.

In the commercial fabrication process, the ion-tracked membrane is submerged into an appropriate etching solution, or etchant, which etches preferentially along the damage track from both sides of the membrane. As a result, cylindrical pores are created. The pore density is determined by the ion-track density, which is governed by the exposure time of the membrane to a collimated beam of high energy particles emanating from a cyclotron or nuclear reactor. Pore diameter is determined by exposure time to the etchant as well as etchant temperature.

This method has been further modified such that membranes containing a single ion damage track can be constructed that provides a substrate for fabricating single nanopores. Such single pores are required for resistive-pulse sensing studies.

Formation of Latent Damage Tracks

As briefly mentioned above, the track-etching of nanopores begins with the irradiation of a membrane with heavy ions (Figure 1-3). That is, the process begins when swift, high-energy particles (1-10 MeV/nucleon), emitted from either a linear accelerator, cyclotron, or nuclear reactor, strafe a polymer membrane. This bombardment with heavy ions cuts completely through the polymer matrix, thereby creating a latent damage track spanning the entire thickness of the
membrane (typically, 5-10 \(\mu m\)). The number of such latent damage tracks formed in this process represents the approximate number of nanopores (typically cylindrical) generated by subsequent chemical etching. In other words, a single ion track yields a single nanopore embedded in the polymer surface. Multiple ion tracks produce many monodisperse nanopores.

Single-track membranes are produced via a technology developed at Gesellschaft fuer Schwerionenforschung (GSI) in Darmstadt, Germany.\(^{57,58}\) In this method, the heavy ion beam is defocused to lower the ion flux. Both a metallic filter containing a 200 \(\mu m\) diameter aperture and automatic shutter, or impenetrable gate, is placed in the beam path and between the ion beam source and the membrane target. An ion detector is placed on the other side of the membrane.

Polymer membranes are typically irradiated in a stack of 5-7 membranes at one time. This stack of membranes is loaded onto a plastic cartridge and placed into an autosampler that enables remote, automated control and efficiency over the irradiation process. When an ion completely traverses the filter aperture and membrane stack, it is detected on the opposite side by the detector and the shutter closes, thereby turning off the ion beam. This prevents the membranes from being further exposed to additional ions. As a result, the polymer membrane contains only one latent damage track. The success of this ion irradiation technology is largely dependent on the energy of the irradiating ions, type of material being irradiated, and post-irradiation storage conditions.\(^{97}\)

**Chemical Development of Damage Tracks**

After irradiation, the latent damage track is etched chemically to obtain conical-shaped nanopores. The process, developed by Apel, et al., begins with the immobilization of the ion-tracked polymer membrane between two halves of an electrochemical, U-tube, cell (Figure 1-4).\(^{59}\) A suitable etching solution that etches the damage track is placed on one side of the
membrane, and a “stopping” solution that “stops,” or neutralizes, the etchant on the other side. The etchant preferentially etches the damage track from the etchant side of the membrane to the stopping solution side. When the etchant has broken through to the stopping solution, neutralization of the etchant occurs. The etching process ends by placing the nanopore membrane briefly in stopping solution and subsequently rinsing with water. This anisotropic etch/neutralization method results in a single, conical-shaped nanopore with a large diameter ("base") opening on the etchant side and a small diameter ("tip") opening facing the stop solution side (Figure 1-5).

During the etching process, a platinum electrode is placed into each half-cell. The positive electrode (anode) is placed in the solution on the base opening (etchant) side. The negative electrode (cathode) is placed in the solution on the tip opening (stopping solution) side. A transmembrane potential difference (typically +1 V) is applied and the ion current across the membrane is measured with a picoammeter. This process serves several key purposes. First, it provides a means of determining exactly when the etchant has broken through the membrane to the stop solution. That is, prior to breakthrough, the current through the growing pore is zero. The moment of breakthrough is signaled by a sudden increase in the ion current (Figure 1-6).

Secondly, after breakthrough, the applied transmembrane potential creates an “electro-stopping” effect. For example, hydroxide and formic acid are commonly used as the etching and stopping solutions, respectively, for etching pores in polymers such as poly(ethylene terephthalate) (PET). When the anode is placed into this etching solution, the hydroxide anions are electrophoretically driven away, or impeded, from the tip opening. Since formic acid neutralizes this lowered hydroxide concentration at the tip, the net effect is a decreased etch rate at the tip opening. This helps generate conical-shaped nanopores having ultrasmall tip diameters.
approaching 1-5 nm. Furthermore, the magnitude of the ion current is related to the pore diameter. Thus, by monitoring the ion current, an approximate pore diameter can be determined (vide infra).

Another benefit of applying a potential during etching is that the cone angle of the conical nanopore in some polymers (e.g., polycarbonate) can be controlled at-will by varying the transmembrane potential applied during etching. That is, cone angle increases with applied potential. However, in other materials, such as high ion track-density PET, this approach is problematic. As a result, etching methods have been developed to control the cone angle in PET. For example, by introducing ethanol into the aqueous hydroxide etchant, the cone angle can be increased by increasing the ethanol/water ratio. As will be discussed later, an entirely non-aqueous method has been developed using potassium hydroxide dissolved in 100% methanol. Additives, such as surfactants, have also been utilized to slow down what is known as the bulk etching rate, \( v_B \).

The geometry of the nanopore is determined by the track-etch ratio, \( \frac{v_T}{v_B} \), where \( v_T \) represents the track-etch rate and \( v_B \) is the bulk-etch rate (Figure 1-7). The track-etch rate is defined as the rate in which the etchant etches down the long axis of, or parallel to, the latent damage track. The track-etch rate is determined by several factors including etching conditions (e.g., etchant composition, concentration, temperature), post-irradiation treatment (e.g., UV treatment), sensitivity of the polymer to ion-tracking, and polymer type.

The bulk-etch rate is the rate in which the etchant etches radially, or perpendicular, to the damage track. The bulk-etch rate is governed by etchant concentration, composition, and temperature. Pore shape is often described using the cone half-angle, \( \alpha \), which is the inverse of
the track-etch ratio, or $\nu_B/\nu_T$. When $\nu_B/\nu_T$ is large, the nanopore is conical-shaped (large $\alpha$). Conversely, when $\nu_B/\nu_T$ is small, a cylindrical pore is obtained (small $\alpha$).

To make functional resistive-pulse sensors using conical nanopores, it is absolutely critical that both the base and tip opening diameters are known and can be reproducibly fabricated and controlled. In other words, validation of any nanopore method of analysis, in terms of instrumentation variability, requires comparable nanopore dimensions from membrane to membrane. The previous described etching method, or “anisotropic etch,” provides excellent control and reproducibility of the base opening diameter. This is accomplished by performing the anisotropic etch for a certain amount of time (i.e., for PET).

For example, to control the base diameter in PET, the polymer is first subjected to UV irradiation ($\lambda = 320$ nm) for ~ 12 hours to sensitize the latent damage track. Then, the anisotropic etch is performed by placing 9 M NaOH on one side of the membrane and 1 M formic acid with 1 M KCl on the other side.$^{59,62}$ A +1 V potential difference is applied across the membrane while the OH- catalyzes the hydrolysis of the ester linkages of PET, thereby leaving the polymer surface populated with carboxylate and hydroxyl groups.$^{110}$ This anisotropic etch is generally performed for a preset amount of time. In the case of PET, this process is allowed to run for 2 hours and produces a base opening diameter of $520 \pm 45$ nm.$^{63}$ However, the tip opening diameter varies much more from membrane to membrane due to the interfacial mixing of reacting etchant and stopping solutions at the tip. Therefore, to solve this problem and fine tune the tip diameter, a second, or isotropic, etch step was developed by Wharton, et al.$^{63}$

This isotropic etch begins with an analogous experimental setup as that used in the previous, anisotropic etch but with two differences.$^{63}$ First, a more dilute etching solution, 1 M NaOH, is placed on both sides of the membrane to facilitate a decreased and controllable bulk
etch rate. It is believed that etching occurs uniformly along the entire length of the conical pore. In other words, both the base and tip opening diameters increase at the same rate. The second difference is that instead of etching for a predetermined amount of time, etching is stopped at a predetermined value of the ion current (Figure 1-8). Thus, the tip opening diameter can be directly correlated to the ion current value as Wharton, et al. showed. If etch time was used instead of ion current, the large variability in tip opening diameter obtained from the anisotropic etch step would be preserved. As a result, this combination of anisotropic and isotropic etching steps provides precise and accurate control over all of the important dimensions of single nanopores.

**Nanopore Materials**

Many materials have been utilized to fabricate nanopores via the track-etch method. Polymer membranes are most commonly used because of their excellent response to the ion-tracking process. They also possess excellent chemical stability and mechanical robustness under a wide range of conditions and are relatively inexpensive. Polymers such as poly(ethylene terephthalate) (PET), poly(imide) (PI, Kapton®), poly(carbonate) (PC), poly(propylene) (PP), and poly(vinylidenefluoride) (PVDF) have been used to fabricate track-etched nanopores. The chemical structure of the 3 most frequently used polymer membranes, PET, PC, and Kapton®, are shown in Figure 1-9. In addition to polymers, conical nanopores have been fabricated in inorganic materials such as glass, muscovite mica, and silicon nitride via the track-etch method.

With such a diverse array of materials available to make nanopores, the etching conditions (i.e., etchant composition, concentration, temperature, stop solution type) vary for each material. As mentioned previously, the latent damage track in PET is etched using 9 M NaOH and a stopping solution comprised of 1 M formic acid and 1M potassium chloride.
Track-etching is performed at ambient temperature using an applied transmembrane potential of +1 V (i.e., anode placed into the etchant). The alkaline etchant catalyzes hydrolysis of the ester bonds in PET. This reaction leaves a nanopore surface populated with both carboxylate and hydroxyl groups. Acid-catalyzed ester hydrolysis also occurs but at a much slower rate due to a more involved reaction mechanism.

Similarly, conical nanopores are fabricated in ion-etched poly(carbonate) using either 9 M NaOH or KOH as the etching solution and 1 M formic acid as the stopping solution. The process is monitored by applying a transmembrane potential of +1 V (i.e., anode placed into the etchant). As mentioned previously, higher potentials can be applied to increase the base diameter and cone angle in low ion-track density (50 ion tracks/cm²) poly(carbonate).

By comparison, ion damage tracks in Kapton® membranes are typically etched using sodium hypochlorite (NaOCl) containing an active chlorine content of 13%, 1-2 M potassium iodide (KI) as the stopping solution, and an applied transmembrane potential of +1 V (i.e., anode placed into the etchant) at a temperature of 50°C. Upon etchant breakthrough at the pore tip opening, an oxidation-reduction reaction takes place in which iodide ions catalyze the reduction of hypochlorite ions to produce chloride ions. The reaction provides iodine which is yellow in color. Thus, this color change signals when membrane breakthrough occurs. This etching process proceeds via the hydrolysis of the imide bonds of Kapton® and, like PET, generates a carboxylate-covered pore surface.

Ion-tracked glass and muscovite mica membranes can also be chemically etched to fabricate conical nanopores. The latent damage track is typically etched with hydrofluoric acid (HF) with a stopping solution of calcium chloride. Such thin glass membranes are very inexpensive, have a small track-etch ratio, and exhibit excellent reproducibility.
Muscovite mica is a material that has received a great deal of attention recently due to its crystallinity, and large degree of hydrophilicity.\textsuperscript{106,146,156} Like glass, ion-tracked mica is etched using a hydrofluoric acid etchant but either a calcium chloride or sodium hydroxide stopping solution (\textit{vide infra}). However, fabrication of conical nanopores in mica is quite challenging due to a relatively high track-etch ratio (large $v_B/v_T$). Thus, the hydrofluoric acid penetrates the entire membrane so quickly that an appreciable amount of bulk material cannot be etched away fast enough to generate any asymmetry in pore shape.

Two approaches have been developed to fabricate conical pores in mica. The first method entails etching a cylindrical pore using hydrofluoric acid.\textsuperscript{106} Then, this pore is completely filled with a material, such as silver, which can be used to slow down the track-etch velocity in a subsequent etch step. In this second step, a mixture of nitric acid and hydrofluoric acid is used. The nitric acid etches away the silver nanowire along the track while the hydrofluoric acid etches away bulk material surrounding the wire. This results in a conical-shaped nanopore.

The second method for making conical pores in mica involves a multi-cycle etching procedure.\textsuperscript{146,156} During each cycle, the ion-tracked mica membrane is etched in concentrated hydrofluoric acid (10 M) on one side of the membrane for a set period of time and then stopped. Sodium hydroxide is used as the stopping solution. In each subsequent etch cycle, the membrane is etched in the same manner. As a result, the degree of asymmetry slowly increases with increasing cycle number, thereby producing a conical-shaped nanopore.

As mentioned previously, the track-etch ratio, $v_B/v_T$, determines the asymmetry of the nanopore. Both the bulk- and track-etch rates vary amongst different materials used for pore fabrication. For instance, the bulk-etch rates of Kapton® and PET are $0.42 \pm 0.04 \mu$m/hour and $\sim 2.17$ nm/min, respectively.\textsuperscript{59,62,99} The track-etch rate of Kapton® is $3.12 \pm 0.65 \mu$m/hour for a
12.5 µm thick Kapton® membrane.\textsuperscript{99} For PET, the track-etch rate is \( \approx 10 \) µm/hour for a membrane having a thickness of 12 µm.\textsuperscript{59} Therefore, PET membranes etch must faster (i.e., have shorter breakthrough times) than Kapton® membranes of comparable thickness. However, since Kapton® has a much larger \( \alpha \) value (\( \nu_B/\nu_T \)), the cone angles and base diameters of nanopores fabricated in Kapton® are much larger than those made in PET.\textsuperscript{59,62,99}

Despite having the ability to fabricate single, conical-shaped nanopores in a wide variety of materials, no material has yet emerged as the one material best-suited for constructing resistive-pulse sensors.

**Nanopore Characterization and Properties**

To develop resistive-pulse sensors using single, conical-shaped nanopores, it is absolutely crucial that the pore dimensions and geometry are accurately known. Without knowing such key parameters as the base and tip opening diameters, it would be quite challenging to successfully construct a functional sensor. As will be described below, an accurate base diameter is required to calculate an accurate tip diameter. This tip diameter must be comparable to the hydrodynamic diameter of the target analyte in order to observe a detectable analyte signal.

Therefore, scanning electron microscopy is typically used to accurately determine the base diameter. As will be discussed below, there are several approaches for doing this. For measuring the tip diameter, an electrochemical method based on the ionic conductance of the electrolyte-filled nanopore is utilized. Moreover, the conical shape presents several advantageous characteristics that make a conical pore more suitable than a cylindrical geometry for resistive-pulse sensing. The conical shape also provides a means for achieving ion current rectification.
Scanning Electron Microscopy

Field-emission scanning electron microscopy (FE-SEM) is generally used to accurately measure the base opening diameter of conical nanopores (Figure 1-10). The base diameter obtained after the anisotropic etch along with the bulk etch rate are determined from FE-SEM measurements on track-etched, multi-pore membranes (i.e., pore density ~10^6 pores cm^-2). Multi-pore membranes are utilized for two reasons. First, it is simply easier to find a pore when the pore density is large. Hence, this makes the imaging more practical. Secondly, intramembrane base diameter and bulk etch rate reproducibility can be more accurately determined by taking such measurements on a large number of pores.

The base opening diameter is governed by the bulk etch rate. Therefore, by multiplying the value for the bulk etch rate times the total etching time, the base opening diameter obtained during the anisotropic etch step can be calculated. For example, as mentioned previously, the reported bulk etch rate for PET is ~2.17 nm/min. This value was obtained from FE-SEM images. Since the anisotropic etch of ion-tracked PET is performed for 2 hours, based on this etch rate, the base diameter should be ~520 nm. Experimentally, a base opening diameter 520 ± 45 nm has been determined.

In addition to using track-etched, multi-pore membranes for measuring pore diameter, two approaches have been developed for imaging much lower pore density membranes. For example, pores in low pore density (50 pores cm^-2) polycarbonate have been imaged by first sputter-coating the membrane surface with a metal. As a result, visible light can only penetrate the membrane by going through the pores. A fluorescent dye, fluorescein isothiocyanate (FITC), is then placed beneath the membrane and allowed to penetrate the pores for subsequent fluorescence microscopy. This enables the isolation of a single pore for FE-SEM.
The second approach was developed in conjunction with the research presented herein. As will be discussed later, this entails first sputter-coating the track-etched membrane with a metal. Then, the membrane is placed on top of a metallic mask that has the same diameter as the membrane. This metallic mask is comparable to the filter aperture utilized for ion-tracking. That is, a 200 μm diameter hole resides in the center of the metallic mask. An ink pen is used to trace this hole onto the center of the sputter-coated membrane. This effectively reduces the search area for subsequent FE-SEM to 200 μm and provides a more efficient way to find and image a pore housed in a single pore membrane.

Despite measuring the base diameter via FE-SEM and calculating the tip diameter using an electrochemical method (vide infra), it is important to accurately determine the geometry of the nanopore. The shape of the nanopore can be characterized by making gold replicas of the pores and imaging them with FE-SEM.64,65,114 These gold replicas are produced using an electroless gold deposition method. This process entails completing filling in the pores with gold and depositing a thin film of gold on both faces of the membrane. To image the gold replicas, two approaches can be used. First, the gold surface layer on both faces on the membrane can be removed, followed by dissolution of the membrane and subsequent filtration of the gold replicas.64,65 In the second approach, only the gold surface layer on the tip side is removed. Dissolution of the membrane reveals an array of gold replicas standing up on the surface. Gold nanocone arrays have been produced this way and constitute part of the research presented herein.65,114 Either strategy provides the means to accurately characterize the geometry of nanopores.
Ionic Conductance Measurements

It is important to know the diameters of both the base and tip openings, the length, and geometry of track-etched nanopores. Because the base diameter is typically large (~500 nm or higher), the base size can be determined by FE-SEM, as mentioned previously. However, resistive-pulse sensing of small analytes often requires tip opening diameters that are too small to be determined via FE-SEM. Therefore, an electrochemical method is utilized. This approach entails mounting the nanopore-containing membrane in an U-tube cell. The setup is analogous to that used in track-etching. An electrolyte of known ionic conductivity is placed into each half-cell and allowed to fill the conical nanopore. A Ag/AgCl electrode is immersed into each half-cell solution. The transmembrane potential is scanned linearly in stepwise fashion while measuring the resulting ion current flowing through the nanopore. As a result, a current-voltage curve is obtained in which the slope represents the ionic conductance, G (in Siemens, S), of the electrolyte-filled nanopore. When the tip diameter is very small, rectification of the ion current can occur (i.e., when the thickness of the electrical double layer is comparable to the pore radius). In this case, the linear portion of the current-voltage curve (i.e., from -0.2 V to +0.2 V) is used to determine the value of G. The ionic conductance of a single, conical-shape nanopore is described by:

\[ G = \frac{\sigma \pi d_{\text{base}} d_{\text{tip}}}{4L} \]  

(Eq. 1.1)

where \( d_{\text{base}} \) is the base opening diameter determined from FE-SEM, \( d_{\text{tip}} \) is the tip diameter, \( L \) represents the length of the nanopore (equivalent to membrane thickness), and \( \sigma \) is the specific conductivity of the electrolyte solution (S m\(^{-1}\)). Therefore, the tip diameter can be calculated from experimental determinations of \( G \) and \( d_{\text{base}} \). The above relationship can be thoroughly
applied to conical nanopores only after the anisotropic etching step. Tip diameters following the anisotropic etch generally range from 1-7 nm.

During the isotropic etch step, the pore is etched at both the base and tip openings at the same rate. Therefore, this must be taken into account when calculating the tip opening diameter. Wharton, et al. developed a mathematical model that accounts for this change and provides the basis for calculating the tip diameter from the ionic conductance (G) obtained after the isotropic, or second, etch step. This model shows that the change in the base diameter is negligible when the tip diameter is very small (e.g., ≤ 50 nm). Thus, the equation above can be used for calculating small tip diameters.

It is important to note that there are three assumptions made when determining the tip diameter of a single, conical-shaped nanopore via Eq. 1.1. First, it is assumed that the conductivity of the electrolyte within the nanopore is comparable to the bulk electrolyte conductivity. Such an assumption is only valid at high salt concentrations. Thus, current-voltage curves are typically obtained using 1 M KCl. As the ionic strength of the bulk electrolyte decreases, the electrical double layer that forms along the interior of the pore begins to play a role in the pore conductivity.

The second assumption is that the pore geometry is that of either a perfect cylinder or cone. For this reason, pore shape is verified by fabricating gold replicas, as described previously. Lastly, it is assumed that the bulk etch rate obtained from multipore-membranes is comparable and transferrable to that for a single nanopore membrane.

**Electric Field Strength and Distribution**

It is important that the nanopore sensing element be conically-shaped for several reasons. First, the conical shape focuses the electric field at the tip and extends the electric field into the immediate bulk solution. For instance, let’s assume a membrane contains a single
nanopore that is cylindrically-shaped and has a pore diameter of 20 nm. When a transmembrane potential difference is applied, the potential is dropped across both in the bulk solution in contact with the pore and the solution inside the pore. Consequently, with such small pore diameters as this, the potential drop occurs mainly inside the pore and less in the bulk solution in contact with the pore. If the pore length is 12 μm and a 700 mV potential difference is applied, then the electric field strength is approximately on the order of $10^4$ V/m inside the entire pore. However, when the pore is conically-shaped, the electric field strength is highly focused at the tip opening and reaches into the solution around the tip. This is because the pore resistance ($R_{\text{pore}} = 1/G$) is inversely proportional to the product of the base and tip diameters (Eq. 1.1). Thus, the potential drop in the solution at the base opening is insignificant when compared to the potential drop at the tip opening. Secondly, simulations show that the electric field strength within the tip opening is enormous.

For instance, Lee, et al. using a finite element approach to simulate the electric field strength magnitude and distribution within a single, conical nanopore ($L = 6 \mu m$) with tip and base opening diameters of 60 nm and 2.5 μm, respectively. The electrolyte was 1 M KCl with an applied potential difference of 1 V. The results of this simulation are shown in Figure 1-11. In Figure 1-11, we see that the electric field strengths in the solutions above and below the conical nanopore are rather small. However, in the expanded view of the tip region, we observe that the electric field strength within the tip opening and in the bulk solution contacting the tip is on the order of $10^6$ V/m. In other words, the potential drop is highly focused at the tip opening of the conical nanopore. Furthermore, when the base diameter is held constant at ~520 nm and the tip diameter is varied between 10-30 nm, such finite element simulations show that the electric field strength inside the tip increases as the tip opening diameter decreases.
As a result, this field focusing effect creates an extremely sensitive sensing zone within the tip opening region.\textsuperscript{75,89,95,96} Only analytes that migrate into this region will have an impact on the ionic current flowing through the pore. This detection mechanism has provided the means to detect small molecules, DNAs, proteins, and nanoparticles as they’re electrophoretically driven into and through the sensing zone. The length of this sensing zone represents the distance from the tip opening where most of the electric field is focused. Heins, et al. defined the sensing zone, or effective length, as the length over which 80\% of the voltage is dropped.\textsuperscript{75} For example, if the base diameter is 5 \( \mu \)m and the tip diameter is 20 nm, finite element simulations show that the conical nanopore has an effective length of 50 nm. The effective length can be tailored to match the size of a target analyte by varying the cone angle of the nanopore.\textsuperscript{97}

**Rectification of Ion Current**

Another important property of single, conical-shaped nanopores is that they can rectify the ion current. This phenomenon is observed when the current-voltage curve obtained for a pore is non-linear.\textsuperscript{18,19} In other words, the absolute value of the ion current measured at a one polarity (e.g., -1 V) is not equal to the absolute value of the ion current at the same voltage, but opposite polarity (e.g., +1 V). This asymmetry is attributed to the preferential transport of either cations or anions through the nanopore tip opening. In fact, the term “rectification” originates from electronics, where it describes devices that conduct electrons in only one direction.\textsuperscript{97} For example, under certain conditions, a conical-shaped nanopore in PET or Kapton preferentially transports cations from the tip opening towards to base opening.\textsuperscript{100-103} This is reflected in a non-linear current-voltage curve.

Although the details are still being debated, several models have been reported to explain ion current rectification in artificial nanopores. When the latent damage tracks in PET and
Kapton membranes are etched, carboxylate groups are produced along the entire nanopore surface.\(^6\) Above the isoelectric point (pI~3 for both PET and Kapton) for the polymer, these carboxylates are deprotonated and create a negative surface charge.\(^6\) As a result, an electrical double layer (EDL) forms along the surface of the nanopore to compensate for this negative charge. The thickness of the EDL is inversely proportional to the ionic strength of the electrolyte.\(^1\) In other words, as electrolyte concentration decreases, the thickness of the EDL increases correspondingly. Consequently, the negatively-charged nanopore becomes cation permselective when the radius of the tip opening is comparable to the thickness of the EDL.\(^1\) That is, the conical pore will preferentially transport cations and reject anions. Therefore, as long as the pore wall is negatively-charged (electrolyte pH > polymer pI), ion current rectification occurs as observed by a non-linear, current-voltage curve.\(^6,99-102\)

In the model developed by Siwy, et al., an electrostatic “ratchet” is described in which an electrostatic trap for cations is created near the tip opening at positive (i.e., anode at base opening) transmembrane potentials.\(^10\) This electrostatic trap hinders the migration of cations, thereby decreasing the ion current flowing through the pore. This is referred to as the “off state” of the conical nanopore. When a negative (i.e., cathode at base opening), transmembrane potential is applied, this electrostatic trap is removed and a larger ion current is observed. This is referred to as the “on state.” This ratchet model applies to negative-charged, conical nanopores. If the surface charge is positive, then the model is reversed.

Three criteria must be met for ion current rectification to occur based on the ratchet model.\(^10\) Such conditions include (1) a conical pore shape, (2) a charged pore wall, and (3) a tip opening radius comparable to the thickness of the EDL. Studies with track-etched cylindrical
nanopores having the same limiting diameter of conical pores show that ion current rectification does not occur.\textsuperscript{165}

A second model explaining ion current rectification in artificial nanopores was developed by Cervera, et al.\textsuperscript{166} Like the ratchet model discussed above, this model requires a conical pore geometry and charged pore wall. This model is based on accumulation and depletion modes of ion transport that are controlled by electrode polarity. For example, let’s consider the case of a negatively-charged nanopore. When the positive electrode (anode) is on the tip side of the pore, cations are transported from the tip side to the base side of the membrane. Anions are transported from the base side towards the tip side. However, anions cannot effectively pass through the tip opening due to electrostatic repulsion caused by the negatively-charged pore wall. Consequently, anions build up in the nanopore, thereby increasing the local concentration of anions. In order to retain electroneutrality, the local concentration of charge-balancing cations must also increase the tip region. As the electrolyte concentration increases, the membrane resistance decreases. As a result, under such conditions (i.e., anode a tip opening), the conical nanopore is in the “on state” and higher ion current is observed.

A different effect is observed when the anode is switched to the base opening of the pore.\textsuperscript{166} When the positive electrode is placed on the base side of the pore, cations are transported from the base side towards tip opening. Anions present within the nanopore are retracted from the pore towards the anode by the electric field. On the tip side of the membrane, anion transport into the tip opening is greatly reduced by electrostatic repulsion from the negative-charged pore wall. Thus, the retracted anions with the pore cannot be effectively replenished and a local salt depletion zone forms. As a result, the membrane resistance increases and an “off state” is
observed with much lower ion current. Bund, et al. reported a similar model based on ion accumulation (i.e., high conductive state) and depletion (i.e., low conductive state).\textsuperscript{155}

The above models are applicable to single, conical nanopores having tip opening diameters that are very small. However, some studies have been done on conical pores with larger tip diameters. For instance, Kovarik, et al. observed ion current rectification with a conical nanopore (in track-etched PET) having a tip opening diameter of 380 nm.\textsuperscript{167} They believe this may be due to geometric affects and/or an electroosmotic flow but further work remains before this phenomenon can be explained.

Yusko, et al. also observed ion current rectification in conical micropores (in borosilicate glass) having tip diameters 500 times larger than the Debye length.\textsuperscript{168} Ion current rectification was achieved by introducing dimethyl sulfoxide (DMSO) into the aqueous electrolyte on the tip side of the pore, thereby increasing the viscosity and reducing the ionic conductance at the tip. Yusko and coworkers proposed an electroosmotic flow contribution to rectification.\textsuperscript{168} That is, when the anode is at the tip, or low conductance, side of the pore, electroosmotic flow drives the low conductance electrolyte into the tip opening. In contrast, when the anode is at the base, or high conductance, side of the pore, electroosmotic flow drives the high conductance electrolyte into the pore.

Jin, et al. demonstrated the rectification of electroosmotic flow in a multi-pore mica membrane containing conical-shaped nanopores.\textsuperscript{156} This phenomenon occurs as a consequence of ion current rectification. As mentioned above, during the “off state” of rectification, there exists a local depletion of electrolyte with the tip region of the pore. This increases the resistivity of the solution. As a result, the velocity of electroosmotic flow ($v_{\text{eof}}$) increases because $v_{\text{eof}}$ is proportional to solution resistivity.
In addition to modeling ion current rectification and its impact on electroosmosis, several studies have been reported on modifying the pore surface to either augment or control ion current rectification. Other studies have focused on using ion current rectification to developing biosensing applications. Harrell, et al. controlled ion current rectification by attaching thiol-terminated DNAs to a gold-coated, conical nanopore in polycarbonate.\textsuperscript{104} By controlling the DNA chain length, the degree of rectification can be controlled. That is, the extent of ion current rectification increases with increasing DNA chain length by increasing the negative surface charge within the tip opening and decreasing the tip diameter. Umehara, et al. reversed the direction of ion current rectification by modifying a conical nanopipette (in quartz) with a positively-charged poly-L-lysine coating.\textsuperscript{169}

Fu, et al. modified a conical nanopipette (in glass) with a fourth generation poly(amide amine) dendrimer (G4-PAMAM).\textsuperscript{170} This dendrimer creates a cationic surface which can bind polyanionic DNA via electrostatic adsorption. As a result, DNA adsorption and hybridization can be detected by monitoring changes in ion current rectification. Vlassiouk, et al. used ion current rectification to develop a sensor for poly-\(\gamma\)-D-glutamic acid (\(\gamma\)DPGA) from \textit{Bacillus anthracis} by modifying a conical pore in track-etched PET with the monoclonal antibody for \(\gamma\)DPGA.\textsuperscript{171} Similarly, sensors for the proteins, avidin and streptavidin, were developed by modifying conical pores with biotin. Furthermore, Vlassiouk and coworkers demonstrated that ion current rectification can be used to determine the isoelectric point of proteins. This was achieved by modifying the tip opening with a small amount of protein and monitoring the change in ion current rectification as the pH is varied. Sexton also used ion current rectification to closely approximate the isoelectric points for bovine serum albumin, amyloglucosidase, and phosphorylase B by modifying track-etched conical nanopores in PET with such proteins.\textsuperscript{172}
Controlling Nanopore Surface Chemistry and Properties

As previously mentioned, the fabrication of conical nanopores in artificial materials provides chemical stability and mechanical robustness under a wide range of conditions. Although this is very important, the analytical utility of such pores is largely determined by controlling the pore surface chemistry and properties. For instance, the non-specific adsorption of biological molecules often has an adverse impact on the sensing capabilities of conical nanopores. Thus, the pore surface is chemically modified to present a biocompatible surface that is more amenable to biosensing. Furthermore, the pore surface can be modified with molecular recognition agents (e.g., antibodies, aptamers, DNA, proteins) to introduce selectivity into the pore.

Several methods exist for controlling the pore surface chemistry (vide infra). The first approach utilizes electroless gold deposition to coat the pore wall with a thin layer of gold which is amenable to subsequent thiol-based functionalization.163 Secondly, the carboxylate groups created during the track-etching of PET are activated and made amine-reactive using 1-ethyl-3-[3-dimethylaminopropyl]carboimide/N-hydroxysufosuccinimide (EDC/sulfo-NHS).173-178 As a result, an amine-terminated species can be covalently linked to the pore surface via an amide bond. In the case of glass nanopores, sol-gel and silane chemistry can be utilized to control the pore surface chemistry.179-181

Electroless Gold Deposition

Electroless deposition of metals, such as gold, inside nanoporous structures provides a useful way for controlling pore diameter and introducing various chemistries into the pore. This approach is also useful for fabricating hollow and solid nanostructures via the template synthesis method. In general, template synthesis entails the deposition of a material into the pores of a template.65,113,114,152,153,182-201 Electroless gold deposition represents a template synthesis method
for depositing gold into nanopores by using a chemical reducing agent to deposit gold from a gold solution onto the pore and membrane surfaces.\textsuperscript{163}

This method begins with the exposure of the track-etched membrane to a sensitizer, Sn\textsuperscript{2+}\textsuperscript{163} (Figure 1-12).\textsuperscript{163} This is achieved by briefly rinsing the nanopore membrane in methanol and then immersing the membrane into a solution of 0.026 M SnCl\textsubscript{2} and 0.07M trifluoroacetic acid in 50/50 water/methanol for 45 minutes. As a result, the Sn\textsuperscript{2+} binds via electrostatic complexation to the negatively-charged functional groups on the pore and membrane surfaces that are created during track-etching.\textsuperscript{163} For instance, track-etching of PET produces carboxylate groups along the surface.\textsuperscript{62} After coating the surface with Sn\textsuperscript{2+}, the membrane surface is thoroughly rinsed with methanol to remove any excess SnCl\textsubscript{2} and subsequently immersed into an aqueous ammoniac solution of 0.029 M AgNO\textsubscript{3} for 7.5 minutes. As a result, a surface redox reaction occurs in which Ag\textsuperscript{+} is reduced to elemental Ag concurrently with the oxidation of surface-bound Sn\textsuperscript{2+} to Sn\textsuperscript{4+}. This generates a membrane surface layer of silver nanoparticles.\textsuperscript{163}

The silver-coated membrane is rinsed in methanol to remove excess silver nitrate. Then, the membrane is placed into a low temperature (4\degree\textsuperscript{o}C), gold plating solution comprised of 0.127 M Na\textsubscript{2}SO\textsubscript{3}, 0.625 M formaldehyde, and 7.9 x 10\textsuperscript{-3} M Na\textsubscript{3}Au(SO\textsubscript{3})\textsubscript{2} and adjusted to pH 10 with dropwise addition of 1 M H\textsubscript{2}SO\textsubscript{4}. Since the standard reduction potential of Au is more positive than that of Ag, Au galvanically displaces the silver nanoparticles on the membrane surface, thereby initiating the formation of a surface layer of gold nanoparticles.\textsuperscript{163} These gold nanoparticles provide excellent catalytic sites to catalyze the subsequent oxidation of formaldehyde with concurrent reduction of Au(I) to Au(0) via:\textsuperscript{163}

\[2 \text{Au(I)} + \text{HCHO}^- + 3\text{OH}^- \longrightarrow \text{HCOO}^- + 2\text{H}_2\text{O} + 2 \text{Au(0)}\]
These catalytic sites on the pore wall are important because to form a surface layer, the reduction of the metal ion needs to occur at the pore surface. This process occurs spontaneously, requires no electrodes, and relies entirely on redox chemistry. Thus, it is appropriately referred to as an “electroless” deposition process.

In the case of conical nanopores, electroless gold deposition results in a gold, conical nanotube and a gold layer on both faces of the membrane. This gold layer is typically too thin to block the nanopore tip opening. To isolate the gold, conical nanotube, the gold surface layer on the membrane faces can be removed via tape-removal (i.e., with Scotch® tape) or ethanolic-swabbing. The diameter of the resulting gold nanotube can be varied at-will by varying the deposition time. In other words, thicker gold layers, or small pore diameters, can be obtained using longer plating times. Gold nanotubes having inner diameters on the order of molecular dimensions (1 nm) can be obtained utilizing this method. Furthermore, by extending the plating time for very long periods of time, solid, gold nanocones can be fabricated. Gold nanocones are described as part of the research presented herein. The advantage of coating a conical nanopore with gold resides in the fact that the gold layer provides an effective means for chemical and biological functionalization using very well-known and versatile gold-thiol chemistry.

**Chemisorption of Thiols on Gold-Coated Nanopores**

To construct functional sensors using conical nanopores, it is important to control the surface chemistry of the pore. By modifying the pore surface, the transport properties of the nanopore can be controlled at-will. For instance, chemisorption has been used to covalently-attach various thiol molecules to the surfaces of gold-coated nanotubes. The process of chemisorption occurs when thiols come in contact with a gold surface. The lone-pair electrons on the sulfur atom form a covalent bond with the electrons from the electron-rich gold surface. This process is commonly utilized to generate self-assembled monolayers anchored via thiols to gold
surfaces.\textsuperscript{202-205} It is also used to functionalize gold nanoparticles with thiol-modified molecules, such as DNA.\textsuperscript{206-209}

Experimentally, chemisorption simply involves immersing the membrane containing a gold nanotube into a solution of the desired thiol for a period of time. The thiol-coated nanotube is then rinsed to remove excess thiol solution. The tip opening diameter of the nanotube can be measured using the electrochemical method, as described previously. Using gold-thiol chemistry, molecules have been attached to nanopores to introduce (1) pH-control over ion selectivity,\textsuperscript{165,200,210-212} (2) chemical selectivity,\textsuperscript{200,211,213-216} (3) size-based selectivity,\textsuperscript{95,117,217,218} and (4) selectivity based on the hybridization of nucleic acids.\textsuperscript{109} Thiol chemisorption has also been used to attach molecular recognition agents onto the pore wall that target specific proteins.

To detect biological molecules with single nanopores, the pore wall must be modified to prevent non-specific adsorption.\textsuperscript{95,117,202-205,219-221} In multi-pore membranes, this has been accomplished by coating the gold-plated pore wall with thiol-modified poly(ethylene glycol) (PEG).\textsuperscript{117} PEG surfaces are typically used to prevent surface adsorption of biomolecules because they are uncharged and hydrophilic. Such PEG coatings have been used on quantum dots\textsuperscript{222} and SPR (Surface Plasmon Resonance) surfaces\textsuperscript{223} to prevent non-specific adsorption. The effectiveness of PEG-coatings in single, conical-shaped nanopores is part of the research presented herein.

**Selective Functionalization using Carboiimide/N-Hydroxysuccinimide Chemistry**

An alternative approach to controlling the surface chemistry of conical nanopores is by using a coupling method based on 1-ethyl-3-\{3-dimethylaminopropyl\}carbodiimide (EDC)/\textit{N}-hydroxysulfosuccinimide (sulfo-NHS) chemistry.\textsuperscript{173-175} This approach is commonly used to couple primary amines, including small molecules, amine-modified DNAs, and proteins, to carboxylates.\textsuperscript{90,172-178,224-229} As previously mentioned, track-etching of both PET and Kapton®
produces free carboxylates along the pore wall. As a result, the pore surface can be modified with primary amines via EDC/sulfo-NHS coupling chemistry.

The procedure generally entails two steps, (1) activation/stabilization and (2) amine conjugation (Figure 1-13). First, the free carboxylate groups along the pore wall are activated with EDC. That is, the negatively-charged oxygen of the carboxylate attacks the electropositive carbon located between the two adjacent nitrogens on EDC. It is electropositive due to inductive withdrawal of electron density by these two nitrogen atoms. As a result, an ω-acylisourea ester is formed which is unstable because of the carbon with three electronegative atoms bonded to it. In other words, this is a high energy intermediate. A number of directions in electron movement would take this intermediate down in energy, thereby producing a more stable species. Thus, the half-life of the ω-acylisourea ester is very short in aqueous solutions. Unless the desired amine reacts with this ester very quickly, the ester is usually converted back to the carboxylate via hydrolysis. Therefore, EDC alone lacks a high degree of efficiency for coupling carboxylates and primary amines. Consequently, the solution to this problem is to convert the unstable ω-acylisourea ester to a more stable, amine-reactive NHS-ester by adding sulfo-NHS.

The formation of a semi-stable intermediate is driven by the formation of the urea byproduct which is very stable relative to EDC and the ω-acylisourea ester. A primary amine is then added to the reaction. Nucleophilic attack from the amine occurs at the electropositive carbon on the carbonyl of the ester. This carbon is electropositive because of all of the electron withdrawing groups nearby (e.g., on the succinimide). Thus, if the pH is high enough such that an appreciable amount of amines are unprotonated (i.e., nucleophilic), then the most stable product, the amide, will dominate at equilibrium. As a result, the desired primary amine is conjugated to the pore wall via a stable amide bond.
Several studies have been conducted on coupling primary amines to conical nanopores using the EDC/sulfo-NHS approach. For instance, Ali, et al. changed the surface polarity of conical nanopores in track-etched Kapton® from negative to positive by coupling ethylenediamine to the pore wall using EDC/NHS. As a result, the ion permselectivity of the nanopore was switched from cation selective to anion selective. This provides a means for controlling the direction of ion current rectification. Vlassiouk, et al. altered the surface charge of conical nanopores in track-etched PET by local modification of the region just inside the tip opening with ethylenediamine. As a result, the direction of ion current rectification was controlled and diode-like behavior observed.

In another study by Ali, et al., conical nanopores in track-etched PET were individually modified with ethylenediamine and propylamine using EDC/pentafluorophenol (PFP) coupling chemistry. PFP was used instead of NHS due to a reportedly higher coupling efficiency. The ethylenediamine changed the polarity of the PET surface from negative to positive. The propylamine provided a more hydrophobic pore surface due to the terminal propyl (\(-\text{C}_3\text{H}_7\)) group. As result, the direction of ion current rectification was controlled. Furthermore, bovine serum albumin (BSA) was adsorbed to the different pore surfaces and detected via current-voltage curves. This provides a means of studying BSA adsorption as a function of pH and pore surface chemistry. As previously mentioned, both Sexton and Vlassiouk, et al. used EDS/sulfo-NHS to couple proteins to conical nanopores in track-etched PET to determine the isoelectric points of proteins. Kececi, et al. utilized EDC/sulfo-NHS to attach ethanolamine to conical nanopores in PET to reduce the negative charge of the pore wall in order to detect small DNAs via the resistive-pulse method. Thus, EDC/sulfo-NHS provides a very versatile route to modifying nanopore surfaces populated with free carboxylates.
Additional Strategies for Surface Functionalization and Controlling Pore Size

In addition to electroless gold deposition and EDC/sulfo-NHS coupling, other methods have been used to chemically modify the pore surface of nanopores and control pore size. Sol-gel chemistry has been used previously to fine tune the pore diameter and surface chemistry.179-181 Briefly, in the sol-gel method, tetraethyl orthosilicate is dissolved in an acidic solution and undergoes hydrolysis for a fixed time interval (e.g., 30 min).179-181 The nanopore is then briefly immersed in this solution and sonicated. Then, the silica sol is cured by removing and rinsing the membrane and placed it in an oven at typically ≥100°C. As a result, a relatively uniform, thin layer of silica is applied to the nanopore surface. This entire method can be performed repeatedly, thereby adding subsequent silica layers and providing a means of controlling the pore diameter. Hillebrenner, et al.179 and Buyukserin, et al.180,181 used sol-gel chemistry to fabricate silica nano test tubes in alumina membranes for eventual use in target-specific delivery and imaging applications.

To control the surface chemistry of the nanopore, silanes have been used. For instance, PEG silanes have been used to enhance surface hydrophilicity and reduce non-specific adsorption. Aldehyde silanes have been used as the first step in coupling molecular recognition agents to the pore wall. First, an aldehyde-terminated silane is coupled to the pore surface. Molecular recognition agents are then coupled via primary amines to the aldehyde group of the silane using Schiff-base chemistry. The stability of the Schiff-base can be improved by reducing the imine bond with NaCNBH3.

Hillebrenner, et al. introduced free amine groups to the open tubular ends of silica nano test tubes via an amine-terminated silane.179 These amine groups were then reacted with aldehyde-modified latex nanoparticles using Schiff-base chemistry. As a result, imine bonds
were formed which linked the nanoparticles to the open ends of the nano test tubes, thereby capping them in a manner akin to corking laboratory test tubes.

In a similar manner, Buyukserin, et al. introduced free amine groups to the interior wall of silica nano test tubes housed in a porous alumina template using an amine-terminated silane. The amine-coated tube wall was then reacted with Alexa 488 carboxylic acid-succinimidyl ester which cross-linked the fluorescent Alexa 488 dye to tube interior while producing 1-hydroxysuccinimide as a reaction by-product. The nano test tubes were then liberated from the alumina template, thereby exposing the tube exterior. These outer walls of the nano test tubes were functionalized with aldehyde groups using an aldehyde-silane. This provided a route to cross-linking antibodies (in this case, rabbit polyclonal IGF-IRα), via the free amine sites on the antibodies, to the aldehyde-coated tube wall using Schiff-base chemistry. Such antibody-coated nano test tubes were used to selectively target MDA-MB-231 breast carcinoma cells and image them via fluorescence microscopy.

**Additional Sensing Strategies Based on Track-Etched Conical Nanopores**

In addition to resistive-pulse sensing, other sensing methods using track-etched conical nanopores have been reported. For instance, Siwy, et al. developed a protein biosensor that operates in an “on” or “off” manner. That is, a single, conical-shaped nanopore was first fabricated via the track-etch method in a polymeric membrane. The pore walls were subsequently coated with a thin, conformal gold layer via electroless deposition. As a proof-of-concept experiment, the gold surface was functionalized with thiol-modified biotin which is a highly selective molecular recognition agent for the protein, streptavidin. The detection paradigm entails passing an ion current through the biotin-coated nanopore. However, unlike the resistive-pulse method, transient current-pulses are not observed. Instead, the protein analyte (in this case,
streptavidin) selectively binds to the biotin immobilized at the tip opening of the conical nanopore. Since the protein and tip opening have comparable diameters, binding of the protein effectively blocks the nanopore tip. This molecular recognition event is detected as a permanent blockage of the ion current. Thus, before target protein addition, the ion current was “on.” Upon introduction of the target protein analyte, the ion current was switched “off.” This sensing paradigm was subsequently applied to two other molecular recognition agent/target analyte systems including protein G/immunoglobulin (IgG), and anti-ricin/ricin. Furthermore, this approach can likely be extended to a wide range of molecular recognition agent/target analyte systems.

In another sensing approach, ion current rectification was used to monitor the adsorption of analyte drug (i.e., Hoechst 33258) molecules to the pore walls in a single track-etched polyimide membrane. Polyimide is relatively hydrophobic, but has free carboxylate groups along the pore wall. The anionic surface, due to these carboxylates, caused the conical pore to rectify the ion current passing through it. Hoechst 33258 is also hydrophobic, but cationic. When the conical pore was exposed to this drug, it adsorbed to the pore surfaces. As a result, the negative surface charge of the pore was reduced along with the extent of ion current rectification. At higher drug concentrations, the surface charge polarity was reversed to positive along with a corresponding reversal in the direction of rectification. Such changes in ion current rectification were related to the drug concentration. Thus, this represents a sensing paradigm incorporating hydrophobic effect-based selectivity using track-etched conical nanopores.

**Dissertation Overview**

The objective of the research efforts presented herein was to investigate resistive-pulse sensing, nanopore properties associated with resistive-pulse sensing and ion transport, as well as nanopore fabrication. Chapter 1 presents an overview of pertinent background information that
supports these research efforts. This includes the resistive-pulse method, prior sensing work with biological and artificial nanopores, ion-irradiation of polymer membranes, the track-etch method, pore materials, nanopore characterization, ion current rectification, and methods for controlling pore size and surface chemistry.

In Chapter 2, an approach for detecting proteins using the resistive-pulse method is reported using a model protein analyte, streptavidin. A single, conical-shaped nanopore in track-etched PET was modified via electroless gold deposition and subsequent chemisorption of thiol-modified PEG. This provided the sensing element to detect individual protein molecules. Current-pulse direction was evaluated as a function of pore surface chemistry. The impact of applied transmembrane potential on the current-pulse frequency was also studied.

In Chapter 3, efforts to detect cationic analytes via the resistive-pulse method are introduced. That is, the resistive-pulse method was applied to the detection of a model cationic analyte, cationic protein-coated gold nanoparticles. These particles were detected using an unmodified, conical nanopore in track-etched PET. This represents a departure from current examples of resistive-pulse sensing in PET, which involved surface-modified nanopores. The impact of particle concentration on current-pulse frequency was investigated. A definition for the detection limit in resistive-pulse sensing was proposed and discussed. Current-pulse direction, duration, and amplitude were also examined.

In Chapter 4, the resistive-pulse method is applied to the detection of polymeric nanoparticles in an alternative polymer-type, polyimide. These particles were detected using an unmodified, conical nanopore in track-etched polyimide. A lower ionic strength electrolyte, relative to electrolytes used previously with polyimide resistive-pulse sensors, was used. A pore loading process based on the use of a wetting agent, with vacuum degassing and perfusion, was
introduced. Efforts towards developing a two-step etch method for single, conical-shaped pores in polyimide were presented. The impact of tip diameter of ion-current rectification was examined. The relationship between current-pulse amplitude, duration, and frequency with applied transmembrane potential was evaluated.

In Chapter 5, an alternative approach to electroless gold deposition for functionalizing the pore surfaces of single, conical-shaped nanopores with poly(ethylene glycol) using EDC/sulfo-NHS coupling chemistry was introduced. The effectiveness of this approach towards reducing non-specific protein adsorption was investigated using three prototype proteins that present different surface reactivity types. Current-voltage curves and X-ray photoelectron spectroscopy (XPS) were used to evaluate protein adsorption.

In Chapter 6, a non-aqueous approach to fabricating and increasing the cone angle of conical nanopores in track-etched PET is presented. This approach provides a route to fabricating single, conical-shaped nanopores with larger cone angles than the commonly used aqueous two-step etch method. The impact of larger cone angle on the electric field strength was modeled via finite element simulations and discussed. The effect of increased cone angle on ionic pore conductance and ion current rectification was examined by holding the tip diameter constant and varying the base diameter. An approach for efficiently finding single pores in single track-etched membranes for imaging was presented. Furthermore, efforts to construct large cone angle pores in high track density PET membranes were examined and discussed. Fabricating randomly distributed gold nanocone arrays of controllable cone height and base diameter was also presented.
Figure 1-1. Diagram detailing the resistive-pulse method (pore not drawn to scale; drawn data used to show concept).

Figure 1-2. The biological protein nanopore, α-Hemolysin, embedded in a lipid bilayer support. [adapted from Bayley, H.; Jayasinghe, L. Molecular Membrane Biology 2004, 21, 209-220.]
Figure 1-3. Schematic of the ion track-etch method. A) Irradiation of a thin membrane with high energy, heavy metal ions results in B) the formation of latent damage tracks along the path of each ion. C) Chemical etching proceeds along each damage track creating pores.

Figure 1-4. Schematic of the electrochemical cell used for track-etching and ionic conductance measurements [adapted from Wharton, J. E.; Jin, P.; Sexton, L. T.; Horne, L. P.; Sherrill, S. A.; Mino, W. K.; Martin, C. R. Small 2007, 3, 1424-1430.]
Figure 1-5. Diagram of a conical nanopore in a polymer membrane showing the base and tip opening diameters (drawing not to scale). [adapted from Wharton, J. E.; Jin, P.; Sexton, L. T.; Horne, L. P.; Sherrill, S. A.; Mino, W. K.; Martin, C. R. *Small* 2007, 3, 1424-1430.]

Figure 1-6. Plot of ion current versus time recorded during the anisotropic etch step for the fabrication of a conical-shaped nanopore in PET. The moment of breakthrough is signaled by the sudden increase in ion current around 75 minutes.
Figure 1-7. A schematic of the track-etch method of fabricating a conical nanopore showing the bulk etch rate, \( v_B \), track etch rate, \( v_T \), and cone half angle, \( \alpha \).

Figure 1-8. Plot of ion current versus time recorded during the isotropic etch step for tailoring the tip opening diameter in PET. The ion current increases with increasing etch time as the tip opening diameter increases.
Figure 1-9. Chemical structures of commonly used polymers for ion track-etching. A) poly(carbonate) (PC), B) poly(ethylene terephthalate) (PET) and C) poly(Imide) (PI, Kapton®).
Figure 1-10. Scanning electron micrographs of nanopores track-etched in various materials. A) Glass, B) PC, C) PET, and D) Kapton®

Figure 1-11. Magnitude and distribution of the electric field across a conical nanopore. The nanopore used for simulations had a base opening diameter of 2.5 μm, a tip opening diameter of 60 nm, and a pore length of 6 μm. 1 V was applied across the nanopore using 1 M KCl. White hash marks are added to the section of the nanopore where the majority of the electric field is focused. [adapted from Lee, S.; Zhang, Y.; White, H. S.; Harrell, C. C.; Martin, C. R. *Analytical Chemistry* **2004**, *76*, 6108-6115.]
Figure 1-12. Schematic of the electroless gold deposition procedure. [adapted from Menon, V. P.; Martin, C. R. Analytical Chemistry 1995, 67, 1920-1928.]

Figure 1-13. Diagram of EDC/Sulfo-NHS coupling chemistry. Formation of a stable amide bond occurs between a carboxylate molecule and a molecule with a terminal primary amine group via EDC/Sulfo-NHS chemistry. [adapted from Pierce Biotechnology, http://www.piercenet.com]
CHAPTER 2
RESISTIVE-PULSE SENSING OF A MODEL PROTEIN USING A CONICAL-SHAPED NANOPORE

Introduction

There is increasing interest in developing resistive-pulse devices for the rapid detection and quantification of small molecules and biological analytes using biological and artificial nanopores. While sometimes referred to as stochastic sensing, the resistive-pulse method begins with a membrane containing a single nanopore having a limiting diameter comparable to the size of the target analyte. This membrane is immobilized between two electrolyte solutions. A transmembrane potential difference is applied and the resulting ion current flowing through the electrolyte-filled nanopore is measured. When the target analyte is added to one of the electrolyte solutions, analyte molecules are driven into the nanopore where they displace a volumetric fraction of electrolyte and transiently block the ion current. The ion current returns to the steady-state when the molecule exits the nanopore, thereby resulting in downward current-pulses. The frequency of the current-pulses is proportional to analyte concentration, and analyte identity, or selectivity, is determined by both the magnitude and duration of the current-pulses.

Most resistive-pulse sensing data reported in the literature have been obtained using α-hemolysin (α-HL), a biological nanopore that self-assembles into supported lipid bilayer membranes. A wide variety of analytes including small molecules, metal ions, DNA, and proteins have been detected using α-HL. As a result, the α-HL nanopore approach represents the benchmark by which devices using alternative pore
materials are evaluated. However, this approach is very limited due to the large fragility of the lipid-bilayer membrane containing the α-HL pore.\textsuperscript{119,143,144} Consequently, this renders lipid bilayer-based systems as impractical. As a result, there is significant research interest in developing artificial analogues (i.e., an abiotic nanopore housed in a chemically stable and mechanically robust artificial membrane) of such biological pores. For instance, ion or electron beam fabrication methods have been utilized to construct nanopores in silicon nitride and silicon films.\textsuperscript{23-25} Such films have been primarily used for the detection of DNA via the resistive-pulse method.

An increasingly popular alternative to biological pores are nanopores fabricated by the track-etch method.\textsuperscript{55-65} This approach has been utilized for decades for the commercial production of nanopores in artificial polymer membranes used for filtration applications.\textsuperscript{148-151} Such membranes have a large nanopore density (i.e., often >10\textsuperscript{6} pores cm\textsuperscript{-2}). However, the resistive-pulse method requires a membrane containing one nanopore. Fortunately, a company in Germany, GSI, developed a single-ion irradiation technique that allows for the fabrication of single nanopores in thin polymer membranes (e.g., polyimide, polycarbonate, poly(ethylene terephthalate), 5-12 μm thick).\textsuperscript{57,58} Both cylindrical- and conical-shaped nanopores can be fabricated in such membranes. Such conical nanopores have been used to detect proteins,\textsuperscript{95} DNA,\textsuperscript{89,90} nanoparticles,\textsuperscript{96} and small molecules.\textsuperscript{74,75}

In this chapter, a resistive-pulse sensor for a model protein, streptavidin, using a conical-shaped nanopore in track-etched poly(ethylene terephthalate) (PET) as the sensing element is described. A conical-shaped nanopore is comprised of two openings, a large-diameter opening at one side of the membrane and a small-diameter opening.
located at the other side of the membrane. The large-diameter opening is referred to as the “base” of the nanopore and the small-diameter opening is known as the “tip” of the nanopore.

The protein, streptavidin, was driven via electrophoresis through the conical nanopore in the direction of tip to base. Protein translocation events were detected as transient blocks, or current-pulses, in the ion current flowing through the nanopore. The frequency of the current-pulses increased with the magnitude of the applied transmembrane potential. The nanopore sensing element was coated with a thin layer of gold via electroless gold deposition. Subsequent chemisorption of a thiol-modified poly(ethylene glycol) on the gold surface was used to reduce non-specific protein adsorption.

Analogous to classic Coulter® Counting, in the PEG-modified nanopore, current-pulses were predominantly downward. In contrast, in an unmodified conical nanopore of comparable tip size, the current-pulses were upward and downward. The results suggest a protein adsorption/desorption event occurring on the unmodified pore wall as proteins translocate the sensing zone.

**Experimental**

**Materials**

Poly(ethylene terephthalate) (PET) films (3 cm diameter, 12 μm thick) were obtained from GSI (Darmstadt, Germany). These films were each irradiated with a single, swift heavy-ion to create a single damage track through the entire film. The model protein, streptavidin (SA), was obtained from Sigma Aldrich and used as received. SA has a molecular weight (MW) of ~60 kDa. A thiol-modified poly(ethylene glycol) (PEG-thiol, MW 5 kDa) was obtained from Laysan Bio (Huntsville, AL). All other chemicals were of reagent grade or better and used as received. All solutions were prepared using
purified water (i.e., obtained by passing in-house-deionized water through a Barnstead, E-pure water purification system at 18 MΩ).

**Fabrication of the Conical Nanopore**

A conical-shaped nanopore was etched into the single-ion tracked poly(ethylene terephthalate) membrane by anisotropic, and subsequent isotropic, chemical etching of the swift heavy-ion irradiated poly(ethylene terephthalate) film. Using the two-step etch method developed by Wharton, et al., the irradiated film was mounted between two halves of an U-tube cell made of Kel-F. An etching solution, 9 M sodium hydroxide (NaOH), was added to one half-cell and a stop solution, 1 M formic acid and 1 M potassium chloride (KCl), was added to the other half-cell. The ion-induced damage track was etched preferentially from the membrane face in contact with the etching solution towards the membrane face in contact with the stopping solution.

Etching was performed until the etch solution completely penetrated through the membrane to the stop solution on the other side. To detect exactly when breakthrough occurred, a platinum wire electrode was placed into each half cell and a transmembrane potential difference of +1 V was applied during etching using a Keithley 6487 voltage source/picoammeter (Keithley Instruments, Cleveland, OH). The electrodes were configured such that the positive electrode (anode) was located in the etching solution side of the membrane. The negative electrode (cathode) was located in the stopping solution side of the membrane. By applying a transmembrane potential difference, the etching process was monitored in real-time. The ion current was initially zero. At the moment of membrane breakthrough, the ion current suddenly increased. For poly(ethylene terephthalate) membrane used herein, breakthrough usually occurred ~1.5 hour. This anisotropic etch procedure was performed for 2 hours. In addition to
fabricating a conical-shaped nanopore, the chemical etching process thinned the membrane slightly from 12 to ~11.76 μm. After etching, the etching solution is removed and replaced with neutralizing stopping solution. Then, both halves of the U-tube were emptied and rinsed thoroughly with water. The membrane was stored overnight in water.

To tailor the tip opening diameter, the second-etch step was performed using an analogous experimental set-up as that used for the anisotropic etch process described above. However, a more dilute etching solution, 1 M NaOH, was placed onto both sides of the membrane. A transmembrane potential difference of +1 V was applied across the membrane with the anode located on the base opening side of the membrane and cathode at the tip opening side. The resulting ion current was measured as a function of etching time. The two-step etch method provides the means for fabricating very reproducible base and tip diameters. As Wharton, et al. demonstrated, the ion current correlates to tip opening diameter. Thus, by stopping the isotropic etching process at an ion current value of 25 nA, the tip diameter was approximated to be 50 nm. The base diameter obtained after the anisotropic etching step and used for this work was 520 ± 45 nm, as obtained via field emission scanning electron microscopy (FE-SEM), per Wharton, et al.

In addition to measuring the base diameter, FE-SEM (Hitachi S-4000) was utilized to evaluate the geometry of the conical nanopores. This was achieved by completely filling in the nanopores (multiporous PET, 10⁶ pores/cm²) with gold using a previously described electroless gold deposition procedure. As a result, gold nanocone replicas of the nanopores are produced. Furthermore, both membrane faces of the poly(ethylene terephthalate) membrane are coated with thin layers of gold during the
deposition process. To image these nanocone replicas, the gold surface layers on both faces of the membrane were removed via mechanical polishing away the gold using a cotton swab wetted with ethanol. The polymer membrane was then removed via dissolution using 1,1,1,3,3,3-hexafluoroisopropanol (HFIP). The liberated nanocones were filtered through a commercially-available anodized alumina membrane filter that had been sputter-coated with Au-Pd.\textsuperscript{64,65}

The same electroless gold deposition method was used to deposit gold along the pore walls of the nanopore to produce a gold-coated conical nanopore.\textsuperscript{95,163} Again, the gold plating process produces a thin, gold surface film on both faces of the membrane which was subsequently removed by mechanically polishing away the gold using a cotton swab wetted with ethanol. As a result, a single, conical-shaped gold nanopore is created. A current-voltage curve obtained after electroless gold deposition was used to measure the tip opening diameter of the resulting gold nanopore (\textit{vide infra}).\textsuperscript{95} No significant change in the base opening diameter occurred due to gold plating.

The gold surface of the nanopore was modified with PEG-thiol (MW 5 kDa) in order to prevent non-specific protein adsorption.\textsuperscript{95,117,217} This was achieved by placing the gold nanopore-containing membrane into a 100 μM solution of PEG-thiol in purified water for ~12 hours at 4°C. The membrane was then carefully rinsed in 2 L of purified water to remove excess PEG-thiol. That was accomplished by suspending the membrane atop of 2 L of purified water and slowly stirring via stirbar. The tip opening diameter of the PEG-modified nanopore was then measured using an electrochemical method based on current-voltage curves (\textit{vide infra}).
Electrochemical Measurements

For measuring tip opening diameter, the single, conical-shaped nanopore membrane was mounted in an U-tube cell made of Kel-F and both half-cells were filled with an electrolyte solution of known conductivity (10.5 – 11.5 S/m) that was 1 M KCl in purified water (pH ~6). A Ag/AgCl electrode was placed into the electrolyte in each half-cell. A transmembrane potential difference was applied across the electrolyte-filled nanopore and the resulting ion current measured. The applied potential was linearly increased in stepwise fashion from -1 V to +1 V while measuring the ion current at each potential step. The slope of the resulting current-voltage was utilized to calculate the tip diameter of the nanopore (vide infra).\(^{59,75,89}\)

For current-pulse measurements, the PEG-modified, conical nanopore membrane was mounted in a U-tube cell in similar fashion. A schematic of the PEG-coated nanopore sensing element is illustrated in Figure 2-1. Both half-cells were filled with ~3.5 mL 0.1 M KCl that was pH 7.4 using a 10 mM phosphate buffer solution. A commercially-available Ag/AgCl electrode (Bioanalytical Systems/BASi, West Lafayette, IN) was placed into the electrolyte in each half-cell and connected to an Axopatch 200B patch clamp amplifier (Molecular Devices Corp., Union City, CA). The Axopatch 200B was utilized to apply a desired transmembrane potential and monitor the corresponding ion current flowing through the electrolyte-filled nanopore. Current recordings were obtained in voltage-clamp mode using a low-pass Bessel filter at 2 kHz bandwidth. The signal was digitized using a Digidata 1233A analog-to-digital converter (Molecular Devices Corporation). Data were recorded and analyzed using pClamp 9.0 software (Molecular Devices Corporation). Unless stated differently, the electrodes were configured as follows: the positive Ag/AgCl electrode (anode) was placed into the
electrolyte in the half-cell located on the base opening side of the conical nanopore and the negative Ag/AgCl electrode (cathode) was placed into the electrolyte at the tip opening side. Because the pH of the sensing buffer (pH ~7.4) used here is above the isoelectric point of streptavidin (pI ~7.0), the protein has a net negative surface charge. Therefore, the protein solution was added at the cathode side of the membrane facing the tip opening of the conical nanopore.

Results and Discussion

Nanopore Characterization

Wharton, et al. demonstrated that the two-step etch method reproducibly produces a base opening diameter of 520 nm for a conical-shaped nanopore in track-etched poly(ethylene terephthalate). Knowing the base diameter allows the tip opening diameter to be calculated from an experimental determination of the ionic conductance of the electrolyte-filled nanopore. If the nanopore possesses a true conical geometry, then the ionic conductance of the nanopore (G) is related to the base diameter (d_{base}), the tip diameter (d_{tip}), the specific conductivity of the supporting electrolyte (σ), and the pore length (membrane thickness after etching, L) via the following equation:

\[
G = \frac{\pi \sigma d_{base} d_{tip}}{4 L} \quad \text{(Eq. 2.1)}
\]

The value of G was experimentally determined by simply measuring the linear current-voltage curve for the electrolyte-filled nanopore. That is, G is the slope of the linear current-voltage curve. With this value of G, all of the other variables in Eq. 2.1 are known. Therefore, the tip opening diameter can be calculated.

To further evaluate the geometry of the nanopore, an electroless gold deposition method was used to completely fill the conical nanopores produced in a comparably-
etched multi-tracked PET membrane with gold.\textsuperscript{163} Multi-tracked PET membranes are used since a single gold replica obtained from a single pore PET membrane is difficult to find. As a result, gold replicas that represent the geometry of the nanopores are produced from multiporous membranes.\textsuperscript{64,65,114} A representative image of one such replica showed ideal conical geometry (Figure 2-2). Furthermore, such replicas can be used to approximate cone angle.

In the case of the PEG-modified nanopore used here, the two-step, anisotropic and isotropic, etch method produced a conical nanopore having a base opening diameter of $520 \pm 45$ nm and a tip opening diameter of $\sim 48$ nm, as determined by the current-voltage curve in Figure 2-3. Electroless gold deposition was used to plate a thin gold film along the walls of the nanopore to provide a reactive surface for PEG-thiol chemisorption. The tip opening diameter after gold deposition was determined via a current-voltage curve to be $\sim 23$ nm (Figure 2-3). Thiol-modified PEG (MW 5 kDa) was then attached to the gold-coated nanopore and the tip diameter re-measured in similar fashion to be $\sim 12$ nm (Figure 2-3).\textsuperscript{95,117,217}

**Resistive-Pulse Sensing of Streptavidin**

A key characteristic of the PEG-modified, conical nanopore sensor is that the ionic current flowing through the pore generates an electric field that is highly focused at the tip opening.\textsuperscript{95,96} Finite element simulations performed by Lee, et al. showed that, when a potential difference of $+1$ V is dropped across the nanopore sensing element, the magnitude of the electric field strength in and around the tip opening is on the order of $10^6$ V m$^{-1}$.\textsuperscript{96} As a result of this field-focusing phenomenon, the ion current flowing through the nanopore tip region becomes very sensitive, thereby creating an analyte sensing zone.\textsuperscript{75,89,96} Therefore, the ion current in this sensing zone becomes highly
sensitive to the presence of a target analyte, such as the protein, streptavidin. This field-focusing feature of conical nanopores makes conical-shaped pores more amendable to resistive-pulse sensing than cylindrical-shaped pores.

In the absence of protein, an applied transmembrane potential of +1000 mV resulted in a steady-state ion current of ~700 pA (Figure 2-4). Without protein, no transient current-pulse events were observed (Figure 2-4). Upon addition of 500 nM streptavidin to the electrolyte solution facing the tip opening of the nanopore, numerous transient current-pulses were observed (Figure 2-5). These current-pulses are due to the electrophoretic transport of streptavidin into the sensing zone at the nanopore tip and through the base of the nanopore. Similar results were previously observed using bovine serum albumin (BSA) as the target analyte for resistive-pulse sensing using a comparable conical nanopore. Such current-pulse data have been interpreted as transient current-blocking events that represent the protein molecules entering and translocating the nanopore sensing element. Furthermore, single nanopores prepared via track etching have produced similar transient current-pulse events attributed to the electrophoretic transport of double-stranded DNAs and porphyrin molecules through tip opening diameters of 40 nm and 4 nm, respectively. However, in these cases, the tip opening diameters were different than that used here because the analytes had different sizes.

Figure 2-6 displays an expanded view of a streptavidin current-pulse. The ion current decreases very sharply at the beginning and then gradually tails upward towards the baseline with increasing data acquisition time. The current-pulse magnitude (Δi) represents the ion current interval between the ion current value at the lowest point in this sharp decrease and the ion current value at the steady-state. The current-pulse duration (τ)
represents the time interval between the very sharp decrease in the ion current and the
time when the ion current returns to its steady-state value. Comparable current-pulse peak
shapes have been observed previously with other charged analytes, such as bovine serum
album (BSA), BSA/anti-BSA complexes, poly(styrene sulfonate), and single-stranded phage DNA, as they were electrophoretically driven in the direction of tip-to-base through conical-shaped nanopores. This peak shape is reasonable because it represents the effectiveness in which analytes block the ion current based on analyte location within the nanopore. That is, the analyte has the largest impact on the ion current while it resides in the tip region. As a result, the analyte is most effective in blocking the ion current in this region. In contrast, the analyte has the smallest impact on the ion current while it’s in the much larger base opening. Thus, the analyte has very little impact on the ion current at the base of the conical nanopore.

Another way of looking at this is to consider the ratio \( \frac{A_{\text{protein}}}{A_{\text{pore},x}} \) of the cross-sectional areas of the protein \( A_{\text{protein}} \) to that of the nanopore \( A_{\text{pore},x} \) at any point, \( x \), which represents the protein’s position along the long axis down the center of the pore. This ratio is greatest at the pore tip opening when the protein first enters the tip. Thus, at large \( \frac{A_{\text{protein}}}{A_{\text{pore},x}} \), the protein is most effective at blocking the ion current. As the protein translocates the sensing zone towards the base, this ratio approaches zero. As a result, the extent in which the protein can affect the ion current diminishes correspondingly.

**Effect of Applied Transmembrane Potential on Current-Pulse Frequency**

The effect of voltage on the current-pulse frequency was determined using applied transmembrane potentials of 400 mV, 600 mV, 800 mV, and 1000 mV (Figure 2-7). The current-pulse frequency \( f_p \) was obtained by counting the number of current-
pulses occurring in 5-min. time intervals and averaging the number of current-pulses from 3 such intervals. As shown in Figure 2-8, there is a threshold potential below which streptavidin current-pulses were not observed. This has been previously observed for other charged analytes using both biological\textsuperscript{83} and artificial nanopores.\textsuperscript{75,89,95} The current-pulse frequency increases exponentially with applied voltage above the threshold potential. This exponential relationship is attributed to an entropic barrier that streptavidin molecules must overcome to enter a nanopore having a tip opening diameter that is comparable to the size of streptavidin.\textsuperscript{75,83,89,95} Therefore, since streptavidin is charged, it lowers its entropy by being driven via electrophoresis into the tip opening of the nanopore.\textsuperscript{75,83,89,95}

**Effect of Pore Surface Chemistry on Current-Pulse Duration**

Current-pulse durations shorter than 100 ms are typically observed with molecular resistive-pulse sensors.\textsuperscript{79,89} For example, current-pulses less than 20 ms were observed for DNA using the biological nanopore, $\alpha$-HL.\textsuperscript{79} Conical nanopore sensors in track-etched polycarbonate produced current-pulses less than 80 ms for large, single-stranded phage DNA.\textsuperscript{89} This is interesting because the diameter of the DNA in solution (~128 nm) is over 3 times larger than the diameter of the nanopore tip (~40 nm).\textsuperscript{89} However, such current-pulse durations for DNA were much smaller than current-pulse duration for the streptavidin (average $\tau = 1$ s) studied here and other proteins studied previously.\textsuperscript{95}

Such long current-pulse durations likely stem from non-specific interactions between the streptavidin and the unmodified gold layer along the pore wall. That is, anionic streptavidin transiently adsorbs to the underlying gold surface via non-specific adsorption. Since a large MW PEG-thiol was used, with respect to the small pore size and
curvature of the pore wall, it is unlikely that a perfectly close-packed monolayer of PEG forms. Thus, for each PEG-thiol, the PEG chain assumes a random coil atop a single thiol, thereby leaving a portion of the underlying gold surface exposed. Any portions of the gold layer that aren’t covered with PEG or the presence of defects in the PEG layer make the pore wall susceptible to non-specific protein adsorption. This phenomenon has been observed previously with bovine serum albumin using a comparable nanopore.\textsuperscript{95} Thus, pore surface chemistry plays a role in current-pulse duration.

**Effect of Pore Surface Chemistry on Current-Pulse Direction**

Despite the conceptual simplicity of constructing resistive-pulse sensors from track-etched conical nanopores, modifying the pore wall via electroless gold deposition and subsequent PEG-thiol chemisorption introduce practical complexity in terms of reproducibly creating a functional sensing element having long-term stability. Therefore, alternative approaches to doing resistive-pulse sensing have been investigated. For instance, a different method for attaching PEG directly to the pore wall using EDC/sulfo-NHS coupling chemistry\textsuperscript{173,174,175} is introduced in Chapter 5.

Here, the resistive-pulse detection of streptavidin using an unmodified, conical nanopore in track-etched poly(ethylene terephthalate) was investigated. Such pores that are not gold-plated offer several advantages. As will be discussed in more detail later, at higher applied transmembrane potentials, the underlying gold layer becomes detached from the pore wall and, after a period time, is completely removed. This is likely due to increased resistive heating that occurs at higher electric field strengths.\textsuperscript{64} The use of higher potentials offers several advantages in resistive-pulse sensing. Theoretically, a higher current-pulse frequency can be obtained at higher electric field strengths that result from increasing the applied transmembrane potential.\textsuperscript{89,95,172} Consequently,
improvements in the limit of detection may be achieved at such higher current-pulse frequencies. A high electric field strength also has been shown to reduce the current-pulse duration.\textsuperscript{23,89,172} Such a reduction may improve selectivity. Thus, the resistive-pulse sensing of streptavidin in an unmodified PET nanopore, which is more amendable to higher transmembrane potentials, was studied.

Using the two-step etch method described previously,\textsuperscript{63} a single, conical-shaped nanopore was fabricated having a base diameter of 520 ± 45 nm and tip diameter of ~12 nm. Using an analogous resistive-pulse experimental setup as that used for the PEG-modified nanopore, streptavidin (500 nM) was electrophoretically driven into the tip opening of the nanopore and detected as transient current-pulses. However, instead of observing the typical downward current-pulses observed for the PEG-coated pore, the current-pulses went both upward and downward (Figure 2-9). That is, the ion current sharply increased above the baseline for a certain time interval and subsequently decreased sharply below the baseline for a certain time interval before returning to the steady-state. This suggests that some additional factor is contributing to the current-pulse aside from what occurs with classic Coulter\textsuperscript{®} counting. Coulter\textsuperscript{®} counting is built on the underlying assumption that an analyte displaces a corresponding volume of electrolyte and, as a result, always results in an increase in ionic resistance (i.e., downward current-pulses). However, at the nanoscale, it is believed that surface chemistry plays a larger role in the resistive-pulse detection of smaller, nanoscale analytes.

Several reports have reported the observation of upward current-pulses instead of downward current-pulses for the resistive-pulse detection of DNA using nanopores.\textsuperscript{35} Such upward current-pulses reflect a decrease in the ionic resistance of the pore upon
DNA translocation. This was attributed to DNA entering the pore along with its charge-balancing counterions. Therefore, due to these additional charge carriers, there is an increase in the local conductivity of the electrolyte within the pore when the DNA is present. As a result, the ion current increases as observed experimentally by an upward current-pulse. The downward current-pulse following the upward pulse can be attributed to pore blocking analogous to the Coulter® case. This explains why current-pulses go upward and downward but doesn’t account for why the upward-downward pulses observed for the unmodified pore here were not observed using the previously used PEG-modified pore.

The results suggest that a transient protein adsorption-desorption process is occurring along the pore wall within the sensing zone at the nanopore tip opening. With PEG present, such a process cannot readily occur because non-specific adsorption has been reduced by the bulky, high MW PEG. It is reasonable to assume that a transient interaction between the unmodified pore wall and the protein would slow down protein translocation, thereby increasing the current-pulse duration. Hence, this is exactly what was observed experimentally. That is, a larger current-pulse duration was observed for the unmodified nanopore compared to that for the PEG-modified pore.

The same streptavidin concentration, electrolyte, and transmembrane potential were used here as used previously for the PEG-modified pore. As shown in Figure 2-9, the current-pulse frequency for streptavidin in the unmodified pore was lower (12 pulses 5 min\(^{-1}\)) than the pulse frequency observed with the PEG-modified pore (23 pulses 5 min\(^{-1}\). One plausible explanation is that the threshold potential for the translocation of streptavidin in the unmodified nanopore is greater than the threshold potential of the
PEG-modified pore. This difference could be attributed to the negative surface charge of the unmodified pore wall relative to the net negative charge of streptavidin. That is, electrostatic repulsion between the carboxylates created along the pore wall as a byproduct of chemical etching and the negatively-charged streptavidin could impede translocation and increase the entropic barrier to translocation. Modifying the pore wall with gold and PEG-thiol reduces some of the surface charge. As a result, there is less electrostatic repulsion and a lower entropy barrier to overcome. Therefore, the current-pulse frequency is somewhat higher in the PEG-modified pore at the applied transmembrane potential of +1000 mV used here. Furthermore, the presence of the bulky, high MW PEG likely reduces the number of available adsorption sites along the pore wall. With less adsorption sites available, less interactions with the pore wall can occur in PEG-coated pores relative to unmodified pores.

Interestingly, if we compare the current-pulse frequency for bovine serum album (BSA) reported by Sexton, et al. to that for streptavidin obtained at the same tip opening diameter (~12 nm), applied transmembrane potential (1000 mV), and electrolyte using a PEG-modified conical nanopore, we observe 6 ± 1 pulses/min. for streptavidin versus ~3 pulses/min. for BSA. However, the concentration of BSA (100 nM) was 5 times smaller than the concentration of streptavidin (500 nM). Current-pulse frequency, $f_p$, can be described by the equation:

$$f_p = \frac{-z F D_t C E (\pi r_{tip}^2 A)}{RT} \quad \text{(Eq. 2.2)}$$

where $z$ is the effective surface charge on the analyte, $D_t$ is the diffusion coefficient associated with analyte transport through the tip opening, $C$ is analyte concentration, $E$ is the electric field strength, $r_{tip}$ is the radius of the tip opening, $A$ is Avogadro’s number,
and the other variables have their usual meanings. Several factors could be attributed to the higher pulse frequency of streptavidin versus that of BSA. Certainly, the higher concentration of streptavidin contributes to the higher current-pulse frequency observed for streptavidin. The cross-sectional area of the tip opening relative to size of the protein is also an important factor. In the case of streptavidin, the protein is about 5 nm in diameter and the pore is 12 nm. Thus, when streptavidin enters the tip opening, it occupies roughly 50% of the cross-sectional area of the tip. In contrast, BSA has a long axis of ~14 nm and a short axis of ~4 nm. Therefore, with a tip opening diameter of 12 nm, BSA transport through the tip becomes hindered because BSA loses a degree of rotational freedom in tips smaller than its long axis. Streptavidin does not experience this. Although a quantitative determination of the effective surface charge wasn’t obtained for either BSA or streptavidin, surface charge undoubtedly impacts the current-pulse frequency as well (Eq. 2.2). The isoelectric points of BSA and streptavidin are ~4.8 and ~7.0, respectively. Thus, both proteins have excess negative surface charge in the pH 7.4 sensing buffer.

**Conclusions**

In this work, it was shown that single, conical-shaped nanopores can be fabricated by chemical etching of ion-tracked polymer membranes. Such nanopores were used to detect a model protein, streptavidin, via the resistive-pulse method in both PEG-modified and unmodified nanopores in poly(ethylene terephthalate). In PEG-coated nanopores, the current-pulses were predominantly downward. However, removing the PEG and underlying gold layers resulted in current-pulses going both upward and downward. This difference was attributed to non-specific interactions (i.e., adsorption and desorption) between the streptavidin and the pore wall. Furthermore, a combination of pore blocking
and enhanced local ionic strength in the sensing zone due to the presence of streptavidin also contributed to the phenomenon observed in bare PET. Such local ionic strength enhancement has been previously observed with resistive-pulse sensors for DNA in artificial nanopores.\textsuperscript{35}

The current-pulse frequency was observed to vary exponentially with increasing applied transmembrane potential. This was due to the entropic penalty that the streptavidin must overcome to enter the tip of the nanopore. The current-pulse frequency of comparable concentrations of streptavidin in the unmodified pore was less than that of the PEG-modified pore. This suggests that the threshold potential for obtaining current-pulses in the unmodified pore is greater than that for the PEG-coated pore.\textsuperscript{95,172}

One of the distinct advantages of track-etched conical pores is that such pores can be coated with gold via electroless gold deposition and subsequently modified with thiols to tailor the pore surface chemistry. As a result, the transport properties of the pore can be controlled. In this work, PEG-thiol was attached via this approach to reduce non-specific adsorption. Another advantage of conical pores is the characteristic conical geometry which causes a field focusing affect within the tip opening that effectively facilitates analyte detection and translocation.

A key requirement for obtaining current-pulses for streptavidin is having the capability to fabricate a tip opening diameter that is comparable to size of streptavidin. The two-step fabrication method provides a way to fine tune the tip diameter to the size of the analyte.\textsuperscript{63} Using an electrochemical method, the tip opening diameter can be measured after each step of the fabrication and pore modification process.\textsuperscript{59,75,89,90,95} This successful detection of streptavidin is a promising step towards expanding the application
of conical nanopores to the resistive-pulse detection of streptavidin-biotin complexes (e.g., streptavidin-biotin-DNA, streptavidin-biotin-aptamer-protein), and additional proteins (e.g., biomarkers).
Figure 2-1. Schematic of the PEG-modified conical nanopore sensing element (*drawing not to scale*).

Figure 2-2. FE-SEM image of a template-synthesized gold nanocone replica prepared in a conical-shaped nanopore in track-etched poly(ethylene terephthalate). The replica represents the geometry of the nanopore sensing element after removal from the PET template.
Figure 2-3. Current-voltage curves obtained in 1M KCl used to calculate the diameter of the tip opening after each step of the resistive-pulse sensor-fabrication process. Blue: track-etched PET, tip diameter ~48 nm, Orange: after deposition of gold surface layer, tip diameter ~23 nm, Black: after attachment of PEG-thiol to gold nanopore walls, tip diameter ~12 nm.

Figure 2-4. Typical current-time transient for the PEG-modified, single conical-nanopore sensor at an applied transmembrane potential of 1000 mV. Electrolyte only; no streptavidin.
Figure 2-5. Typical current-time transient for the PEG-modified, single conical-nanopore sensor at an applied transmembrane potential of 1000 mV. Electrolyte contained 500 nM streptavidin.

Figure 2-6. Expanded view of a typical current-pulse reflecting the tip-to-base translocation of 500 nM streptavidin through a PEG-modified conical nanopore with a tip diameter of 12 nm.
Figure 2-7. Streptavidin current-time transient as function of applied transmembrane potential. Tip diameter = 12 nm. [streptavidin] = 500 nM. PEG-coated, conical nanopore. Applied transmembrane potentials: (A) 1000 mV, (B) 800 mV, (C) 600 mV, (D) 400 mV.

Figure 2-8. Streptavidin current-pulse frequency versus transmembrane potential. Tip diameter = 12 nm. [streptavidin] = 500 nM. Error bars represent standard deviations obtained by averaging the number of current-pulses during three 5-minute intervals of current-pulse data.
Figure 2-9. Current-time transients of 500 nM streptavidin at an applied transmembrane potential of 1000 mV using an (A) unmodified conical nanopore in PET, tip diameter ~12 nm, and (B) PEG-modified conical nanopore in PET, tip diameter ~12 nm.

Figure 2-10. Histograms of streptavidin current-pulse amplitude (left) and duration (right). Tip diameter = 12 nm (with PEG attached). [streptavidin] = 500 nM. Applied transmembrane potential = 1000 mV.
CHAPTER 3  
RESISTIVE-PULSE SENSING OF A MODEL CATIONIC ANALYTE WITH A CONICAL NANOPORE SENSOR

Introduction

There is increasing interest in the concept of using nanopores in artificial or biological membranes as resistive-pulse sensors for small molecule and biopolymer analytes. The resistive-pulse method, which when applied to such target analytes is sometimes called stochastic sensing, entails mounting a membrane containing a nanopore between two electrolyte solutions, applying a transmembrane potential difference, and measuring the resulting ion current flowing through the electrolyte-filled nanopore. In simplest terms, when the analyte enters and translocates the nanopore, it transiently blocks the ion current, resulting in a downward current-pulse. The frequency of such analyte-induced current-pulse events is proportional to the concentration of the analyte. Analyte identity, or selectivity, is encoded in the magnitude and duration of current-pulses.

The majority of such molecular resistive-pulse sensing work has been done using a biological nanopore, α-hemolysin (α-HL), embedded in a supported lipid-bilayer membrane as the sensor element. Numerous analytes including metal ions, DNA, proteins, and various small molecules have been detected with the α-HL nanopore. These studies have shown unequivocally that resistive-pulse sensing using a nanopore as the sensor element is a promising sensing paradigm.

However, because the supported lipid-bilayer membrane that houses the α-HL nanopore is very fragile, it seems unlikely that any practical, real-world, sensing devices will be possible with this bilayer technology. One approach for solving this
problem is to replace the bilayer membrane and biological nanopore with a mechanically robust and chemically stable artificial membrane containing an artificial micro- or nanopore. Prototype artificial micro- and nanopore sensors have been prepared by the track-etch method, by inserting a carbon nanotube into an epoxy/silicon nitride support, by electron-beam lithography in silicon membranes, by ion-beam sculpting in silicon dioxide and nitride membranes, and by a femtosecond-pulsed laser method in a glass membrane. There artificial nanopore sensors have been used, with the resistive-pulse method, to detect DNA, polystyrene nanoparticles, small molecules, proteins, and virus particles. Furthermore, like the α-HL-based sensor, chemical selectivity can be introduced by biofunctionalization of the artificial nanopore sensor element.

In addition to selectivity, a key question that must be addressed for any proposed new sensing method is – what detection limits can be achieved with this technology? To date, the lowest detection limits that have been reported with nanopore resistive-pulse sensors for ionic, small molecule, protein, or DNA analytes are at the nanomolar level, although quantitative evaluations of the detection limit are often not reported. This work represents a step towards such a description. That is, this research explores the issue of resistive-pulse detection limits using a single, conical-shaped nanopore, prepared by the track-etch method, in a poly(ethylene terephthalate) (PET) membrane as the sensor element. Results are reported here for an ideal, nano-sized analyte, 5 nm-diameter poly-L-lysine-coated cationic gold nanoparticles. Particles were detected at sub-nanomolar concentrations, which are lower than that reported in any ionic, molecular, or macromolecular resistive-pulse sensing experiment.
Experimental

Materials

Poly(ethylene terephthalate) (PET) membranes (3 cm diameter, 12 μm thick), that had been irradiated with a single, swift heavy ion to create a single damage track through the membrane, were obtained from Gesellshaft fur Schwerionenforschung (GSI), Darmstadt, Germany. Poly-L-lysine-conjugated, cationic gold nanoparticles (5 nm diameter, particle concentration: $5 \times 10^{13}$ particles/mL) were obtained from Ted Pella. All other chemicals were of reagent grade. Solutions were prepared in purified water (Barnstead, E-pure) and the buffer used to prepare the analyte solutions was filtered through Durapore (Millipore) filters.

Preparation of the Conical-Nanopore Sensing Element

The single damage track in the PET membrane was converted into a conical-shaped nanopore using the two-step chemical etching procedure described previously. Briefly, the first etch step entails placing the membrane between a 9 M NaOH etch solution and a stop-etch solution comprised of 1 M formic acid and 1 M KCl in a U-tube cell made of Kel-F (3.5 mL half-cell volume). This yields a single, conical-shaped nanopore with the large diameter (or base) opening facing the etch solution and the small diameter (or tip) opening facing the stop-etch solution. To determine when the etchant had broken through to the stop-etch solution, and a contiguous pore had been obtained, a platinum wire electrode was placed in each half-cell solution and a potential difference of +1 V (using a Keithley 2487 voltage source/picoammeter, Keithley Instruments, Cleveland, OH) applied across the membrane. The electrodes were configured such that the positive electrode (anode) was placed in the half-cell containing the etch solution and the negative electrode (cathode) was placed in the half-cell containing the stop solution.
Before breakthrough, the transmembrane ion current was zero and breakthrough was signaled by a sudden rise in the ion current. The first etch step yielded a conical nanopore with a base diameter of 520 ± 45 nm, as determined by scanning electron microscopy. Wharton, et al. previously validated the first step etch for reproducibly producing base diameters of 520 ± 45 nm in single ion-irradiated PET membranes.63

The second etch step is used to adjust and fine tune the size of the tip opening.63 This step entails placing a more dilute (i.e., 1 M NaOH) etch solution on both sides of the membrane. Again, a potential difference of +1 V was applied across the membrane and the transmembrane ion current was monitored during the etching process. Excellent reproducibility in the tip diameter (relative standard deviation less than 10%) can be obtained by stopping the second etch at a prescribed value of the transmembrane ion current. For the research reported here, the second etch was stopped at an ion current value of ~4.6 nA yielding a conical nanopore with a tip diameter of ~10 nm. The tip diameter was determined using an electrochemical method59,75,89 based on current-voltage curves (i.e., ionic pore conductance) as described below.

Resistive-Pulse Sensing

The single, conical-nanopore membrane was placed between two halves of a Kel-F conductivity cell comparable to that used for pore fabrication (Figure 3-1). Both half-cells were filled with a pH 7, 10 mM phosphate buffer solution that also contained 0.1 M KCl. A Ag/AgCl electrode (Bioanalytical Systems/BASi, West Lafayette, IN) was placed into each half-cell solution, and an Axopatch 200B (Molecular Devices Corp., Union City, CA) was used to apply the desired transmembrane potential and measure the resulting ion current flowing through the electrolyte-filled nanopore. The electrode polarity was configured such that the cathode (negative electrode) was in the solution
facing the base opening and the anode (positive electrode) was in the solution facing the
tip opening. The Axopatch amplifier was used in voltage clamp mode with a lowpass
Bessel filter (2 kHz bandwidth). Data were obtained using a Digidata 1322x analog-to-
digital converter (10 kHz sampling frequency) and pClamp 9.0 software (both from
Molecular Devices Corp.)

The 5 nm diameter, poly-L-lysine-conjugated gold nanoparticles (a model
cationic analyte) were diluted in the pH 7 buffered electrolyte described above. Because
the pH is below the pKa of the lysine amine groups (pKa = 10.0), the analyte
nanoparticles have excess cationic surface charge. The analyte solution was placed in the
half-cell facing the tip opening, and the nanoparticles were driven by electrophoresis
through the nanopore from the tip side to the base side of the conical pore-containing
membrane.

Typically, in resistive-pulse sensing, translocation of the analyte through the
nanopore sensor element results in a downward (decreasing below the baseline) current-
pulse; i.e., the analyte transiently blocks the ion current flowing through the
nanopore. However, there have been recent reports of upward (increasing above
the baseline) current-pulses associated with translocation of highly charged analytes
through the nanopore sensor element. Upward current-pulses are observed because the
highly charged analyte brings its charge-balancing counterions with it as it translocates
the pore (Figure 3-2). This results in a transient increase in the ionic strength, and thus the
ionic conductivity, of the solution within the nanopore. Upward current-pulses were
observed for the cationic nanoparticles investigated here (Figure 3-3). The current-pulses
in three 5 minute recordings were analyzed at each concentration of analyte used. This
analysis yielded the average current-pulse duration ($\Delta \tau$), magnitude ($\Delta i$), and frequency ($f_p$).

**Results and Discussion**

**Nanopore Characterization**

Knowing the tip diameter of the conical nanopore is critical to developing functional resistive-pulse sensors. As Sexton, et al. demonstrated, the current-pulse frequency depends on tip diameter within a certain range. In order to determine the tip opening diameter, the base opening diameter must be known. Wharton, et al. showed that the two-step etch method, used in this work, reproducibly produces base diameters of 520 $\pm$ 45 nm. Knowing this value for the base diameter, the tip diameter can be obtained via calculation based on an electrochemical determination of the ionic conductance of the electrolyte-filled nanopore. Since previous studies have shown that pores etched with the two-step etch method produce truly conical-shaped pores, the ionic conductance of the pore ($G$) is related to the base diameter ($d_{base}$), tip diameter ($d_{tip}$), specific conductivity of the electrolyte ($\sigma$, 10.5-11.5 S/m for 1 M KCl), and the length of the pore ($L$, membrane thickness) via the following equation:

$$G = \frac{\pi \sigma d_{base} d_{tip}}{4 L} \quad \text{(Eq. 3.1)}$$

The value for $G$ was determined by obtaining the linear current-voltage curve for the electrolyte-filled nanopore. This was done by mounting the pore-containing membrane between both halve-cells of a U-tube cell comparable to that used for pore fabrication and filling each half-cell with electrolyte (1 M KCl). A Ag/AgCl electrode was placed into each half-cell and the applied transmembrane potential was linearly scanned from -1 V to
+1 V while measuring the ion current flowing through the conical pore at each voltage step (Figure 3-4). The slope of the resulting current-voltage curve is \( G \). If the slope isn’t linear between -1 V and +1 V, then the slope obtained from a smaller voltage range (e.g., -200 mV to +200 mV) is used.\(^{95}\)

**Why Conical-Shaped Nanopores**

In a conical-shaped nanopore, the voltage drop caused by the ion current flowing through the electrolyte-filled pore is focused to the electrolyte solution in the tip opening.\(^{95,96}\) Indeed, the electric field strength in the solution within the nanopore tip can be greater than \( 10^6 \text{ V m}^{-1} \), when the total voltage drop across the nanopore membrane is only 1 V.\(^{96}\) A consequence of this field-focusing effect is that the nanopore ion current is extremely sensitive to analyte species in the nanopore tip. That is, there is an ‘analyte sensing zone’ just inside the tip opening where detection occurs.\(^{75,89,95,96}\) This property makes conical-shaped nanopores more ideally suited for resistive-pulse sensing applications than cylindrical-shaped pores which lack this field-focusing effect. This has been demonstrated with prototype conical-nanopore sensors for analyte species ranging in size from small molecules,\(^{75}\) to DNA,\(^{89,90}\) proteins,\(^{95}\) and nanoparticles.\(^{96}\)

**Proposed Definition for the Detection Limit in Resistive-Pulse Sensing**

Because the cationic analyte nanoparticles are driven by electrophoresis through the nanopore, the flux (\( J \)) of analyte through the pore is given by:\(^{89}\)

\[
J = \frac{zFDCe}{RT}
\]  

(Eq. 3.2)

where \( z \) is the effective charge on the particle, \( D \) is the diffusion coefficient associated with particle transport through the tip opening, \( C \) is the particle concentration, \( E \) is the
electric field strength, and the other terms have their usual meanings. Multiplying both sides of Eq. 3.2 by the cross sectional area of the nanotube tip (\(A_t\)) converts the flux into the moles of analyte per second translocating the tip. Multiplying by Avogadro’s number (\(A_g\)) converts this to the number of molecules translocating the nanopore tip per second, which is equivalent to the frequency of the analyte-induced current-pulses, \(f_p\) by:

\[
f_p = \frac{zFDEA_t A_g}{RT}
\]

(Eq. 3.3)

Equation 3.3 suggests that \(f_p\) should be linearly related to the analyte concentration. This has been verified in studies of DNA analytes, as well as other studies.\(^89\) The linear dependence of \(f_p\) on concentration means that the detection limit for the resistive-pulse method must be defined in terms of how long the analyst is willing to wait to detect a current-pulse due to the analyte. At low analyte concentrations, the frequency becomes prohibitively low, or put another way, the average time interval between pulses becomes prohibitively long.

To define the detection-limit concentration, \(C_{dl}\), an agreement must be reached on how long the analyst is willing to wait to see a current-pulse due to the analyte. It is proposed here that the detection limit be defined as that concentration that yields, on average, a current-pulse every 60 sec. This definition was chosen because analysts will undoubtedly want to record and analyze at least 5 to 10 analyte current-pulses. Hence, with this definition, the total analysis time per sample would be 5 to 10 minutes. If the analysts can tolerate lower sample throughput than this, then a more liberal definition of the detection limit (e.g., a pulse every 2 minutes) could be employed.
What Order of Magnitude Detection Limit Can Be Anticipated?

With the definition proposed here, $C_{dl}$ is that analyte concentration that gives a current-pulse frequency of 0.017 Hz; we call this the detection-limit frequency, $f_{dl}$, and rearranging Eq. 3.3 yields:

$$C_{dl} = \frac{RT f_{dl}}{EFDA_s}$$

(Eq. 3.4)

If we assume that $E$ is $10^4$ V cm$^{-1}$, that $D$ for our 5 nm cationic nanoparticles is $9.7 \times 10^{-7}$ cm$^2$ s$^{-1}$ (calculated from the Stokes-Einstein equation, $D = kT/6\pi\eta r$), and that the charge on the particle is $z = 100$, the theoretical $C_{dl}$ for our particles using a conical nanopore having a tip diameter of 10 nm is 1 pM. However, while we use the same applied transmembrane potential of +1 V as used in the determination of the electric field strength via finite element simulations, $E$ is undoubtedly higher in our nanopore than the $10^4$ V cm$^{-1}$ calculated by these simulations because our tip diameter is smaller. Finite element simulations by Sexton, et al. showed that the electric field strength increases with decreasing tip opening diameter and constant base diameter. Furthermore, we do not yet have an accurate value for $z$ for our nanoparticles. Hence, this $C_{dl}$ of 1 pM should be regarded only as a rough approximation.

Analysis of the Current-Pulse Data

With an applied transmembrane potential of +1 V, the conical nanopore sensor yielded, in the absence of analyte, a steady-state ion current of ~1050 pA (Figure 3-5). Addition of the analyte nanoparticles resulted in upward current-pulses associated with translocation of the particles through the nanopore tip (Figure 3-6), and as expected, current-pulse frequency, $f_p$, increases with analyte concentration (Figure 3-7). Although the current-pulse frequency was very low, current-pulses were observed at 100 fM.
concentration level (Figure 3-8). However, over the concentration range studied in Figure 3-7), $f_p$ does not increase linearly with concentration, as predicted by Equation 3.3. Instead, over this concentration range, $f_p$ is related to $C$ via the empirical relationship:

$$y = -80.23664 \exp(-x/0.10971) + 84.03351$$

(Eq. 3.5)

which was determined via curve fit using Origin Software, version 8.1 SR2 (OriginLab, Massachusetts). Despite the lack of data points between 100 pM and 10 nM concentration levels, Eq. 3.5 and Fig. 3-7 suggest that a saturation phenomenon is occurring at higher concentrations for the cationic nanoparticle analyte. This was not the case for the DNA analyte investigated previously, where the predicted linear relationship (Eq. 3.3) was observed; however, a much smaller concentration range, 5 to 25 nM, was investigated.\(^{89}\)

The nature of this saturation phenomenon is the subject of on-going research. Similar saturation of ion conductance is known to occur in glycine receptor and sarcroplasmic reticulum channels.\(^\text{17}\) That is, with these channels, a comparable saturation phenomenon occurs when the binding-unbinding steps associated with ion permeation become rate limiting. Such behavior has been described empirically using Michaelis-Menton curves.\(^\text{17}\)

By analogy, it is possible that the cationic nanoparticles undergo electrostatic binding-unbinding with the anionic pore wall. Thus, at some very high nanoparticle concentrations, it is possible that the rate of particle entry into the pore exceeds the maximum rates of unbinding with the pore wall.

Figure 3-9 shows a scatter plot of the current-pulse magnitude ($\Delta i$) versus current-pulses duration ($\Delta \tau$), for the current-pulses obtained at the 10 nM nanoparticle concentration (Fig. 3-6). Figure 3-10 show histograms representing the current-pulse magnitude and duration at different particle concentrations. When the scatter plot is
compared to analogous scatter plots obtained by us and others for DNA analytes,\textsuperscript{89} we find that the spread in \( \Delta i \) was significantly small for the nanoparticle analyte. However, the spread in \( \Delta \tau \) was unexpectedly higher for the nanoparticles. For example, for a single-stranded DNA analyte 7250 bases long, \( \Delta \tau \) varied over a factor of 25 (5 ms to 75 ms) and \( \Delta i \) varied over a factor of 3.7 (300 pA to 1100 pA).\textsuperscript{89} In contrast, \( \Delta i \) for the nanoparticle analyte varied by only a factor of 0.5 (190 pA to 260 pA) (Fig. 3-9). However, \( \Delta \tau \) varied over a wide range from 60 ms to 4360 ms which is much worse than that observed for DNA.

The better reproducibility of \( \Delta i \) observed for the nanoparticle analyte (Fig. 3-9) undoubtedly results because the nanoparticle is a hard sphere, and thus does change conformation as it approaches and enters the nanopore tip. This is not the case for the DNA analyte, which had a radius of gyration larger than the radius of the nanopore tip.\textsuperscript{89} Hence, the Au nanoparticle-analyte is, in this regard, a better model system for fundamental investigations of nanopore resistive-pulse sensing such as those reported here. A related advantage is that such nanoparticles can be obtained commercially over a large size range and with a variety of different surface charges and chemistries.\textsuperscript{241,242}

The decrease in reproducibility of \( \Delta \tau \) for the cationic nanoparticles relative to that for DNA likely results from electrostatic interactions between the negatively-charged pore wall and the positively-charged nanoparticles. The resistive-pulse sensing of the DNA was conducted using a high ionic strength electrolyte (1 M KCl)\textsuperscript{89} whereas the nanoparticles were detected using a much lower ionic strength electrolyte (0.1 M KCl and 0.010 M phosphate). As a result, in the case of the pore used to detect DNA, the negative surface charges of the pore wall were screened by the high salt concentration.\textsuperscript{164} In
contrast, for the nanopore used to detect the nanoparticles, the charges along the pore wall aren’t screened as effectively and are available to interact more readily with the particles having opposite surface charge. Therefore, an electrostatic binding and release mechanism likely contributes to the broad distribution in $\Delta \tau$ for the cationic nanoparticle analyte. To get the best of both worlds (i.e., narrow distributions of both $\Delta \tau$ and $\Delta i$), a surface passivation technique is required for future studies.

Furthermore, three aspects of the conical nanopore system used here may augment the electrophoretic flux of the cationic nanoparticles. First, single, conical-shaped nanopores in track-etched PET membranes having small tip diameters (i.e., $< 15$ nm) are known to rectify the ionic current flowing through the electrolyte-filled nanopore in low ionic strength electrolyte (e.g., 0.01 M – 0.1 M KCl) when the electrolyte pH is above the isoelectric point (pI) of the membrane surface (pI ~3 for PET). As a result, cations, such as the cationic particles, are preferentially transported and anions are rejected.

Secondly, based on the model of ion current rectification proposed by Cervera, et al., when the electrodes are configured such that anode is placed at the tip opening and the cathode is placed at the base opening, migrating anions are driven from the base opening towards the tip opening. Since the pore surface has fixed anionic surface charge, anions cannot effectively pass through the tip due to electrostatic repulsion with the pore wall. Consequently, the local concentration of anions just inside the tip increases. In order to maintain electroneutrality, the local concentration of charge-balancing cations increases at the tip as well. Thus, the local ionic strength of the electrolyte in the tip region increases and a higher ionic conductance state is observed.
This is often referred to as the “on state” of the conical nanopore. A comparable electrode configuration was used to detect the cationic nanoparticles. Thus, it is reasonable to expect some degree of augmentation of the electrophoretic flux of the particles due to this higher ion conductance state. Lastly, Jin, et al. showed that electroosmotic flow occurs with conical-shaped nanopores in both the tip-to-base (i.e., anode at the tip) and base-to-tip (i.e., anode at base) directions. Although the impact of these 3 phenomena on electrophoretic flux were not investigated here, based on previous studies, it is believed that they contribute to an enhanced current-pulse frequency in the case where the analyte and electrode polarity at the tip side of the membrane are both opposite in polarity to the pore wall (as was the case for the cationic nanoparticles).

**Conclusions**

The model cationic, nanoparticle analyte studied here was detected at sub-nanomolar concentration levels which are lower than the lowest concentrations previously reported for small-molecule and macromolecule analytes obtained via resistive-pulse sensing. A simple definition for the detection limit in resistive-pulse sensing was proposed and a simple model for calculating what detection limits should be possible via this sensing paradigm was presented.

A narrow distribution in current-pulse amplitude was obtained which was attributed to the use of a hard-sphere nanoparticle analyte of narrow size distribution that does not have the conformational flexibility akin to many biological analytes. However, a broad distribution in current-pulse duration was observed. This was likely due to electrostatic binding and release between the cationic nanoparticle analyte and the anionic pore surface during translocation. It is believed that modifying the pore wall in a way that prevents such electrostatic interactions will undoubtedly improve the...
distribution in current-pulse duration, thereby making metallic nanoparticles the ideal analyte system for fundamental studies on resistive-pulse sensing. Furthermore, a saturation in the electrophoretic flux was observed at a high particle concentration. This suggests that at high concentrations, the flux becomes rate-limiting. This phenomenon remains the subject of on-going research.

It is believed that ion current rectification and electroosmotic flow may contribute to varying extents to an enhanced current-pulse frequency in the case where both (1) the analyte and (2) electrode polarity at the tip side of the membrane are opposite in polarity to the pore wall (as was the case for the cationic nanoparticles). This represents a departure from previous resistive-pulse studies using conical nanopores, like those with BSA$^{95}$ and streptavidin (Chapter 2), where the pore wall, analyte, and electrode polarity at the tip were all of the same polarity (i.e., negative for studies on BSA and streptavidin).
Figure 3-1. General conductivity cell setup used for resistive-pulse experiments.

Figure 3-2. Schematic of the process by which the local ionic strength within the tip region is increased as the cationic gold nanoparticle introduces its charge-balancing counterions.
Figure 3-3. Upward current-pulses reflecting the translocation of cationic gold nanoparticles through the tip opening of a conical nanopore. Expanded view of a typical current-pulse. Tip diameter: ~10 nm; Base diameter: 520 ± 45 nm.

Figure 3-4. Current-voltage curve determination of tip opening diameter. Slope of the linear current-voltage curve is equivalent to the ionic conductance (G) of the pore which, with a known base diameter of 520 ± 45 nm, was used to calculate the tip diameter.
Figure 3.5. Steady-state ion current using a single, conical-shaped nanopore in track etched poly(ethylene terephthalate). No particles present. Electrolyte: pH 7, 10 mM phosphate buffer that was also 0.1 M KCl. Applied transmembrane potential: +1 V.

Figure 3.6. Resistive-pulse sensing of 5 nm diameter cationic gold nanoparticles using a single, conical-shaped nanopore in track etched poly(ethylene terephthalate). Particle concentration: 10 nM. Electrolyte: pH 7, 10 mM phosphate buffer that was also 0.1 M KCl. Applied transmembrane potential: +1 V.
Figure 3-7. Plot of current-pulse frequency versus particle concentration taken at 5 minute intervals. Smaller plot (inset) is an expansion of the lower concentration data points contained in the larger plot. Curve fit performed using Origin version 8.1 SR2.
Figure 3-8. Resistive-pulse sensing of 5 nm diameter cationic gold nanoparticles using a single, conical-shaped nanopore in track-etched poly(ethylene terephthalate). Particle concentration: 100 fM. Electrolyte: pH 7 10 mM phosphate buffer that was also 0.1 M KCl. Applied transmembrane potential: +1 V.
Figure 3-9. Scatter plot of the magnitude of current block ($\Delta i$) versus the duration of that block ($\Delta \tau$). Particle concentration: 10 nM. Applied transmembrane potential: +1 V.

Figure 3-10. Histograms of current-pulse magnitude (left) and duration (right) data for cationic nanoparticles. Particle concentration: 10 nM (red), 100 pM (green), 40 pM (blue).
CHAPTER 4
RESISTIVE-PULSE SENSING OF NANOPARTICLES USING A CONICAL-SHAPED NANOPORE IN TRACK-ETCHED POLYIMIDE

Introduction

The utilization of sensing paradigms derived from nanopores has become increasingly popular in recent years. Such nanopore-based sensing devices generally employ a biological or artificial nanopore embedded in a membrane which is immobilized between two halves of a U-tube cell. As a transmembrane potential difference is applied, an ionic current passes through the electrolyte-filled nanopore which drives the electrophoretic translocation and corresponding detection of analyte molecules as they transiently block and traverse the pore sensing zone. The magnitude and duration of such transient blocks in the ion current provide identity information of the analyte molecules. This approach is often referred to as resistive-pulse sensing; however, the technique has been called stochastic sensing as well.

The most commonly used biological nanopore, α-hemolysin, is a bacterial protein which self-assembles into lipid bilayer membranes. Both native and genetically engineered forms of α-hemolysin have been used to detect DNA, polymers, small molecules, proteins, and metal ions via the resistive-pulse method. Resistive-pulse sensing based on α-hemolysin provides an excellent demonstration of the nanopore-based sensing concept; however, replacement of the fragile lipid bilayer membrane with an artificial pore construct is required to develop more mechanically robust and chemically stable devices.
A wide variety of approaches have been developed to fabricate such artificial nanopores for sensing applications including focused ion-beam sculpting of silicon oxide and nitride,\textsuperscript{26,38,43} embedded carbon nanotubes,\textsuperscript{49-50} electron-beam lithography and chemical etching of silicon membranes,\textsuperscript{28,54} femtosecond-pulsed laser drilling of glass,\textsuperscript{51-53} and track-etching of polymeric membranes.\textsuperscript{74,75,89,90,95,96} Such artificial pores have been used to detect DNA,\textsuperscript{26,31-36,41} polystyrene nanoparticles,\textsuperscript{48,96} small molecules,\textsuperscript{75} proteins,\textsuperscript{38,39,42,95} and viruses.\textsuperscript{51} Track-etched conical nanopores in polymeric membranes are of particular interest because of the control and reproducibility of pore size, tailorable pore surface chemistry,\textsuperscript{63} and cost-effectiveness (i.e., in terms of fabrication and materials). Conical nanopores have been fabricated in a variety of ion-tracked polymers including poly(ethylene terephthalate),\textsuperscript{59,62,63,90,91,95} polycarbonate,\textsuperscript{64,89,96,97} and polyimide.\textsuperscript{60,62,74,75,158}

Polyimide is particularly attractive because of its better transport properties (i.e., less noisy and more stable baseline) compared to poly(ethylene terephthalate).\textsuperscript{99} To date, only two analytes, anionic porphyrins\textsuperscript{75} and double-stranded DNA,\textsuperscript{243} have been sensed via the resistive-pulse method using single, conical-shaped nanopores in track-etched polyimide. Both of these analytes were detected under high salt conditions (i.e., 1 M KCl).\textsuperscript{74,243} This work presents an example of resistive-pulse sensing of a prototype analyte, 20 nm diameter fluorescent nanoparticles, using a conical nanopore in track-etched polyimide under lower salt conditions (i.e., 0.1 M KCl). A 2-step etching process and pore-loading procedure based on perfusion are also presented.
Experimental

Materials

Polyimide membranes (Kapton-50 HN, DuPont, 3 cm diameter, 12 μm thick) which contained a single, heavy ion-induced damage track, were obtained from Gesellschaft fuer Schwerionenforschung (GSI), Darmstadt, Germany. The carboxylated, fluorescent nanoparticles (carboxy-modified Fluospheres®, cat. #F8787, particle diameter = 20, concentration = 2.63 x 10^{15} particles/mL) were obtained from Invitrogen (Eugene, OR). Sodium hypochlorite (13% active chloride ion, Sigma) and all other chemicals (certified A.C.S. grade, Fisher Scientific) were used as received. Solutions were prepared using purified (18 MΩ) water (obtained by passing in-house distilled water though a Barnstead E-pure water purification system).

Fabrication of the Conical Nanopore

A conical-shaped nanopore was etched into the single-ion tracked polyimide membrane via anisotropic chemical etching of the heavy-ion induced damage track.\textsuperscript{60,62,75,99} This process entails mounting the irradiated membrane between two halves of a U-tube cell. An etch solution (sodium hypochlorite) was added to one half-cell and a neutralizing, or stop, solution (2 M KI) was added to the other half-cell.\textsuperscript{60,62,75,99} Chemical etching was performed at 50 °C using a temperature-feedback controlled hotplate. To monitor etching progress and detect membrane breakthrough, a platinum wire electrode was placed into each half-cell solution and a potential difference of 1 V was applied during etching using a Keithley 6487 voltage source/picoammeter (Keithley Instruments, Cleveland, OH). The electrodes were configured such that the positive electrode (anode) was placed in the etch solution and the negative electrode (cathode) placed in the stop solution.
During this etching process, the latent damage track was preferentially etched from the membrane face in contact with the etch solution towards the membrane face in contact with the stop solution. \(^6^{0,62,75,99}\) Before breakthrough, the transmembrane ion current was initially zero, but increased exponentially after the etch solution breaks through the membrane into the stop solution (\textit{vide supra}). The etching process was stopped upon reaching an ion current of \(\sim 100\) pA. The etch time required to reach this current value varied greatly but typically took between 2-4 hours. The membrane was then rinsed briefly with stop solution and subsequently with purified water.

This procedure produces a single, conical-shaped nanopore having a larger diameter, or base, opening on the etch solution side of the membrane and a small diameter, or tip, opening on the stop solution side of the membrane. The base diameter (in this case, 1255 nm) was determined via calculation based on the bulk-etch rate for polyimide as experimentally measured by field-emission scanning electron microscopy (FE-SEM). FE-SEM (JEOL JSM-6335F) was used to measure pore diameter of multi-ion tracked polyimide membranes that were chemically etched for different times (1, 2, and 3 hours). A previously described electrochemical method was used to determine the tip diameter. \(^59,75,89,90,95\) As will be discussed below, a second, or isotropic, etch step was developed for polyimide to tailor the tip opening to the desired diameter. After this step, the final tip opening diameter was \(\sim 21\) nm.

\textbf{Current-Pulse Measurements}

The single, conical nanopore membrane was mounted into a U-tube cell comparable to the cell used for pore fabrication. Both half-cells were filled with \(\sim 3.5\) mL of electrolyte solution comprised of 10 mM phosphate buffer solution (pH = 7.4) and 100
mM KCl. A Ag/AgCl electrode (Bioanalytical Systems/BASi, West Lafayette, IN) was immersed into each half-cell solution. The electrodes were connected to an Axopatch 200B (Molecular Devices Corp., Union City, CA) patch-clamp amplifier. The Axopatch served as the voltage source/picoammeter to apply a constant transmembrane potential difference and measure the resulting ion current flowing through the electrolyte-filled nanopore. The ion current was recorded in voltage-clamp mode on the Axopatch with a low-pass Bessel filter at 2 kHz bandwidth. A Digidata 1233A analogue-to-digital converter (Molecular Devices Corp.) was utilized to digitize the signal at a sampling rate of 10 kHz. Data were recorded and analyzed using pClamp 9.0 software (Molecular Devices Corp.).

The Ag/AgCl electrodes were configured such that the Ag/AgCl anode was placed in the electrolyte solution facing the base opening and the Ag/AgCl cathode in the solution facing the tip opening. The carboxylated-nanoparticles have a negative effective surface charge in the pH 7.4 sensing buffer. Thus, the nanoparticles were added to the electrolyte solution facing the nanopore tip and subsequently driven via electrophoresis in the direction of tip-to-base through the conical nanopore.

Results and Discussion

Determination of the Bulk-Etch Rate

To determine the bulk-etch rate of polyimide, 3 ion-tracked polyimide membranes (ion track density = $10^6$ ions/cm$^2$) were chemically etched for etch times of 1, 2, and 3 hours. Each membrane was then analyzed via FE-SEM and the pore size measured. The 1-hour membrane had a mean pore diameter of 478 ± 16 nm (Figure 4-1A). The 2-hour and 3-hour membranes had mean pore diameters of 981 ± 29 nm (Figure 4-1B) and 1426 ± 59 nm (Figure 4-1C), respectively. The bulk-etch rate was determined to be 0.48 ± 0.02
μm/hour which is slightly faster that the published value of 0.42 ± 0.04 μm/hour. This value was then used to calculate the base diameter of the conical nanopore used for resistive-pulse sensing.

**Two-Step Etching Method for Ion-Track Polyimide**

In resistive-pulse sensing, a tip opening diameter comparable to the size of the target analyte is required. To achieve this, control and reproducibility of the tip opening diameter are a critical requisite of the pore fabrication process. Wharton, et al. introduced a two-step etching method for ion-tracked poly(ethylene terephthalate) (PET) which provides a process for controlling and reproducing tip diameters of 50 nm or less. In step one, PET is anisotropically etched (with 9 M NaOH) from one membrane face for a fixed, predetermined amount of time (i.e., 2 hours) to achieve a certain base diameter. In step two, PET is isotropically etched from both membrane faces using a dilute etch solution (i.e., 1 M NaOH, or ~11% of the step one etchant concentration). Using a similar approach, a two-step etching approach for fabricating conical nanopores in ion-tracked polyimide was developed.

In step one, a heavy ion-irradiated polyimide membrane is etched to a predetermined value of the ion current around 100 pA. Using a fixed etch time for the first step (as used for PET) is problematic for polyimide for two reasons. First, for polyimide, when the etch solution breaks through to the stop solution, there is an exponential increase in the transmembrane ion current along a short time scale (Figure 4-2A). Thus, the tip opening diameter changes dramatically during a brief time interval. In contrast, the ion current detected upon breakthrough in PET increases at a much lower rate (Figure 4-2B). Secondly, the breakthrough time varies greatly between polyimide
membranes (Figure 4-2A). With PET, the breakthrough time is more consistent. As a result, in step one, polyimide is etched to an ion current value of ~100 pA.

In step two, a slower bulk-etch rate is desired. Since the bulk-etch rate is largely governed by etchant concentration, the optimal etch solution concentration had to be determined. To accomplish this, 4 ion-tracked polyimide membranes were etched via step one to an ion current value of ~100 pA. Each membrane was subsequently etched isotropically using 1 M KCl spiked with different concentrations (i.e., 1%, 3%, 6%, and 10%) of the step one etchant at ambient temperature using an applied transmembrane potential of 1V. Figure 4-3A shows the impact of etch time on the transmembrane ion current at each etch solution concentration. At the 1% level, very little change in the ion current occurred. However, at the 10% level, the ion current increased very dramatically (65 nA in 1 hour). By comparison, the transmembrane ion current during a typical second etch step for PET using ~11% of the first etch step etchant concentration is illustrated in Figure 4-3B. With PET, the ion current at this level increases ~25 nA in 1 hour. Thus, two intermediate etch solution concentrations at 3% and 6% were used to etch polyimide and slower etch rates of 5 nA and 20 nA in 1 hour were observed, respectively (Figure 4-3A).

**Nanopore Characterization**

In order to determine the tip opening diameter, the base opening diameter must be known. The base diameter for conical nanopores is determined primarily from the bulk-etch rate and FE-SEM. For the conical nanopore used in the sensing work described here, the base diameter was determined, using the bulk-etch rate for polyimide, to be 1255 nm. With this value, the tip diameter can be calculated via experimental
determination of the ionic conductance (G) of the electrolyte-filled conical nanopore via the following equation:

\[ G = \frac{\pi \sigma d_{\text{base}} d_{\text{tip}}}{4L} \]  

(Eq. 4.1)

where \( d_{\text{base}} \) represents the base diameter, \( d_{\text{tip}} \) is the tip diameter, \( \sigma \) is the conductivity of the electrolyte (pH 6, 1 M KCl, \( \sigma = 10.5 - 11.5 \) S/m), and \( L \) is the pore length (or membrane thickness). The value of \( G \) was determined experimentally by a linear scan of the applied transmembrane potential from -1 V to +1 V and measuring the resulting ionic current flowing through the pore at each potential step. As a result, a current-voltage curve is obtained in which the slope is \( G \).

**Controlling the Tip Diameter in Ion-Tracked Polyimide via Isotropic Etching**

Single, ion-tracked polyimide was etched using the first step etch described above and stopped less than 100 pA. A current-voltage curve was obtained using 1 M KCl (pH 6); however, no ion current was observed (blue trace, Figure 4-4). This was likely due to stopping the first etch step short of complete breakthrough occurring. The membrane was etched isotropically using the 3% etchant solution in 1 M KCl until a transmembrane ion current value of 6 nA was achieved. The tip opening diameter was calculated via current-voltage curve to be 13 nm (red trace, Figure 4-4). The conical nanopore was subsequently etched to final ion current values of 9 nA, 16 nA, and 19 nA with corresponding tip diameters of 15 nm (grey trace, Figure 4-4), 25 nm (black trace, Figure 4-4), and 28 nm (green trace, Figure 4-4), respectively. Figure 4-5 shows the relationship between final nanopore ion current and tip opening diameter. This demonstrates the great promise of applying the two-step etch method to controlling the tip opening diameter of track-etched, conical nanopores in polyimide.
Interestingly, the conical nanopore exhibited ion current rectification at all tip diameters studied using 1 M KCl (Figure 4-4). Ion current rectification occurs when there is asymmetric flow of cations and anions through the electrolyte-filled nanopore.\textsuperscript{100,101,102,103} It is observed experimentally as a non-linear current-voltage curve. In general, this phenomenon occurs when the pore radius is comparable to the thickness of the electrical double layer.\textsuperscript{102} To increase the extent of ion current rectification, either the pore radius must be decreased (e.g., by decreasing the tip diameter\textsuperscript{102}) and/or the thickness of the electrical double layer must be increased (e.g., by lowering the ionic strength of the electrolyte\textsuperscript{164}). In this case, the electrical double layer thickness is negligible because of the high ionic strength of the 1 M KCl electrolyte.\textsuperscript{164} The extent of ion current rectification is measured by determining the rectification ratio (R. R.) using the following equation:\textsuperscript{104}

\[
R. R. = \frac{|i_{E=-1V}|}{|i_{E=+1V}|} \quad \text{(Eq. 4.2)}
\]

where \(i_{E=-1}\) represents the value of the transmembrane ion current at an applied potential of -1 V, and \(i_{E=+1}\) is the value of the ion current at +1 V. In Figure 4-6, the rectification ratios in 1 M KCl were plotted as a function of tip opening diameter. The largest rectification ratio was found to be 4 for the smallest tip diameter (13 nm) and decreased linearly with increasing tip diameter through a tip diameter of 28 nm (Figure 4-6). Ion current rectification under high ionic strength electrolyte (i.e., 1 M KCl) conditions is due to the increased negative surface charge density along the pore surface. Jin, et al. observed a large degree of ion current rectification in 1 M KCl using conical nanopores in mica membranes.\textsuperscript{156} Harrell, et al. tailored the pore surface of conical nanopores in track-etched polycarbonate with DNA chains of varying lengths attached to the pore wall.
The degree of ion current rectification was observed to increase with increasing DNA chain length (i.e., increasing negative surface charge).

**Resistive-Pulse Sensing of Carboxylated, Fluorescent Nanoparticles**

An important property of the conical nanopore sensing element described here is that the voltage drop attributed to the ion current flowing through the electrolyte-filled nanopore is focused in and around the nanopore tip opening.\(^{95,96}\) Finite element simulations performed by Lee, et al. indicate that the focused electric field strength located in the region just inside the tip is on the order of \(10^6\) V/m when a transmembrane potential of +1 V is applied.\(^{96}\) As a result, a very sensitive detection zone forms at the tip opening. The ion current flowing through this detection zone is very sensitive to the presence of any molecular species present in or near this region.\(^{75,89,95,96}\) This is one advantage of using conical-shaped nanopores for resistive-pulse sensing applications instead of cylindrical-shaped nanopores.

A conical nanopore was fabricated in polyimide using the 2-step method described previously (*vide supra*). The base opening diameter was 1255 nm and the tip opening diameter was determined via a current-voltage curve to be 28 nm (blue trace, Figure 4-7). No current-pulses were initially observed using this conical nanopore. It was believed that this was due to the poor wetting behavior of polyimide. Therefore, a wetting agent, methanol, was used to first wet the nanopore for 10 minutes. The methanol was removed and quickly replaced with sensing buffer that was pH 7.4 10 mM phosphate buffer and 100 mM KCl. The U-tube cell was placed briefly into a vacuum chamber and a vacuum applied for 10 minutes. The sensing buffer was alternately removed and replaced with new buffer on each side of the cell several times via perfusion *while* keeping the membrane wet. After this process, a second current-voltage curve was
obtained and the tip diameter determined to be 41 nm (red trace, Figure 4-7). The difference between this tip value and the previous tip value of 28 nm is believed to be due to a more complete filling of the pore with electrolyte after wetting. In other words, the pore wasn’t completely filled with electrolyte and perhaps, a surface conduction mechanism along the negatively-charged pore wall dominated the previously measured ion current.

At an applied transmembrane potential of +200 mV, in the absence of analyte, a steady-state ion current of ~2500 pA that was free of current-pulses was observed (Figure 4-8A). Upon addition of 100 nM carboxylated, fluorescent nanoparticles (20 nm diameter) to the solution on the tip side of the membrane, transient current-pulse events were observed (Figure 4-8B). Current-pulses have been observed for proteins and DNA using track-etched, conical nanopores in PET. Similarly, current-pulses have been observed for small molecules and DNA using conical pores in polyimide. Such current-pulse data have been attributed to the transient blockage of the transmembrane ion current as the analyte translocates the detection zone of the nanopore.

Figure 4-9B shows an expanded view of typical current-pulses for the fluorescent nanoparticles. The ion current increases sharply at the start and is sustained for a certain pulse duration, and then decreases sharply back to the baseline. That is, the current-pulses are upward (i.e., increasing above the steady-state baseline) and square-shaped. Upward current-pulses have been observed previously with DNA and cationic, protein-coated nanoparticles (Chapter 3) and have been attributed to an increase in the local ionic strength of the electrolyte in the tip opening upon nanoparticle translocation. That is, the negatively-charged, carboxylated nanoparticle carries its charge-balancing counterions
with it into the tip opening of the nanopore. These additional charge carriers result in a transient increase in the local electrolyte concentration in the tip region upon nanoparticle entry and translocation. As a result, ion current enhancement is observed as an upward current-pulse.

Another plausible explanation is local charge inversion. This phenomenon occurs when a charged analyte transiently increases or decreases the magnitude of the surface charge along the pore wall within the tip opening. In the case of the fluorescent nanoparticles used here, the conical nanopore in track-etched polyimide rectifies the ion current in 1 M KCl. Therefore, the negative surface charge of the pore wall must be very large. Although a quantitative determination for the surface charge of the pore wall is not available, it is possible that local charge inversion occurs upon the transient adsorption/desorption of a less negative particle onto the pore wall within the tip opening.

Interestingly, the only two reports of resistive-pulse sensing using polyimide nanopores used a high ionic strength electrolyte (pH 7-8, 1 M KCl). In these cases, analyte (i.e., porphyrin and double-stranded DNA) translocation was observed as transient current-pulses that were downward, as opposed to the upward pulses observed here for nanoparticles in 0.1 M KCl (pH 7.4). This suggests that the current-pulse direction is very sensitive to electrolyte concentration and ionic strength along the pore wall.

In Figure 4-8A, the steady-state background ion current was ~2500 pA using an applied transmembrane potential of +200 mV. In contrast to a conical nanopore in track-etched PET, the transmembrane ion current is two times larger than that observed with
comparable PET pores for which a higher potential of 1 V and identical supporting electrolyte were used. One cause for this difference is the lower pore resistance in the polyimide nanopore. The ionic resistance of the nanopore ($R_{pore}$) is related to the tip opening diameter ($d_{tip}$), and base diameter ($d_{base}$) via the following equation:

$$R_{pore} = \frac{1}{G} = \frac{4L}{\pi \sigma d_{tip} d_{base}}$$

(Eq. 4.3)

where $\sigma$, $L$, and $G$ are as previously described. Eq. 4.3 shows that $R_{pore}$ is inversely proportional to the product of the tip and base opening diameters. Thus, for example, for a PET nanopore having a $d_{base}$ of 520 nm, $d_{tip}$ of 17 nm, and $L$ of 12 $\mu$m, the ionic resistance of the pore is higher than that for the polyimide nanopore used in this work ($d_{base} = 1255$ nm, $d_{tip} = 41$ nm, $L = 12$ $\mu$m). Consequently, higher ion currents can be achieved at lower $R_{pore}$ and larger cone angles (Chapter 6). Another benefit of using conical nanopores with large cone angles is that lower potentials can be used to drive electrophoresis.

Figure 4-10 shows that the current-pulses observed for the nanoparticles are due to electrophoretic transport. At an applied potential of +300 mV, when the electrode polarity was reversed for 1 hour, no current-pulses attributed to nanoparticle translocation were observed. This is because with the polarity reversal, the anionic nanoparticles are driven electrophoretically away from the nanopore. Interestingly, some large spikes in the ion current occurred while the electrode polarity was reversed. While the origin of these spikes is unknown, they appear to occur much more frequently upon electrode polarity reversal and less frequently using the normal electrode polarity (i.e., anode at the base opening and cathode at the tip opening) and in the presence of anionic nanoparticles. The
difference in the steady-state ion current between electrode polarities (i.e., reversed vs. normal) was due to ion current rectification. As previously discussed, ion current rectification was observed by measuring current-voltage curves under high ionic strength conditions (1 M KCl) (Figure 4-4). In the case of resistive-pulse sensing, the ionic strength of the sensing buffer is much lower (pH 7.4 10 mM phosphate and 100 mM KCl). Therefore, ion current rectification undoubtedly occurs because the electrical double layer thickness increases as the ionic strength of the supporting electrolyte decreases from 1 M KCl to 0.1 M KCl. Upon returning the electrodes to the normal polarity, an induction time was observed. That is, a period of time prior to the start of current-pulses occurred and was less than 4 min.

The change in current-pulse signature as a function of applied transmembrane potential was determined at potentials of 50 mV, 100 mV, 200 mV, 300 mV, 400 mV, and 500 mV. In Figures 4-11A to 4-15A (see Figure 4-8A for E = 200 mV), the steady-state background ion current at each value of applied potential is shown. At each potential, the background ion current was free of current-pulses attributed to nanoparticle translocation. However, in a few cases (i.e., at E = +50 mV and E = +100 mV), a few of the current spikes comparable to those observed during the electrode polarity reversal were observed. These could be simply due to someone walking near the instrument during data acquisition since the Axopatch 200B is highly sensitive to the local environment. Steady-state background ion currents of approximately 700 pA, 1325 pA, 2500 pA, 3600 pA, 4600 pA, and 5500 pA were observed at applied transmembrane potentials of 50 mV, 100 mV, 200 mV, 300 mV, 400 mV, and 500 mV, respectively (Figure 4-8A, 4-11A to 4-15A).
Upon addition of 100 nM anionic nanoparticles to the solution side facing the tip opening of the pore, upward current-pulses were observed at each applied potential (Figures 4-8B, 4-11B to 4-15B). The current-pulse signature is comprised of the current-pulse amplitude and duration. The current-pulse amplitude ($\Delta i$) represents the change in ion current above or below the steady-state baseline current. The current-pulse duration ($\tau$) represents the time interval between the beginning of each current-pulse and when the ion current returns to the baseline. Table 4-1 shows the current-pulse amplitude and duration values at applied transmembrane potentials of 50 mV, 100 mV, 200 mV, 300 mV, 400 mV, and 500 mV.

At the lower potentials of 50 mV, 100 mV, and 200 mV, the current-pulse duration decreased from $418 \pm 245$ ms (at $E = 50$ mV), to $210 \pm 71$ ms (at $E = 100$ mV), and $164 \pm 54$ ms (at $E = 200$ mV). Figure 4-16 shows histograms of current-pulse duration at these potentials. This decrease was attributed to the increase in the electric field with increasing applied transmembrane potential. This has been observed previously in resistive-pulse sensing studies on DNA. The current-pulse duration ($\tau$) is inversely proportional to the electric field strength via the following equation:

$$\tau = \frac{l_D k T}{|z| e E D}$$

(Eq. 4.4)

where $l_D$ represents the length of the detection zone, $k$ is Boltzmann’s constant, $T$ is temperature, $z$ is the effective surface charge on the nanoparticle, $E$ is the electric field strength, and $D$ is the diffusion coefficient associated with nanoparticle transport through the tip. Interestingly, the percent relative standard deviation in the current-pulse amplitude decreased from 59% at 50 mV to 34% and 33% at 100 mV and 200 mV, respectively.
The current-pulse amplitude increased from 73 ± 2 pA (at $E = 50$ mV), to 113 ± 5 pA (at $E = 100$ mV), and 173 ± 4 pA (at $E = 200$ mV). Figure 4-17 shows histograms of the current-pulse amplitude at 50 mV, 100 mV, and 200 mV. This increase was due to the increase in the ion current at these increasing applied transmembrane potentials. In other words, the volumetric fraction of electrolyte displaced by the presence of a nanoparticle in the tip region is the same at each potential (i.e., nanoparticle and tip size are constant). However, since the ion current increases at these applied potentials, the translocation of each nanoparticle is reflected by a larger current-pulse amplitude. Furthermore, the relative standard deviation in current-pulse amplitude ranged from 2-4 % at these 3 potentials. This is undoubtedly expected because the current-pulse amplitude reflects the size of the analyte. In the case of the anionic nanoparticles studied here, the particles generally do not adopt multiple conformations akin to biological analytes (e.g., some proteins). Consequently, this resulted in a highly consistent (i.e., low percent relative standard deviation) current-pulse amplitude. Furthermore, as the applied transmembrane potential was increased further to 300 mV, 400 mV, and 500 mV, the current-pulse amplitude continued to increase to 232 ± 5 pA, 289 ± 5 pA, and 332 ± 6 pA, respectively. Figure 4-18 shows the relationship between current-pulse amplitude and applied transmembrane potential. The plot shows the relationship observed at 50 mV, 100 mV, and 200 mV was extended to 300 mV, 400 mV, and 500 mV.

A scatter plot of current-pulse amplitude versus current-pulse duration at potentials of 50 mV (green), 100 mV (red), and 200 mV (blue) is shown in Figure 4-19. From this plot, the occurrence of current-pulses having longer pulse durations decreases with increasing transmembrane potential. Additionally, this scatter plot shows the
narrow distribution of current-pulse amplitude which reflects the use of a nanoparticle analyte.

The current-pulse frequency, which relates to analyte concentration, describes the rate of electrophoretic transport of the analyte through the conical nanopore. This is related to the electrophoretic flux ($J$, in mol s$^{-1}$ cm$^{-2}$) via the following equation:\textsuperscript{89,95}$\textbf{J} = \frac{-z F D_c C E}{RT}$ \hspace{1cm} (Eq. 4.5)

where $z$ represents the effective charge of the nanoparticle, $F$ is Faraday’s constant, $D_c$ is the diffusion coefficient associated with diffusive transport through the tip opening, $C$ is the nanoparticle concentration, $E$ is the electric field strength focused at the tip opening, $R$ is the universal gas constant, and $T$ is temperature. By multiplying both sides of Eq. 4.5 with Avogadro’s number ($A$) and the cross-sectional area of the tip opening ($\pi r_{tip}^2$), Eq. 4.5 becomes the following equation for current-pulse frequency ($f_p$):\textsuperscript{89,95}$\frac{molecules}{s} = f_p = \frac{-z F D_c C E (\pi r_{tip}^2) A}{RT}$ \hspace{1cm} (Eq. 4.6)

From Eq. 4.6, the current-pulse frequency is directly proportional to the electric field strength focused at the tip opening of the conical nanopore. As the applied transmembrane potential increased, the current-pulse frequency for the anionic, fluorescent nanoparticles increased from 53 events/min at 50 mV, to 72 events/min and 85 events/min at 100 mV and 200 mV, respectively (Table 4-1). However, as the potential was increased further to 300 mV, 400 mV, and 500 mV, the current-pulse frequency decreased and pulse amplitude increased. This presents a perplexing issue that warrants further investigation before any definitive conclusions can be made. One possibility is that the applied electrophoresis current pins the nanoparticles on the
polyimide surface on the tip side of the membrane. By increasing the electrophoresis current at higher potentials, the electrophoretic force acting on these nanoparticles increases as well. Therefore, the entry of these nanoparticles into the tip opening of the conical nanopore is impeded. Lee, et al. observed the pinning of nanoparticles to the membrane surface. However, their particles were larger than the tip opening diameter. Another plausible explanation is that at higher potentials, de-wetting of the nanopore occurs and the seal between half-cells is either lost or reduced. In fact, the background ion current in the presence of nanoparticles at the highest potential studied, 500 mV (Figure 4-15), decreased with increasing data acquisition time.

Conclusions

A single, conical-shaped nanopore was fabricated in polyimide using a two-step etching method that was developed to tailor the tip opening diameter. Despite variability in the track-etch rate that leads to variability in the membrane breakthrough times, this two-step process is an important step towards reproducibly fabricating conical nanopores of desired dimensions in ion-tracked polyimide. This approach was used to fabricate a conical nanopore which was used for the resistive-pulse sensing of 20 nm diameter carboxylated, fluorescent nanoparticles. Unlike the two previous reports of resistive-pulse sensing using conical nanopores in polyimide with a high ionic strength sensing buffer, a lower ionic strength sensing buffer was used for sensing nanoparticles. At first, no current-pulse events were observed. However, after a wetting agent was utilized to facilitate more efficient filling of the nanopore with sensing buffer via perfusion, upward current-pulse events were observed. These upward events were attributed to either (1) a local enhancement of the ionic strength at the tip opening due to the transient
introduction of additional charge carriers\textsuperscript{35} or (2) local charge inversion\textsuperscript{245} induced by the translocating anionic nanoparticles.

 Compared to the small cone angle typically observed in PET, the cone angle inherent to nanopores fabricated in track-etched polyimide is larger due to a faster bulk-etch rate and slower track-etch rate.\textsuperscript{59,60,62,99} As a consequence, lower applied transmembrane potentials were used to electrophoretically drive nanoparticles through the nanopore tip opening. Current-pulses were observed at applied potentials of 50 mV, 100 mV, 200 mV, 300 mV, 400 mV, and 500 mV. In agreement with theory,\textsuperscript{89,95} the mean current-pulse duration decreased and pulse frequency increased as the potential was increased from 50 mV to 100 mV and 200 mV. The current-pulse amplitude increased in near linear fashion with increasing potential over the entire range of applied transmembrane potentials studied. At the higher values (i.e. 300 mV, 400 mV, and 500 mV) of applied potential, the current-pulse duration worsened (i.e., increased both in magnitude and distribution) and the pulse frequency decreased. Such perplexing results could be attributed to de-wetting of the pore or pinning of nanoparticles to the membrane face on the tip side of pore due to increased electrophoretic force present at increasing transmembrane potentials. However, further studies are needed to investigate this phenomenon further.

 Moreover, this report represents the first resistive-pulse sensor using a conical-shaped nanopore in track-etched polyimide for (1) the detection of an analyte other than double-stranded DNA\textsuperscript{243} and porphyrins\textsuperscript{75} and (2) detection under lower ionic strength sensing conditions which is desirable for macromolecules (e.g., proteins, disease
biomarkers). Thus, this work is a progressive step forward in the development of track-
etched conical nanopores in polyimide for use in resistive-pulse sensing devices.
Figure 4-1. Electron micrographs of multiple nanopores etched into multiple ion-tracked polyimide membranes at different etch times. Etch times were (A) 1 hour, (B) 2 hours, and (C) 3 hours. Mean pore diameters were $478 \pm 16$ nm (A), $981 \pm 29$ nm (B), and $1426 \pm 59$ nm.
Figure 4-2. Ion current-time recordings for monitoring breakthrough in (A) polyimide and (B) PET membranes during the first-step etch. For polyimide (A), 3 different membranes were etched as reflected by the different colored traces.

Figure 4-3. Ion current-time recordings for tailoring the tip opening diameter during the second-etch step in (A) polyimide and (B) PET membranes. For polyimide (A), 4 different membranes were second step-etched using different concentrations of the first-step etchant, 10% (blue trace), 6% (green trace), 3% (light blue trace), and 1% (red trace) in 1 M KCl. For PET (B), 11% of the first-step etchant concentration was used in the second-step etch.
Figure 4-4. Current-voltage curves obtained in 1 M KCl used to calculate the diameter of the tip opening after each round of second-step etching of polyimide. Blue: first-step etch stopped short of breakthrough (no ion current flow). Red: second-step etch to 6 nA (d_{tip} ~13 nm). Grey: second step etch to 9 nA (d_{tip} ~15 nm). Black: second step etch to 16 nA (d_{tip} ~25 nm). Green: second step etch to 19 nA (d_{tip} ~28 nm).

Figure 4-5. Plot of tip opening diameter versus final nanopore ion current during the second-, isotropic, etch step for single track-etched polyimide.
Figure 4-6. Plot of rectification ratio versus tip opening diameter in 1 M KCl (pH 6) after the second-step etch of a conical pore in polyimide.

Figure 4-7. Current-voltage curves obtained in 1 M KCl (pH 6) after the second-step etch (blue, $d_{\text{tip}} \sim 28$ nm) and after treatment with a wetting agent and vacuum (red, $d_{\text{tip}} \sim 41$ nm).
Figure 4-8. Ion current-time transients (A) in the absence of nanoparticles and (B) with 100 nM carboxylated, fluorescent nanoparticles. Applied transmembrane potential = 200 mV.
Figure 4-9. Ion current-time transients of (A) 100 nM carboxylated, fluorescent nanoparticles and (B) expanded view of (A). Applied transmembrane potential $= 200$ mV.
Figure 4-10. Ion current-time transients of 100 nM carboxylated, fluorescent nanoparticles with (A) a reversed transmembrane potential of -300 mV for 60 min. and (B) transmembrane potential of +300 mV.
Figure 4-11. Ion current-time transients (A) in the absence of nanoparticles and (B) with 100 nM carboxylated, fluorescent nanoparticles. Applied transmembrane potential = 50 mV.
Figure 4-12. Ion current-time transients (A) in the absence of nanoparticles and (B) with 100 nM carboxylated, fluorescent nanoparticles. Applied transmembrane potential = 100 mV.
Figure 4-13. Ion current-time transients (A) in the absence of nanoparticles and (B) with 100 nM carboxylated, fluorescent nanoparticles. Applied transmembrane potential = 300 mV.
Figure 4-14. Ion current-time transients (A) in the absence of nanoparticles and (B) with 100 nM carboxylated, fluorescent nanoparticles. Applied transmembrane potential = 400 mV.
Figure 4-15. Ion current-time transients (A) in the absence of nanoparticles and (B) with 100 nM carboxylated, fluorescent nanoparticles. Applied transmembrane potential = 500 mV.
Figure 4-16. Histograms of current-pulse duration for 100 nM carboxylated, fluorescent nanoparticles at applied transmembrane potentials of (A) 200 mV, (B) 100 mV, and (C) 50 mV.
Figure 4-17. Histograms of current-pulse amplitude for 100 nM carboxylated, fluorescent nanoparticles at applied transmembrane potentials of (A) 200 mV, (B) 100 mV, and (C) 50 mV.
Figure 4-18. Plot of current-pulse amplitude versus applied transmembrane potential for 100 nM carboxylated, fluorescent nanoparticles.

Figure 4-19. Scatter plot of current-pulse amplitude ($\Delta i$) versus current-pulse duration ($\tau$) for 100 nM carboxylated, fluorescent nanoparticles at applied transmembrane potentials of (A) 50 mV (green), (B) 100 mV (red), and (C) 200 mV (blue).
Table 4-1. Tabulated data for current-pulse amplitude (Δi), current-pulse duration (τ), and current-pulse frequency (fp) of 100 nM carboxylated, fluorescent nanoparticles as a function of applied transmembrane potential (E).

<table>
<thead>
<tr>
<th>E (mV)</th>
<th>Δi (pA)</th>
<th>τ (ms)</th>
<th>f_p (events/2 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>73 ± 2</td>
<td>418 ± 245</td>
<td>106</td>
</tr>
<tr>
<td>100</td>
<td>113 ± 5</td>
<td>210 ± 71</td>
<td>144</td>
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<td>200</td>
<td>173 ± 4</td>
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</tr>
<tr>
<td>300</td>
<td>232 ± 5</td>
<td>297 ± 213</td>
<td>112</td>
</tr>
<tr>
<td>400</td>
<td>289 ± 5</td>
<td>521 ± 351</td>
<td>71</td>
</tr>
<tr>
<td>500</td>
<td>332 ± 6</td>
<td>515 ± 304</td>
<td>66</td>
</tr>
</tbody>
</table>
CHAPTER 5
DIRECT COUPLING OF AMINE-MODIFIED POLY(ETHYLENE GLYCOL) TO PORE SURFACES OF CONICAL NANOPORES FOR PREVENTING NON-SPECIFIC PROTEIN ADSORPTION

Introduction

In recent years, there has been increasing interest in utilizing nanoscale pores as resistive-pulse sensors to develop new analytical tools for a wide variety of target analytes. Such nanopores have been developed in materials comprised of both biological and artificial building blocks. Biological nanopores, such as α-hemolysin, have been utilized to detect DNA, small molecules, and metal ions. Abiotic, or artificial, nanopores have been used to detect DNA, proteins, small molecules, viruses, and particles. Despite the excellent reproducibility in pore diameter provided by biological pores, biological pores suffer from several limitations. For example, the most widely used biological pore, α-hemolysin, self-assembles and inserts itself into lipid bilayer membranes which are very fragile. Such membranes cannot endure the increased transmembrane potentials required to provide a sufficient level of sensitivity without rupture. Additionally, the limiting pore diameter (i.e., 2 nm) of α-hemolysin restricts its analytical utility to ions, small molecules, and threading nucleic acids (i.e., single-stranded DNA) via the resistive-pulse method. Such drawbacks can be circumvented by using a pore construct that is more chemically stable and mechanically robust over a much wider range of applied potentials and that provides a means of tailoring the pore diameter, thereby expanding application to a broader size-range of target analytes. Hence, artificial materials, such as silicon nitride and
oxide, carbon nanotubes, silicon, glass, and polymeric membranes have been used to fabricate nanopores for developing resistive-pulse devices.

Track-etched polymeric membranes are of particular interest due to the relative ease and cost-effectiveness of fabrication and controllable pore size. Furthermore, the pore walls of such nanopores can be modified at-will to control the surface properties of the nanopore as well as introduce selectivity. One method of modifying the pore wall involves the electroless deposition of a thin gold film along the pore wall and subsequent chemisorption of thiols onto the gold surface. For instance, Siwy, et al. coated a single, conical-shaped nanopore in poly(ethylene terephthalate) with gold, followed by gold surface modification with thiol-modified biotin. This provided a mechanism for the selective recognition of the protein, streptavidin. In a similar fashion, Harrell, et al. modified a gold-coated conical pore in polycarbonate with thiol-modified DNA for studies on ion-current rectification. Sexton, et al. and Yu, et al. coated single- and multi-nanopore membranes with gold, followed by thiol-modified poly(ethylene glycol), respectively, in order to prevent non-specific protein adsorption. A similar approach was used in Chapter 2 for constructing resistive-pulse sensors for streptavidin.

In recent years, there has been increasing interest in directly coupling molecules to the pore wall of track-etched nanopores, thereby obviating the need for electroless gold deposition. Such direct coupling approaches take advantage of the functional groups produced as a result of track-etching. That is, the chemical etching of the latent damage track produces a pore surface populated with functional groups. Thus, simple coupling...
methods can be utilized to conjugate molecules directly to these functional groups on the pore surface. For example, free carboxylate groups are produced on the pore surface as a by-product of etching on both poly(ethylene terephthalate) and polyimide nanopores. A coupling reaction, such as that using EDC/sulfo-NHS, can be used to couple amines to these carboxylate groups on the pore wall. For instance, Kececi, et al. modified conical pores in track-etched poly(ethylene terephthalate) with ethanolamine via EDC/sulfo-NHS to reduce to negative surface charge of the pore surface. This strategy facilitated more effective detection of small DNAs. Using comparable coupling chemistry, Vlassiouk, et al. coupled ethylenediamine to nanopores in poly(ethylene terephthalate) to switch the polarity of the pore wall from negative to positive charge, thereby creating a nanofluidic diode.

Coating surfaces with poly(ethylene glycol) to prevent undesirable surface adsorption is a widely studied phenomenon. However, no systematic study focused on non-specific protein adsorption in single, conical-shape nanopores has been conducted. To advance studies for using nanopores in biosensing applications, minimizing, or eliminating, the non-specific adsorption of biomolecules is absolutely critical. The primary reason for this, in resistive-pulse sensing, is because the current-pulse frequency is proportional to the cross-sectional area of the tip opening. It is believed that any reduction in the electrophoretic flux at the tip opening due to non-specific interactions between the translocating analyte molecules and the pore wall will undoubtedly result in a decreased current-pulse frequency. Furthermore, non-specific interactions between proteins and the pore wall have been implicated in increased current-pulse duration and the occurrence of ion current rectification. It is believed that
both phenomena adversely impact the analytical utility of nanoscale resistive-pulse sensing. In this work, amine-modified poly(ethylene glycol) was directly coupled to single, conical-shaped nanopores in PET. The non-specific adsorption of three model proteins, bovine serum album, fibrinogen, and lysozyme, was studied via ionic pore conductance (i.e., current-voltage curves) and X-ray photoelectron spectroscopy.

**Experimental**

**Materials**

Poly(ethylene terephthalate) (PET) membranes (3 cm diameter, 12 \( \mu \)m thick) that were irradiated with a swift heavy-ion to produce a single damage track through the membrane were obtained from Gesellschaft fuer Schwerionenforschung (GSI, Darmstadt, Germany). Amine-modified poly(ethylene glycol) (PEG-amine), MW 550 Da (PEG-550), was obtained from Laysan Bio (Huntsville, AL). The 1-ethyl-3-[3-dimethylaminopropyl]carboiimide hydrochloride (EDC), \( N \)-hydroxysulfosuccinimide (sulfo-NHS), and 2-\( (N\)-morpholino)ethanesulfonic acid-buffered saline (MES) were obtained from Pierce (IL). Bovine serum albumin (BSA), lysozyme, and fibrinogen were obtained from Sigma (MO). All other chemicals were reagent grade or better and used as received. Purified water (18 M\( \Omega \), obtained by sending house-distilled water through a Barnstead E-pure water purification system) was used to prepare all solutions.

**Fabrication of Conical Nanopores**

Single, conical-shaped nanopores were etched into single ion-tracked PET membranes by anisotropic and subsequent isotropic chemical etching, also referred to as the two step-etch method.\(^6\) This process entails mounting the irradiated PET membrane between two halves of a U-tube cell. An etch solution (9 M NaOH) was placed in one half-cell and a stop, or neutralizing, solution (1 M formic acid with 1 M KCl) was placed
in the other half-cell. To monitor the etching process, a platinum wire electrode was placed into both half-cells with the anode in the etch solution and cathode in the stop solution. A transmembrane potential difference of 1 V was applied, and the resulting ion current measured using a Keithley 6487 voltage source/picoammeter (Keithley Instruments, Cleveland, OH). Initially, the ion current was zero, and upon breakthrough, the ion current suddenly increased. Breakthrough generally occurred after 60-90 minutes from the start of etching. As etching proceeds, the latent ion-induced damage track is preferentially etched in anisotropic fashion from the PET surface in contact with the etch solution to the PET surface contacting the stop solution. This anisotropic etch step was continued for 2 hours. Previous studies have shown that this process produces a base opening diameter of ~520 nm in PET.63

The pore was then etched in isotropic fashion by placing 1 M NaOH on each side of the single pore PET membrane.63 A transmembrane potential difference of 1 V was again applied by placing the platinum wire anode in the etch solution located at the base opening and the cathode at the tip opening of the pore. This second-etch step was monitored by measuring the transmembrane ion current flowing through the etchant-filled conical nanopore. As reported by Wharton, et al., this provides a means of monitoring the increasing tip diameter in real-time because the tip opening diameter correlates to the value of the ion current.63 Typically, this fabrication step was stopped at an ion current value of ~25 nA which provided a tip opening diameter of ~40 - 50 nm as determined electrochemically via current-voltage curves. The conical nanopore was then rinsed with and stored in water.
Ionic Pore Conductance Measurements

Two approaches were utilized in-tandem to study non-specific adsorption of three model proteins, fibrinogen, BSA, and lysozyme, on the pore surface and impact on tip diameter. For each protein, 3 single, conical-shaped nanopores in track-etched PET were fabricated having a tip opening diameter of ~45-55 nm. As will be discussed below, an electrochemical method was used to measure the tip size in 1 M KCl. The conical pores were then exposed overnight to a solution containing 100 nM protein in pH 7.4 10 mM phosphate that contained 100 mM KCl. The protein solution was then completely discarded and the pore thoroughly rinsed with water. The tip diameter was re-measured after protein exposure using the same electrochemical method based on ionic pore conductance. All pore conductance measurements were obtained with the nanopore-containing membrane mounted in a U-tube cell. After measuring the tip diameter, the membrane area around the pore was analyzed via X-ray photoelectron spectroscopy (vide infra).

To confirm PEG-amine attachment and evaluate the behavior of PEG-amine-modified nanopores towards protein adsorption, single, conical-shaped nanopores were fabricated in track-etched PET having a tip opening diameter of ~45-55 nm. PEG-amine was coupled via amide bond formation between the amine group on the PEG chains and the free carboxylates on the pore surface via well established EDC/sulfo-NHS coupling chemistry. The tip diameter was measured via current-voltage curves before and after PEG-amine modification. XPS was used to confirm PEG-amine attachment.

In experiments analogous to the protein exposure experiments describe above, the PEG-amine-coated nanopores were exposed overnight to a solution containing 100 nM protein in pH 7.4 10 mM phosphate that contained 100 mM KCl. The protein solution
was then completely discarded and the pore thoroughly rinsed with water. The tip
diameter was then re-measured to evaluate the impact of the PEG layer on protein
adsorption.

As a control experiment, 3 comparable PET pores were fabricated and treated in
similar fashion as the protein-exposed pores but were not exposed to protein. The tip
opening diameters for these nanopores were monitored for a period of several days.

**X-Ray Photoelectron Spectroscopy**

A PHI 5100 XPS (Perkin-Elmer, Waltham, MA) was used to investigate non-
specific protein adsorption on single, conical-shaped nanopores in track-etched PET.
More specifically, XPS was used for two reasons. First, XPS was used to verify
attachment of the PEG-amine to the PET surface by comparing the N 1s signal of
unmodified PET to that for PEG-amine-modified PET. Secondly, XPS was utilized to
verify that any change observed in the tip opening diameter (i.e., in unmodified PET) was
due to protein adsorption by again comparing the N 1s signal for nanopores exposed to
protein to that for pores not exposed to protein.

**EDC/Sulfo-NHS Coupling of Amine-Modified Poly(ethylene glycol) to PET**

The track-etch process of fabricating conical nanopores in PET membranes yields
free carboxylate groups along the pore walls and membrane surfaces. A commonly-
used conjugation technique, using EDC chemistry, was used to cross-link amine-
modified PEGs to these free carboxylates via formation of amide bonds. This entailed
first equilibrating the single pore-containing membrane in 20 mL of pH 5.5, 0.1 MES
buffer for 1 hour. To this solution, 10 mL of both 10 mM sulfo-NHS and 4 mM EDC in
pH 5.5, 0.1 MES buffer were added. The membrane was immersed into this activating
solution for 1 hour. Then, the membrane was removed, briefly immersed into pH 7.4 10
mM phosphate buffer for rinsing, and subsequently immersed in fresh pH 7.4 10 mM phosphate buffer which contained 100 mM PEG-amine. The coupling reaction was allowed to proceed for 2 hours at room temperature with gentle stirring. Then, the membrane was removed, rinsed with phosphate buffer, and stored in purified water overnight.

**Results and Discussion**

**Nanopore Characterization**

Conical-shaped nanopores have both a large diameter, or base, opening and a small diameter, or tip, opening. The base opening diameter for these studies was ~520 nm based on the two-step etch method for reproducibly etching conical pores in PET reported by Wharton, et al.\(^{63}\) Knowing the base diameter allows the tip opening diameter to be determined using a electrochemical method based on the ionic conductance of the electrolyte-filled nanopore.\(^{59,63,75,89,90,95}\) For conical pores, the tip diameter (\(d_{\text{tip}}\)) is related to the base diameter (\(d_{\text{base}}\)), pore length (\(L\), membrane thickness), conductivity of the electrolyte (\(\sigma\), 10.5 – 11.5 S/m for 1 M KCl), and ionic conductance of the pore (\(G\)) via the following equation:\(^{59,63,75,89,90,95}\)

\[
G = \frac{\pi \sigma d_{\text{tip}} d_{\text{base}}}{4L} \quad \text{(Eq. 5.1)}
\]

The value for \(G\) was experimentally determined via current-voltage curves obtained by scanning the applied transmembrane potential from -1 V to +1 V. That is, the pore-containing membrane was mounted into a U-tube cell, and 1 M KCl was placed into both half-cells along with Ag/AgCl electrodes (Bioanalytical Systems/BASi, West Lafayette, IN). A Keithley 6487 voltage source/picoammeter (Keithley Instruments, Cleveland, OH)
was used to obtain the current-voltage curves. This pore characterization procedure was utilized throughout this study on non-specific protein adsorption.

**Tip Diameter Stability of Unmodified Conical Pores in PET**

As a control experiment, the stability of the tip opening diameter was evaluated by monitoring the tip size for 3 track-etched single pores in PET for a period of 4 days. That is, 3 single ion-tracked PET membranes were etched to tip diameters of ~38-41 nm. All 3 pore-containing membranes were left mounted in comparable U-tube cells for the duration of the stability evaluation. Current-voltage curves were obtained for each membrane for 4 consecutive days in 1 M KCl to measure the tip diameter. Figure 5-1 to 5-3 show composite current-voltage curves for all 4 days for each membrane. Table 5-1 summarizes the tip size data and shows that the tip diameter for each pore changed very little (i.e., the greatest change was 3 % r.s.d.) over the 4-day time interval. Thus, single pores were deemed stable for the duration of these experiments.

**XPS of Unmodified PET Membranes**

As a control experiment for XPS studies, 6 PET membranes were analyzed via XPS. Figures 5-4 to 5-9 shows XPS surveys for each membrane. The unmodified PET membranes show strong signals for carbon (C 1s) and oxygen (O 1s). This is to be expected since the atomic percentages of carbon and oxygen in the PET monomer, \( \text{C}_{10}\text{O}_{4}\text{H}_{8} \), are 71.4% and 28.6%, respectively (XPS does not detect hydrogen). Table 5-2 summarizes the XPS data for all 6 PET membranes. Excellent reproducibility in the XPS data was observed amongst the 6 membranes. The percent relative standard deviations for the C 1s and O 1s signals were 1.1% and 2.8%, respectively. One PET membrane exhibited weak signals for Si 2s and Si 2p while all other membranes showed none. These peaks were attributed to some sample contamination during handling. Auger peaks
for oxygen and carbon were observed at 720 eV and 990 eV, respectively. Furthermore, the XPS revealed the absence of any detectable nitrogen (N 1s) signal in all 6 membranes. This is to be expected since unmodified PET does not contain any nitrogen.

No sodium (Na 1s) signal was observed for any of the 6 PET membranes because the membranes were not chemically etched. Kececi, et al. observed a Na 1s XPS signal for PET as a consequence of etching with NaOH. That is, the positively-charged sodium from NaOH binds to the negatively-charged carboxylate groups generated along the pore wall during track-etching. Aside from this, the C 1s and O 1s data obtained for all 6 membranes were consistent with the XPS results obtained by Kececi, et al. for unmodified PET.

**Impact of Non-Specific Protein Adsorption on Tip Diameter in Unmodified PET**

Three model proteins, BSA, fibrinogen, and lysozyme, were used to evaluate the impact of non-specific protein adsorption on the tip opening diameter of single, conical-shaped nanopores in track-etched PET. These 3 proteins were selected in an effort to expose a variety of different protein interactions to the nanopore surface. BSA is a 66 kDa protein (isoelectric point (pI) ~4.8) from the albumin family that is widely used in many biochemical applications (e.g., ELISA). Fibrinogen is a 340 kDa protein (pI ~6.0) involved in blood clotting and was chosen for its propensity to stick to surfaces in a manner akin to sticky serum proteins. Lysozyme is a 14 kDa enzyme (pI ~12.0) that damages the cell wall of bacteria and was chosen as a model cationic protein.

Single, conical-shaped nanopores were track-etched in PET using the two-step method to tip opening diameters of ~38-45 nm as measured via current-voltage curves in 1 M KCl. Each pore was then thoroughly rinsed with water. Then, the pore was
immersed overnight in 100 nM protein in pH 7.4, 10 mM phosphate buffer that also
contained 100 mM KCl. The pore was then removed from protein solution, rinsed
thoroughly with water, and the tip diameter re-measured in 1 M KCl. Any change in tip
diameter was attributed to non-specific adsorption of the protein to the pore surface.
Presence of the protein on the PET surface was verified via measuring the N 1s signal
using XPS.

Figures 5-10 to 5-12 show the results for BSA. Before BSA exposure, the tip
opening diameters were 40 nm, 41 nm, and 39 nm for pore A (Figure 5-10), pore B
(Figure 5-11), and pore C (Figure 5-12), respectively. After BSA exposure, the tip
diameters decreased to 27 nm (pore A), 29 nm (pore B), and 29 nm (pore C). After
measuring the tip size, the single pore-containing membranes were rinsed thoroughly
with water, allowed to dry, and analyzed via XPS (vide infra).

The results for fibrinogen are shown in Figures 5-13 to 5-15. Before fibrinogen
exposure, the tip opening diameters were 38 nm, 38 nm, and 39 nm for pore A (Figure 5-
13) pore B (Figure 5-14), and pore C (Figure 5-15), respectively. After fibrinogen
exposure, the tip diameters decreased to 26 nm (pore A), 20 nm (pore B), and 23 nm
(pore C). Again, after measuring the tip diameter, the pore-containing PET membranes
were rinsed with water, allowed to dry, and analyzed via XPS (vide infra).

Figures 5-16 to 5-18 shows the results for lysozyme. Prior to lysozyme exposure,
the tip diameters were 45 nm, 41 nm, and 42 nm for pore A (Figure 5-16), pore B (Figure
5-17), and pore C (Figure 5-18), respectively. Following lysozyme exposure, the tip
opening diameters decreased to 36 nm (pore A), 34 nm (pore B), and 34 nm (pore C).
After measuring the tip diameter, the membranes were rinsed with water, allowed to dry, and analyzed via XPS (vide infra).

**Analysis of Non-Specific Protein Adsorption to PET Membranes via XPS**

After measuring the pore tip diameter before and after exposure to proteins, each membrane was analyzed via XPS. More specifically, the membrane face around the tip opening was analyzed for nitrogen (N 1s). Since nitrogen was not present in any of the unmodified PET membranes, any protein present on the PET surface would be detected by a positive nitrogen signal. This nitrogen is largely due to the nitrogen-containing peptide backbone of proteins. Figures 5-19 to 5-21 show the XPS results for the 3 pore-containing PET membranes exposed to BSA. The N 1s signals were 9.1%, 10.0%, and 8.6% for pore A (Figure 5-19), pore B (Figure 5-20), and pore C (Figure 5-21), respectively. Figures 5-22 to 5-24 show the XPS results for the 3 pore-containing membranes exposed to fibrinogen. The N 1s signals were 14.6%, 13.3%, and 15.2% for pore A (Figure 5-22), pore B (Figure 5-23), and pore C (Figure 5-24), respectively. Some sodium (from etching), silicon (contaminant), and chlorine signal were observed for pores A and B but not pore C. Lastly, Figures 5-25 to 5-27 exhibit the XPS data obtained for the membranes exposed to lysozyme. The N 1s signal was 10.8%, 11.3%, and 9.6% for pore A (Figure 5-25), pore B (Figure 5-26), and pore C (Figure 5-27), respectively. Thus, each protein adsorbed to the PET surface. Table 5-3 summarizes these findings.

**XPS of PEG-Amine-Modified Single Pore-Containing PET**

XPS was utilized to verify the attachment of PEG-amine to the single, conical-shaped nanopores in PET. For the non-specific adsorption studies, a small PEG-amine (MW 550 Da, PEG-550) was used. Conical pores comparable to those used above were
fabricated in 2 PET membranes via the two-step etch method. Using the previously discussed EDC/sulfo-NHS coupling method, PEG-550 was attached to the pore surface and membrane faces. After PEG-550 attachment, the membrane was rinsed thoroughly with water, allowed to dry, and analyzed via XPS. Figures 5-28 and 5-29 show that after PEG-550 attachment, the nitrogen (N 1s) signal was detected at 1.7% and 1.4% for pore A and pore B, respectively. This reflected the successful coupling of the PEG-550 to the PET surface.

**Impact of Non-Specific Protein Adsorption on Tip Diameter in PEG-amine modified PET**

To investigate the impact of non-specific protein adsorption on pore tip diameter in the PEG-550-coated pores, several sets of current-voltage curves were obtained in 1 M KCl to measure the tip diameter. First, the tip diameters of the unmodified single pores were obtained. Three conical pores per protein were evaluated. Then, the pores were modified with PEG-550 and the tip opening diameters were re-measured. These PEG-coated pores were then immersed overnight in 100 nM protein in pH 7.4, 10 mM phosphate that was also 100 mM in KCl. The pores were removed from the protein solution, rinsed thoroughly with water, and the tip size re-measured a second time to determine the extent to which the PEG-550 layer affected non-specific adsorption.

For BSA, Figure 5-30A shows the current-voltage curves before and after PEG-550 modification of the pore and membrane surfaces. That is, the tip opening diameter before PEG-550 attachment was 54 nm. After attachment, the tip diameter decreased to 47 nm. This pore was then exposed to BSA. After BSA exposure, the tip diameter was re-measured to be 46 nm (Figure 5-30B). Thus, the tip size didn’t change much. Two additional conical pores, pores 2 and 3, were treated in a similar manner. Pore 2 (Figure
5-31A) showed pre- and post-PEG-550 modification tip sizes of 45 nm and 36 nm, respectively. After BSA exposure, the tip diameter of pore 2 was 34 nm (Figure 5-31B). Pore 3 (Figure 5-32A) exhibited pre- and post-PEG-550 modification tip diameters of 45 nm and 37 nm, respectively. After BSA exposure, the tip size of pore 3 was 38 nm (Figure 5-32B). Since there is ~10% error in the tip size, this change is negligible.

Similarly, for fibrinogen, Figure 5-33A shows the current-voltage curves obtained before and after PEG-550 modification of the pore and PET membrane surfaces (pore 1). The tip opening diameter before and after PEG-550 attachment was 41 nm and 25 nm, respectively. After exposure of the PEG-550-coated pore to fibrinogen, the tip size was 18 nm (Figure 5-33B). Pore 2 (Figure 5-34A) had pre- and post-PEG-550 modification tip sizes of 44 nm and 36 nm, respectively. After fibrinogen exposure, the tip diameter of pore 2 was 27 nm (Figure 5-34B). Pore 3 (Figure 5-35A) had pre- and post-PEG-550 modification tip diameters of 40 nm and 26 nm, respectively. After exposure to fibrinogen, the tip size was re-measured to be 20 nm (Figure 5-35B).

Lastly, lysozyme adsorption onto PEG-550-coated single pores was studied. Figure 5-36A shows the current-voltages curves obtained before and after PEG-550 modification of the pore and membrane surfaces (pore 1). The tip opening diameter before and after PEG-550 attachment was 42 nm and 34 nm, respectively. After lysozyme exposure to the PEG-550-coated pore, the tip diameter was 30 nm (Figure 5-36B). Pore 2 (Figure 5-37A) had pre- and post-PEG-550 modification tip sizes of 45 nm and 35 nm, respectively. After lysozyme exposure, the tip size of pore 2 was 32 nm (Figure 5-37B). Pore 3 (Figure 5-38A) had pre- and post-PEG-550 modification tip diameters of 48 nm and 41 nm, respectively. After exposure to lysozyme, the tip size was
measured to be 36 nm (Figure 5-38B). Table 5-4 summarizes the data for BSA, fibrinogen, and lysozyme. By attaching PEG-550 to the pore wall and membrane faces, non-specific protein adsorption was reduced but not eliminated. Although the adsorption of all 3 proteins was reduced in the PEG-550-coated pores, the PEG-550 layer was more efficient at blocking BSA and lysozyme adsorption than that of fibrinogen (Tables 5-4 and 5-5).

It is important to discuss why PEG is used and the impact of non-specific protein adsorption on the pore wall for developing resistive-pulse sensors. Such non-specific binding is generally due to different types of interactions between the proteins and the pore surface.\textsuperscript{222,223} First, proteins tend to adhere more readily to hydrophobic surfaces than hydrophilic surfaces.\textsuperscript{222} PEG reduces this tendency by making the surface more hydrophilic. Secondly, electrostatic interactions between charged proteins and the charged pore wall can also lead to non-specific adsorption.\textsuperscript{222} PEG decreases the ability of proteins to interact with the pore surface in this manner because the PEG molecules reduce the surface charge of the pore wall. Consequently, PEG can also impact the electrical double layer.\textsuperscript{222}

Bentzen, et al. studied the effect of PEG length on non-specific adsorption of quantum dots in live cell assays.\textsuperscript{222} They discovered that the length of the PEG chain was not important until it was shorter than 14 units. Thus, PEG-350 and PEG-550 conjugates were able to reduce non-specific binding by 70% and 60%, respectively. Longer PEG chains, such as PEG-2000, decreased binding even further (i.e., 90% reduction). Steric hindrance was not a problem until the PEG chain length was increased to MW 2000 Da or greater. As a consequence, Bentzen, et al. observed a inverse relationship between the
number of PEG chains bound and chain length.\textsuperscript{222} That is, as chain length increased, steric hindrance increased, thereby effectively reducing the number of cross-linked PEG chains. While this work dealt with quantum dots, it does suggest that PEG chain length, steric hindrance, and PEG coverage are key factors to consider in eliminating non-specific protein adsorption in conical nanopores.

During resistive-pulse sensing with conical nanopores, it is important that the cross-sectional area of the tip opening remain constant. Without a fixed tip diameter, the electrophoretic flux through the nanopore varies and, in some cases, may decrease due to a decrease in pore size caused by non-specific adsorption of proteins to the pore wall. For instance, Yu, et al. observed a decrease in the electrophoretic flux of proteins through an unmodified, multipore membrane due to non-specific adsorption.\textsuperscript{217} The electrophoretic flux ($J$, mol s$^{-1}$ cm$^{-2}$) through the tip opening is directly proportional to analyte concentration via the following equation:\textsuperscript{89,95}

$$J = -\frac{zF D_t C E}{RT}$$  \hspace{1cm} (Eq. 5.2)

where $z$ is charge on the analyte, $D_t$ is the diffusion coefficient of the analyte through the tip opening, $C$ is analyte concentration, and $E$ is the electric field strength in the tip. $F$, $R$, and $T$ have their usual meanings. Eq. 5.2 can be converted to current-pulse frequency ($f_p$) by multiplying both sides of Eq. 5.2 with the cross-sectional area of the tip opening ($\pi r_p^2$) and Avogadro’s number ($A$). As a result, Eq. 5.2 becomes the following equation:\textsuperscript{89,95}

$$\text{molecules/s} = f_p = \frac{-zF D_t C E (\pi r_p^2) A}{RT}$$  \hspace{1cm} (Eq. 5.3)

Eq. 5.3 shows that the current-pulse frequency, which reflects the analyte concentration, is proportional to the cross-sectional area of the tip opening. If non-specific protein
adsorption occurs, the current-pulse frequency is adversely impacted. For instance, Sexton, et al. reported a tip-size dependence of the current-pulse frequency for BSA.\(^9\) It is believed that this can lead to other problems such a poor pore-to-pore reproducibility, and wide distributions in current-pulse duration. Thus, non-specific interactions must be minimized.

An obvious question is why discard the previous PEGylation method of coating the pore surface with gold and subsequently modifying the gold layer with thiol-modified PEG?\(^{9,117,217}\) Eq. 5.3 shows that the current-pulse frequency is proportional to the electric field strength. To obtain improved limits of detection, higher current-pulse frequencies are needed. One way to achieve this is by increasing the electric field strength by applying higher transmembrane potentials. The problem is the gold layer delaminates at increased potentials. In Figure 5-39, a single, conical-shaped nanopore in track-etched PET was fabricated with a tip opening diameter of 42 nm. Electroless gold deposition\(^{163}\) was used to coat the pore surface with a thin gold film, thereby decreasing the tip diameter for 4 nm. In Figure 5-40, an applied transmembrane potential of 10 V was applied and the impact on the tip diameter measured as a function of time. After 2 minutes, the tip opening diameter increased from 4 nm to 12 nm. The tip size continued to increase with time while the high transmembrane potential was applied. This was attributed to a delamination of the gold layer due to resistive-heating.\(^6\) To mitigate this problem, an alternative approach for directly modifying the pore surface with PEG, based on EDC/sulfo-NHS coupling chemistry, has been presented here. Although this doesn’t offer any conclusive evidence of the stability of PEG-coated pores via EDC/sulfo-NHS at
such high potentials, it does indicate that gold-coated PET pores at higher transmembrane potentials are not stable.

Conclusions

Non-specific protein adsorption is problematic in resistive-pulse sensing due to the dependence of the cross-sectional area of the tip opening on the current-pulse frequency. Three model proteins, lysozyme, BSA, and fibrinogen, were found to absorb to the pore surface and decrease the tip opening diameter. In previous studies, tip size was experimentally found to impact current-pulse frequency, in agreement with theory. Furthermore, a surface adsorption phenomenon has been previously implicated in longer current-pulse durations. This is believed to adversely impact selectivity. Thus, PEG is attached to the pore wall to help mitigate these adsorption problems.

Taking advantage of the free carboxylates generated along the pore wall as a result of chemical etching, a direct coupling method using EDC/sulfo-NHS chemistry was presented to modify the pore surface with amine-modified PEG (MW 550 Da). Successful modification was verified via XPS. The ability of such PEG-amine-PET pores to resist non-specific adsorption of BSA, lysozyme, and fibrinogen was evaluated. The PEG-amine layer reduced the adsorption of all 3 proteins to varying extents.

The previous method of coating conical pores in PET with PEG by first depositing a gold surface film and subsequently attaching thiol-modified PEG suffers from instability at higher potentials. This approach also suffers from poor reproducibility. It is believed that the direct cross-linking of PEG-amine to the pore wall via an amide bond provides a more stable and reproducible PEG-coating of the PET surface. Further improvements in the biocompatibility of the pore surface may be gained by considering
PEG chains of different lengths, surface layers comprised of mixed PEG lengths, and PEG coverage density.
Figure 5-1. Current-voltage curves obtained for 4 consecutive days in pH 6, 1 M KCl (pore 1). Curves for days 0, 1, 2, 3, and 4 are overlaid.

Figure 5-2. Current-voltage curves obtained for 4 consecutive days in pH 6, 1 M KCl (pore 2). Curves for days 0, 1, 2, 3, and 4 are overlaid.
Figure 5-3. Current-voltage curves obtained for 4 consecutive days in pH 6, 1 M KCl (pore 3). Curves for days 0, 1, 2, 3, and 4 are overlaid.

Figure 5-4. XPS spectra of unmodified PET membrane 1.
Figure 5-5. XPS spectra of unmodified PET membrane 2.

Figure 5-6. XPS spectra of unmodified PET membrane 3.
Figure 5-7. XPS spectra of unmodified PET membrane 4.

Figure 5-8. XPS spectra of unmodified PET membrane 5.
Figure 5-9. XPS spectra of unmodified PET membrane 6.

Figure 5-10. Current-voltage curves obtained in 1 M KCl for pore A before exposure (blue trace, $d_{tp} = 40$ nm) and after exposure (red trace, $d_{tp} = 27$ nm) to BSA.
Figure 5-11. Current-voltage curves obtained in 1 M KCl for pore B before exposure (blue trace, \(d_{tp} = 41\) nm) and after exposure (red trace, \(d_{tp} = 29\) nm) to BSA.

Figure 5-12. Current-voltage curves obtained in 1 M KCl for pore C before exposure (blue trace, \(d_{tp} = 39\) nm) and after exposure (red trace, \(d_{tp} = 29\) nm) to BSA.
Figure 5-13. Current-voltage curves obtained in 1 M KCl for pore A before exposure (blue trace, $d_{tip} = 38$ nm) and after exposure (red trace, $d_{tip} = 26$ nm) to fibrinogen.

Figure 5-14. Current-voltage curves obtained in 1 M KCl for pore B before exposure (blue trace, $d_{tip} = 38$ nm) and after exposure (red trace, $d_{tip} = 20$ nm) to fibrinogen.
Figure 5-15. Current-voltage curves obtained in 1 M KCl for pore C before exposure (blue trace, \(d_{tip} = 39 \text{ nm}\)) and after exposure (red trace, \(d_{tip} = 23 \text{ nm}\)) to fibrinogen.

Figure 5-16. Current-voltage curves obtained in 1 M KCl for pore A before exposure (blue trace, \(d_{tip} = 45 \text{ nm}\)) and after exposure (red trace, \(d_{tip} = 36 \text{ nm}\)) to lysozyme.
Figure 5-17. Current-voltage curves obtained in 1 M KCl for pore B before exposure (blue trace, \(d_{\text{tip}} = 41 \text{ nm}\)) and after exposure (red trace, \(d_{\text{tip}} = 34 \text{ nm}\)) to lysozyme.

Figure 5-18. Current-voltage curves obtained in 1 M KCl for pore C before exposure (blue trace, \(d_{\text{tip}} = 42 \text{ nm}\)) and after exposure (red trace, \(d_{\text{tip}} = 34 \text{ nm}\)) to lysozyme.
Figure 5-19. XPS spectra of chemically-etched PET about a single nanopore exposed to BSA (pore A).

Figure 5-20. XPS spectra of chemically-etched PET about a single nanopore exposed to BSA (pore B).
Figure 5-21. XPS spectra of chemically-etched PET about a single nanopore exposed to BSA (pore C).

Figure 5-22. XPS spectra of chemically-etched PET about a single nanopore exposed to fibrinogen (pore A).
Figure 5-23. XPS spectra of chemically-etched PET about a single nanopore exposed to fibrinogen (pore B).

Figure 5-24. XPS spectra of chemically-etched PET about a single nanopore exposed to fibrinogen (pore C).
Figure 5-25. XPS spectra of chemically-etched PET about a single nanopore exposed to lysozyme (pore A).

Figure 5-26. XPS spectra of chemically-etched PET about a single nanopore exposed to lysozyme (pore B).
Figure 5-27. XPS spectra of chemically-etched PET about a single nanopore exposed to lysozyme (pore C).

Figure 5-28. XPS spectra of chemically-etched PET about a single nanopore modified with PEG-550-amine (pore A).
Figure 5-29. XPS spectra of chemically-etched PET about a single nanopore modified with PEG-550-amine (pore B).

Figure 5-30. Current-voltage curves of pore 1 obtained in 1 M KCl for (A) an unmodified nanopore (blue trace, $d_{tip} = 54$ nm), amine-PEG-550-modified nanopore (red trace, $d_{tip} = 47$ nm) and (B) the same PEG-550-modified nanopore (red trace, $d_{tip} = 47$ nm) before BSA exposure and after BSA exposure (grey trace, $d_{tip} = 46$ nm).
Figure 5-31. Current-voltage curves of pore 2 obtained in 1 M KCl for (A) an unmodified nanopore (blue trace, $d_{tip} = 45$ nm), amine-PEG-550-modified nanopore (red trace, $d_{tip} = 36$ nm) and (B) the same PEG-550-modified nanopore (red trace, $d_{tip} = 36$ nm) before BSA exposure and after BSA exposure (grey trace, $d_{tip} = 34$ nm).

Figure 5-32. Current-voltage curves of pore 3 obtained in 1 M KCl for (A) an unmodified nanopore (blue trace, $d_{tip} = 45$ nm), amine-PEG-550-modified nanopore (red trace, $d_{tip} = 37$ nm) and (B) the same PEG-550-modified nanopore (red trace, $d_{tip} = 37$ nm) before BSA exposure and after BSA exposure (grey trace, $d_{tip} = 38$ nm).
Figure 5-33. Current-voltage curves of pore 1 obtained in 1 M KCl for (A) an unmodified nanopore (blue trace, $d_{tip} = 41$ nm), amine-PEG-550-modified nanopore (red trace, $d_{tip} = 25$ nm) and (B) the same PEG-550-modified nanopore (red trace, $d_{tip} = 25$ nm) before fibrinogen exposure and after fibrinogen exposure (grey trace, $d_{tip} = 18$ nm).

Figure 5-34. Current-voltage curves of pore 2 obtained in 1 M KCl for (A) an unmodified nanopore (blue trace, $d_{tip} = 44$ nm), amine-PEG-550-modified nanopore (red trace, $d_{tip} = 36$ nm) and (B) the same PEG-550-modified nanopore (red trace, $d_{tip} = 36$ nm) before fibrinogen exposure and after fibrinogen exposure (grey trace, $d_{tip} = 27$ nm).
Figure 5-35. Current-voltage curves of pore 3 obtained in 1 M KCl for (A) an unmodified nanopore (blue trace, \(d_{\text{tip}} = 40 \text{ nm}\)), amine-PEG-550-modified nanopore (red trace, \(d_{\text{tip}} = 26 \text{ nm}\)) and (B) the same PEG-550-modified nanopore (red trace, \(d_{\text{tip}} = 26 \text{ nm}\)) before fibrinogen exposure and after fibrinogen exposure (grey trace, \(d_{\text{tip}} = 20 \text{ nm}\)).

Figure 5-36. Current-voltage curves of pore 1 obtained in 1 M KCl for (A) an unmodified nanopore (blue trace, \(d_{\text{tip}} = 42 \text{ nm}\)), amine-PEG-550-modified nanopore (red trace, \(d_{\text{tip}} = 34 \text{ nm}\)) and (B) the same PEG-550-modified nanopore (red trace, \(d_{\text{tip}} = 34 \text{ nm}\)) before lysozyme exposure and after lysozyme exposure (grey trace, \(d_{\text{tip}} = 30 \text{ nm}\)).
Figure 5-37. Current-voltage curves of pore 2 obtained in 1 M KCl for (A) an unmodified nanopore (blue trace, \(d_{\text{tip}} = 45\) nm), amine-PEG-550-modified nanopore (red trace, \(d_{\text{tip}} = 35\) nm) and (B) the same PEG-550-modified nanopore (red trace, \(d_{\text{tip}} = 35\) nm) before lysozyme exposure and after lysozyme exposure (grey trace, \(d_{\text{tip}} = 32\) nm).

Figure 5-38. Current-voltage curves of pore 3 obtained in 1 M KCl for (A) an unmodified nanopore (blue trace, \(d_{\text{tip}} = 48\) nm), amine-PEG-550-modified nanopore (red trace, \(d_{\text{tip}} = 41\) nm) and (B) the same PEG-550-modified nanopore (red trace, \(d_{\text{tip}} = 41\) nm) before lysozyme exposure and after lysozyme exposure (grey trace, \(d_{\text{tip}} = 36\) nm).
Figure 5-39. Current-voltage curves obtained in 1 M KCl for a single, conical-shaped nanopore fabricated in track-etched PET having tip diameters of 42 nm after etching (blue trace) and 4 nm after electroless gold deposition (red trace).

Figure 5-40. Current-voltage curves obtained in 1 M KCl for a single, conical-shaped nanopore fabricated in track-etched PET. The initial tip opening diameter was 4 nm after electroless gold deposition (red trace). As an applied transmembrane potential of 10 V was applied, the tip diameter increased from 4 to 22 nm.
Table 5-1. Summary of the tip opening diameter stability for 3 single, conical-shaped nanopores in track-etched PET over a period of 4 days.

<table>
<thead>
<tr>
<th>Day</th>
<th>Pore 1</th>
<th>Pore 2</th>
<th>Pore 3</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>41</td>
<td>38</td>
<td>41</td>
</tr>
<tr>
<td>1</td>
<td>41</td>
<td>38</td>
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<td>41</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>38</td>
<td>40</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>mean</th>
<th>s.d.</th>
<th>% r.s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tip Opening Diameter (nm)</td>
<td>40</td>
<td>1</td>
<td>3</td>
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</tbody>
</table>

Table 5-2. Summary of XPS spectra for 6 unmodified PET membranes.

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<tr>
<th>Membrane</th>
<th>%O 1s</th>
<th>%N 1s</th>
<th>%C 1s</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28.5</td>
<td>0.0</td>
<td>70.7</td>
</tr>
<tr>
<td>2</td>
<td>28.7</td>
<td>0.0</td>
<td>71.3</td>
</tr>
<tr>
<td>3</td>
<td>29.8</td>
<td>0.0</td>
<td>70.2</td>
</tr>
<tr>
<td>4</td>
<td>27.5</td>
<td>0.0</td>
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</tr>
<tr>
<td>5</td>
<td>29.4</td>
<td>0.0</td>
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</tr>
<tr>
<td>6</td>
<td>29.0</td>
<td>0.0</td>
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<table>
<thead>
<tr>
<th></th>
<th>mean</th>
<th>s.d.</th>
<th>% r.s.d.</th>
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<tbody>
<tr>
<td>XPS of Unmodified PET</td>
<td>28.8</td>
<td>0.8</td>
<td>2.8</td>
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</table>
Table 5-3. Summary of XPS spectra of chemically-etched PET about the nanopore exposed to BSA, fibrinogen, and lysozyme (3 single pore-containing membranes per protein).

<table>
<thead>
<tr>
<th>Membrane</th>
<th>%O 1s</th>
<th>%N 1s</th>
<th>%C 1s</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA/Pore 1</td>
<td>23.5</td>
<td>9.1</td>
<td>65.7</td>
</tr>
<tr>
<td>BSA/Pore 2</td>
<td>24.2</td>
<td>10.0</td>
<td>65.8</td>
</tr>
<tr>
<td>BSA/Pore 3</td>
<td>23.5</td>
<td>8.6</td>
<td>67.9</td>
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<tr>
<td><strong>mean</strong></td>
<td><strong>23.7</strong></td>
<td><strong>9.2</strong></td>
<td><strong>66.6</strong></td>
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<tr>
<td><strong>s.d.</strong></td>
<td><strong>0.4</strong></td>
<td><strong>0.7</strong></td>
<td><strong>1.2</strong></td>
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<td><strong>% r.s.d.</strong></td>
<td><strong>1.7</strong></td>
<td><strong>7.7</strong></td>
<td><strong>1.9</strong></td>
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<table>
<thead>
<tr>
<th>Membrane</th>
<th>%O 1s</th>
<th>%N 1s</th>
<th>%C 1s</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Fibrin./Pore 1</td>
<td>18.1</td>
<td>14.6</td>
<td>66.1</td>
</tr>
<tr>
<td>Fibrin./Pore 2</td>
<td>19.1</td>
<td>13.3</td>
<td>66.1</td>
</tr>
<tr>
<td>Fibrin./Pore 3</td>
<td>20.0</td>
<td>15.2</td>
<td>64.8</td>
</tr>
<tr>
<td><strong>mean</strong></td>
<td><strong>19.1</strong></td>
<td><strong>14.4</strong></td>
<td><strong>65.7</strong></td>
</tr>
<tr>
<td><strong>s.d.</strong></td>
<td><strong>1.0</strong></td>
<td><strong>1.0</strong></td>
<td><strong>0.8</strong></td>
</tr>
<tr>
<td><strong>% r.s.d.</strong></td>
<td><strong>5.0</strong></td>
<td><strong>6.8</strong></td>
<td><strong>1.1</strong></td>
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</tbody>
</table>

<table>
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<tr>
<th>Membrane</th>
<th>%O 1s</th>
<th>%N 1s</th>
<th>%C 1s</th>
</tr>
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<tbody>
<tr>
<td><strong>Lyso./Pore 1</strong></td>
<td>21.9</td>
<td>10.8</td>
<td>67.3</td>
</tr>
<tr>
<td>Lyso./Pore 2</td>
<td>21.1</td>
<td>11.3</td>
<td>67.6</td>
</tr>
<tr>
<td>Lyso./Pore 3</td>
<td>23.5</td>
<td>9.6</td>
<td>66.9</td>
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<tr>
<td><strong>mean</strong></td>
<td><strong>22.2</strong></td>
<td><strong>10.6</strong></td>
<td><strong>67.3</strong></td>
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<td><strong>1.2</strong></td>
<td><strong>0.9</strong></td>
<td><strong>0.4</strong></td>
</tr>
<tr>
<td><strong>% r.s.d.</strong></td>
<td><strong>5.5</strong></td>
<td><strong>8.3</strong></td>
<td><strong>0.5</strong></td>
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</tbody>
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*Fibrin. = Fibrinogen  
**Lyso. = Lysozyme

Table 5-4. Summary of the impact of non-specific adsorption of BSA, fibrinogen, and lysozyme on single, conical-shaped nanopores in track-etched PET (3 single pore-containing membranes per protein).

<table>
<thead>
<tr>
<th>Protein</th>
<th>(d_{tip \ initial \ (nm)})</th>
<th>(d_{tip \ after \ protein \ (nm)})</th>
<th>(\Delta d_{tip \ ^*bare \ PET \ (nm)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>40</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>29</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>29</td>
<td>10</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>38</td>
<td>26</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>23</td>
<td>16</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>45</td>
<td>36</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>41</td>
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</tr>
<tr>
<td></td>
<td>42</td>
<td>34</td>
<td>8</td>
</tr>
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</table>

*bare = unmodified
Table 5-5. Summary of the effect of amine-PEG-550 modification on the tip opening diameter and non-specific adsorption of BSA, fibrinogen, and lysozyme.

<table>
<thead>
<tr>
<th>Protein</th>
<th>( d_{tip} ) pre-PEG-NH(_2) (nm)</th>
<th>( d_{tip} ) post-PEG-NH(_2) (nm)</th>
<th>( \Delta d_{tip} ) PEG (nm)</th>
<th>( d_{tip} ) after protein (nm)</th>
<th>( \Delta d_{tip} ) PEG-PET (nm)</th>
</tr>
</thead>
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<td>38</td>
<td>7</td>
<td>37</td>
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<tr>
<td></td>
<td>54</td>
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<td>7</td>
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<td>45</td>
<td>38</td>
<td>9</td>
<td>34</td>
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<tr>
<td>Fibrinogen</td>
<td>41</td>
<td>25</td>
<td>16</td>
<td>18</td>
<td>7</td>
</tr>
<tr>
<td></td>
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<td>40</td>
<td>28</td>
<td>14</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>Lysozyme</td>
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CHAPTER 6
FABRICATION OF LARGER CONE ANGLE NANOPORES IN PET FOR STUDIES ON THE AFFECT OF CONE ANGLE ON ELECTRIC FIELD STRENGTH, IONIC PORE CONDUCTANCE, AND ION CURRENT RECTIFICATION

Introduction

There is increasing interest in utilizing artificial nanopores as the sensing element in resistive-pulse sensors for analyzing a wide variety of target analytes including small molecules, nucleic acids, proteins, and particles. Resistive-pulse sensing entails placing a membrane containing a single nanopore having a limiting diameter comparable to the size of the analyte between two electrolyte solutions. A potential difference is then applied across the nanopore, thereby generating an ion current flowing through the electrolyte-filled pore. By measuring this ion current, analytes are detected when they are driven through nanopore. That is, when an analyte enters and translocates the nanopore, the analyte transiently blocks the ion current (i.e., increases the pore resistance), resulting in a downward current-pulse. The frequency of these current-pulses is proportional to analyte concentration. Analyte identity, or selectivity, is encoded in the current-pulse signature, which is comprised of both the average current-pulse duration and magnitude.

Single, conical-shaped nanopores in track-etched polymeric membranes are of particular interest because of the control and reproducibility of pore diameter, cost-effective fabrication and materials, controllable surface chemistry, and the increased stability of polymeric membranes compared to lipid bilayer-embedded pores under a variety of conditions (e.g., increased transmembrane potentials). These nanopores have a larger opening, or base, diameter at one face of the membrane and a smaller opening, or tip, diameter at the other
The conical shape of such nanopores makes them ideally suited for resistive-pulse sensing. For instance, Lee, et al. used the finite element simulation method to simulate the magnitude and distribution of the electric field strength within an electrolyte-filled conical nanopore. Their simulation showed that the electric field strength is highly focused at the tip opening and is on the order of $10^6$ V/m. As a result, a sensing zone is formed at the tip opening that is highly sensitive to the presence of any molecular species that enters the tip. Thus, conical-shaped pores are better suited for developing resistive-pulse sensors than cylindrical-shaped pores.

The electric field strength is a key factor that governs current-pulse frequency and current-pulse frequency determines analyte concentration over the dynamic range for any respective analyte. One strategy for increasing the current-pulse frequency is to simply increase the electric field strength by increasing the value of the applied transmembrane potential. In fact, Sexton used this approach to increase the current-pulse frequency of BSA.

An alternative strategy presented here is based on a nanopore shape-mediated approach. In other words, increasing the cone angle (i.e., increasing the base opening diameter at fixed tip diameter) was found, via finite element simulations, to have a dramatic impact on the magnitude of the electric field strength. These simulations also showed that the magnitude of the electric field strength was more sensitive to increasing cone angle than increasing transmembrane potential. Interestingly, very few, if any, studies have been reported on studying the impact of cone angle on conical nanopore-based transport, particularly resistive-pulse sensing. However, this strategy is ahead of its
time because fabrication methods for controlling and reproducing larger cone angle nanopores must first be developed and validated.

Harrell, et al. utilized very high transmembrane potentials during chemical etching of conical pores in multiple ion-tracked polycarbonate to vary the base diameter. Some control over cone angle was afforded by this approach but ideal pore asymmetry was arguably degraded. Furthermore, polycarbonate membranes are often difficult to handle and, without chemical treatment (e.g., with PVP), are highly hydrophobic. Scopece, et al. studied the impact of etch solutions comprised of varying ethanol-to-water ratios on the cone angle in track-etched poly(ethylene terephthalate). They discovered that larger cone angles were obtained by increasing the ethanol-to-water ratio.

In this work, a non-aqueous etchant was used to fabricate conical-shaped nanopores in ion-tracked PET membranes having cone angles that are larger than such pores produced via the aqueous two-step etch method. Ionic pore conductance and ion current-rectification were evaluated in single, conical-shaped pores having comparable tip open diameters but different base diameters. An efficient approach for finding and imaging single nanopores in single ion-irradiated membranes is also presented.

**Experimental**

**Materials**

Poly(ethylene terephthalate) (PET, 3 cm diameter, 12 μm thick) membranes containing single ion- and multiple ion-induced damage tracks were obtained from GSI (Darmstadt, Germany). Single ion-irradiated PET was used for the ionic pore conductance, ion current rectification, and pore imaging work. Multiple ion-irradiated PET (10⁶ ions/cm²) was used for the pore fabrication studies of base diameter and cone
height of gold nanocones. A ion track density of 10^5 ions/cm^2 was used for the high potential etching of PET membranes. HFIP (1,1,1,3,3,3-hexafluoroisopropanol) was obtained from Sigma (St. Louis, MO) and used as received. All other chemicals were of reagent grade and used as received. Purified water (obtained by sending house-distilled water through a Barnstead E-pure water purification system) was used to prepare all solutions.

**Fabrication of the Conical Nanopores and Gold Nanocones**

Several different etch methods were used for this work. For the work dealing with the use of increased transmembrane potentials during etching, conical nanopores were fabricated in multiple ion-irradiated PET membranes (10^5 ion tracks/cm^2) by mounting the membrane in between two half-cells of a U-tube cell. One half-cell was filled with etch solution (9 M NaOH) and the other half-cell filled with stop solution (1 M formic acid and 1 M KCl). A platinum wire electrode was placed into each solution and a transmembrane potential difference was applied during the entire etching process. In this case, anisotropic etching was allowed to proceed for 2 hours. The membrane was then rinsed briefly with stop solution, followed by water. The resulting nanopores were completely filled with gold by electroless gold deposition overnight (~15 hours) using a previously described electroless gold plating method. This process also deposited gold on both faces of the membranes. These gold layers were removed via swabbing the membrane faces with cotton swabs wetted with ethanol. The PET membrane was then completely dissolved using HFIP. The liberated gold nanocones were collected onto an alumina membrane (Anodisc®, Whatman) via filtration.

For the work dealing with the control of base diameter and nanocone height, a non-aqueous etch method was used. An etching cell setup comparable to that used
above was used except that the etch solution was 5 M KOH in 100% methanol and the stop solution used was the same (i.e., 1 M formic acid and 1 M KCl). Anisotropic etching was allowed to proceed as a function of etch time. No transmembrane potential was applied. After each etching time interval, the conical pores in the PET membrane were completely filled with gold by electroless gold deposition overnight (~15 hours) using the previously described electroless gold plating method.\textsuperscript{163} Instead of liberating the resulting gold nanocones from the membrane, only the gold surface film on the membrane face at the tip side was removed via ethanolic swabbing.\textsuperscript{65,114} As a result, the gold surface film on the membrane face at the base side was attached to a piece of double-sided copper tape and mounted onto a scanning electron microscope (SEM) stub. The PET membrane was then carefully dissolved away via dropwise addition of HFIP. As a result, a randomly distributed array of gold nanocones standing on a gold surface film was obtained.\textsuperscript{114}

For the ionic pore conductance, pore imaging, and ion current rectification work, single, conical-shaped nanopores were fabricated in single-ion irradiated PET membranes. An etching cell setup comparable to that used above was used but the etch and stop solutions used were varied for the larger and smaller base opening diameters (i.e., larger and smaller cone angles) for pores having comparable tip opening diameters. For the conical pores having the larger base diameters, a non-aqueous etch method was used.\textsuperscript{114} The etch solution was 5 M KOH in 100% methanol and the stop solution was 1 M formic acid and 1 M KCl. A platinum wire electrode was placed into each half-cell (i.e., anode immersed in the etch solution and cathode in the stop solution) and a transmembrane potential difference of 1 V was applied using a Keithley 6487 voltage source/picoammeter (Keithley Instruments, Cleveland, OH). This provided a means of
monitoring the etching process and determining when to stop etching by monitoring the ion current. Initially, the ion current was zero. When etch solution broke through to the stop solution, a sudden increase in the ion current was observed signaling breakthrough. In this case, etching was stopped when the ion current reached a value of ~50-150 pA. The pore was then briefly rinsed with stop solution followed by water.

For the conical pore having the smaller base diameter, the previously described two-step etch method was used. The etch solution was 9 M NaOH and the stop solution was 1 M formic acid and 1 M KCl. A platinum wire electrode was placed into each half-cell (i.e., anode placed in the etch solution and cathode in the stop solution) and a transmembrane potential difference of 1 V was applied. This anisotropic etching process was allowed to proceed for 2 hours. Then, the isotropic etching step of the two-step etch method was used to tailor the tip opening diameter to match that obtained for the larger base diameter pores. Etch solutions of 1 M NaOH were placed on each side of the pore-containing membrane during this step and an applied potential of 1 V was used. The platinum electrodes were configured such that the anode was immersed in the solution facing the base opening and the cathode was in the solution facing the tip opening.

**Field-Emission Scanning Electron Microscopy**

A JEOL 6335F (JEOL, Ltd.) FE-SEM was used to measure the base opening diameters of the single nanopores using a method presented below for finding single pores. FE-SEM (Hitachi S-4000) was also used to the measure the base diameter of multi-pore membranes for the high potential etching work and for measuring the base diameter and height of gold nanocones. The fabrication of these gold structures is described above.
Finite Element Simulations

The electric field strength at the tip of the electrolyte-filled, conical nanopore was simulated using COMSOL Multiphysics v.3.3a software (COMSOL, Inc.). This program was run using a Dell Optiplex GX520 (Pentium D CPU, 3.2 GHz, 2 GB RAM) (Dell, Inc.) computer. This program has been previously described in simulations of the electric field strength magnitude and distribution in conical nanopores. The COMSOL software utilizes the finite element method to solve partial differential equations that govern a system. Laplace’s equation, \( \nabla^2 \varphi = 0 \), was solved for the electrostatic potential, \( \varphi \).

An electrolyte layer (600 \( \mu \)m thick) on both sides of the membrane was included in the simulation. The electrolyte-filled, conical nanopore was positioned in between these layers. The conical pore length was assumed to be 12 \( \mu \)m long (equivalent to the membrane thickness). Two conical nanopores were simulated. The first pore had a base diameter of 5000 nm and a tip diameter of 6 nm. The second pore had a base diameter of 520 nm and a tip diameter also of 6 nm. The electric field strength was simulated for each of these pores using applied transmembrane potentials ranging from 1–20 V. Each nanopore was divided in two along its long axis (i.e., axis of symmetry), and the simulation was done for only one of the halves. By doing so, a larger number of elements could be used, which improved accuracy. The number of elements used to compute each result ranged between 150,000 and 165,000.
Results and Discussion

Finite Element Simulations: The Impact of Cone Angle on the Electric Field Strength

As previously described, the main determinant of analyte concentration is the current-pulse frequency.\(^{89,95}\) The current-pulse frequency \((f_p)\) can be described by the following equation:\(^{89,95}\)

\[
molecules/s = f_p = \frac{-zF D_t C E (\pi r_{tip}^2) A}{RT}
\]

(Eq. 6.1)

in which \(z\) represents the charge on the analyte, \(D_t\) is the diffusion coefficient associated with analyte transport through the tip opening, \(C\) is the analyte concentration, \(E\) is the electric field strength, \(\pi r_{tip}^2\) is the cross-sectional area of the tip opening, and \(A\) is Avogadro’s number. \(F, R,\) and \(T\) have their usual meanings. Eq. 6.1 shows that the current-pulse frequency is directly proportional to the electric field strength.

The electric field strengths in two conical-nanopores having different base diameters (i.e., 5000 nm and 520 nm) and identical tip diameters of 6 nm were simulated using the finite element method.\(^{95,96}\) The electric field strength for each of these pores was simulated at applied transmembrane potentials ranging from 1-20 V. This was done to simultaneously evaluate the impact of increasing the applied potential and increasing the cone angle of the electric field strength.

Figure 6-1 shows the results of the simulations. These simulations show that a much smaller increase in the magnitude of the electric field strength occurs in the smaller cone angle nanopores compared to that of the larger cone angles pore as the applied potential increases from 1 to 20 V. In other words, for any change in the value of the transmembrane potential within this range, the electric field strength is more sensitive to
changes in cone angle than potential. For instance, for the pore having the smaller cone angle (i.e., smaller base diameter), the magnitude of the field strength at 20 V is slightly less than the field strength of the pore having the larger cone angle (i.e., larger base diameter) at 3 V. Thus, dramatic gains in the magnitude of the electric field strength can be achieved at lower applied transmembrane potentials if a larger cone angle pore is used.

One caveat of using this approach in resistive-pulse sensing is that as the electric field strength is increased, the current-pulse duration decreases correspondingly.\textsuperscript{89,95} Thus, it is believed that higher sampling frequencies may needed to operate at higher current-pulse frequencies. However, before this approach can be experimentally evaluated in resistive-pulse sensing, methods for fabricating and reproducibly controlling larger cone angle nanopores must be developed and validated.

**Nanopore Characterization**

For experiments involving multiple ion-tracked PET membranes, SEM was used for characterization of gold replicas of the nanopores. For single, conical-shaped nanopores in single ion-irradiated PET membranes, base opening diameter for the smaller cone angle pores was 520 nm as described previously for the two-step etch method.\textsuperscript{63} The base diameter for the larger cone angle pores was determined via SEM using the imaging approach described below. Knowing the base opening diameter allows the tip opening diameter to be determined via an electrochemical method based on the ionic conductance of the electrolyte-filled nanopore.\textsuperscript{59,63,75,89,90,95} That is, the ionic conductance (G) of the nanopore is related to the tip opening diameter (d\textsubscript{tip}), base opening diameter (d\textsubscript{base}), specific conductivity of the electrolyte (\(\sigma\), 10.5 – 11.5 S/m for 1 M KCl), and the length of the conical nanopore (L) via the following equation:\textsuperscript{59,63,75,89,90,95}
The value for $G$ was obtained by first mounting the conical nanopore-containing membrane between two halves of a U-tube cell and filling each half-cell with electrolyte. A Ag/AgCl electrode (Bioanalytical Systems/BASi, West Lafayette, IN) was then placed into each half-cell and an applied transmembrane potential was scanned linearly from -1 V to +1 V while measuring the ion current flowing through the electrolyte-filled nanopore at each potential step. The slope of the resulting current-voltage curve is $G$ (in amp/volt). With this value of $G$, all other values in Eq. 6.2 are known, and $d_{\text{tip}}$ can be calculated.

**Etching Multiple Ion-Track ed PET at High Potentials**

To fabricate single, conical-shaped nanopores with large cone angles in ion-tracked PET membranes, a method similar to that reported by Harrell, et al. was first used. Multiple ion-irradiated PET membranes ($10^5$ ion tracks/cm$^2$) were etched using 9 M NaOH as the etch solution and 1 M formic and 1 M KCl as the stop solution. A platinum wire electrode was immersed into the etch solution (anode) and in the stop solution (cathode). Transmembrane potentials of 5, 10, 15, and 20 V were applied for the duration of the etching process which took 2 hours. FE-SEM was used to characterize the pore diameter and gold replicas were used to evaluate the pore shape. Figure 6-2 shows a plot of the base diameters obtained for PET membranes etched at 5, 10, 15, and 20 V.

The excellent linearity observed by Harrell, et al. for a similar plot of base opening diameter versus etching potential for 50 ion tracks/cm$^2$ polycarbonate was not observed here with PET. Instead, some degradation of the PET membrane was observed particular at 20 V and the PET membrane melted at 30 V.
When the anode is placed in the etch solution, the hydroxide ions are
electrophoretically retracted from the tip opening. As a result, the local concentration
of hydroxide ions in the tip region becomes depleted. Thus, etching proceeds more
slowly in the tip region. When the value of the applied transmembrane potential is
increased, a higher ionic current flows through the nanopore during etching. Harrell, et al.
proposed that this increased ionic current causes resistive-heating of the solution inside
the nanopore. Since the etch rate is known to increase with temperature, the resistive-
heating causes the etch rate at the base opening side of the membrane to increase. Thus, a
linearly increase in base opening diameter (for polycarbonate) was observed with
increasing etching potential (i.e., 0-30 V). However, the extent to which this occurs in
PET isn’t clear. The ion-track density of the PET was 4 orders of magnitude larger. Thus,
the membrane resistance \( R_{\text{membrane}} \) of the PET membrane was much lower than that of
the polycarbonate membrane used by Harrell, et al. With a much lower \( R_{\text{membrane}} \), the
ionic current flowing through the nanopore is undoubtedly much higher. Harrell, et al.
described an equation for calculating the heat \( q \) dissipated through the membrane during
etching using the following equation:

\[
q = \frac{U^2}{R_{\text{membrane}}} t = c \Delta T m
\]

(Eq. 6.3)

where \( U \) represents the applied transmembrane potential, \( t \) is etch time, \( c \) is the specific
heat of the NaOH (\( c = 0.88 \text{ cal K}^{-1} \text{ g}^{-1} \), or 3.68 J K^{-1} g^{-1} \), and \( m \) is the mass of the NaOH
solution obtained from an etch solution volume of 3.5 mL and an etch solution density of
1.3 g mL^{-1}.

Since current-voltage curves were not obtained for the PET membranes,
calculating an accurate value for \( q \) wasn’t possible. However, since identical etch and
stop solutions were used for the polycarbonate experiments by Harrell, et al. and the PET experiments here, an approximate comparison can be made with regards to q. Since \( R_{\text{membrane}} \) is undoubtedly much lower (i.e., due to the higher pore density) with the PET membranes used here than the polycarbonate membranes, q is higher for the PET membranes. This is likely the reason why high pore density PET membranes are not amenable to etching at very high transmembrane potentials (i.e., \( E > 20 \text{ V} \)). This was what was observed experimentally. Thus, lower track density PET membrane must be used or an alternative approach to etching with high transmembrane potentials. Eq. 6.3 does not take into account the heat released from the neutralization reaction that occurs when the etch and stop solutions react at the tip opening. Figure 6-3 shows a representative SEM image of a gold replica of a pore fabricated in the PET membrane using 20 V during etching. This shows a much less than ideal conical shape and a truly conical shape is needed to accurately calculate the tip opening diameter using Eq. 6.2.

**Etching Multiple Ion-Track PET With Non-Aqueous Etchant**

Two etch rates govern the cone angle in conical nanopores.\(^{59,60,62,99}\) The bulk, or radial, etch rate (\( v_B \)) describes the rate at which bulk material is etched away. This determines the etching rate of the base diameter. The track etch rate (\( v_T \)) is the rate at which the latent damage track resulting from ion irradiation is etched. This determines how fast the etch solution penetrates the membrane. Thus, a small track etch ratio (\( v_T/v_B \)) is needed to construct large cone angle nanopores. Decreases in this ratio result in increases in the cone angle.

Since very limited success was observed etching with the high transmembrane potential approach, an alternative approach using a 100% non-aqueous etchant was
studied. That is, conical nanopores were etched in PET membranes \((10^6 \text{ ion tracks/cm}^2)\) using 5 M KOH in 100% methanol as the etch solution and 1 M formic acid with 1 M KCl as the stop solution.\(^{114}\) Etching was performed at ambient temperature as a function of etch time using etch times of 50 s, 100 s, 175 s, 250 s, and 500 s. No transmembrane potential was applied. The resulting conical nanopores were completely filled with gold to create gold replicas of the pores (Figure 6-4). As described in more detail previously, dissolution of the PET membrane left a randomized array of gold nanocones standing up on the base end on top of a gold surface film.\(^{114}\)

Figure 6-5 shows that the base opening diameter varied linearly with etch time. From this relationship, a bulk etch rate of \(4.7 \pm 0.1 \text{ nm s}^{-1}\) was obtained. At an etch time of 500 s, a base diameter of \(~2.3 \mu\text{m}\) was obtained which is 4 times larger than the base diameter of 520 nm typically obtained via the aqueous, two-step etch method.\(^{63}\) Furthermore, the gold replicas showed that the pore shape was truly conical. Thus, larger cone angle nanopores were fabricated using the non-aqueous method. The cone height of the gold nanocones was also observed to vary linearly over the etch times studied (Figure 6-6). From this relationship, a track etch rate of \(21.2 \pm 0.4 \text{ nm s}^{-1}\) was obtained. The gold nanocones are shown in Figure 6-7. Thus, the track-etch rate was much faster than the bulk etch rate using the non-aqueous etching approach. Similar etching rate behavior was observed using aqueous etching conditions in prior etching studies.\(^{59,60,62,99}\) Despite not obtaining the large base opening diameter used in the finite element simulation, larger base diameters than those typically observed were obtained by using the non-aqueous etch approach. Thus, a variation of this method was transferred to single ion-irradiated
PET membranes for evaluating the impact of cone angle on ionic pore conductance and ion current rectification.

**Impact of Increased Cone Angle on Ionic Pore Conductance in PET**

Single, conical-shaped nanopores having 3 different base diameters were fabricated using the non-aqueous etch method (for single pores) described in detail above. The base opening diameters of these nanopores were determined via FE-SEM to be 1541 nm (pore A), 1475 nm (pore B), and 1370 nm (pore C) (Figure 6-8). The SEM images were obtained using an approach for finding single pores described in detail below. The ionic conductance of each electrolyte-filled pore was used to determine the tip opening diameters for each pore. Tip diameters of 10 nm, 17 nm, and 19 nm were determined for pores A, B, and C, respectively.

Single, conical nanopores having comparable tip opening diameters to those obtained for pores A, B, and C but base diameters of 520 nm were fabricated using the two-step etch method. Wharton, et al. reported that this method reproducibly produces base opening diameters of 520 nm in single ion-irradiated PET membranes. Figure 6-9 compares the ionic pore conductance (G from Eq. 6.2) for pore A ($d_{base} = 1541$ nm, $d_{tip} = 10$ nm) to that of the pore having base and tip diameters of 520 nm and 10 nm, respectively. Larger ionic pore conductance (i.e., higher ion currents) were obtained for the pore having the larger base diameter (i.e., larger cone angle). Similar results were observed for pores B and C. Figure 6-10 compares the ionic pore conductance for pore B ($d_{base} = 1475$ nm, $d_{tip} = 17$ nm) to that of the pore having a base diameter of 520 nm and a tip diameter of 18 nm. Figure 6-11 compares the ionic pore conductance for pore C ($d_{base} = 1370$ nm, $d_{tip} = 19$ nm) to that for the pore having base and tip sizes of 520 nm and 18
nm, respectively. Both Figures 6-10 and 6-11 showed comparable results to that of Figure 6-9. That is, ionic pore conductance increased with increasing cone angle.

One explanation for this observation is that the higher electric field produced as a result of increasing the cone angle, as shown by the simulation, produces an increased ion current. Another way of explaining this is in terms of pore resistance. Taking the inverse of the ionic conductance of the electrolyte-filled nanopore, G (Eq. 6.2), gives pore resistance ($R_{\text{pore}}$) described by the following equation:

$$R_{\text{pore}} = \frac{4L}{\pi \sigma d_{\text{base}} d_{\text{tip}}} \quad \text{Eq. 6.4}$$

where each variable has the same meaning as in Eq. 6.2. From Eq. 6.4, the value for $R_{\text{pore}}$ decreases as the product of the tip and base diameters is increased. Thus, by increasing the base opening diameter and maintaining a constant tip diameter, the pore resistance decreases. As a result, the ion current increases and that was what was observed experimentally.

**Finding Single Nanopores in Single-Ion Irradiated PET Membranes for Imaging**

Imaging nanopores via SEM is typically reserved for high pore density membranes because the pores of single pore-containing membranes are challenging to find. Harrell, et al. introduced an approach for finding, isolating, and imaging single pores in polycarbonate membrane having a pore density of 50 pores/cm$^2$.\(^{165}\) This approach entails sputter-coating the surface of the porous membrane with a thin metallic coating. A small drop of fluorescent dye is then placed onto a glass slide. The metal-coated membrane is then applied (i.e., with metal side facing upward) onto this drop of dye and the dye wicks up through the pores. Because the dye expands, or blossoms, above the pore, the pores can be found and isolated using fluorescence microscopy. Once
found, the vicinity of the pore is inscribed using a Sharpie® pen. A mask (Scotch® tape) containing a 3 mm diameter hole is placed around the inscribed area for isolation. Thus, a 3 mm search area exists for imaging. Because the pore density is very small, single pores can be isolated.

An alternative approach is presented here for single pore-containing membranes that circumvents the need for fluorescence microscopy and reduces the search area from 3 mm to 200 μm. Figure 6-12 shows a detailed schematic of this process. First, a single pore-containing membrane is sputter-coated with a thin metallic coating on the membrane face that is to be imaged. A metallic mask, having a 200 μm diameter hole in its center, is placed beneath the metal-coated membrane with the metal-coated surface facing upwards. This metallic mask is an exact replica of the filter-aperture mask used during the single swift heavy-ion irradiation of the membrane.\textsuperscript{57,58} In other words, the ion has to pass through such a 200 μm diameter aperture during the ion irradiation process. Therefore, the pore has to be within this area of the membrane. Once the mask is lined up perfectly beneath the membrane, an ultrafine-tip Sharpie® pen is then used to inscribe a 200 μm diameter circle on the metal-coated surface. The process enables a very efficient search of the membrane surface for finding the pores in single pore-containing membranes for imaging.

**Impact of Increased Cone Angle on Ion Current Rectification in PET**

Two of the single, conical-shaped nanopores having the larger cone angles were utilized to evaluate the effect of increasing cone angle on ion current rectification at electrolyte concentrations of 0.01 M KCl, 0.1 M KCl, and 1 M KCl. Several models have been proposed to explain ion current rectification. According to the model proposed by
Cervera, et al., if a conical-shaped pore has excess negative surface charge and a tip opening diameter that is sufficiently small (i.e., electrical double layer thickness $> \text{pore radius}$), then the tip region will preferentially transport cations and reject anions. In other words, the fraction of the ion current carried by migrating cations, often represented by the cation transference number ($t_+$), is much greater than the fraction of the ion current carried by migrating anions (i.e., anion transference number, $t_-$). The sum of the cation and anion transference numbers equals 1. As a result, asymmetric ion migration is observed as a non-linear current-voltage curve. In this case, both an “on state” and “off state” exists for the conical nanopore based on electrode polarity.

When the electrode polarity is such that cathode is located at the base opening and the anode is located at the tip opening, migrating cations are transported from the anode (tip side) towards the cathode (base side). Anion transport occurs in the opposite direction, from cathode towards the anode. However, electrostatic repulsion from the negatively-charged pore wall at the tip region reduces, or prevents, anion transport through the tip. As a result, the local concentration of anions in the tip increases. To maintain electroneutrality, an increased local concentration of cations are required in the tip region to balance the excess anionic charge. Consequently, the local electrolyte concentration in this region increases and membrane resistance decreases. Thus, an increased ionic current, or “on state,” is obtained when the electrode polarity is configured in this manner.

Conversely, when the polarity of the electrodes is reversed such that anode is on the base side and the cathode is at the tip, migrating cation transport occurs in the base-to-tip direction in the conical nanopore. Anions are retracted from the nanopore and
into the bulk solution on the base side of the pore by the ion current. On the tip side of the pore, anion transport through the tip opening is inhibited due to electrostatic repulsion with the anionic surface charge of the pore wall. The net result is a local decrease in the electrolyte concentration in the tip region and increase in membrane resistance. As a result, a decreased ionic current, or “off state,” is obtained when electrode polarity is arranged in this way. If instead the pore wall has excess positive charge, both the “on state” and “off state” are reversed.

However, this model does not take into account the surface conduction pathway available for migrating cations (i.e., for pores having excess negative surface charge such as PET\textsuperscript{62}). Thus, a brief discussion is included here. The transference number for species \( i \) can be calculated using the following equation:\textsuperscript{164} 

\[
t_i = \frac{|z_i| u_i C_i}{\sum_j |z_j| u_j C_j}
\]  
(Eq. 6.5)

where \( |z_i| \) represents the magnitude of the ion charge, \( u_i \) is the mobility of the ion in an electric field, and \( C_i \) is the concentration of the ion.

Consider two cases. In Case I, let’s assume the conical nanopore has no surface charge. Thus, without surface charge, no electrical double layer forms. Assuming a simply electrolyte such as KCl is used (i.e., \( u_{K^+} \) is comparable to \( u_{Cl^-} \), \( |z_{K^+}| = |z_{Cl^-}| = 1 \)) The transference numbers for the cations and anions can be approximated using the following equations:

\[
t_+ = \frac{u_{K^+} C_{K^+}}{u_{K^+} C_{K^+} + u_{Cl^-} C_{Cl^-}}
\]  
(Eq. 6.6)

\[
t_- = \frac{u_{Cl^-} C_{Cl^-}}{u_{K^+} C_{K^+} + u_{Cl^-} C_{Cl^-}}
\]  
(Eq. 6.7)
In the case of a neutral pore surface and this electrolyte, $t_+$ and $t_-$ are both 0.5. Thus, a linear current-voltage curve is expected since no ion current rectification occurs (i.e., $t_+ = t_-$).

In Case II, let’s assume the conical nanopore has fixed, excess negative surface charge, as is the case with pores fabricated in PET membranes. Under low ionic strength electrolyte (e.g., dilute KCl) conditions, an electrical double layer that is greatly enriched with cations forms along the pore surface. In an electric field, these double layer cations migrate from the anode towards the cathode. As a result, a pore surface conduction pathway, based on these migrating double layer cations, is available for cations but not anions. Thus, for Case II, the cation and anion transference numbers can be approximated using the following equations:

\[
\begin{align*}
\Delta t_+ &= \frac{\frac{u_{K+,bulk} C_{K+,bulk}}{u_{K+,bulk} C_{K+,bulk} + u_{Cl-,bulk} C_{Cl-,bulk} + u_{K+,double layer} C_{K+,double layer}}}{\frac{u_{K+,bulk} C_{K+,bulk} + u_{Cl-,bulk} C_{Cl-,bulk} + u_{K+,double layer} C_{K+,double layer}}{}} \\
\Delta t_- &= \frac{u_{Cl-,bulk} C_{Cl-,bulk}}{u_{K+,bulk} C_{K+,bulk} + u_{Cl-,bulk} C_{Cl-,bulk} + u_{K+,double layer} C_{K+,double layer}}
\end{align*}
\]

(Eq. 6.8)

(Eq. 6.9)

As long as the contribution of cations (in the electrical double layer) to the ion current is significant relative to that carried by cations in bulk solution, the cation transference number will be greater than the anion transference number and ion current rectification will occur.

Ion current rectification is typically quantified by determining the rectification ratio using the following equation:

\[
\text{Rectification Ratio} = \frac{|i_{E=-4V}|}{|i_{E=+4V}|}
\]

(Eq. 6.10)
where $i_{E = -1 \text{ V}}$ is the ion current value at an applied transmembrane potential of -1 V and $i_{E = +1 \text{ V}}$ is the ion current value at an applied potential of +1 V. The rectification ratios for conical nanopores having base diameters of 1370 nm (Pore 1) and 1541 nm (Pore 2) were determined as a function of electrolyte concentration and compared to that obtained for pores have comparable tip sizes but a smaller base diameter of 520 nm. Pore 1 and pore 2 had tip diameters of 19 nm and 10 nm, respectively. Figures 6-13, 6-14, and 6-15 show the current-voltage curves obtained for pore 1 compared to those obtained for a smaller base diameter pore at 0.01 M KCl, 0.1 M KCl, and 1 M KCl, respectively. Both pores had comparable tip diameters. Higher rectification ratios were observed for the smaller base diameter (i.e., smaller cone angle) pore. The rectification ratio for the small base diameter pore increased with decreasing electrolyte concentration; however, for the larger base diameter pore, a slightly larger rectification ratio of 1.44 was observed for 0.1 M KCl than the rectification ratio of 1.32 observed for 0.01 M KCl.

This was unexpected since the thickness of the electrical double layer increases with decreasing ionic strength of the electrolyte. Thus, for a fixed pore radius, greater rectification ratios (i.e., larger ion current rectification) are expected at lower electrolyte concentrations. Several factors could have contributed to this unexpected result. For instance, tip diameter is generally measured using 1 M KCl. By subsequently obtaining current-voltage curves in 0.01 M KCl, salt carryover could have occurred even though the pore was rinsed with water in between electrolyte exchange. Another possible problem is salt leeching from the electrode. That is, each Ag/AgCl electrode is contained in a glass housing containing 3 M NaCl. A porous frit separates 3 M NaCl from 0.01 M KCl.
KCl and some leaching of the highly concentrated salt solution from the electrode has been known to occur. This result was not observed for the second pore.

Figures 6-16, 6-17, and 6-18 show the current-voltage curves obtained for pore 2 compared to those obtained for a smaller base diameter pore at similar concentrations of KCl. Both pores had comparable tip diameters. Again, higher rectification ratios were observed for the smaller base diameter (i.e., smaller cone angle) pore at all electrolyte concentrations. The rectification ratio for both pores increased with decreasing electrolyte concentration. Table 6-1 summarizes the data obtained for all of the conical nanopores. Interestingly, a rectification ration of 0.87 was observed for the larger cone angle pore using 1 M KCl. As mentioned previously, the pore resistance decreases with increasing base diameter for pores having comparable tip size. Thus, this could be attributed to scanning the voltage under such reduced pore resistance conditions too quickly. In other words, a fraction of the ion current at one voltage step could carry over to the subsequent voltage step if the scan rate is too fast. This may be exacerbated at lower pore resistance observed with increasing cone angle because the ion current is higher.

The ion current rectification model proposed by Cervera, et al. can be used to propose an explanation for the lower ion current rectification observed in the larger cone angle nanopores.\(^{166}\) This model assumes that the thickness of the electrical double layer (\(l_{DL}\)) is comparable to the radius of the pore (\(r_{pore}\)), \(l_{DL} \approx r_{pore}\) and ignores any contribution of surface conduction due to migrating double layer cations. Although the tip opening diameters of both the larger and smaller base diameter nanopores were comparable, the region just inside the tip is changing. That is, as the cone angle is increased, \(r_{pore}\) increases in the local region inside the tip towards the base opening. For a fixed electrolyte
concentration (i.e., fixed $l_{DL}$), increasing $r_{pore}$ with increasing cone angle means $r_{pore} > l_{DL}$. As a result, the cation transference number, using the model proposed by Cervera, et al., decreases. As a result, ion current rectification decreases. This is assuming that etching under 100% non-aqueous conditions (i.e., 5 M KOH in 100% methanol) does not alter the surface charge on the conical nanopore.

**Conclusions**

Single, conical-shaped nanopores having different cone angles were fabricated in single ion-irradiated PET membranes. The two-step (aqueous) etch method was utilized to produce conical pores having base diameters of 520 nm. A non-aqueous etch method was presented and used to fabricate conical pores having base diameters that were ~3 times larger than those obtained via the two-step etch method. With comparable tip opening diameters, the impact of increasing cone angle (i.e., larger base diameter) on ionic pore conductance and ion current rectification was investigated.

The larger cone angle pores showed reduced ion current rectification which was attributed to an increase in the pore radius in the region just inside the tip continuing towards the base with increasing cone angle. As a consequence, the cation permselectivity was reduced (i.e., $t_+ \text{ decreased}$) and rectification decreased. Assuming the non-aqueous etch process didn’t alter the negative surface charge of the pore, this suggests that some geometric control of ion current rectification in conical nanopores can be achieved by varying the cone angle but further studies are needed in PET and other materials.

The larger cone angle nanopores also exhibited higher ionic pore conductance which was attributed to lower pore resistance. Finite element simulations suggest that a higher electric field strength also occurs in larger cone angle pores. Furthermore,
simulations showed that the electric field strength, which is a main determinant of current-pulse frequency in resistive-pulse sensing,\textsuperscript{89,95} is much more sensitive to increasing cone angle than increasing transmembrane potential.

Fabricating large cone angle nanopores in PET using large ion-track density membranes and high transmembrane potentials was not successful and is not a good strategy because the decreased membrane resistance (due to the large pore density) generates a large degree of resistive heating. Such heating adversely impacted both the structural integrity of the polymer membrane as well as produced pores having less-than-ideal conical shape, particular at higher potentials. Heating can be reduced by using much lower ion track density, or even single ion-tracked, PET membranes.

For single-ion irradiated PET membranes, the non-aqueous etching approach provided a better process for fabricating larger cone angle single pores. An approach for quickly finding and imaging single nanopores was successfully applied to single pores having base diameters less than 1600 nm. Furthermore, the non-aqueous etching method provided a means for fabricating randomly distributed gold nanocone-arrayed surfaces of controllable base diameter and cone height. It is believed that the non-aqueous approach presented here, or a derivative thereof, provides a suitable approach for fabricating larger cone angle pores for developing resistive-pulse sensors capable of producing high current-pulse frequencies.
Figure 6-1. Plot of electric field strength in the tip opening of the conical nanopore obtained from finite-element simulations versus applied transmembrane potential for nanopores having a large base diameter of 5000 nm (red trace) and a smaller base diameter of 520 nm (blue trace). Identical tip diameters of 6 nm were used for each pore.

Figure 6-2. Plot of the base diameter obtained for multiple ion-tracked PET membranes etched at 5, 10, 15, and 20 V for 2 hours.
Figure 6-3. SEM image of a gold replica of a pore fabricated in a multiple-ion irradiated PET membrane at an applied transmembrane potential of 20 V applied during etching for 2 hours.

Figure 6-4. SEM image of randomly distributed gold nanocone replicas of conical pores fabricated in multiple-ion irradiated PET membrane using a non-aqueous etch method.
Figure 6-5. Plot of gold nanocone base diameter versus etch time. Error bars encompass data obtained for three membranes prepared in an identical manner.

Figure 6-6. Plot of gold nanocone height versus etch time. Error bars encompass data obtained for three membranes prepared in an identical manner.
Figure 6-7. SEM images of randomly distributed arrays of gold nanocones obtained from pores etched at ambient temperature for (A) 30 s, (B) 100 s, (C) 175 s, (D) 250 s, and (E) 500 s.
Figure 6-8. SEM images of the base opening diameters of single, conical-shaped nanopores fabricated in single ion-tracked PET. Base diameters of (A) 1541 nm (pore A), (B) 1475 nm (pore B), and (C) 1370 nm (pore C) were obtained using the filter aperture-mask approach to finding pores for imaging.
Figure 6-9. Current-voltage curves obtained in 1 M KCl for a conical nanopore (Pore A) having a base diameter of 1541 nm and tip diameter of 10 nm (red trace) and a second conical pore having a base diameter of 520 nm and a tip diameter of 10 nm (blue trace).

Figure 6-10. Current-voltage curves obtained in 1 M KCl for a conical nanopore (Pore B) having a base diameter of 1475 nm and tip diameter of 17 nm (red trace) and a second conical pore having a base diameter of 520 nm and a tip diameter of 18 nm (blue trace).
Figure 6-11. Current-voltage curves obtained in 1 M KCl for a conical nanopore (Pore C) having a base diameter of 1370 nm and tip diameter of 19 nm (red trace) and a second conical pore having a base diameter of 520 nm and a tip diameter of 18 nm (blue trace).

Figure 6-12. Schematic detailing an approach for finding single nanopores in single ion-irradiated membranes uses a replica of the filter aperture-mask used during single swift heavy-ion irradiation.
Figure 6-13. Current-voltage curves obtained in 0.01 M KCl for pore 1 (red trace, \(d_{\text{base}} = 1370 \text{ nm}, d_{\text{tip}} = 19 \text{ nm}\)) and a smaller cone angle pore (blue trace, \(d_{\text{base}} = 520 \text{ nm}, d_{\text{tip}} = 18 \text{ nm}\)).

Figure 6-14. Current-voltage curves obtained in 0.1 M KCl for pore 1 (red trace, \(d_{\text{base}} = 1370 \text{ nm}, d_{\text{tip}} = 19 \text{ nm}\)) and a smaller cone angle pore (blue trace, \(d_{\text{base}} = 520 \text{ nm}, d_{\text{tip}} = 18 \text{ nm}\)).
Figure 6-15. Current-voltage curves obtained in 1 M KCl for pore 1 (red trace, $d_{\text{base}} = 1370$ nm, $d_{\text{tip}} = 19$ nm) and a smaller cone angle pore (blue trace, $d_{\text{base}} = 520$ nm, $d_{\text{tip}} = 18$ nm).

Figure 6-16. Current-voltage curves obtained in 0.01 M KCl for pore 2 (red trace, $d_{\text{base}} = 1541$ nm, $d_{\text{tip}} = 10$ nm) and a smaller cone angle pore (blue trace, $d_{\text{base}} = 520$ nm, $d_{\text{tip}} = 10$ nm).
Figure 6-17. Current-voltage curves obtained in 0.1 M KCl for pore 2 (red trace, $d_{\text{base}} = 1541$ nm, $d_{\text{tip}} = 10$ nm) and a smaller cone angle pore (blue trace, $d_{\text{base}} = 520$ nm, $d_{\text{tip}} = 10$ nm).

Figure 6-18. Current-voltage curves obtained in 1 M KCl for pore 2 (red trace, $d_{\text{base}} = 1541$ nm, $d_{\text{tip}} = 10$ nm) and a smaller cone angle pore (blue trace, $d_{\text{base}} = 520$ nm, $d_{\text{tip}} = 10$ nm).
Table 6-1. Tabulated summary of the effect of increasing base diameter in pores 1 and 2 relative to that of pores 3 and 4 with comparable tip diameters (pores 1 and 3, pores 2 and 4) and the effect of electrolyte concentration on the ion current rectification ratio.

<table>
<thead>
<tr>
<th>Pore</th>
<th>$d_{\text{tip}}$ (nm)</th>
<th>$d_{\text{base}}$ (nm)</th>
<th>0.01 M KCl</th>
<th>0.1 M KCl</th>
<th>1 M KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19</td>
<td>1370</td>
<td>1.32</td>
<td>1.44</td>
<td>1.07</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>520</td>
<td>3.02</td>
<td>2.36</td>
<td>1.13</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>1541</td>
<td>1.21</td>
<td>1.01</td>
<td>0.87</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>520</td>
<td>2.59</td>
<td>2.32</td>
<td>1.07</td>
</tr>
</tbody>
</table>
CHAPTER 7
CONCLUSION

The objective of this research was to develop sensors based on the resistive-pulse method using conical-shaped nanopores in track-etched polymeric membranes, investigate properties of such pores that impact their sensing capabilities, and investigate nanopore fabrication. Chapter 1 introduced an overview of pertinent background information for this dissertation, including nanopore materials, ion-irradiation of polymer membranes, the track-etch method, nanopore characterization, the resistive-pulse method, previous resistive-pulse sensing work with biological and artificial nanopores, and methods for controlling pore size and surface chemistry.

In Chapter 2, resistive-pulse sensing of a model protein, streptavidin, was achieved using a single, conical nanopore fabricated via the two-step etch method in poly(ethylene terephthalate) (PET). Thus, the two-step etch method was indeed an effective way of tailoring the tip size to that of the analyte for constructing functional resistive-pulse sensors. The conical nanopore surface was first coated with a thin, conformal layer of gold via electroless gold deposition and subsequently functionalized, via chemisorption, with thiol-modified poly(ethylene glycol) (PEG-SH) of large molecular weight (5 kDa). The PEG-coated nanopore sensing element detected current-pulses for 500 nM streptavidin. The frequency of these current-pulses was found to follow an exponential dependence on the applied transmembrane potential. Such current-pulses were predominantly downward (i.e., decreasing below the baseline), and had a tailing peak shape which was attributed to protein position within the nanopore. Removal of the PEG and underlying gold layers changed the current-pulses such that each pulse consisted of
both an upward (i.e., increasing above the baseline) and a downward pulse. Furthermore, the absence of the PEG- and gold-layers reduced the current-pulse frequency obtained using comparable tip diameters, sensing buffers, protein concentration, and applied potentials by ~50%. This suggested that the threshold potential for the PEG-coated sensing element was different (i.e., lower) than that for the unmodified sensing element.

In Chapter 3, a model cationic analyte, poly-L-lysine-conjugated gold nanoparticles were detected via the resistive-pulse method using a single, conical nanopore in track-etched PET. This work represented a departure from previous resistive-pulse studies using track-etched PET for two reasons. First, an unmodified nanopore was used. Previous studies used either a PEG-SH-modified gold coated nanopore or an ethanolamine-coated pore. Secondly, both the electrode polarity at the tip side of the membrane and the net surface charge of the analyte were opposite in polarity (i.e., positive) relative to the anionic pore wall. Particles were detected as transient, upward current-pulses at nanomolar, picomolar, and femtomolar concentration levels although the current-pulse frequency was really low at the femtomolar level. Such upward-shaped, current pulses reflect the current-enhancing effect of the counter-ions accompanying each nanoparticle into and through the nanopore sensing zone.

A definition for the detection limit in resistive-pulse sensing was proposed and discussed. A narrow current-pulse amplitude was observed which was attributed to the use of a hard-sphere model analyte that doesn’t have the conformational flexibility akin to many biological molecules. A wide current-pulse duration was observed which was attributed to an electrostatic binding and release between the cationic particles and the anionic pore wall. It is believed that an effective surface passivation technique that (1)
removes the negative surface charge of the pore surface and (2) maintains a high degree of hydrophilicity will undoubtedly decrease the wide spread in current-pulse duration.

In Chapter 4, an alternative polymer type, ion-tracked polyimide, was investigated for use in resistive-pulse sensing. Carboxylated nanoparticles that were also fluorescent were detected using an unmodified, conical nanopore in polyimide. To fabricate the nanopore sensing element, a two-step etch method for tailoring the tip diameter to a size comparable to that of the particles was presented. In all resistive-pulse sensing work, controlling the tip opening diameter during fabrication is absolutely critical to constructing functional resistive-pulse sensors. The tip diameter was observed to scale linearly with the final value of the ion current during the two-step etch. The extent of ion current rectification, as described by the rectification ratio, was inversely related to the tip opening diameter at the tip sizes evaluated. An often overlooked aspect of resistive-pulse experimentation is technique. For instance, what is the best approach to properly and completely fill a single nanoscopic pore with aqueous electrolyte? This work suggests that by combining the use of a wetting agent, vacuum degassing, and perfusion, better filling of the nanopore with sensing buffer can be facilitated.

Current-pulses were exclusively upward and detected using much lower applied transmembrane potentials than those typically used for resistive-pulse sensors housed in track-etched PET. For instance, current-pulses were detected with potentials as low 50 mV although the current-pulse frequency was very low. This was attributed to the large cone angle and correspondingly lower pore resistance characteristic of conical pores fabricated in polyimide. A narrow distribution in current-pulse amplitude was observed and attributed to the use of a hard-sphere nanoparticle which does not possess the
conformational flexibility akin to many biomolecules. The current-pulse amplitude was observed to increase linearly over all potentials studied. The current-pulse frequency increased with increasing transmembrane potential for the first three potentials used; however, the pulse frequency showed unexpected behavior at the higher three potentials used. The current-pulse duration followed a similar trend by decreasing with increase potential over the lower three potentials and showing wide variability over the higher three potentials. It is believed that a de-wetting of the pore may have occurred at higher transmembrane potentials.

In Chapter 5, an alternative strategy to electroless gold deposition for functionalizing the surfaces of single, conical nanopores with PEG based on EDC/sulfo-NHS coupling chemistry was introduced. Minimizing non-specific pore surface adsorption is absolutely critical in resistive-pulse sensing for two reasons. First, since current-pulse frequency, the primary determinant of analyte concentration, is governed in part by the cross-sectional area of the tip opening, a constant tip size is essential to obtaining reproducible data and low limits of detection. Secondly, non-specific interactions between the pore wall and translocating analyte molecules have been implicated as a key contributor to wide distributions in current-pulse duration, a component of selectivity, observed in prior studies and those presented herein. Thus, by eliminating, or significantly reducing, non-specific interactions between translocating proteins and the pore wall, it is believed that improvement in current-pulse duration can be achieved.

In this work, the stability of the tip opening diameter of single conical pores in track-etched PET membranes was demonstrated over a 4-day time interval. Three model
proteins, BSA, fibrinogen, and lysozyme, were found to reduce the tip diameters of unmodified conical pores via non-specific adsorption. Such adsorption of the three proteins was verified via X-ray photoelectron spectroscopy (XPS). Comparable conical nanopores were functionalized with PEG-NH$_2$ using EDC/Sulfo-NHS coupling chemistry which cross-links the amine group on the PEG to the free carboxylates on the pore wall via an amide bond. Such cross-linking was confirmed using XPS and current-voltage curves. The PEG-modified conical pores were then exposed to the 3 model proteins and a reduction in non-specific adsorption was observed to varying extents for each protein.

Furthermore, the prior approach of coating single pores in PET with PEG by first depositing a gold surface film and subsequently attaching PEG-SH was shown the suffer from instability at higher potentials. It is believed that the direct cross-linking of PEG-NH$_2$ to the pore wall via EDC/sulfo-NHS coupling chemistry provides a more stable and reproducible PEG-coating of the PET pore surface. Further improvements in the biocompatibility of the pore surface may be gained by considering PEG chains of different lengths, surface layers comprised of mixed PEG lengths, and PEG coverage density.

In Chapter 6, the electric field strength distributions inside two single, conical-shaped nanopores having identical tip diameters but different base diameters (i.e., one large and one small) were evaluated via finite element simulations. These simulations show the electric field strength, which is directly proportional to current-pulse frequency, increases more with increasing cone angle than with increasing transmembrane potential. Thus, this provides the impetus for fabricating larger cone angle nanopores. However,
before doing resistive-pulse sensing, methods for fabricating, controlling and reproducing large cone angle nanopores are needed.

A high voltage approach for fabricating large cone angle nanopores in high track density PET membranes wasn’t successful due to increased resistive-heating. Thus, a non-aqueous approach to fabricating single, conical nanopores having larger cone angles than pores typically produced via the aqueous two-step etch method was presented. Using this approach, the effect of increasing cone angle on ionic pore conductance and ion current rectification was evaluated. Increased ionic pore conductance and decreased ion current rectification were observed with the larger cone angle pores relative to those having a smaller cone angle.

Although the field of molecular scale, resistive-pulse sensing remains very much in its infancy, the research presented herein demonstrates that such sensors can be constructed in track-etched polymeric membranes. This work stresses the importance of controlling and optimizing the pore surface properties and pore geometry to truly realize the analytical utility that can potentially be derived from resistive-pulse devices based on conical nanopores. It is hoped that this work provides the impetus for continued research efforts in these critical areas. Furthermore, since no one material has yet emerged has the best material for fabricating conical nanopores for use in resistive-pulse sensors, continued research in this area is also encouraged. Single molecule, resistive-pulse sensors constitute a conceptually simple, label-free detection paradigm that is very promising for developing a variety of useful applications.
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BIOGRAPHICAL SKETCH

Lloyd Peyton Horne, Jr. was born in Durham, NC. For his undergraduate studies, he attended the University of North Carolina at Chapel Hill and earned a B.S. in chemistry in the spring of 1997. He then spent 7 years working in the pharmaceutical industry in the areas of analytical research and development, technology transfer, manufacturing process optimization, and drug product commercialization. Lloyd conducted master’s research at East Carolina University on the characterization and optimization of pulsed-amperometric waveforms in HPLC for the detection of biomolecules and earned a M.S. certificate in 2002. In January of 2005, Lloyd started the doctoral program in analytical chemistry at the University of Florida, and joined the research group of Prof. Charles R. Martin. He completed his research in the spring of 2010, and obtained a Doctor of Philosophy in chemistry.