



Enumerating Solute Transport across Bacterial outer membrane

by

Jayesh Arun Bafna

PhD Proposal

Approved Proposal Committee

Prof. Dr. Mathias Winterhalter

Prof. Dr. Roland Benz

Date of Defense:

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Abstract:

The outer membrane of a Gram-negative bacteria acts as a selective permeable barrier, which plays a crucial role in antibiotic resistance. Due to the growth of antibiotic resistance, and its effect on the transport across the barrier, have forced scientists and researchers to develop new methods and tools to understand transport mechanism *in vitro*. As a physicist, we try to develop reliable technique to study the translocation of antibiotic by applying biophysical methods to elucidate molecular details of the translocation of antibiotics. Here the precise mechanism of the antibiotic transport across a bacterial membrane is of keen interest, as the understanding of transport would lay the foundation for the development and designing of new antibiotics.

I have worked on two different projects where the main idea was to understand translocation of the substrate across the membrane porins, the first project focusses on the electro-osmotic transport of antibiotic and its entry channel. The experiment precisely involves reconstitution of a single OmpF, the most abundant nonspecific porin in the outer membrane of *E. coli*; into a planar lipid bilayer followed by time-resolved ion current measurement in the presence of individual substrate namely norfloxacin, levofloxacin, ciprofloxacin and the Beta-lactamase inhibitor (Clavulanic acid). From the results, a systematic interaction of OmpF with the substrate was observed, showing an effect of electro-osmosis over the transport of these substrates from the pore OmpF. The result demonstrates the ability to study the molecules along the passage pathway inside the nanopore, and allows identifying the exact exit and entry points and thus providing a convincing proof of the molecules translocating through the pore.

The second part of the project was to characterize the outer membrane pore of *Pseudomonas aeruginosa* OprD, a human pathogen responsible for many hospital-acquired infectious diseases, over the uptake of basic amino acids and carbapenems. The main aim of this work is to understand the structural changes observed when the conductance of the pore is measured at different salt concentration, and then try to understand the uptake of solute molecules. Using single and multi-channel measurements, first we reconstituted single OprD channel in a planar lipid bilayer and characterized the interaction with imipenem, meropenem, arginine and glutamic acid. The statistical analysis of single channel data at high salt did not reveal any concrete information on the uptake of solute molecules. Later we employed reversal potential technique, which was developed in our lab to measure the transport of charged substrate across the barrier. Reversal potential of arginine, glutamic acid and lysine were

measured and their respective permeability were extracted by fitting the reversal potential in Goldman-Hodgkin-Katz flux equation. This technique gives us a direct method to quantify the transport of the charged species. Preliminary data obtained on OprD can be used as a starting point for understanding structural changes and its specific substrate permeation.

Introduction:

Bacteria constitute a large kingdom of prokaryotic microorganisms. They exist in different shapes ranging from spheres to rod and of length scales around a micrometer. Bacteria are majorly divided into two groups i.e. Gram-positive and Gram-negative bacteria [1]. The basic difference between both the groups of bacteria is that Gram-positive bacteria have a single layer of lipid membrane, which separates the outer environment from the cell cytoplasm. Whereas, the Gram-negative bacteria is made up of two layers of lipid membranes, and are classified as outer membrane and the inner membrane, between both the layer there, exists a periplasm space which is a concentrated gel-like matrix, and it constitutes up to 40% of total cell volume in Gram-negative bacteria [1, 2]. The outer cell wall acts as a protecting layer against the penetrations or diffusion of toxic compound [1-3]. The multiplex structure of the membrane in Gram-negative bacteria fortifies its survival by allowing a channel for the nutrients into and from the cell interior. The transport is largely governed by the channels called as porins [4-6]. Porins are the gateway that extends to the outer membrane of Gram-negative bacteria and allows the diffusion of hydrophilic molecules [4, 5]. In the case of Gram-positive bacteria, it lacks the outer membrane and instead has a thicker peptidoglycan layer, which is porous to allow diffusion of a broad range of metabolites into the plasma membrane[1]. Apart from numerous types of nutrient molecules and energy components, there is a set of class of antibiotics as β -lactams are known to use outer membrane channels to the reach target site [7].

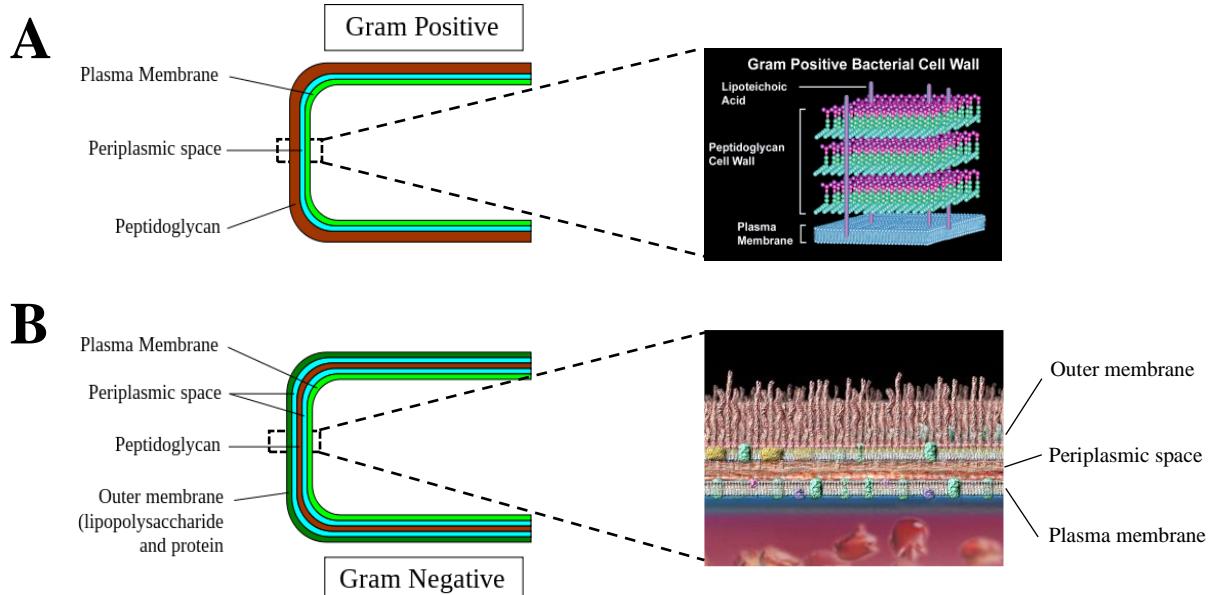


Figure1 (A): Scheme of Gram positive membrane and its well resolved structural detail [8], (B) Scheme of Gram negative membrane and its double barrier showing the outer and inner membranes of Gram-negative bacteria [9].

To quantify the transport of antibiotics and other molecules, a single porin is reconstituted on to a lipid membrane and using single molecule detection technique one can extract binding rates of antibiotics with the porin [10-15]. The technique of single molecule detection dates back to a technique called Resistive Pulse Technique (RPT), based on the observation of change in conductance of the nanopore a molecule displaces volume equal to its size when it zips through the nanopore [16, 17]. The amplifier detects this change in the volume of the electrolyte, and a corresponding dip in the current signal is observed. The origin of RPT took birth in the late 1940's by Wallace H. Coulter who invented a micro pore based cell counter with a small pore in the cellophane cigarette wrapper with a hot needle, and Joseph R. Coulter helped Wallace H. Coulter in developing the electronics [16-18]. This technique of counting cells made a drastic revolution in the field of clinical pathology labs in contrast to old age methods of counting blood cells, which were to prepare on microscopic slides, and staining them for each patient, this technique consumed much time for analysis and led to significant errors of all type. In 1970 DeBlois and C.P. Bean made a development in the counting technology by making a submicron pore by track etched method for virus translocation and also developed the model for the calculation of the particle size [19, 20].

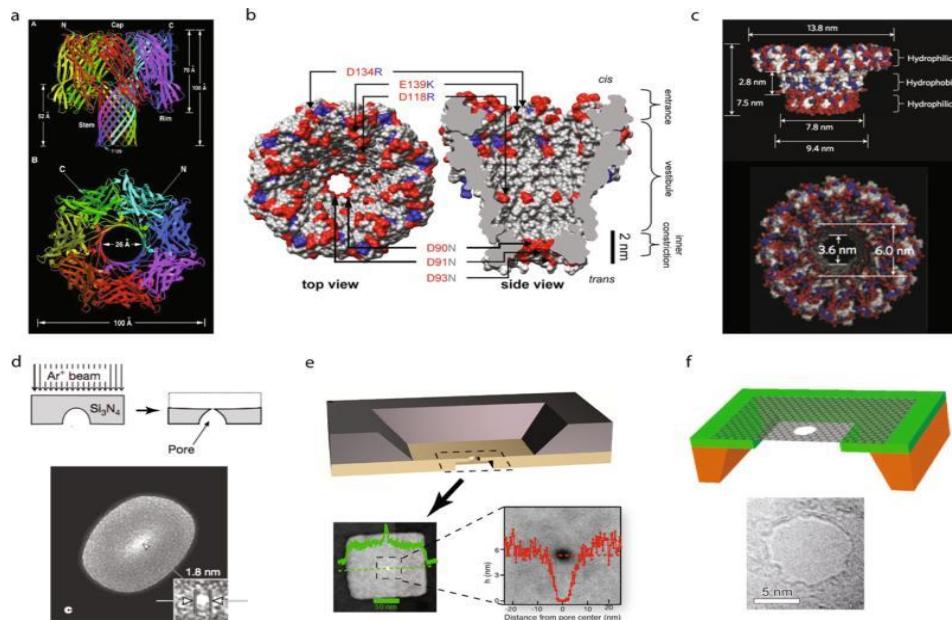


Figure 2 (A-F) : A) The X-ray structure of toxin α -hemolysin from *Staphylococcus aureus* [21]. B) X-ray structure of MspA from *Mycobacterium smegmatis* [22]. C) X-ray structure of engineered phi29 viral packaging motor [23]. D) Ion-sculpted pores in silicon nitride membrane. D) Ion-sculpted pores in silicon nitride membrane [24]. E) Scheme of sub-10 nm thick solid-state pores generated by dry etching a selected area of a silicon nitride membrane and electron-beam pore drilling [17]. F) Pores in a suspended single-layer graphene membrane [25].

The birth of nanopore was not until 1996 when a toxic alpha-hemolysin which is obtained from *Staphylococcus aureus* which is a transmembrane channel and it is characterized under biological nanopore [21]. Since the length of the channel is very long, so it added to a disadvantage to record current form every nucleotide of a DNA when it zips through the nanopore. Later in 2008 MspA was introduced as a nanopore which is obtained from *Mycobacterium smegmatis*, MspA added advantage over alpha-Hemolysin as the length of the constriction in MspA is ~ 1 nm and pore diameter of ~2 nm which led experimentalist to electronically detects individual nucleotides of ssDNA [22]. Alpha hemolysin and MspA were used for detection of small molecules like ssDNA and RNA, in 2009 bacteriophage phi29 DNA packaging motor which was embedded into lipid membrane and was used as a new bio nanopore for electronic detection of dsDNA with inner construction wide enough to about 3.6nm to 6nm for dsDNA to translocate [23]. Since it was not easy to find bio nanopores with aperture size greater than alpha hemolysin [16], so the need of artificial nanopore emerged, and the first solid state nanopore was made via a technique called ion beam sculpting. Here prefabricated bowl geometry on a silicon nitride membrane and argon ions were bombarded for thinning the membrane on the opposite face of the bowl geometry till the small opening of the nanopore is seen, and the only disadvantage of the technique is that it does not allow us to have a precise control of pore diameter [24]. To overcome this issue, in 2003 a new method of fabricating silicon based nanopore was introduces which made use of TEM and allowed us to have a precise control over the pore diameter in real time fabrication and it was shrunk below 10nm which stands as a potential candidate for detection individual molecule with good spatial resolution [17, 26]. For detection of current from single nucleotide of dsDNA, silicon based nanopore added to disadvantage as they could detect currents from few tens of nucleotide because of the length of the channel was too long to detect single nucleotide [27].

With the development of single molecule detection technique scientists are branching out this field in many different areas of research and one is towards the sensing of antibiotics through specific and selective porins. The need for the development of new design in drug manufacturing has become very important to fight the resistance of antibiotics through bacteria [28]. β -lactam antibiotics (beta-lactam antibiotics) are the broad class of antibiotics, consisting of all antibiotic agents that contain a beta-lactam ring in their molecular structures. This includes penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems. Most β -lactam antibiotics work by inhibiting cell wall biosynthesis in the bacterial organism and are the most widely used group of antibiotics. More than half of all

commercially available antibiotics in use were β -lactam compounds [2, 28-30]. Bacteria often develop resistance to β -lactam antibiotics by synthesizing a β -lactamase, an enzyme that attacks the β -lactam ring. To overcome this resistance, β -lactam antibiotics are often given with β -lactamase inhibitors [28, 30]. The results from the single channel measurement of bacterial porin can be applied to the transport of solute across eukaryotic cell membrane, as the underlying principle of transport across the membrane remains the same. As the problem of transport mechanism remains unanswered, a good starting point for understanding the transport mechanism would be looking at the single molecule detection results, which are quite complicated sometimes and it requires detailed and careful analysis of the obtained results. But on the other hand all thanks to the X-ray crystallographer and computer modeler for giving us the detailed single atom resolution structure of each and every porin which helps us in better understanding of single molecule detection data.

Transport of antibiotics plays a crucial step in curing any bacterial disease, as the antibiotic has to reach its target to kill the bacteria. As a physicist, I would be more interested in looking closely to the surface of the channel, because at nanoscale, the surfaces are so close to each other that charge residues inside the channel and its interaction with the buffer in the surrounding creates a complicated system. Recent results on single channel measurement have turned out to be really uncertain, but carefully looking at the data revealed a strong additional force acting on the antibiotic transport apart from electrophoretic force, this additional force is called the electro-osmotic force (EOF). Electroosmotic flow is the bulk liquid motion that results when an externally applied electric field interacts with the excess charged ions in the diffuse part of an electrical double layer, which is formed inside the channel. Such results have turned out to be very interesting as this could be one possible key solution for the transport of antibiotics across the membrane (*in vitro*). Taking it up further with care full analysis will help me to contribute in the field of antibiotic transport and will help researchers and scientist to design and develop new drugs for efficient permeation across the barrier.

Material and Methodology

Phospholipid bilayer is formed with 1,2-diphytanoyl-sn-glycero-3-phosphatidylcholine (DPhPC) by employing the classic Montal and Muller technique [31]. Lipid bilayer is the basic structural element of cell membranes and can be assembled from lipids, as a single planar structure that separates the two aqueous phases. The electrical capacity of these bilayers exactly matches that of biological membranes, and thus allows the formation of asymmetric membranes. Eventually, this technique allows incorporating membrane protein into the lipid bilayer. The experimental technique involves a Teflon cell (scheme Figure 3A) with two compartments separated by a thin wall 25- μm Teflon film with an approximately 50- μm diameter aperture, the size of this aperture is small enough to form a stable bilayer, with possibility of protein insertion. The planar lipid bilayer was formed using the monolayer technique of Montal and Mueller [31].

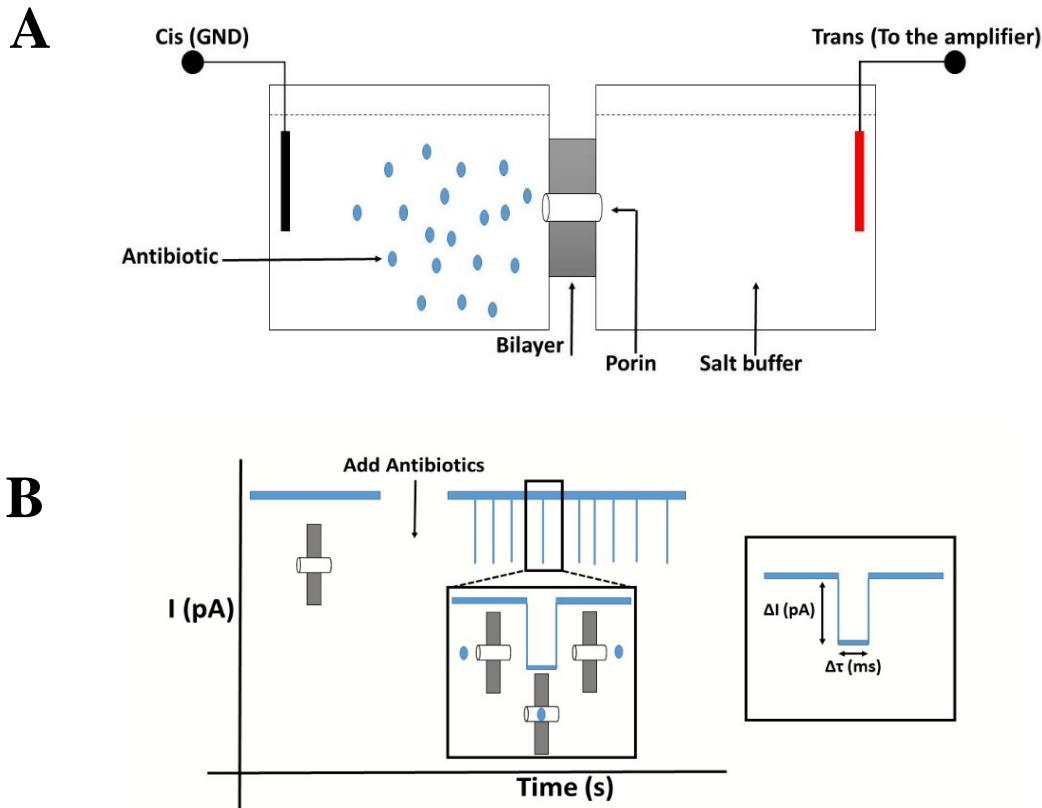


Figure3: A) Experimental scheme consisting of lipid bilayer and single channel reconstitution separating the two fluid chambers into cis and trans, antibiotics are added to the cis side of the fluid chamber. B) Under the applied potential we see modulation of current signal when antibiotic is added to the fluid chamber and the zoom of the modulated signal gives us the information on change in the signal strength and the residence time when antibiotics translocate through it.

The method involves the formation of the bilayer via simultaneous formation of two monolayers. Prior to bilayer membrane formation, the aperture is prepainted with a 1% solution of n-hexadecane in n-hexane to make it lipophilic. After being dried for about 10 min, both chambers were filled with buffer (electrolyte), and a lipid bilayer is prepared by spreading a solution of 5 mg/mL solution of lipid (1, 2-diphytanoyl-sn-glycero-3-phosphocholine in a solvent mixture of n-pentane) [28]. Our studies were mainly performed using 1M KCl (Potassium Chloride) that was added to both sides of the chamber. Standard silver chloride electrodes were placed in each chamber to detect the ion current. For single molecule detection, a single channel was made to insert into the lipid bilayer, under the applied potential ionic current was recorded and amplified using Axopatch 200B amplifier (Axon Instruments) in the voltage clamp mode (Scheme Figure 3B). Signals were filtered by an on board low pass Bessel filter at 10 kHz and was further recorded onto computer hard drive with a sampling frequency of 50 kHz. Amplitude, probability and noise analyses were performed using Origin (Microcal Software Inc.), Clampfit (Axon Instruments) and custom made LabVIEW data analysis software.

Theoretical calculation regarding the net ion transport through the channel is governed by Nernst-Planck equation. The motion of an individual particle/ion in dense fluid does not allow to follow a straight path, as it travels, the particle is jostled by the random collision with other solvent molecules, this leads to follow a random correlated path. The force experienced by the particle/ion under the application of electric field is given by equation 1,

$$F_{ele} = z \cdot e \cdot E \quad (1)$$

In fluid, in addition to applied field, there is an additional force experienced by the particle called the drag force (F_{drag}), from Newtons II law the net force experienced by the particle is given by equation 2,

$$F_{net} = F_{ele} + F_{drag} \quad (2)$$

On further substitution in equation 2 in place of F_{ele} and F_{drag} , we can derive the equation called the Nernst-Planck equation, which governs the ion flux through the channel, and it is given by equation 3 [32],

$$j_i = -D_i \left[\frac{dc_i}{dx} - \frac{z_i \cdot E \cdot F}{R \cdot T} \right] \quad (3)$$

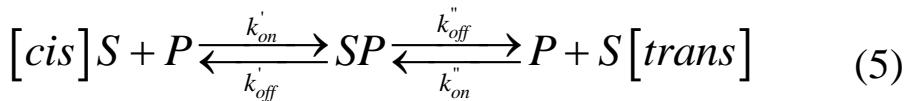
Here, j_i is the ion flux in Am^{-2} , D_i is the diffusion constant in m^2s^{-1} , c_i (mol/L) is the concentration of ionic species, z is the charge of the ionic species, E is the applied field in (V/m), F is the Faraday's constant, T is the temperature and R is the gas constant. The above equation 3, describes the transport of particles across the channel through diffusion as well as under the applied electric field.

From equation 3, we can get the conductance of the pore and compare it with the experimental value obtained from the linear fit of the IV curve. Theoretical calculation on the conductance of the channel is given by equation 4,

$$G_{pore} = \frac{g(c) \cdot \pi \cdot r_{pore}^2}{L_{pore}} \quad (4)$$

Here G_{pore} is the conductance of the channel in (pS), $g(c)$ is the conductivity of the salt buffer used in the experiment, r_{pore} is the radius and L_{pore} is the length of the channel.

To understand the chemical kinetics, binding constant and the net flux of a substrate, we can consider a substrate entering a channel, binding and leaving the channel as a chemical adsorption-desorption process between the substrate (S) and pore (P). Since there is a binding site in the channel, we assume that the channel gives access to the substrate from both cis and the trans side and may be subjected to different activation energy barrier and it is defined as 2 barrier and 1 binding site model. The chemical reaction for substrate entering from cis and trans is given as:



Considering pore and substrate cause two chemical reaction on the 'cis' and 'trans' side which are same. Therefore, there is only one overall binding constant (K) and it is given in equation 6 [33],

$$K = \frac{k_{on}'}{k_{off}'} = \frac{k_{on}''}{k_{off}''} = \frac{k_{on}'}{k_{off}'} + \frac{k_{on}''}{k_{off}''} \quad (6)$$

Defining the rate constants, the k_{on} is the association rate and k_{off} is the dissociation rate constants, where K stands for the overall thermodynamic binding constant. The equilibrium binding constant as a function of the various rate constants can be expressed as further, from

the single channel recordings, the values for the k_{on} and k_{off} are basically determined from the number of blocking events and the duration of the events, also known as residence time respectively. This blockage event, mainly occurs on subsequent addition of substrate (antibiotic) on either cis or trans side of the lipid bilayer[33-35].

$$k_{on} = E/n.(c) \quad (7)$$

$$k_{off} = 1/\tau \quad (8)$$

Where, E is the number of binding events per second, n is the number of monomeric channel and c is the antibiotic concentration in molar and τ is the resident time in (ms), the unit for $k_{on} = M^{-1}s^{-1}$ and $k_{off} = s^{-1}$.

There are several methods to analyze the single channel data and one being the Power spectrum method, it is important sometimes, especially when the time resolution of the acquired data is not good enough to resolve each binding event independently.

To study the power spectrum of such data and extract its association and dissociation rates, we first subtract the power spectrum of blank single channel data (no substrate) with the power spectrum of substrate. If the interaction of substrate with the channel is a two-state Markov model, then the power spectrum reads as shown below equation [34, 35] (9):

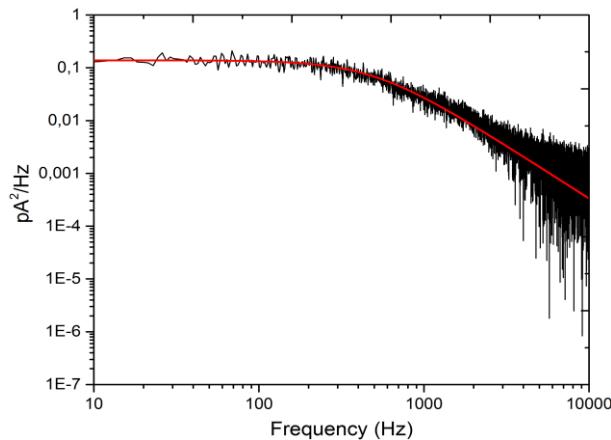


Figure 4: The black line is the blank subtracted PSD from 0.5mM norfloxacin current trace PSD and the solid red line is the fit of Lorentzian model.

$$S(f) = \frac{a}{1+(2\pi f \tau_c)^2}; \quad (9)$$

$$a = 4N_c(\Delta j)^2 c k_{on} k_{off} \tau_c^3; \quad (10)$$

$$\tau_c = \frac{1}{k_{off} + c k_{on}} \quad (11)$$

Equation 9 is the Lorentzian model [33, 35, 36], Here a is the Lorentzian factor and τ_c is the inverse corner frequency which is the fitting parameter extracted from the model. Using these fitting parameters we can estimate the on and off rates of the substrate (for further detail on the equation check [35, 36].

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Transport of small charged solute through OmpF, a membrane channel from *E. coli*.
(Work done in collaboration with I. Ghai)

Abstract:

A major challenge over the designing of the novel antibiotics contrary to Gram-negative bacteria is to achieve suitably fast permeation to avoid high doses, resulting towards toxic side effects. So far, suitable assays for enumerating uptake of charged molecules into bacteria are limited. We hereby compare flux of a small charged β -lactamase-inhibitors clavulanic acid using two electrophysiological methods, which includes zero-current assay and single channel event blockage in-order to understand at molecular level over interactions between the nanopore OmpF and the charged solute. We determine that at zero-membrane potential a concentration gradient of 50 mM of clavulanic acid created permeability rates P_{K^+} / P_{solute} of 4 : 0.7 under bionic conditions. Whereas sensing at single channel level in the presence of 1M KCl the transport with the gradient of 50 mM shows approximately 80 molecules per second per monomeric OmpF channel at an applied potential of 25 mV.

Introduction:

Here, we investigate and compare the permeation of charged solute through OmpF channel of *E. Coli* by examining the ion current fluctuations induced in the presence of solute and compared the obtained blocking events[1, 2] with the novel electrophysiological reversal potential assay [3, 4]. Unfortunately, most small solute molecules do not show easily detectable changes in the ionic currents while passing through the nanopore but for this case we observed well resolved ionic current blockages [5]. Further, the obtained statistics were analyzed and compared with the macroscopic turnover number obtained experimentally using reversal potential assay[6, 7].

Materials and Methods

Materials:

Clavulanic acid or Clavulanate potassium or Potassium clavulanate (Figure 1) VETRANALTM, analytical standard CAS Number 61177-45-5 was obtained from Sigma Aldrich Germany and was consumed within 1 week of opening the bottle, 1,2-diphytanoyl-sn-glycero-3-phosphocholine was

procured from Avanti Polar Lipids (Alabaster, AL) and all other chemicals used were procured from AppliChem,

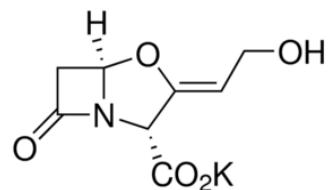


Figure 1: Molecular structure of Potassium Clavulanate, Molecular formulae $\text{C}_8\text{H}_8\text{NO}_5\text{K}$, Molecular weight 237.25 g/mol.

Methods:

Planar Lipid Bilayer and Electrical Recording:

Planar lipid bilayer conferring to Montal and Mueller were formed as defined in detail briefly[3, 4, 6, 8-10], an aperture in a Teflon septum with a diameter of 100-120 μm was pre-painted with hexadecane dissolved in n-hexane at 1-5% (v/v) and the cuvette compartments were dried for 30-35 min, in-order to eliminate the solvent. Bilayer were made with 1,2- diphyanoyl-sn-glycerophosphocholine at a concentration of 4-5 mg/ml in n-pentane. Stock solutions of the outer membrane porin OmpF (1 mg – 2 mg protein/ml) was added to the cis (ground) side for all the measurements. Standard Ag/AgCl electrodes were used to detect the ionic current. Further, for measuring electrophysiological reversal potential assay, for asymmetric condition we used commercial calomel electrodes (Metrohm). The cis side electrode of the cell was grounded, whereas the trans side electrode was linked to the headstage of an Axopatch 500B amplifier, used for the conductance measurements in the voltage clamp mode. Signals were filtered by an on board low pass Bessel filter at 10 kHz and logged onto a computer hard drive with a sampling frequency of 50 kHz. Examination of the current recordings was completed using Clampfit (Axon Instruments), and with a custom made analysis code in LabView 2015 (National Instrument). The current voltage relation of the individual experiments was calculated from single averaged currents at the given voltage. The relative permeability of cations vs solute anions in the tri-ionic case ($P_{\text{K}^+}/P_{\text{Cl}^-}/P_{\text{solute}^-}$) and bionic conditions ($P_{\text{K}^+}/P_{\text{solute}^-}$) were obtained by fitting of the experimental I-V-curves with the Goldman-Hodgkin-Katz current equation [3, 4].

Results :

Electrophysiological measurements:

Purified OmpF channel was reconstituted into planar lipid bilayer. The trimeric channel in 1M KCl, 20 mM MES buffer at pH 6 revealed a conductance of $\bar{G}_{trimer} = 4 \pm 0.5 \text{ nS}$, in particular agreement with previous study [11]. In the absence of solute clavulanic acid, the channel current measurements from a bilayer at $V_m = \pm 100 \text{ mV}$ did not reveal frequent channel blockages (Figure 2 A). Further over addition of Clavulanic acid significant channel gating/blocking events were observed. These events were specifically observed in a systematic manner where over cis side addition of Clavulanic acid over OmpF reconstituted bilayer channel currents blocking was observed only at negative applied potential. Whereas over trans side addition the events were observed at positive applied potentials [3, 4]. The interaction of Clavulanic acid in a concentration range from 0.16 to 50 mM to cis followed by trans side of the membrane comprising single trimeric OmpF channel were measured. Well resolved blockage/events with $f_{gating} \approx 10 - 15 \text{ s}^{-1}$ for cis side addition at -100 mV and $f_{gating} \approx 8 - 13 \text{ s}^{-1}$ for trans side addition at +100 mV of the OmpF monomeric channel at a concentration of 2.5 mM clavulanic acid addition were observed. This reveals a systematic behavior of transport as a function of electro-osmotic flow over applied electric field across channel on the translocation of negatively charged solute against the applied electrophoretic force. As, electro osmotic flow is generated due to the interface formed between the electrolyte and the walls of the nanopore which is called a double layer in equilibrium. It is a non-zero surface charge, screened by a very thin diffused layer mainly composed of counter ion of width λ (Deby screening length) which increases with decreasing salt concentration. Under the application of tangential electric field the deby layer which is composed of the counter ions will drag the fluid to produce an effective slip velocity ($u_l = -\mu_E E_l$) [7]. This phenomena plays a major role in transport of analyte through the nanopores[6], especially when the pore dimensions are as close as the thickness of electrostatic double layer [6].

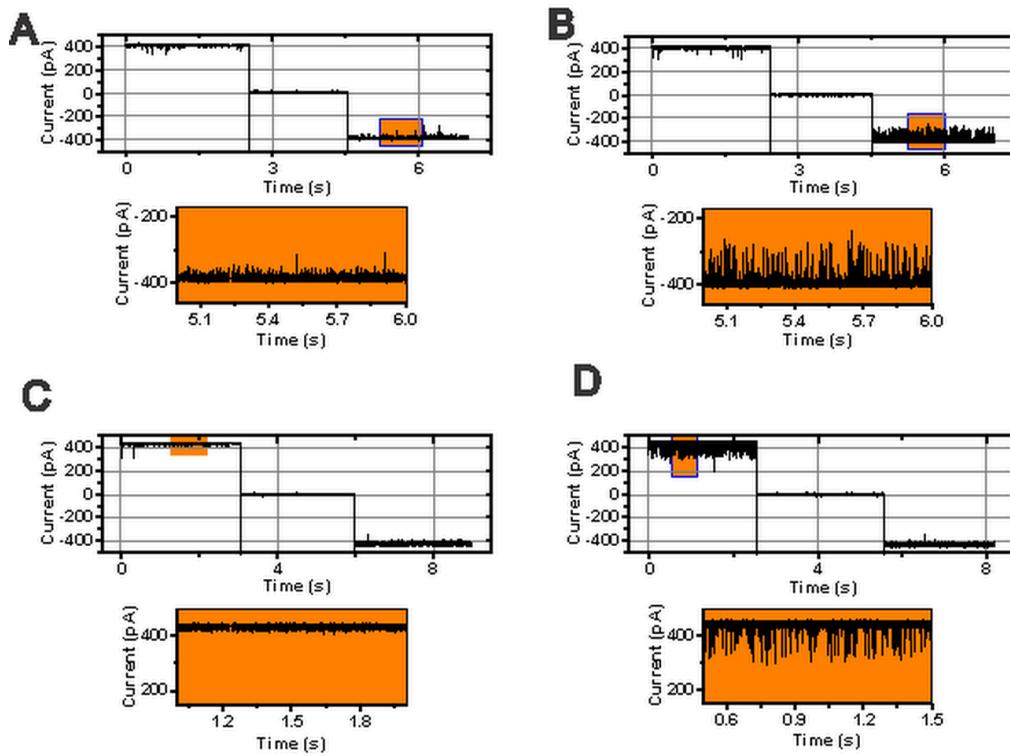


Figure 2: Single channel current recordings from bilayer containing a single active reconstituted trimeric OmpF channel.

Left: Current recordings (A and C) Blank (no substrate) (B) Addition of 2.5 mM Clavulanic acid *cis* side (C) Addition of 2.5 mM Clavulanic acid *trans* side. (*cis* = Ground connected side). *cis/trans* Clavulanic acid addition were measured in separate experiments.

Though, clavulanic acid carries out a partial charge of $n = -1$ at pH 6 thus, at this pH more directly measure its permeation via OmpF by applying electrophysiological reversal potential permeation assay [3, 4] using concentration gradients of clavulanic acid under bi and tri-ionic conditions [3, 4].

Electrophysiological permeation assay: Method-Outline

We are concerned over the information on the selectivity of the membrane transport of charged solute which are however mostly obtainable in limited amounts and thus can only be used in trace quantities within lower μM to mM concentrations[12, 13]. To resolve this issue, we apply an investigational setup where a grouping of symmetric salt at low concentrations (tri-ionic conditions) table 1 on both sites of the membrane are complemented with low concentrations

potassium chloride and followed by specific solute at one side of the membrane. Bionic conditions were measured where the measurement includes initial low concentration of solute symmetric cis and trans followed by solute addition to cis side (Table 2).

Substrate*	Substrate cis (mM)	KCl (cis/trans)	V_{rev} (mV)	$P_{K^+} / P_{Cl^-} / P_{solute}$
Clavulanic acid	50 mM	10	29±5	4: 1: 0.6

Table 1: Experimental reversal potential values and calculated permeability ratios of Clavulanic acid through the OmpF pore under tri-ionic conditions. The permeability ratio of $P_{K^+} / P_{Cl^-} = 4: 1$ for OmpF has been determined independently under bionic conditions and was fixed during fitting of V_{rev} (tri-ionic) additional to salt pH 6.0, buffered with 10 mM MES.

Substrate*	Substrate cis/trans (mM)	V_{rev} (mV) (n=3)	P_{K^+} / P_{solute}
Clavulanic acid	80/30	18 ± 4	4: 0.7

Table 2: Experimental reversal potential values and calculated permeability ratios of Clavulanic acid through the OmpF pore under Bi-ionic conditions pH 6.0, buffered with 5 mM MES.

Electrophysiological permeation assay calculations

Assuming single channel recording from a bilayer with an arbitrary channel having the following arbitrary properties

tri-ionic conditions:

Permeability: $P_{K^+} = 4; P_{Cl^-} = 1.0; P_{Solute^-} = 1.0;$

Cation : $z_{K^+} = 1; c_{K^+cis} = 60 \text{ mM}; c_{K^+trans} = 10 \text{ mM}$

Anion: $z_{Cl^-} = -1.0; c_{Cl^-cis} = 10 \text{ mM}; c_{Cl^-trans} = 10 \text{ mM}$

Solute: $z_{Solute} = -1.0$; $c_{Solute-cis} = 50mM$; $c_{Solute-trans} = 00mM$

Zero current potential: $V_{rev} = 29.0 \text{ mV}$ (*experimental value*)

Considering that the assumptions of the GHK-theory are valid and the ion fluxes considered to be independent² we can calculate the expected current voltage relation for the above membrane channel for any combination of the bi -or tri-ionic concentrations:

1. $I_{K^+}(V) = I(V, P_{K^+}, z_{K^+}, c_{K^+cis}, c_{K^+trans})$,
2. $I_{Cl^-}(V) = I(V, P_{Cl^-}, z_{Cl^-}, c_{Cl^-cis}, c_{Cl^-trans})$
3. $I_{Solute^-}(V) = I(V, P_{Solute^-}, z_{Solute^-}, c_{Solute^-cis}, c_{Solute^-trans})$
4. $\sum I(V) = I_{K^+}(V) + I_{Cl^-}(V) + I_{Solute^-}(V)$

Bi-ionic conditions:

Permeability: $P_{K^+} = 4$; $P_{Solute^-} = 0.7$;

Cation : $z_{K^+} = 1$; $c_{K^+cis} = 80 \text{ mM}$; $c_{K^+trans} = 30 \text{ mM}$

Solute: $z_{Solute} = -1.0$; $c_{Solute^-cis} = 50mM$; $c_{Solute^-trans} = 00mM$

Zero current potential: $V_{rev} = 18.0 \text{ mV}$ (*experimental value*)

Considering that the assumptions of the GHK-theory are valid and the ion fluxes considered to be independent² we can calculate the expected current voltage relation for the above membrane channel for any combination of the bi -or tri-ionic concentrations:

1. $I_{K^+}(V) = I(V, P_{K^+}, z_{K^+}, c_{K^+cis}, c_{K^+trans})$,
3. $I_{Solute^-}(V) = I(V, P_{Solute^-}, z_{Solute^-}, c_{Solute^-cis}, c_{Solute^-trans})$
4. $\sum I(V) = I_{K^+}(V) + I_{Cl^-}(V) + I_{Solute^-}(V)$

Further, in-order to prove the robustness of the methodologies we extended our approach towards verification of the existence of particle translocation using reversal potential technique and Single Channel Electrophysiology. The obtained event rates of Clavulanic acid with single OmpF channel were compared with both methods showing effective proof of molecule translocation across the OmpF membrane channels.

Discussion

We have shown that recording nanopore I-V curves and fitting the results with the GHK equation gives the zero-current potential and thus allows the calculation of the relative permeability for the ions present in solution. In particular, with nanometer sized pores at low mM concentrations of current carrier electrolytes like KCl the nanopore permeability of large charged solute molecules like the β lactamase inhibitors anions of clavulanic acid can be screened under tri ionic conditions using low mM concentrations of the charged large solute molecules. Beside the principal difficulties underlying the GHK constant field theory, which assumes independent movement of the ions through the pores (see references for a detailed discussion) [3, 4] we have demonstrated that the methodology can be used to obtain semi-quantitative measures for permeation of charged drugs through nanopores. The method presented can, after a suitable miniaturization and parallelization, serve as a basis for a simple, fast and sensitive permeability screen of nanopores for charged molecules. Further quantification of the flux requires calculation of the individual contribution of each ion species to the total flux passing the single channel. Despite the principal difficulties underlying the GHK constant field theory, using this approach the experimental I-V curves can be divided into the fluxes of the individual ions, which then allows a semi-quantitative estimation of turnover numbers for each of the ions. Comparing our bi ionic and tri ionic measurements and the respective GHK analysis surrounds in a coarse qualitative consistency of the data, which sanctions our conclusions to be qualitatively valid. Further, we performed a systematic investigation over modulation of ion-channel currents through the pore of the *E. coli* OmpF porin. As the observed frequency of blocking events $f_{gating} \approx 10 - 15 s^{-1}$ with single OmpF channel in the presence of clavulanic acid with background conditions of 1M KCl, at pH 6.0 was observed. Whereas on comparison with zero-current potential assay both methods sort to be complementing each other proving towards an optimized approach towards nanopore transport sensing.

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The effect of electro-osmosis on the transport of fluoroquinolones across Outer membrane porins F

Abstract:

We report on the ability to control the dynamics of a single antibiotic capture and passage across a voltage-biased, OmpF nanopore, under conditions that the electroosmotic force exerted on the analyte dominates the electrophoretic transport. The interpretation of transport is still misunderstood, and it is a hot topic for debate within researchers. At the nanoscale regime, inside the channel, the applied electric field acting on the substrate and the electro-osmotic flow (EOF) which is a fluid flow along the direction of applied field, contributes strongly to the transport of antibiotics. We perform experiments at two different pH i.e. pH 6 and pH 9, at these pH OmpF is cation selective. To demonstrate the strong presence and the effect of EOF, we choose to work with three different substrates (Norfloxacin, Ciprofloxacin and Levofloxacin). At pH 6 the translocation of antibiotic was due to both applied electric field and EOF, whereas at pH 9 the translocation of antibiotic was purely due to EOF. Statistical analysis of the resolvable dwell-times reveals rich kinetic details at these two pH regarding the direction and rates of stochastic movement of antibiotics inside the nanopore. The presented approach demonstrates the ability to shuttle and study molecules along the passage pathway inside the nanopore, and allows to identify the exact exit and entry points and thus providing a convincing proof of a molecule translocating through the pore.

Introduction:

Bacteria are mainly classified into Gram-positive and Gram-negative bacteria. The common difference between them is Gram-positive bacterial shell is made up of single layer of lipid membrane which separates the outer environment from the inner machineries. Whereas the Gram-negative bacteria is made up of two layers of lipid membranes, and they are classified as outer membrane, and the inner membrane. Between both the layer there, exists a periplasm space which is a concentrated gel-like matrix, and it constitutes up to 40% of total cell volume in Gram-negative bacteria [1, 2].

The outer membrane of bacteria is very distinct in its composition when compared to its cytoplasmic inner membrane, the outer envelope of the outer membrane is mainly made up of complex lipopolysaccharide whose lipid portion acts as an endotoxin. The outer membrane of bacteria is embedded with lots of porins, which are beta like barrel shape and are naturally reconstituted across a cellular membrane and act as a pathway for molecules to diffuse. Unlike other transport proteins, these porins are large enough to allow pure diffusion of antibiotics and nutrients across the membrane [3, 4]. They act like selective porins that are particular to different types of molecules (e.g., maltoporin has a binding site for malto-oligosaccharides for the uptake into the bacteria) and some porins facilitate non-specific diffusion (OmpF and OmpC from *E. coli*) [5].

Antibiotic transport is the fundamental process in the biological cell [6] as it facilitates the uptake of antibiotic for infection healing which is purely governed by the process of diffusion [7]. In *E. coli* the general outer membrane porin's like OmpF and OmpC have a special region which is called the bottle neck area. This area (Constricted region) is decorated with charged residues and acts as the barrier for the translocation of antibiotics and various energy components [8]. Such barrier in the outer membrane protein also filters selectively for the translocation of molecules through the porin's when they are *invivo*. In biology, to maintain the effectiveness of the antibiotic, the antibiotic has to diffuse through the outer membrane porin and target the infected area by again crossing the barrier of periplasm and inner [9]. However, the mechanism of translocation of small molecules remains mystery [10].

Here we show the single molecule translocation of analyte across the wild type outer membrane porinF at pH = 6 and pH = 9. We show the dominance of electro-osmotic flow on the translocation

of our analyte against the applied electrophoretic force. EOF is a flow generated in micro or nano fluidic channels or porous material under the applied tangential electric field. Electro Osmotic flow arises due to instantaneous counterion formation on the solid –liquid interface. When the surface gets in contact with the liquid, the counterion (positive or negative ion, depending on the surface charge) screens the surface due to coulombs attraction, thus forming a thin layer of net charge called the electrostatic double layer. When a tangential electric field is applied to the surface, the counterion moves along the direction of the electric field and the velocity of the Electro Osmotic flow is given by relation $u_I = \mu_E E_I$ [11, 12]. Electro-osmotic flow plays a major role in transport of analyte through the nanopores [13], especially when the pore dimensions are as close as the thickness of electrostatic double layer.

Exploitation of electro-osmotic flow has been done in many application like studying the force extension studies of polymers in narrow channels [14, 15], blood related diseases identification [16], segregation of chemical species [17]. As EOF is a surface driven phenomenon, it is only studied from surface related effect but here in this article we demonstrate most important the role of EOF in charged antibiotic transport by simple single channel electrophysiology experiment. Recently, the role of electro-osmosis in the porins have drawn immense interest for researchers and scientists as the claim and several proof of the transport through electro osmotic flow [12, 18-20].

Material and Methods:

Chemicals:

The following chemical reagents used in this study are as follow: Potassium Chloride (KCl), 2-(N-morpholino)ethane sulfonic acid (MES), 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) all these chemicals were purchased from Sigma-Aldrich (USA). For bilayer formation, 1,2-diphytanoyl-sn-glycero-3-phosphocholine was purchased from Avanti Polar Lipid (Alabama, USA). N-Pentane, n-hexane, and hexadecane were procured from Sigma-Aldrich (USA). Lipid bilayer was formed using a technique published previously [21] on a 25 μ m thin Teflon membrane which separates liquid flow cell into 2.5ml volumes each. An aperture in the Teflon membrane with a diameter of 50–70 μ m was pre-painted with purified hexadecane dissolved in n-hexane at a concentration of 1-2% (v/v) and was allowed to cure for about 20 min in-order to annihilate the solvent. Both chambers of the liquid flow cell were filled with 1M KCl with 20 mM 2-(N-

morpholino) ethane sulfonic acid (MES) as a buffer for pH6 and CAPS buffer for pH 9. Formation of Bilayer was done using 1,2- diphyanoyl-sn-glycerophosphocholine at a concentration of 5 mg/ml dissolved in n-pentane. The stock of the outer membrane porin F (OmpF) 1mg/ml was diluted 100 times to get the exact concentration for getting a single channel embedded into bilayer (0.3 μ L) was added to the Ground side of the flow cell for all the measurements and standard Ag/AgCl electrodes were used for the measurement. All current measurements were made using Axon 200B amplifier (Axon Instruments, USA) in voltage clamp mode and the current signals were filtered by an on board low pass Bessel filter set at 10 kHz with a sampling frequency of 50kHz and data acquisition was done using Axon 1440A digitizer. Analysis of the ion trace was done using custom made LabVIEW analysis code.

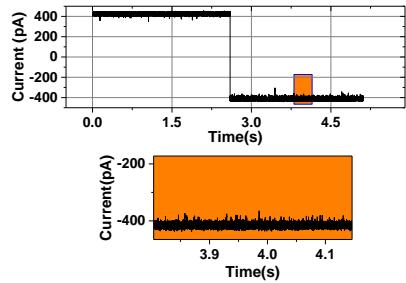
Results and Discussion:

Interaction of Analyte (Norfloxacin, Ciprofloxacin and Livofloxacin) with single outer membrane channel OmpF at pH 6 and pH9 is shown in the Figure1-4. Analyte was added to cis side of the liquid flow cell for all the experiments. Concentration of the analyte used in the experiment was 0.5mM in 1M KCl solution at pH6 and pH9. Considering the situation at pH6 (Fig 1-2), under the application of applied potential across the membrane, the ionic current was monitored through the pore over time for both positive and negative potential Fig1(i) represents the blank current trance at both \pm 100mV. At positive applied potential no translocation of analyte was seen (see Fig1(ii)) as the applied electric field drives the particle away from the pore vicinity (see schematic Fig1(iii) for the direction of EOF and applied field). Whereas, at negative bias both Electric field and EOF drives the particle one by one from Cis side of the pore to the trans side. Clear and distinct blockages are seen [Fig1(ii)], the direction of EOF and E-Field is shown in Fig1(iv) we can clearly see from the schematic that the direction of EOF and Analyte translocation is in the same direction as that of E field. Similarly, when analyte was added to the trans side of the membrane, interactions were observed on the positive applied potential.

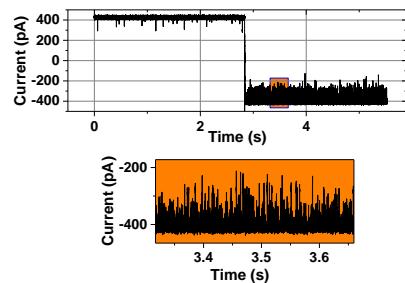
Figure 1:

pH = 6

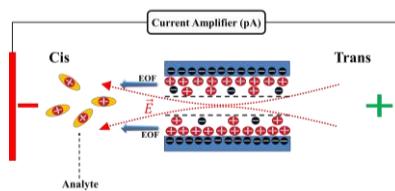
1(i)



1(ii)



1(iii)



1(iv)

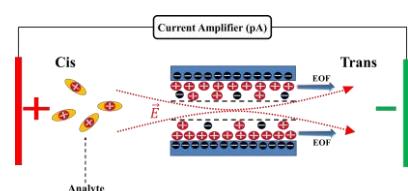


Figure 1: 1(i) Single channel current recording from reconstituted OmpF porin at both $\pm 100\text{mV}$ (no substrate). 1(ii) Single channel current recording of OmpF porin showing real-time detection of Norfloxacin at $\pm 100\text{mV}$ with a concentration of 0.5mM in 1M KCl at $\text{pH } 6$ and the zoom of the negatively applied voltage trace shows clear and distinct blockages appeared at -100mV , here at -100mV . Figure 1(iv) experimental scheme clearly supports the data, as the applied field and EOF is in the direction of translocating molecule and 1(iii) is the schematic of the direction of electric field and electro osmotic flow at $+100\text{mV}$.

Figure : 2

pH 6

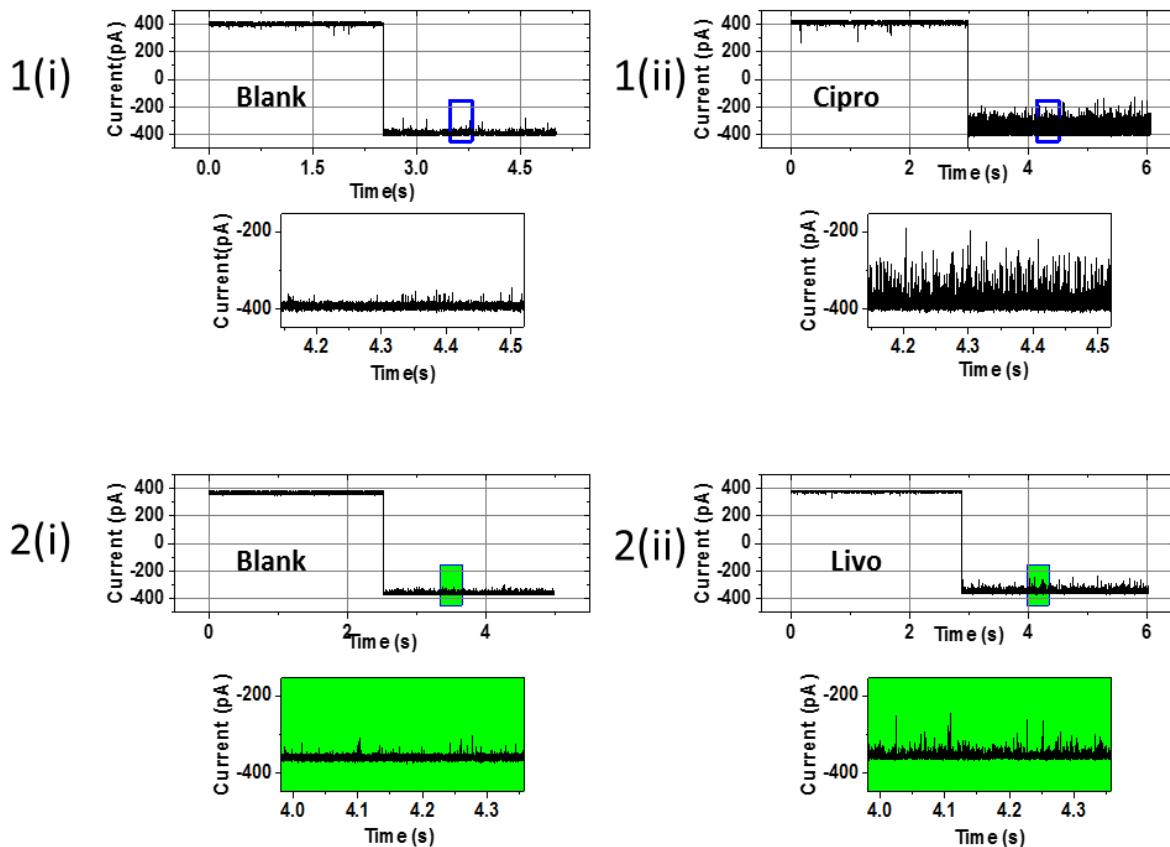


Figure 2: 1(i) and 2(i) Single channel blank trace of OmpF porin for both Ciprofloxacin and Levofloxacin at $\pm 100\text{mV}$. 1(ii) and 2(ii) Single channel trace of OmpF porin showing real-time detection of both Ciprofloxacin and Levofloxacin at $\pm 100\text{mV}$ with substrate concentration of 0.5mM in 1M KCl at pH 6 (for schematic of EOF at pH 6 please see Figure 1(iii) and 1(iv)).

Now, to see the sole effect of EOF on the translocation of analyte across the pore we changed the net charge of the analyte by changing the pH of the buffer i.e. from pH 6 to pH 9. But the net charge of pore was maintained negative as the pI of OmpF is 4.5, so any pH above 4.5 will have always net negative charge on the system. By changing pH from 6 to 9, the charge of the analyte is changing from +ve net charge to -ve net charge. Fig3(i) represents the blank current trace at pH 9. Surprisingly, at positive applied potential no translocation of analyte were seen (Fig3(ii)). Such behavior of analyte forced us to investigate further and helped us in drawing conclusion about the presence of strong electro osmotic flow. See Fig3(iii) for the direction of EOF and applied field when the pore is maintained at positive potential. When the bias voltage

was switched to negative bias potential across the membrane, large number of blockages were seen (Fig3(ii)), despite the fact that electric field would drive the analyte away from the pore vicinity. Such surprising effect were due to the presence of strong EOF which drives the analyte against the applied field [see schematic Fig3(iv)], similar behavior was also seen on ciprofloxacin and levofloxacin (See Figure 4). Further, we compared the event rate and the dwell times of all the analyte at both the pH 6 and 9 (see Table 1 for event rates and Table 2 for dwell times). From the analysis we found that at pH 9 event rate drops down by an order of magnitude compared to the translocation at pH 6. Such drop in the event rate was very obvious as at pH 6 the analyte translocation is due to both electrophoretic and electro osmotic forces so both the forces are vectorial addition on the analyte and thus shorter dwell times were achieved. Whereas, at pH 9 the analyte translocation is against the electrophoretic force and thus, the analyte slows down by a factor of 3 compared to the translocation at pH 6. Because of interplay between electrophoretic force and electro-osmotic flow it is possible to reverse the direction of translocation by just changing the pH across the pI of analyte.

Figure 3: pH = 9

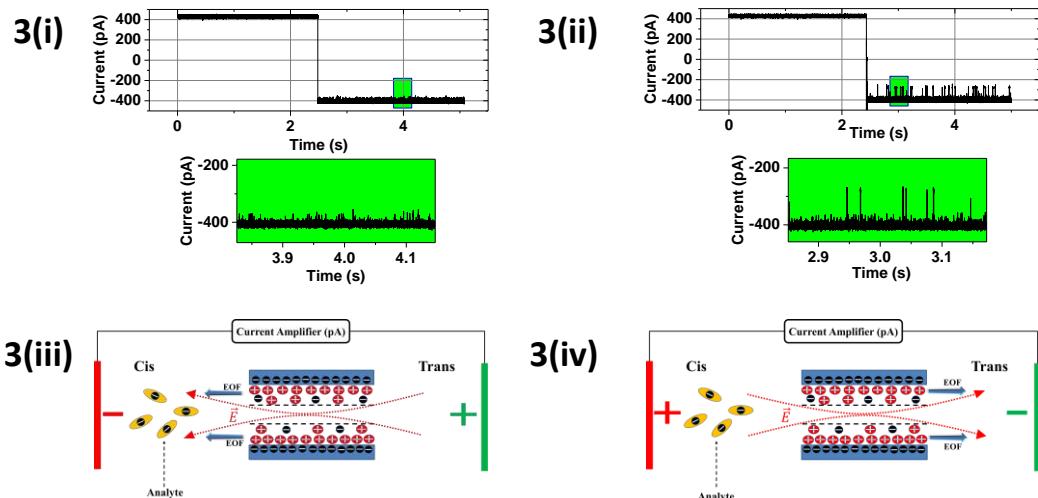


Figure 3: 3(i) Single channel blank current recording from reconstituted OmpF porin at both $\pm 100\text{mV}$. 3(ii) Single channel current recording of OmpF porin showing real-time detection of Norfloaxacin at $\pm 100\text{mV}$ with a concentration of 0.5mM in 1M KCl at pH 9 and the zoom of the negatively applied voltage trace shows the clear and distinct blockages appeared at -100mV . 3(iv) experimental scheme clearly supports the data as the EOF is in the direction of molecule

translocation. 3(iii) is the schematic of the direction of electric field and electro osmotic flow at +100mV.

Figure : 4

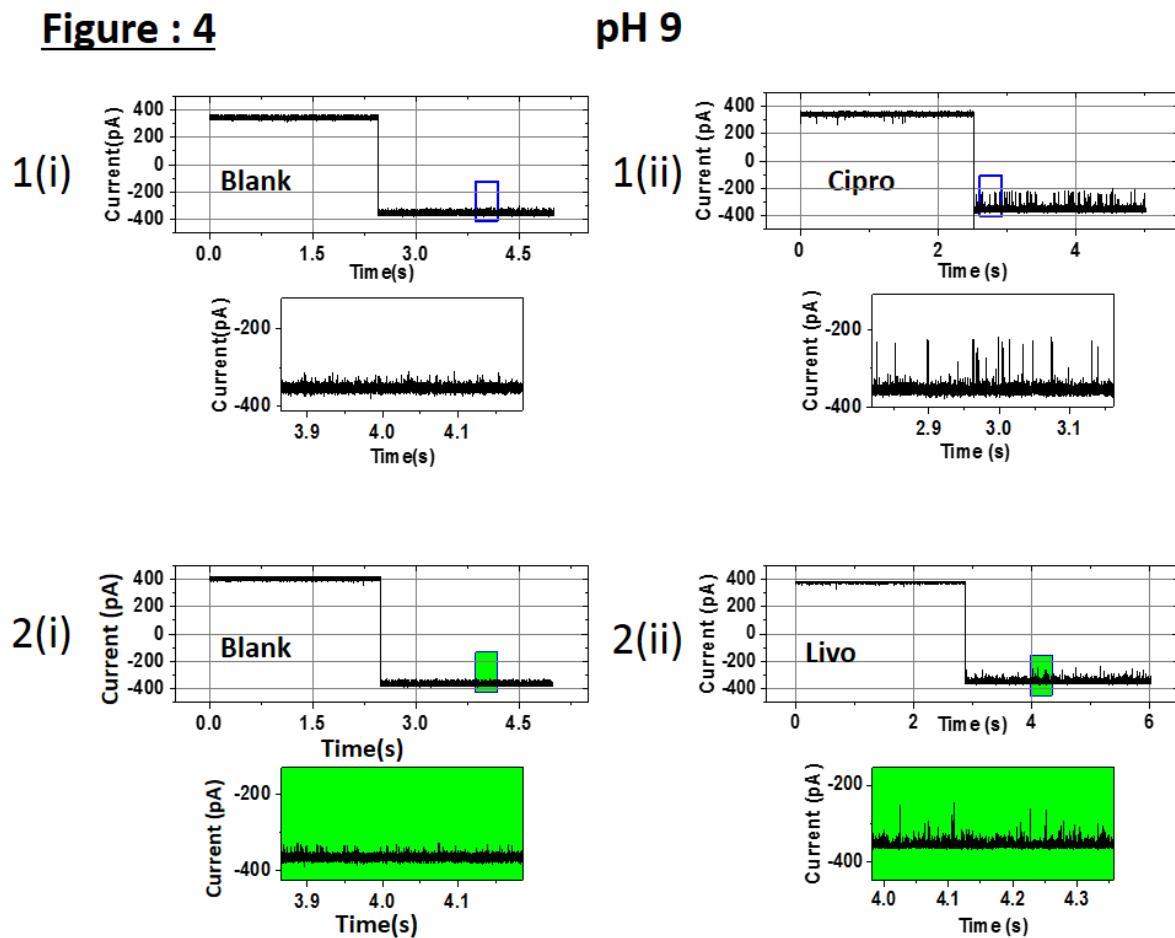


Figure 4: 1(i) and 2(i) Single channel blank trace of OmpF porin for both Ciprofloxacin and Levofloxacin at $\pm 100\text{mV}$. 1(ii) and 2(ii) Single channel trace of OmpF porin showing real-time detection of both Ciprofloxacin and Levofloxacin at $\pm 100\text{mV}$ with a concentration of 0.5mM in 1M KCl at pH 6 (for schematic of EOF at pH 9 please see Figure 3(iii) and 3(iv)).

Table 1:

Substrate	Event Rates Per Monomer (s^{-1})			
	pH 6		pH 9	
	Cis_-100mV	Trans_+100mV	Cis_-100mV	Trans_+100mV
Norfloxacin	47±14	35±8	7 ±2.5	0.4±0.2
Ciprofloxacin	81±22	55±16	8±3	0.5±0.2
Livofloxacin	14±5	5±2	0.5±0.2	0.5±0.3

Table 2:

Substrate	Residence Time (ms)			
	pH 6		pH 9	
	Cis_-100mV	Trans_+100mV	Cis_-100mV	Trans_+100mV
Norfloxacin	0.22±0.06	0.11±0.03	0.42 ±0.08	0.14±0.03
Ciprofloxacin	0.1±0.03	0.11±0.04	0.32±0.07	0.17±0.05
Livofloxacin	0.11±0.03	0.114±0.03	N/A	N/A

Now, the same experiment was repeated at different voltages from $\pm 12.5\text{mV}$ to $\pm 150\text{mV}$ and Event rates for both positive and negative voltages are plotted against the applied potential for both pH 6 and pH 9 (Fig 5 and Fig 6). We clearly see a rise in the event rates when negative bias is applied for both pH 6 and pH 9 experiments, at pH 6 the translocation is due to both electrophoretic and electro osmotic driven but for pH 9 the translocation is purely electro-osmotic. Now, comparing their event rates in figure 5 and figure 6, we see almost 5 fold decrease in event rate in pH 9 compared to pH 6. In pH 9 the analyte has to over come the energy barrier laid by the applied electrophoretic force so this leads to a drop in event rates and a significant effect on dwell time was also observed, which can be clearly seen in figure 5 and 6 for respective pH. We see almost 3 fold increase in dwell time at pH 9, this confirms that the analyte slows down at pH 9 due to opposing force offered by the applied electric field.

Figure 5: pH = 6

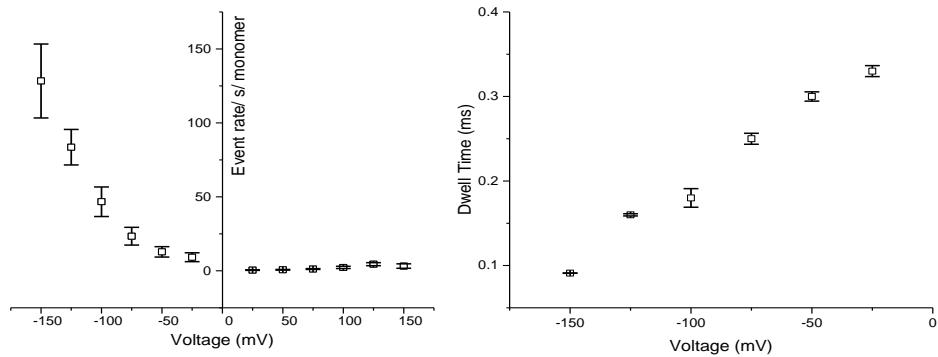


Figure 5: Plot of event rate versus applied potential for the addition of 0.5mM norfloxacin on the cis side of the membrane and its associated plot of dwell times (right).

Figure 6: pH = 9

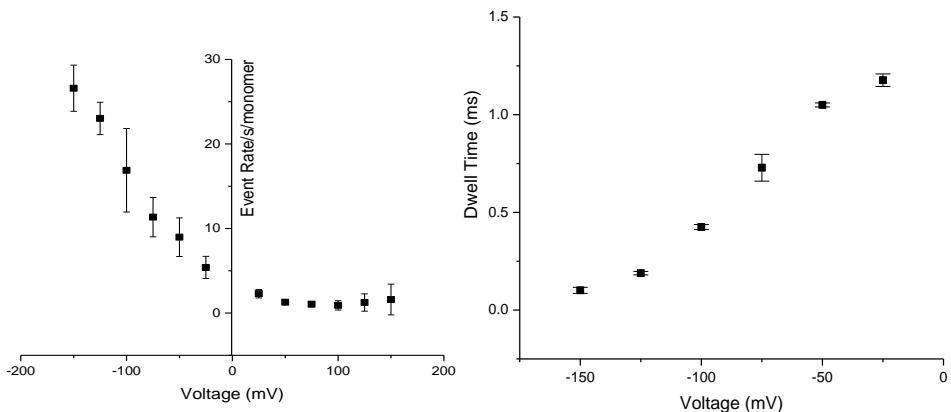


Figure 6: Plot of event rate versus applied potential for the addition of 0.5mM norfloxacin on the cis side of the membrane and its associated plot of dwell times (right).

Conclusion :

The ability to capture a single antibiotic inside a non-homogenous channel have allowed researchers to answer open questions like how a molecule is captured and translocated from one side of the nanopore to the other side. We show here the presence of strong electro-osmotic flow which creates a strong absorbing region at the pore openings and its ability to capture the molecule against the applied electric field. Once the EOF captures the molecule inside the pore, the molecule continuously move under the strong EOF by over coming the energy barrier laid by the repelling electric-field, such behavior have been seen for the first time in the outer membrane channels, and statistics analysis reveals rich kinetic details about the translocating molecule. We see extended dwell time and significant reduction in event rates when the charge of antibiotic is change from net positive to net negative charge. Such nonlinear effect sheds light to the problem of transport and EOF could be one possible way to study the transport mechanism in further detail. This similar result was also first demonstrates on polymers translocating via α - Hemolysin [20]. Such interesting result also lay a foundation in the field of antibiotic translocation which proves that there is highest probability that the particle translocate when such nonlinear effects dominates the transport of analytes, and also helps us in determining the precise entry and exit point of the analyte when captured by the pore [20] .

Finally we believe that our results strongly hints that the transport of analyte is mainly due to EOF, as from our analysis it seems to support EOF to be strong driving force when pH 9 data is compared to pH 6 data.

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Future Task:

Projects	Timeline of completion
<p>The effect of Electro-osmosis on the transport of fluoroquinolones across Outer membrane porins F.</p> <ul style="list-style-type: none"> • The measurement on OmpF has revealed that electroosmotic flow can enhance or even dominate antibiotic transport over the electrophoretic motion (<i>in vitro</i>). • To check the presence and effect of EOF, I would like to perform single channel experiment on OmpC with fluoroquinolones at pH 6 and pH 9 and validate the role of EOF on both OmpF and OmpC. • To perform reversal potential measurement on both OmpF and OmpC with fluoroquinolones and correlate the obtained flux values with single channel data. • To quantifying electro osmotic flow rates and correlate them to the residence time of the substrate, this would prove the effect of EOF on the transport of substrate molecules through channels. • Preliminary results clearly shows that EOF dominates the electrophoretic transport and consequently suppress or even reverse the electrophoretic transport. • Such results would give a new dimension for physicist to understand the transport of antibiotics across the membrane (<i>in vitro</i>) 	November 2017 - March 2018
<p>OprD interaction study with carbapenams.</p> <p>Preliminary results on OprD have forced me to perform some basic experiments as the foundation on OprD is not well established</p> <ul style="list-style-type: none"> • To study the structural changes of OprD at different salt concentration. • To perform single channel experiment on basic amino acids and carbapenams at low salt conditions • To perform reversal potential measurement on basic amino acids and carbapenams. 	November 2017 - September 2018

To develop and study the membrane potential using Inner field compensation technique <ul style="list-style-type: none"> • To develop the method for measuring membrane potential using electro compression of bilayer, which results in change in capacitance of the bilayer and can be correlate to the change in the membrane potential. 	March 2018 - March 2019
Thesis writing and submission	September 2019