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Voltage-Driven Polyelectrolyte Complexation Inside a Nanopore

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**VOLTAGE-DRIVEN POLYELECTROLYTE COMPLEXATION INSIDE A
NANOPORE**

A Dissertation Presented

by

PRABHAT TRIPATHI

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2018

Chemistry

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VOLTAGE-DRIVEN POLYELECTROLYTE COMPLEXATION INSIDE A NANOPORE

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by

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DEDICATION

I dedicate this work to Mrs. Sunita Tripathi, my elder sister.

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ABSTRACT

VOLTAGE-DRIVEN POLYELECTROLYTE COMPLEXATION INSIDE A NANOPORE

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Directed by: Professor Murugappan Muthukumar

This thesis investigates how a pair of oppositely charged macromolecules can be driven by an electric field to form a polyelectrolyte complex inside a nanopore. To observe and isolate an individual complex pair, a model protein nanopore, embedded in artificial phospholipid membrane, allowing compartmentalization (*cis/trans*) is employed. A polyanion in the *cis* and a polycation in the *trans* compartments are subjected to electrophoretic capture by the pore. We find that the measured ionic current across the pore has a distinguishable signature of complex formation, which is different from the signature of the passage of individual molecules through the pore. The ionic current signature allows us to detect the interaction between the two oppositely charged macromolecules and thus, enables us to measure the lifetime of the complex inside the nanopore.

After showing that we can isolate a complex pair in the nanopore, we studied the effects of molecular identity on the nature of interaction in different complex pairs. In contrast to the irreversible conductance state of the alpha-hemolysin (α HL) channel in the complexation of poly-styrene-sulfonate (PSS) and poly-L-lysine (PLL), a reversible

conductance state is observed during complexation between single stranded DNA (ssDNA) and PLL. This suggests that there is a weak interaction between ssDNA and PLL, when compared to the interaction in a PSS–PLL complex.

Analysis of the PSS-PLL complexation events and its lifetime inside the nanopore supports a four step-mechanism: (i) The polyanion is captured by the pore, (ii) the polyanion starts threading through the pore. (iii) The polycation is captured, a complex pair is formed in the pore, and the polyanion slides along the polycation. (iv) The complex pair can be pulled through the pore into the *trans* compartment or it can dissociate. Additionally, we have developed a simple theoretical model, which describes the lifetime of the complex inside the pore.

The observed reversible two-state conductance across α HL channel during ssDNA-PLL complexation, is described as the binding/unbinding of PLL during the translocation of ssDNA. This enables us to evaluate the apparent rate constants for association/dissociation and equilibrium dissociation constants for the interaction of PLL with ssDNA.

This thesis throws light on the behavior of polyelectrolyte complexes in an electric field and enhances our understanding of the electrical aspects of inter-macromolecular interactions, which plays an extremely important role in the organization of macromolecules in the crowded and confined cellular environment.

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CHAPTER 1

INTRODUCTION

1.1 Macromolecular Complexation

If we zoom inside the cells of any living organisms, we are likely to meet large and electrically charged molecules (Figure 1.1 (a)). These molecules are the building blocks of life and are called macromolecules, which include proteins, DNA, and RNA, for example. In the crowded cellular environment these macromolecules do not work in isolation. On the contrary, they usually assemble into larger functional units, consisting of anything from several molecules to a few hundreds or even thousands of individual macromolecules. Many of these macromolecular complexes can be thought of as molecular machines, in the sense that they are modular and, complex, they have moving parts that carry out the same process many times over, and they consume energy¹. For the survival of living organism macromolecules need to perform essential tasks in the cell, such as reading out and translating the genetic code, generating or converting metabolic energy, generating force to enable the cell to move, taking up, synthesizing or secreting metabolites and other macromolecules, and recognizing and reacting to signals from the outside world¹. The success and failure of all these essential tasks relies on the nature and strength of the inter-macromolecular interactions. Ultimately, the sum of all the intermolecular interactions and assemblies of macromolecules defines the uniqueness of a cell, an organism or an individual. Understanding these natural nano-machines and how they work is not only a fundamental and fascinating problem but also one of the most challenging tasks for the future of basic science and medicine. From a conceptual point of view, the challenge for understanding arises from the fact that the whole system is strongly correlated and every

Figure 1.1 (a) A cell is a crowded "Coulomb soup," with charged macromolecules and their assemblies moving between different compartments. Image is adapted from "The world of the cell" (Benjamin/Cummings Series in the Life Sciences). (b) Sketch of the natural polyelectrolyte, RNA. Many factors contribute to the structures, and functions of such polyelectrolytes, including, chain connectivity, charges on the polymer, counterions, salt ions, excluded volume effects, hydrogen bonding, and structure of water².

macromolecule interacts with every other one in the system.

Let us consider an isolated molecule of RNA in an electrolyte solution. A small portion of single-stranded RNA (ssRNA) molecule is sketched in Figure 1.1 (b). The primary confounding forces operative in polyelectrolyte systems are evident even in such a single molecule situation. One of the most important properties of these macromolecules is that they carry electrical charges; The system is endowed with long-ranged electrostatic forces due to charged group along the chain (the phosphate groups for RNA, in conjunction with highly mobile charges due to counterions, and salt ions). Additionally, the chain can interact through hydrogen-bonding, dipolar interactions (*via* water), van der Waals interactions (*via* excluded volume effects), chain connectivity and confinement². While there has already been huge progress made in understanding the behavior of a single polyelectrolyte chain in an electrolyte solution^{2,3}, when multiple chains of different kinds

are present in the system, the challenge increases exponentially. The collective behavior of the above-mentioned interactions is not yet fully understood.

To take a step closer towards understanding the assembly of macromolecules and their dynamics in cellular environments, we must know how two oppositely charged macromolecules (polyelectrolytes) interact with each other to form a complex. In order to build intuition about the energetics of the complexation of two oppositely charged polymers in an electrolyte solution, let us consider the simplest known case; the energetics associated with the formation of a small ion-pair.

1.2 Ion-Pair Formation

Consider the electrostatic interaction energy U_{ij} between a pair of oppositely charged ions with charges $z_i e$ and $-z_j e$ separated by a distance r_{ij} in a medium with uniform dielectric constant ϵ (in the absence of any other charges). This interaction is given by Coulomb's law

$$\frac{U_{ij}}{k_B T} = -z_i z_j \frac{e^2}{4\pi \epsilon_0 \epsilon k_B T} \frac{1}{r_{ij}}, \quad (1)$$

where $k_B T$ is the Boltzmann constant times the absolute temperature, z_i is the valency of the i^{th} ion, e is the electronic charge, and ϵ_0 is the permittivity of the vacuum. The middle factor in the above equation has the dimension of length and is called the Bjerrum length $(l_B)^4$,

$$l_B = \frac{e^2}{4\pi \epsilon_0 \epsilon k_B T}, \quad (2)$$

If the separation distance r_{ij} is comparable to l_B , then the electrostatic interaction energy of two monovalent ions is comparable to the thermal energy $k_B T$, as evident from Eq. 1. Thus, for oppositely charged ions, if the separation is shorter than l_B , the attraction is stronger than the thermal energy ($k_B T$) and the ions are likely to form ion pairs, rather than being completely dissociated ions. The Bjerrum length sets the scale for energy. It is inversely proportional to ϵT , where ϵ is also temperature dependent. For aqueous solutions at 25°C, the Bjerrum length (with $\epsilon = 80$) is $l_B \simeq 0.7$ nm.

1.3 Question of Polyion-Pair Formation (Polycation-Polyanion Complexation)

Now, let us consider the electrostatic interaction energy U_{ij} between a pair of oppositely charged macromolecules (Figure 1.2). The individual behavior of the polycation and polyanion; during pair formation is difficult to imagine, and hence the interaction energy is also difficult to predict. The only thing we can imagine is that if a complex form, then the interaction energy will be greater than the thermal energy $k_B T$. Therefore, we need an experimental measurement to gain more insight about the electrical aspects of the interactions between the polycation and polyanion.

Another important point is that for ion-pair formation as in the case of atomic salts, the assumption that the ions are point charge is reasonable. However, if we look at the chemical structure (Figure 1.2 (b), (c) and (d)) of natural (ssDNA/RNA) and synthetic polystyrenesulfonate (PSS), poly-L-lysine (PLL) polyions, their monomers are not like point charges and the effective dielectric constant in the neighborhood of the chain backbone can be quite different and

Figure 1.2 (a) A cartoon representation for the formation of a polyanion–polycation pair. (b–d) Sketches of the chemical structures of (b) poly-styrene-sulfonate (PSS), (c) single-stranded DNA (ssDNA) and (d) poly-L-lysine (PLL).

therefore, the interaction energy in the formation of poly-ion pair can quite different from the solvent. Therefore, the interaction energy in formation of a poly-ion pair can be different. For example, consider two polycation-polyanion complex pairs: (i) the complex of negatively charged PSS with positively charged PLL (ii) the complex of negatively charged ssDNA with positively charged PLL. In both cases the complex pairs might be considered as electrically identical; - meaning that if one replaces monomers with point charge then one would expect to have same interaction energy in both cases, since the net electric charge present on each monomer is identical. One can also imagine that based on the identity of the macromolecule, that steric effects and the local effective dielectric constant in the complexes are different and therefore the interaction energies in the different complex pairs may also be different.

Since, we are interested in understanding the electrical aspects of macromolecular interactions, it is also reasonable to ask what happens to a complex of a polycation and

polyanion when an external electric field is applied. We discuss the possible behaviors of the polyelectrolyte complex in an electric field in the next section.

1.4 Polyelectrolyte Complex in an Electric Field

Let us consider a already formed, isolated complex of polycation-polyanion in an aqueous electrolytic medium. the polycation would like to go in the direction of electric field, where as polyanion would like to go in the direction of opposite to the electric field

Figure 1.3 A cartoon representation for the behavior of polyanion-polycation complex in an electric field².

-(Figure 1.3). Thus, we can expect three possibilities: (i) there may exist a threshold electric field at which complex may dissociate. (ii) the charges on the chain may be neutralized and thus entire complex may act as a neutral particle; it would not be influenced by electric field. (iii) There may be the formation of a collection of dipoles and the behavior of the complex in electric field will be dictated by the collective response of these dipoles. An experimental observation for the behavior of the polycation–polyanion complex in an electric field may give us valuable insight about the energetics of complex formation. For example, the threshold electric field at which a complex dissociates may give us insight

about the net interaction energy and the effects of the local dielectric constant on the interactions.

That being said, observing the behavior of the polyelectrolyte complex in an electric field is extremely difficult, if we simply mix two aqueous solutions of oppositely charged polymers, it is very difficult to isolate a single polyelectrolyte complex pair as every macromolecule electrostatically interacts with many others. Additionally, to observe all of the electrical aspects of the interactions, we must apply a sufficiently high electric field. In an electrolyte solution, an electric field can be induced by applying an electric potential gradient given by:

$$\vec{E} = -\frac{\partial V}{\partial x} \hat{x}, \quad (3)$$

Where $\hat{x}$ is unit vector in the direction of the region of higher electric potential to the region of lower electric potential. Applying an electric potential gradient in a beaker across long distances is not going to create large enough electric field; we must find a way to apply the potential gradient across shorter distances, so that we can produce an enough electric field that is stronger than the interaction forces in the complex pair.

To correct for both of these issues we must isolate two oppositely charged polymers in close proximity to each other and we must apply the potential gradient across very narrow displacements (nm) such that induced electric field is high enough.

Fortunately, recent developments in nanopore technology have allowed for single-molecule analysis of macromolecules like DNA, RNA, proteins and synthetic polyelectrolytes⁶⁻²⁹. Additionally, several studies have focused on manipulating the dynamics of polyelectrolytes passing through a nanopore³⁰⁻³⁸, which is called

translocation. A number of approaches for single – molecule translocation have been introduced, including use of biological nanopores, like α - hemolysin, aerolysin, MspA^{18,19}, CsgG²⁹ or a solid state nanopores like those made of SiN, graphene, HfO₂^{7,20,21}. These nanopore technologies are widely regarded as “third generation sequencing” instruments that can sequence a mammalian genome for ~\$1,000 in about 24 hours¹². The initial concept is based on the threading of a single polyelectrolyte molecule through a nanopore under an applied electric potential and recording changes in the ionic current across the pore. In the next section, we will formally introduce the basic idea of nanopore and we will try to understand the some of the basic principle of polymer translocation through a nanopore. Finally, we will propose how it can be used to study the behavior of a polyelectrolyte complex in an electric field.

1.5 Polymer Translocation Through a Nanopore

In a nanopore setup, an electric potential gradient is applied across a membrane separating two compartments *cis* (donor) /*trans* (acceptor) (Figure 1.4). Each compartment contains an electrolyte solution, therefore incorporating a small hole or nanopore (typically diameter on the nanoscale) into the membrane allow ions to flow across the compartments and lead to a non-zero ionic current. Due to the presence of an electric potential gradient (on the order of 10mV to hundreds of mV) across the membrane (thickness on the order of 5nm), there is very high electric

Figure 1.4 Representation of the schematic for the polymer translocation through nanopore.

-field (on the order of 10^7 V/m) induced inside the nanopore. When a relatively larger macromolecule enters the pore, it blocks the space inside the pore, and therefore blocks the passage of small ions flowing across the compartments. Since the mobility of the macromolecule is much slower; the observed ionic current will also be lower. When the macromolecule exits the pore either by successful translocation or by returning back to the initial compartment, the ionic current -will return to its initial value- This process allows us to detect single-molecules and study their translocation properties.

The general process of the polymer translocation through nanopore may be divided into several separate parts (Figure 1.5). There are three essential steps associated with the transit of a polymer through a nanopore (i) Drift-diffusion (ii) Capture, and (iii) Translocation³.

1.5.1 Drift-diffusion

In the first step (Figure 1.5 (a,i)), far away from the pore), the polymer undergoes a combination of drift, due to the externally imposed electric field, and diffusion arising from the collisions with solvent molecules. The drift—diffusion behavior of the polymer is established by the structure and size of the polymer, the nature of the background fluid (such as solvent quality and ionic strength), and influences from the external electric field. This will persist until the polymer finds the nanopore opening.

1.5.2 Polymer Capture

Once the entire chain finds the pore mouth, it must rearrange until one end of the chain enters the pore. This is known as capture and is very complex. Some effects at the pore mouth include, force fields generated by chemical decoration of the inside surface of the pore, a steep electric potential gradient may occur due to the dielectric mismatch between the layer in which the pore is embedded and the rest of the system, and strong flow currents may arise at the pore mouth due to the movement of water through the pore. For situations dealing with charge-bearing pores containing electrolyte solutions, this flow force, called the electroosmotic force, can be quite significant. Since, the flux of water flow must be continuous in the system, and since there is only a narrow passage for fluid flow inside the pore, strong velocity gradients may develop at the pore mouth. and facilitates translocation. Additional effects influencing capture include ionic strength, and pressure gradients. Depending on the details of these contributing factors, the nature of the flow field within a range r_c from the pore can be qualitatively different from that outside that range. By experiencing such forces within r_c , the polymer approaches the pore mouth,

designated (Figure 1.5 (a, ii)). The capture of the polymer at the pore mouth is controlled by the strength of the sucking force at the pore entrance, by the range of the flow field in front of the pore where the velocity gradients are strong, and by the typical time scales for conformational fluctuations of the polymer chain (Figure 1.5 (a, iii)).

Figure 1.5 (a) (i) The charged polymer drifts into the capture zone, defined by the radius r_c , and is pulled toward the pore by an applied electric field; (ii) the polymer arrives at the pore with its ends unregistered with the pore entrance; (iii) one end of the strand aligns with the pore entrance and enters the pore; (iv) the strand begins to uncoil as the end moves through the pore; and (v) the strand crosses the pore through a combination of electrophoretic drift and diffusion. Each step corresponds to a different stage on a free-energy landscape (b) The coordinate values 0 and L correspond to the points when the chain's head and tail enter and exit the pore, respectively. The transitions between the various stages are typically associated with a free-energy barrier of $10\text{--}15 k_B T$. Here, ΔF is the net free energy gain by the translocating molecule⁵.

1.5.3 Polymer Translocation

In general, when the polymer is caught at the pore mouth, it is in a jammed state without any initial correlation between the chain ends and the pore mouth. The chain needs to unravel itself to place one of its ends at the pore mouth for the single-file translocation to occur, and then to thread through the pore. This step of translocation consists of three stages: (a) chain-end localization, (b) nucleation, and (c) threading. An entropic barrier must be overcome in placing one of the chain ends at the pore mouth from a jammed coil

state (designated as), to enable the eventual single-file translocation. This is due to the requirement that one end must be at a specific special location, instead of all possible locations, whereby the chain end is losing its translational entropy. After the localization of one chain end, there is an additional entropic barrier. Only if enough monomers crosses this “nucleation barrier” can the chain undergo further translocation. The final stage of the translocation is a downhill threading process, which is a drift-diffusion process. The chain is finally kicked out of the pore into the receiver compartment as the ultimate step.

1.6 Thesis Overview

We can take advantage of our understanding of single polymer translocation, to further understand the behavior of a polyelectrolyte complex in an electric field. We know that we can bring a charged polymer inside a nanopore; that in this region, the electric field is sufficiently high; and that we can monitor the transport process by measuring the ionic current signature. Thus, if we load the *cis* chamber with a polyanion, as in Figure 1.4, and simultaneously load the *trans* compartment with a polycation, we may be able to detect polycation–polyanion complexation and also might be able to measure the lifetime of the complex inside the pore; in a region where electric field is very high. This can give us insight about the behavior of the polyelectrolyte complex in an electric field at the single complex pair resolution. We note that in the case of complexation experiments we denote the *cis/trans* compartments with respect to the polyanion; the *cis* compartment is that in which the polyanion is loaded.

In this thesis, we propose and demonstrate the use of nanopore technology to drive a pair of oppositely charged macromolecules to form a complex. The confinement of nanopore in conjunction with the applied electric field provide the necessary precision to form an isolated complex pair. Based on the ionic current measurements across the pore, we identify a unique signature for the complexation process, which captures the dynamics of the polyelectrolyte complex under non-equilibrium conditions.

Figure 1.6 Idea of polycation-polyanion complexation inside the nanopore. At high voltage complex may dissociate whereas at lower it may get stuck inside the pore.

At the single-pair level of investigation on the behavior of polycation–polyanion complex in an electric field, this thesis throws light on the following issues:

- (1) Distinguishability of the ionic current signature of polycation–polyanion complex inside the pore from individual passages of the polycations and polyanions.
- (2) Measurement of the life-time of the complex inside the pore.
- (3) Behavior of lifetime of the complex with respect to the applied electric field.
- (4) Theoretical description for the life-time of the complex.

- (5) Effect of the macromolecular identity on the behavior of the polyelectrolyte complex.

In Chapter 2, by adding the polyanion PSS in the *cis* compartment, the polycation PLL in the *trans* compartment, and using an α -HL protein nanopore on a phospholipid bilayer, we are going to address the first four issues above. In Chapter 3, we replace the polyanion PSS by ssDNA and repeat the experiments to address the 5th point above. In Chapter 4, we discuss additional topics on translocation of polyelectrolytes that were studied. These include effects of the identity of the salt on the translocation behavior of PSS, interactions of the β -crystallin protein with the α -HL protein nanopore, the effects of macromolecular crowding by creating osmotic stresses by putting high concentration of PEG in the *cis* compartment with the polyanion, and finally the effects of an oscillating electric field. Finally, we conclude in Chapter 5 with a discussion of outlooks and future prospects.

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CHAPTER 2

POLYCATION-POLYANION COMPLEXATION INSIDE A NANOPORE

2.1 Introduction

Survival of living organisms relies on macromolecular assembly and their transport across cellular compartments in an electrolytic medium. For instance, protein import into mitochondria^{1, 2}, bacterial conjugation³, and transport through nuclear pore complexes^{4, 5}. These processes occur repeatedly, and energy is injected into the system by ATP, thus the biological system is driven away from equilibrium. Although nature's biochemical machinery has inspired chemist to develop artificial molecular machines to mimic the form and function of their biological analogs⁶, we still lack the fundamental understanding of the non-equilibrium dynamics that occur in the confined and crowded cellular environment.

One of the simplest components of the biological system is a charged polymer in an electrolyte solution^{7, 8}. There has been huge progress made in understanding the behavior of a single polyelectrolyte chain in an electrolyte solution^{7, 8}. To take a step closer towards an understanding of the assembly of macromolecules and their dynamics in cellular environments, we must know how two oppositely charged polyelectrolytes interact with each other to form a complex pair. Apart from biological importance, polyelectrolyte complexes must be understood to design new materials^{9, 10} for separation science, flocculation, coating, and gene therapy applications^{11, 12}.

If we simply mix two aqueous solutions of oppositely charged polymers, it is very difficult to isolate a polyelectrolyte complex pair as every macromolecule electrostatically interacts

with many others. To simplify this effect and to observe the interaction between only two polyelectrolytes, we must isolate two oppositely charged polymers in close proximity to each other. Fortunately, recent developments in nanopore technology have allowed for single-molecule analysis of macromolecules like DNA, RNA, proteins and synthetic polyelectrolytes¹³⁻³⁶. Additionally, several studies have focused on manipulating the dynamics of polyelectrolytes passing through a nanopore³⁷⁻⁴⁵.

In this chapter, we propose using nanopore technology to drive a pair of oppositely charged macromolecules to form a complex. The confinement of nanopore in conjunction with an applied electric field provide the necessary precision to form an isolated polyelectrolyte complex. Our ionic current measurements across the pore identify a unique signature, which captures the dynamics of a polyelectrolyte complex under non-equilibrium conditions. We identify a four-stage process that include detectable polycation-polyanion complex formation event, revealing a sliding mechanism in complexation which has not been observed in bulk solution.

A schematic of our experimental setup is shown in Figure 1(a). We form phospholipid bilayer which creates two compartments, cis/trans, in our experimental chamber. To create a channel between chambers, we embedded a protein nanopore in the membrane. By loading polyanion in the cis, polycation in the trans chambers, and, by applying a voltage, we bias dynamics such that the oppositely charged polymers may meet inside the pore. This process can be monitored by measuring ionic current across the pore. When the macromolecule undergoes translocation through the pore, the ionic current is reduced to an average value of I_b (occupied pore current) from the open pore current value of I_0 . Therefore, the ratio I_b/I_0 gives the state of pore while a molecule is passing through. For the

Figure 2.1. (a) Concept of voltage driven complexation in an α -HL pore, negatively charged NaPSS (red) on the cis side and positively charged PLL (blue) on trans side are subjected to electrophoretic capture by the same pore. (b) Representative ionic current traces when NaPSS and PLL were both subjected to electrophoretic capture. (c) Corresponding distribution of ionic current blockages (I_b/I_0). Three distinct peaks are observed in the distribution. The first two peaks centered on $I_b/I_0 = 0.45$ and 0.25 are identical with isolated NaPSS translocation events. The observed sharp peak at $I_b/I_0 = 0$ (pore is completely blocked), is attributed to complexation between NaPSS and PLL inside the pore. (d) Expanded view of ionic current levels in complexation between NaPSS and PLL. (e) Schematic of the observed four stages of complexation. All the measurements were carried out in 1M KCl, at 7.5 pH and 30°C.

nanopore we employ the protein staphylococcal α -haemolysin (α -HL) because of its well characterized nature. At its narrowest constriction, α -HL is 1.5 nm in diameter, Sodium

polystyrene sulfonate (NaPSS) was used as a model polyanion and poly-L-lysine (PLL) as a model polycation. NaPSS has been historically studied due to its electric and geometric resemblance to that of ssDNA with charge of $-0.30e$ per monomer in 1M KCl solution and backbone thickness of 0.9 nm ⁸. PLL has thickness of roughly 1 nm along its backbone, therefore only a single polymer can pass through the narrowest constriction (1.5 nm) but not a complex pair. Previous work has established the ability of α -HL pore, to distinguish between successful and unsuccessful translocations of NaPSS^{13, 35}. We take advantage of this capability to illuminate, for the first time, voltage directed complexation of two oppositely charged macromolecules. We performed all the experiments in 1M KCl, at 7.5 pH and at 30°C .

Before studying complex formation, we performed two control experiments; translocation of only NaPSS and translocation of only PLL. In next section we are going to discuss about the study of polyanion (PSS) translocation.

2.2 Polyanion (PSS) Translocation Through the Nanopore

As described in earlier reports^{13, 35, 45}, for PSS there are three different types of events observed during the translocation. At 140 mV , we observed three types of events with two main populations of I_b/I_0 (Figure 2.2). The duration of blockage which corresponds to $I_b/I_0 < 0.35$, is proportional to the molecular weight of NaPSS and inversely proportional to the voltage Figure 2.2 and 2.3). Therefore, only these blockages correspond to successful translocation events.

Figure 2.2. (a) Representative ionic current trace at 140mV, 7.5 pH, 30°C in 1M KCl, when 8 μM NaPSS(16kD) were present on *cis* compartment (b) Close look of different type of events in ionic current trace (c) Histogram of current blockade levels observed and (d) event diagram for NaPSS translocation at same condition. Histogram and event diagram shows two distinct population of observed events, one corresponds to successful ($I_b/I_0 < 0.35$) and other is unsuccessful translocation ($I_b/I_0 > 0.35$). Event type 1 and 121 are due to presence of NaPSS in vestibule.

Figure 2.3. Histogram of duration of (a) Event122 (b) Event121 (c) Event1 for 16kD NaPSS at 140 mV, 7.5pH, 30°C, in 1M KCl. Most probable duration as a function of voltage (d) for Event122 (e) for Event 121 (f) for Event1. Most probable duration as a function of molecular weight of NaPSS at 140 mV (g) for Event122 (h) for Event 121 (i) for Event1. Since, the populations of Event2 were very few they were analyzed together with Event 122. Error bars represent standard deviation from three independent experiments. The durations of events of type 122 and 2 are proportional to molecular weight of NaPSS and inversely proportional to voltage. Therefore, only type122 and type2 of events are successful translocation events.

2.3 Polycation (PLL) Translocation Through the Nanopore.

For PLL, no clear translocation events are observed; only spike-like current blockages (duration less than 0.5 ms) were found corresponding to $I_b/I_0 > 0.40$ (SI: Figure 2.4).

Figure 2.4. (a) Representative ionic current trace at 140mV, 7.5 pH, 30⁰C in 1M KCl, when 23.3 μ M PLL (1.6kD) were present on *trans* compartment (b) close look of events, mostly spike like event observed. (c) Event diagram showing depth of blocked pore current and its duration. (d) Histogram of the dwell time of PLL.

Figure 2.5 Capture rate of NaPSS (16 kD) and PLL (1.6 kD) as a function of their concentration in *cis* and *trans* compartment respectively, at 140 mV, 7.5pH, 30°C, in 1M KCl. Error bars represent standard deviation from three independent experiments. Since, capture rate of NaPSS is 85 times more frequent compare to capture of PLL, therefore the probability that PLL will find an unoccupied pore is least.

2.4 Observation of Complexation Between Polycation and Polyanion Inside a Nanopore

To observe complex pair formation, NaPSS was added to *cis* chamber, PLL was added to *trans* chamber, and a potential of 140mV to 240mV was applied. We observed different types of transient current blockages (Figure 1 (b) and (c)). In addition to the NaPSS events

(i and ii), an event where the pore is nearly completely blocked ($I_b/I_0 \approx 0$) is observed (iii). A representative current trace with such an event is shown in Figure 1(d), with very long blockage durations (in some cases longer than a minute) and four distinct current level indicating four stages (Figure 1(e)). The NaPSS enters the nanopore in vestibule (i) and gets conformationally trapped before reaching to the narrowest constriction (ii), upon entering narrowest constriction (β -barrel) trying to navigate through the pore, while PLL is approaching the pore from trans side (iii) PLL gets captured and makes complex with NaPSS which leads to complete blockage of the pore (i.e. $I_b/I_0 \approx 0$) and depending upon strength of applied voltage NaPSS-PLL complex pair can be pulled through the pore or dissociate. In the following sections, we analyze each of these stages in detail.

2.4.1 Stage i, Polyanion Capture in Vestibule: The first stage is indicated by blockade of the open pore current ($I_0 = 162 \pm 2.2$ pA) to level i (Figure 1(d)), which has exactly same conductance value ($\frac{I_b}{I_0} = 0.45$) as isolated NaPSS in the vestibule of the pore (SI: Figure S (1)).

2.4.2 Stage ii, Polyanion Enters into β -barrel: In next step the current drops further to level ii (Figure 1(d)), which has exactly the same conductance value ($\frac{I_b}{I_0} = 0.25$), as NaPSS threading through narrowest constriction of the pore (SI: Figure S (1)), and into the β -barrel.

2.4.2 Stage iii, Complexation: The pore becomes completely blocked indicated by an ionic current that nearly vanishes ($\frac{I_b}{I_0} \approx 0$, Figure 1(d)). We attribute this to complex formation between NaPSS and PLL molecules inside the nanopore. We rarely observe these

types of events below 120 mV, which corresponds to the voltage threshold for successful NaPSS translocation. We also observe that populations of these completely blocked pore events depend upon molecular weight of NaPSS (SI: Figure S5). These observations suggest that complexation is occurs in the β -barrel. This hypothesis is further supported by the fact that the narrowest constriction of α -HL is too small to allow both NaPSS and PLL to pass through together.

To test this hypothesis more systematically, we denote these completely blocked pore events as complexation events and define a conditional probability as:

$$P_{\text{cmp}} = \frac{\text{Number of complexation events}}{\text{Number of NaPSS succesfull translocation events}} ;$$

In this equation, we assume in every complexation event the NaPSS successfully translocates. We analyze the molecular weight and concentration dependences of this probability.

Figure 2.6. Probability of complexation (P_{cmp}) event as a function of (a) Molecular weight of NaPSS (b) Concentration of NaPSS. (c) Molecular weight of PLL. (d) Concentration of PLL. Error bars represents standard deviation obtained from three independent measurements.

According to our hypothesis, P_{cmp} depends on both the NaPSS residence time inside the β -barrel and PLL capture rate. Therefore, the use of NaPSS with higher molecular weight and enhancement of PLL capture, will lead to a higher P_{cmp} . Figure 2 shows the experimental measurements of P_{cmp} . We found that indeed P_{cmp} increases with the NaPSS molecular weight (Figure 2 (a)) and is independent of NaPSS concentration (Figure 2(b)).

Moreover, P_{cmp} is an increasing function of PLL concentration (Figure 2(d)) and shows weak dependence on PLL molecular weight (Figure 2(c)). Weak dependency of P_{cmp} on PLL molecular weight can be explained by the small changes in capture rates of PLL for the three different chain lengths employed. All the above results clearly validate that these long, completely clogged pore events are due to a complex pair formation between NaPSS and PLL in the β -barrel.

2.5 Measurement of the Life-Time of the Complex Inside a Nanopore

Given a complex pair forms in the pore, one can now ask about its dynamics. To examine the dynamics of the complex pair, we observe the duration of complexation event (τ_{cmp}) as shown in Figure 1(d). We fit the histogram of τ_{cmp} with log-normal (SI: Figure S6) and obtained a time constant $\tau_{\text{cmp peak}}$ as a representative number, which characterizes the complex pair dynamics, the τ_{cmp} is two orders of magnitude longer compare to isolated NaPSS translocation (SI: Figure S6 and S2), suggesting that two oppositely charged molecules are being electrostatically trapped inside the pore. As shown in Figure 3(a), τ_{cmp} decreases exponentially as a function of applied voltage and increases with the molecular weight of NaPSS. These results clearly indicate that during complexation events (Fig. 1(d)), NaPSS must be pulled through the pore into *trans* compartment. This further supports our assumption that in every complexation event NaPSS successfully translocate.

Figure 2.7. Event diagram of current blockade level when $8\mu\text{M}$ of different molecular weight of NaPSS were taken on *cis* side and $23.3\mu\text{M}$ of PLL_{1.6kD} were taken on *trans* side at 140 mV, 7.5 pH, 30°C in 1M KCl. We can see from the event diagrams that, for higher molecular weight of NaPSS there are more population of complexation event. However, when the molecular weight of NaPSS decreases, population of complexation event also decreases. This is because longer NaPSS stay for longer time in the β -barrel and chances of its complexation with PLL increases.

As NaPSS is pulled into the trans chamber, it must slide along the PLL chain (Figure 1(e), iii).

This sliding motion is accompanied by a friction due to electrostatic interactions between NaPSS and PLL. This sliding mechanism can be observed in an increase in τ_{cmp} with increasing molecular weight of PLL (Figure 3(b)). As longer PLL molecule can create more electrostatic frictional force against NaPSS translocation. The value of τ_{cmp} does not depends on the concentration of NaPSS, but increases with concentration of PLL (Figure 3(c) and 3(d)). The former fact aligns with intuition that complexation occurs with a single molecule of NaPSS and PLL in the pore. The increase in τ_{cmp} with PLL concentration can be understood

Figure 2.8. Histogram of log of complexation event duration at different voltages, NaPSS(16kD) and (23.3 μ M) PLL₁₀. Peak position of the distribution was obtained by fitting the histogram with log-normal function.

because capture of PLL occurs more quickly leaving the complex more time to remain in the pore. However, we also observe that the life-time of the complex in the pore behave differently for different molecular weight of PLL (Fig.3 (d)). The exit ring of the β -barrel has 7 negative charges, which can also interact electrostatically with PLL, and can make PLL to be fixed in the pore while NaPSS is sliding.

2.5.1 Stage iv, Complex Pair Translocation or

Dissociation: Based on information that in every complexation event, NaPSS is successfully translocated via sliding along PLL, there can be two possibilities (Fig. 1(e)). One possible case is that the whole NaPSS-PLL complex pair is pulled out of the pore into the *trans* compartment. This is likely the case when the applied voltage is not large enough to break the complex pair apart. When the applied voltage is above a threshold, PLL will dissociate with NaPSS and move into *cis* compartment (as illustrated in Fig. 1(e)).

Figure 2.9. $\tau_{\text{cmp peak}}$ (time constant obtained from fit of the histogram of τ_{cmp} with log-normal), as a function of voltage for (a) different molecular weight of NaPSS, (b) different molecular weight of PLL. Dotted line represents exponential fit (c) Most probable complexation event duration as a function of concentration of NaPSS and (d) concentration of PLL. Error bars represents standard deviation obtained from three independent measurements.

Given, in 90% case of the complexation events, the blocked pore current directly recovers to the open pore current (iii→iv Fig. 1(d)), it is more likely that NaPSS-PLL complex pair translocate to *trans* side. If any complex pair dissociation is occurring in the pore, then one would expect to see at least one current level in between iii and iv (Fig. 1(d)). However, it could also be possible that after the dissociation of the complex pair, the dwell time of the

individual polyelectrolytes in the pore are so small that we cannot measure it in given experimental resolution.

2.6 Fokker-Planck Description for The Lifetime of the Complex Inside the Pore

To describe the strong dependence of life-time of the complex in the pore on voltage, molecular weight of PSS and PLL, we developed a simple analytical model for our system. We follow the theory of reference (46,47 and 48), which describe the free energy of negatively charged polymer translocating through a nanopore. The theory predicts the free energy and translocation time with respect to two parameters:(i) effective charge on individual monomer of the polymer (q) and (ii) effective pore-polymer interaction (ϵ). The presence of PLL inside the β -barrel can be modeled (Fig. 4 (a)) as a pore having two different pore-polymer interactions (ϵ_1 and ϵ_2). This can be further manifested by presence of negatively charged amino acid residues at the end of β -barrel, which can help PLL to bind with the pore. Therefore, creating a net pore having two distinct regions with different pore-polymer interactions. Although, based on ionic current traces observed in experiment, there can be possibility of complex pair translocation, this model assumes that complex pair dissociates. We also assume that the electric potential drops linearly across the pore. The translocation of NaPSS through a nanopore having regions of two different pore-polymer interaction consists of five stages (shown in Fig). We derive the free energy landscape for the translocation process. For an effective charge q on individual translocating monomer, N_1 monomers of NaPSS, N_2 monomers of PLL and M pore length, the free energy landscape for the translocation process with respect to translocation coordinate m is given by equation 1,

Figure 2.10 (a) Theoretical model describing the presence of positively charged PLL inside the β -barrel as a pore having two different regions, interacting differently with translocating negatively charged polymer (NaPSS). (b) Five stages in the translocation of NaPSS through the pore. (c) A representative free energy landscape for the translocation process as a function of translocation co-ordinate m . $\epsilon_1 = 0.25$, $\epsilon_2 = 1.5$, $q = 0.30$, $N_1 = 78$ (33.4kD NaPSS) and $N_2 = 10$ (1.6 kD PLL). (d) Free energy landscape during the ejection stage of the polymer (16kD NaPSS) for PLL_{1.6kD} (Black, $\epsilon_2 = 1.505$), PLL_{3.3kD} (Blue, $\epsilon_2 = 1.550$) and PLL_{4.9kD} (Red, $\epsilon_2 = 1.630$). $\epsilon_1 = 0.05$, $q = 0.30$, $N_1 = 78$, $N_2 = 10$ (e) Free energy landscape during the ejection of the polymer (5.18kD NaPSS) according to the model described in main text ($\epsilon_1 = 0.05$, $\epsilon_2 = 1.115$, $q = 0.30$, $N_1 = 25$, $N_2 = 10$). Energy barrier (∇f) is $5.702 K_B T$. (f) Free energy landscape during the ejection of the polymer (33.4kD NaPSS) according to the model described in main text ($\epsilon_1 = 0.05$, $\epsilon_2 = 1.731$, $q = 0.30$, $N_1 = 162$, $N_2 = 10$). Energy barrier (∇f) is $11.862 K_B T$. (g) Free energy landscape during the ejection of the polymer (126.7kD NaPSS) according to the model described in main text ($\epsilon_1 = 0.05$, $\epsilon_2 = 1.965$, $q = 0.30$, $N_1 = 615$, $N_2 = 10$). Energy barrier (∇f) is $14.202 K_B T$.

$$\frac{F(m)}{k_B T} = \begin{cases} -\epsilon_1 * m - \frac{q * V}{2 * 25.7 * M} * m^2, & 0 \leq m \leq M - N_2 \\ -\epsilon_1 * (M - N_2) - \epsilon_2 * (m - (M - N_2)) - \frac{q * V}{2 * 25.7 * M} * m^2, & M - N_2 \leq m \leq M \\ -\epsilon_1 * (M - N_2) - \epsilon_2 * N_2 - \frac{q * V}{2 * 25.7} * M - \frac{q * V}{25.7} * (m - M), & M \leq m \leq N_1 \\ -\epsilon_1 * (N_1 + M - m - N_2) - \epsilon_2 * N_2 - \frac{q * V}{2 * 25.7 * M} * (M^2 - (m - N_1)^2) - \frac{q * V}{25.7} * (m - M), & N_1 \leq m \leq N_1 + M - N_2 \\ -\epsilon_2 * (N_1 + M - m) - \frac{q * V}{2 * 25.7 * M} * (M^2 - (m - N_1)^2) - \frac{q * V}{25.7} * (m - M), & N_1 + M - N_2 \leq m \leq N_1 + M \end{cases} \quad (1)$$

where k_B is boltzman constant and T is temperature and $k_B T/e$ is taken as 25.7 mV at room temperature and e is charge of an electron. For such strong electric fields (20-50 mV/nm) in our experiments, the contribution from conformational entropy of the polymer outside the pore is negligible and thus is ignored in the above equation. For $q=0.30$ (Based on the manning argument), $\epsilon_1=0.25$ and $\epsilon_2=1.5$ a typical landscape is given in Fig. 4(c), where $V = 140\text{mV}$, $M = 15$ (corresponding to the length of β -barrel 5 nm), $N_2 = 10$ (PLL 1.6kD) and $N_1 = 78$ (corresponding to 16kD NaPSS). The role of the attractive electrostatic interaction between the NaPSS and PLL is manifested as an attraction between NaPSS and pore, which lead to a barrier (about $15k_B T$) in the last stage of the translocation.

We calculated the τ_{cmp} using free energy of last three stages ($m>M$). We employ the theoretical machinery most suited to connecting the free-energy landscape and the time evolution of the probability of realizing a state $W_m(t)$ is the Fokker-Planck formalism. In general, the Fokker- Planck equation for the situation presented here is given by equation 2 as,

$$\frac{\partial W_m(t)}{\partial t} = D \frac{\partial}{\partial m} \left[\frac{\partial \left(\frac{F(m)}{k_B T} \right)}{\partial m} W_m(t) + \frac{\partial W_m(t)}{\partial m} \right] \quad (2)$$

where D is the monomer diffusion coefficient related to local friction inside the pore (46,47,48). Substitution of Eq. 1 into Eq. 2 gives the time dependence of the probability of realizing a value of m in the reaction coordinate. Using standard procedures in the Fokker-Planck formalism, the average time, τ_{cmp} , for crossing state of M to N_1+M is given by equation

$$\tau_{cmp} = \frac{1}{D} \int_M^{N_1+M} dm \int_M^m dm' e^{\left[\frac{F(m)}{k_B T} - \frac{F(m')}{k_B T}\right]} \quad - (3)$$

where $F(m)$ is given by Eq. 1. In defining τ_{cmp} , we used the reflecting boundary condition at $m=M$ and the absorbing boundary condition at $m=N_1+M$, which are evident from our experimental observation as, the polyanion very rarely retracts back into the donor compartment after some monomers are threaded through the pore.

Our model describes the voltage response of the life-time of the complexation events for all molecular weight of NaPSS and PLL employed with ϵ_2 as an adjustable parameter (Fig. 4(d), 4(e) and SI: S7-S14). Increasing the molecular weight of NaPSS and PLL can be modeled as an increase in the net electrostatic attraction with the pore (ϵ_2). An increase in molecular weight of NaPSS and PLL may lead to an increase in ϵ_2 and therefore increase in the energy barrier in the landscape during the ejection of negatively charged polymer. A list of ϵ_2 and energy barrier during the ejection are given in Table 1 obtained from the best-fit for the τ_{cmp} , calculated from the model and observed in the experiments.

Figure 2.11. Experimentally observed and calculated values of τ_{cmp} are plotted together. (a) The black dot with error bars represents experimental data for the 5.18 kD NaPSS and 1.6kD PLL. The orange dash curve represents fit from the model described in main text ($\epsilon_1=0.05$, $\epsilon_2=1.115$, $q=0.30$, $N_1=25$, $N_2=10$). (b) The black dot with error bars represents experimental data for the 16 kD NaPSS and 1.6kD PLL. The orange dash curve represents fit from the model described in main text ($\epsilon_1=0.05$, $\epsilon_2=1.505$, $q=0.30$, $N_1=78$, $N_2=10$). (c) The black dot with error bars represents experimental data for the 33.4 kD NaPSS and 1.6kD PLL. The orange dash curve represents fit from the model described in main text ($\epsilon_1=0.05$, $\epsilon_2=1.731$, $q=0.30$, $N_1=162$, $N_2=10$). (d) The black dot with error bars represents experimental data for the 126.7 kD NaPSS and 1.6kD PLL. The orange dash curve represents fit from the model described in main text ($\epsilon_1=0.05$, $\epsilon_2=1.965$, $q=0.30$, $N_1=615$, $N_2=10$). (e) The black dot with error bars represents experimental data for the 16 kD NaPSS and 3.3 kD PLL. The orange dash curve represents fit from the model described in main text ($\epsilon_1=0.05$, $\epsilon_2=1.550$, $q=0.30$, $N_1=78$, $N_2=10$). (f) The black dot with error bars represents experimental data for the 16 kD NaPSS and 4.9 kD PLL. The orange dash curve represents fit from the model described in main text ($\epsilon_1=0.05$, $\epsilon_2=1.630$, $q=0.30$, $N_1=78$, $N_2=10$).

N_{PLL}	N_{PSS}	$\varepsilon_2(k_B T)$	$\Delta f(k_B T)$
10	25	1.115	5.702
10	78	1.505	9.602
10	162	1.731	11.862
10	615	1.965	14.202
20	78	1.550	10.052
30	78	1.630	10.852

Table 2.1. Values of interaction energy parameters ε_2 and free energy barrier Δf , obtained as a result of best fit of calculated τ_{cmp} from the model and experimentally observed τ_{cmp} .

2.6.1 Another Model

To disentangle between complex pair translocation and complex pair dissociation, the dynamics of complexation in a nanopore is described as: Sliding of polyanion along polycation through the nanopore (I→II), peeling off the polyanion from the pore till the number of monomers of polycation and polyanion in the pore are same (II→III), and then either translocation of complex pair (III→IV (1)) or dissociation (III→IV (2)) as illustrated in Figure 1. We assumed that the time taken for filling the pore during complexation is small and does not contribute to τ_{cmp} . We follow the theory of Muthukumar (1,2,3) and derive the free energy landscape for the process by considering the effective attractive interaction energy between the pore-polyanion ($\epsilon_1/\text{monomer}$), pore-polycation ($\epsilon_2/\text{monomer}$), polycation-polyanion (ϵ_{12}) and the drift force on each polyelectrolyte from the externally imposed electric field recognizing that when charged monomers are transferred from the donor to the receiver compartment, there is a net

Figure 2.12 Theoretical model describing the presence of positively charged PLL inside the β -barrel as a pore having two different regions, interacting differently with translocating negatively charged polymer (NaPSS). Theoretical model describing dynamics of complex pair in the nanopore.

electrochemical potential gain. In present study, we assumed that the number of monomer of polycation (N_2) is smaller compare to maximum number of monomer that can be accommodated inside the pore (M). We also assumed that the voltage drop across the pore is linear. The value of the effective charge/monomer for each polyelectrolyte in the cis/trans compartment can in principle be quite different from the value inside the pore. Let the $-q_{11}$, q_{21} be the effective charge/monomer on polyanion, polycation inside the pore and $-q_{12}$, q_{22} is outside the pore in units of electronic charge. The free energy landscape for complex pair dynamics for N_1 monomer of polyanion through the pore as a function of coordinate (m) is given by

$$\frac{F(m)}{k_B T} = \begin{cases} -\epsilon_1 M - \epsilon_2 N_2 - \epsilon_{12} - \frac{q_{11}V}{51.4}M + \frac{q_{21}V}{51.4M}(M^2 - (M - N_2)^2) - \frac{q_{12}V}{25.7}(m - M), & \text{if } \mathbf{M} \leq \mathbf{m} \leq \mathbf{N}_1 \\ -\epsilon_1(N_1 + M - m) - \epsilon_2 N_2 - \epsilon_{12} - \frac{q_{11}V}{51.4M}(M^2 - (m - N_1)^2) + \frac{q_{21}V}{51.4M}(M^2 - (M - N_2)^2) - \frac{q_{12}V}{25.7}(m - M), & \text{if } \mathbf{N}_1 \leq \mathbf{m} \leq \mathbf{N}_1 + \mathbf{M} - \mathbf{N}_2 \\ -\epsilon_1(N_1 + M - m) - \epsilon_2(N_1 + M - m) - \epsilon_{12} - \frac{q_{11}V}{51.4M}(M^2 - (m - N_1)^2) + \frac{q_{21}V}{51.4M}(M^2 - (m - N_1)^2) - \frac{q_{12}V}{25.7}(m - M) \\ \quad + \frac{q_{22}V}{25.7}(N_2 - (N_1 + M - m)), & \text{if } \mathbf{N}_1 + \mathbf{M} - \mathbf{N}_2 \leq \mathbf{m} \leq \mathbf{N}_1 + \mathbf{M} \text{ and for IV(1)} \\ -\epsilon_1(N_1 + M - m) - \epsilon_2 N_2 - \epsilon_{12} \left(\frac{N_1 + M - m}{N_2} \right) - \frac{q_{11}V}{51.4M}(M^2 - (m - N_1)^2) + \frac{q_{21}V}{51.4M}(M^2 - (M - N_2)^2) \\ \quad - \frac{q_{12}V}{25.7}(m - M), & \text{if } \mathbf{N}_1 + \mathbf{M} - \mathbf{N}_2 \leq \mathbf{m} \leq \mathbf{N}_1 + \mathbf{M}, \text{ and for IV(2)} \end{cases} \quad -(4)$$

Where k_B is boltzman constant and T is temperature and $k_B T/e$ is taken as 25.7 mV at room temperature and e is charge of an electron. For such strong electric fields (20-50 mV/nm) in our experiments, the contribution from conformational entropy of the polymer outside the pore is negligible and thus is ignored in the above equation. A typical landscape is given in Figure 2, where $V = 140\text{mV}$, $M = 15$ (corresponding to the length of β -barrel 5 nm), $N_2 = 10$ (PLL 1.6kD) and $N_1 = 78$ (corresponding to 16kD NaPSS).

The role of the attractive interactions between the pore-polycation can be seen as a barrier of $2.193 k_B T$ (Figure 3) during the stage of ejection of the complex pair from the pore (IV (1)). Whereas polyanion-polycation interaction determine the barrier of $8 k_B T$ in dissociation of complex pair (IV (2)). Furthermore, at 140mV, the barrier of complex pair dissociation is much larger compare to the barrier of ejection of complex pair from the pore. Which suggest that at this voltage complex pair is pulled through the pore instead of dissociating. However, as we increase

Figure 2.13 (a) Calculated free energy landscape for the dynamics of complex pair in the pore. (b) Free energy landscape for last stage for both path IV (1) and IV (2) at 140 mV (c) at 180mV (d) at 220mV. As from figure we can see that barrier is much higher for dissociation (IV (2)) to occur than complex pair translocation (IV (1)). The calculation was performed for the parameter given in Table 2.2.

-the voltage the barrier of dissociation decreases (Figure 4 and 5) and therefore it suggests that after some threshold voltage (around 200mV) dissociation of complex pair and translocation of pair both are equally probable. We find an excellent match with predicted τ_{cmp} with experimental data for free energy of IV (1) (Figure 6). However, for higher voltage we also find nice match with predicted time calculated from the free energy of IV (2), as it is evident from the free energy calculation that at higher voltages barrier for dissociation decreases, therefore complex pair can find dissociation as another possible path. Which suggest that, there may exist a threshold voltage (around 200 mV) at which complex start dissociating.

Figure 2.14 Fit of experimental τ_{cmp} with calculated value of τ_{cmp} using Fokker-Planck formalism for free energy of the model. At lower voltages (below 200mV) we find excellent match of our calculation with experimental data for path IV (1). After 200 mV, good agreement with path IV (2) is also observed. Which indicates that there exists a threshold voltage at which complex starts dissociating. The calculation was performed for the parameter given in Table 2.2

N_2	M	q_{11}	q_{21}	q_{12}	q_{22}	$\epsilon_1(k_B T)$	$\epsilon_2(k_B T)$	$\epsilon_{12}(k_B T)$	$1/D(\text{ms})$
10	15	0.365	0.888	0.888	0.888	0.5	1.362	38.294	1.075

Table 2.2. Values of parameters used in calculation of free energy and τ_{cmp} . Note that value of N_2 and M chosen for chain length of PLL (1.6 kD) and length of β -barrel. Whereas q_{11} , q_{21} , q_{12} , q_{22} , ϵ_1 , ϵ_2 , ϵ_{12} and $1/D$ are generated from best-fit algorithm based on the predicted τ_{cmp} from our model and experimentally observed τ_{cmp} for $N_1=78$ (16kD NaPSS).

2.7 Statistical Model for the Lifetime of the Complex Based on Double-Exponential Distribution Functions

Although the time constant in the log-normal fits of the τ_{cmp} histograms provide the qualitative behavior of the complex lifetime, it is insufficient for understanding the underlying physical processes and thus making quantitative predictions. A physical basis for the choice of log-normal probability density function for fitting is also absent. Therefore, in this section, to enhance our understanding, we will improve our analysis procedure and develop a statistical model for the complex dwell time based on a physical chemistry principle.

Given that the complex is already inside the pore at time t , the probability of not finding the complex inside the pore in a time interval t to $t+\Delta t$, is proportional to Δt at first order. Therefore, the probability of finding the complex inside the pore in a time interval t to $t+\Delta t$ is:

$$p(t \rightarrow t + \Delta t) = 1 - \alpha \Delta t; \quad (5)$$

where, α is a constant independent of time.

Let the probability density of finding a complex with dwell time t be $p(t)$. The probability density $p(t+\Delta t)$ of the complex having dwell time $t+\Delta t$ will be proportional to:

- (i) The probability density $p(t)$ of finding the complex with dwell time t .
- (ii) The probability $p(t \rightarrow t + \Delta t)$ of finding the complex inside the pore in the following time interval, t to $t+\Delta t$.

The above two events are mutually independent, therefore:

$$p(t + \Delta t) = p(t)(1 - \alpha \Delta t); \quad (6)$$

$$p(t + \Delta t) - p(t) = -\alpha \Delta t p(t); \quad (7)$$

$$\frac{p(t + \Delta t) - p(t)}{\Delta t} = -\alpha p(t); \quad (8)$$

By definition of the derivative, in the limit of $\Delta t \rightarrow 0$, the above equation becomes:

$$\frac{d p(t)}{dt} = -\alpha p(t); \quad (9)$$

and by integration we obtain:

$$p(t) = a e^{-\alpha t}; \quad (10)$$

The quantity α has a unit of inverse of time can be written as $1/\tau$, where τ is characteristic time constant for the complex life time distribution. The constant a is determined by normalization to be $a = \alpha$.

However, the histogram of complex life-time gives a poor fit with single exponential probability density function (Figure 2.15 (a)). Therefore, we implement double exponential forms and we describe probability density function for the complex life-time having two-time constants, given by

$$f(t) = \frac{a_1}{\tau_1} e^{-\frac{t}{\tau_1}} + \frac{a_2}{\tau_2} e^{-\frac{t}{\tau_2}}; \quad (11)$$

We find perfect match of the complex life-time histogram with double exponential forms (Figure 2.15 (b)); this is indicative of two underlying physical processes, occurring with time constant τ_1 and other with time constant τ_2 .

Figure 2.15. (a) Histogram of life-time of the complex measured at 140mV for 16kD PSS and 1.6kD PLL. Black solid line represents the fit with single exponential function. (b) Same histogram as mentioned in a but here black solid line represents fit with the double-exponential probability density function. Both histograms are generated for 3079 events.

To assign the observed time constants τ_1 and τ_2 , we generated histograms with respect to applied voltage across the membrane and molecular weight of NaPSS. As mentioned previously, there can be possibility of either complex pair translocation or complex dissociation. In this section by analyzing the histograms based on double exponential probability density function with respect to molecular weight of NaPSS and voltage, we will assign the time constants τ_1 and τ_2 to either of the complex translocation or dissociation (Figure 2.16). The histograms and its fit with the double exponential probability density function for different molecular weight of PSS/PLL, [PLL], and Voltages are given in appendices (Figure 2.21, Figure 2.22, and Figure 2.23). We find that in almost at every condition of molecular weight of PSS, PLL, [PLL], and voltage, the obtained τ_1 is one order of magnitude smaller compare to τ_2 .

Figure 2.16. An explanation for observed two-time constants τ_1 and τ_2 , from fitting of the histogram of life-time of the complex with double exponential probability density function. The faster time constant τ_1 may be due to complex pair translocation and slower time constant τ_2 may be due to the complex pair dissociation.

One can imagine that during complex pair dissociation life time of the complex inside the pore will be higher compare to the case of complex pair translocation, as it will require to cross the barrier for the dissociation. Suggesting that τ_1 may be life-time of the complex during complex pair translocation, whereas τ_2 is the life-time of the complex during complex pair dissociation. The time constant τ_1 and τ_2 both increases with the molecular weight of NaPSS. Suggesting that in both cases either it is complex translocation or dissociation, involves sliding of PSS along PLL, this sliding step contributes significantly to the life-time of the complex inside the pore. Upon increasing the molecular weight of NaPSS, the sliding time increases. Interestingly, both time constant τ_1 and τ_2 roughly scale square of molecular weight of PSS (Figure 2.17 (a) and (b)). The quadratic scaling of translocation time in diffusion limited region have been theoretically addressed⁸. The time constant τ_1 depends weakly upon applied voltage. Suggesting that the mechanism with characteristic complex dwell time τ_1 may be due to complex pair translocation.

Because, τ_1 does not affected by electric field induced due to applied voltage, or in other words suggesting that the complex acting like neutral particle and diffuses out of the pore. The time constant τ_2 decreases with the applied voltage, suggesting that upon increasing the voltage it is easier to cross the barrier for the dissociation. The time constant τ_1 does not depend upon [PLL], however the time constant τ_2 increases with [PLL]. This may be because in the time constant τ_1 there is only 1:1 complexation of PSS and PLL, whereas in case of complex with time constant τ_2 there may be cooperativity in binding of PLL with single PSS or in other words there may be two or more PLL molecules involved in complex with the single PSS. The fraction of events corresponds to the probability density function with time constant τ_1 is given by $a_1/(a_1+a_2)$. The fraction of event with time constant τ_1 is plotted as a function of voltage for different molecular weight of PSS and PLL in Figure 2.18.

Our, interpretation is based on the idea that the slower time constant occur due to the dissociation of complex pair, which may involve a free energy barrier as discussed in section 2.6. To interpret our hypothesis of time constant τ_2 may be due to the complex dissociation, we describe the behavior of τ_2 with respect to voltage, as the model we described in section 2.6. That is, we use Fokker-Planck formalism to fit the experimentally observed τ_2 . We will find the parameters in the free energy expression given in section 2.6. The fit for the model and τ_2 are given for different molecular weight of PSS and PLL (Figure 2.19). There is discrepancy at higher voltage for higher molecular weight of PSS, and we observe the deviation from the crude theoretical model. The question can be raised if slower time constant τ_2 is due to the dissociation of complex pair, then why τ_1 is complex translocation. In other words, why, the time taken for complex pair translocation should be

faster compare to the dissociation time scale. This may be thought as, for the complex pair translocation it must diffuse out of the pore in trans compartment and time required for this diffusion should be smaller compare to dissociation as there is less barrier for the complex pair translocation via diffusion. The fraction of event with faster time constant τ_1 is approximately 33% at 140mV for 16kD molecular weight of NaPSS and 1.6kD PLL and 67 % are for slower time constant.

We observed slight decrease in the fraction of events with faster time constant τ_1 for the 126.7 kD molecular weight of NaPSS. At 140mV we observed 24 % fraction of events with faster time constant for 126.7kD PSS. However, the fraction of events with faster time constant is 48% and 34 % for 5.18kD PSS and 33.4 kD PSS respectively. We observed that the fraction of events also depends weekly on [PLL]. At 140mV the fractions of events with faster time constant are 46.16 %, 41.31 %, 32.7 % and 40.98 % respectively.

Figure 2.17. Effect of molecular weight of NaPSS on time constant (a) τ_1 for 23.3 μ M 1.6kD PLL (b) τ_2 23.3 μ M PLL 1.6kD. Effect of voltage on time constant (c) τ_1 and (d) τ_2 for different molecular weight of PLL (23.3 μ M) at a fixed molecular weight of PSS (16kD). (e) τ_1 with voltage for different molecular weight of PLL. (f) τ_2 with voltage for different molecular weight of PLL. (g) Dependence of concentration of PLL on time constant for a fixed molecular weight of PSS (16kD) and PLL (1.6kD).

Figure 2.18 Dependence of fraction of event with time constant τ_1 on voltage, for all molecular weight of PSS and PLL (23.3 μM).

Figure 2.19 Experimentally observed and calculated values of τ_2 are plotted together. (a) The black dot with error bars represents experimental data for the 5.18 kD NaPSS and 1.6 kD PLL (23.3 μM). The orange dash curve represents fit from the model described in section 2.6 ($\varepsilon_1 = 0.05$, $\varepsilon_2 = 0.15$, $q = 0.30$, $N_1 = 25$, $N_2 = 10$). (b) 16 kD NaPSS and 1.6 kD PLL (23.3 μM). ($\varepsilon_1 = 0.05$, $\varepsilon_2 = 0.775$, $q = 0.30$, $N_1 = 78$, $N_2 = 10$). (c) 16 kD NaPSS and 3.3 kD PLL (23.3 μM). ($\varepsilon_1 = 0.05$, $\varepsilon_2 = 0.973$, $q = 0.30$, $N_1 = 78$, $N_2 = 10$). (d) 16 kD NaPSS and 4.9 kD PLL (23.3 μM). ($\varepsilon_1 = 0.05$, $\varepsilon_2 = 1.08$, $q = 0.30$, $N_1 = 78$, $N_2 = 10$). (e) 33.4 kD NaPSS and 1.6 kD PLL (23.3 μM). ($\varepsilon_1 = 0.05$, $\varepsilon_2 = 1.160$, $q = 0.30$, $N_1 = 162$, $N_2 = 10$). (f) 126.7 kD NaPSS and 1.6 kD PLL (23.3 μM). ($\varepsilon_1 = 0.05$, $\varepsilon_2 = 1.41$, $q = 0.30$, $N_1 = 615$, $N_2 = 10$).

N_{PLL}	N_{PSS}	$\varepsilon_2(k_B T)$	$\Delta f(k_B T)$
10	25	0.15	0.103
10	78	0.775	2.256
10	162	1.160	6.152
10	615	1.41	8.65
20	78	0.973	4.34
30	78	1.08	5.352

Table 2.3. Values of interaction energy parameters ε_2 and free energy barrier Δf , obtained as a result of best fit of calculated τ_2 from the model described in section 2.6 and experimentally observed τ_2 .

N_{PLL}	N_{PSS}	$\varepsilon_2(k_B T)$	$\Delta f(k_B T)$
10	25	0.01	0.009
10	78	0.11	0.05
10	162	1.0	4.58
10	615	1.25	7.05
20	78	0.78	2.79
30	78	0.85	3.31

Table 2.4. Values of interaction energy parameters ε_2 and free energy barrier Δf , obtained as a result of best fit of calculated τ_1 from the model described in section 2.6 and experimentally observed τ_1 .

Figure 2.20 Experimentally observed and calculated values of τ_1 are plotted together. (a) The black dot with error bars represents experimental data for the 5.18 kD NaPSS and 1.6 kD PLL (23.3 μ M). The orange dash curve represents fit from the model described in section 2.6 ($\epsilon_1 = 0.05$, $\epsilon_2 = 0.01$, $q = 0.30$, $N_1 = 25$, $N_2 = 10$). (b) 16 kD NaPSS and 1.6 kD PLL (23.3 μ M). ($\epsilon_1 = 0.05$, $\epsilon_2 = 0.11$, $q = 0.30$, $N_1 = 78$, $N_2 = 10$). (c) 16 kD NaPSS and 3.3 kD PLL (23.3 μ M). ($\epsilon_1 = 0.05$, $\epsilon_2 = 0.78$, $q = 0.30$, $N_1 = 78$, $N_2 = 10$). (d) 16 kD NaPSS and 4.9 kD PLL (23.3 μ M). ($\epsilon_1 = 0.05$, $\epsilon_2 = 0.85$, $q = 0.30$, $N_1 = 78$, $N_2 = 10$). (e) 33.4 kD NaPSS and 1.6 kD PLL (23.3 μ M). ($\epsilon_1 = 0.05$, $\epsilon_2 = 1.0$, $q = 0.30$, $N_1 = 162$, $N_2 = 10$). (f) 126.7 kD NaPSS and 1.6 kD PLL (23.3 μ M). ($\epsilon_1 = 0.05$, $\epsilon_2 = 1.25$, $q = 0.30$, $N_1 = 615$, $N_2 = 10$).

2.8 Summary and Conclusions

We have demonstrated that how oppositely charged macromolecules can be driven by electric field to form a polyelectrolyte complex inside a nanopore. Our ionic current measurements across the pore identify a unique signature of complex formation, which captures the dynamics of a complex pair in non-equilibrium condition. In total, we observe four stages in the complexation process, and we have interpreted a macromolecular description for each. We find that the histogram of life-time complex obeys double-exponential probability density function, indicative of occurrence of two different processes. Our analysis of complexation events suggests that, after complexation in the pore, the NaPSS slides along PLL against a frictional force due to electrostatic attraction. Sliding phenomenon of SSB proteins over ssDNA has been recently studied^{49, 50}, where protein sliding over DNA is described by reptation mechanism. The two-time constants obtained from the fitting of the histogram with the double exponential probability density function, tend to increase with molecular weight of PSS with $\tau \sim MW_{\text{pss}}^2$. Detection of signature and a theoretical description for complex dissociation are future scope of this work. Our simple crude theoretical model describes the life-time of the complex inside the pore and indicates that small changes in interaction between NaPSS and PLL can account for substantial increase in life-time.

2.9 Materials and Methods

Formation of perpendicular bilayer: We used 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DphPC, purchased from Avanti Polar Lipid.) to form perpendicular phospholipid bilayer across an aperture (diameter, 50 μm) in a Teflon film separating the

two compartments (1 ml each cis/trans) of the polytetrafluoroethylene (PTFE) flow cell apparatus (purchased from Nanopore Solutions, Portugal).

Buffer solution: 1M potassium chloride (KCl) solutions in 10 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES) in deionized water (18 MΩcm) were prepared for measurements. The pH of the buffer solution was adjusted to 7.5 by adding potassium hydroxide (KOH) or hydrochloric acid (HCl).

αHL pore: Wild-type αHL monomers, α-toxin from staphylococcus aureus were purchased from EMD Millipore (MA). A stock solution of αHL were prepared by adding 50:50 (v/v) of water and glycerol mixture to make final concentration of 0.5 mg/ml. This resulting solution was further diluted to by 1M KCl buffer solution to make final concentration of 0.5 μg/ml. A portion of the resulting αHL protein solution (2 μl) was added to the cis compartment of a bilayer apparatus to form a single-channel. Once the single pore is formed, the cis chambers is flushed with 1M KCl solution to avoid the formation of multiple channels.

NaPSS and PLL: The polyanion used in this work, sodium polystyrene sulfonate (NaPSS) was purchased from Scientific Polymer Products NY, with weight-averaged molecular weights M_w = 1.5, 5.18, 16, 33.4, 126.7, and 262.8 kD. The polydispersity indices are 1.12, 1.13, 1.13, 1.17, 1.17 and 1.20, respectively. The polycation used, poly-L-lysine (PLL) was purchased from Alamanda polymers with average molecular weights of 1.6 (PLL10), 3.3 (PLL20), and 4.9 kD (PLL30). After the insertion of a single αHL pore from the cis compartment, the solutions of cis/trans chambers were replaced with the KCl buffer solutions containing desired concentration of NaPSS and PLL respectively.

Electrical measurements and data analysis: Ionic currents were measured by using Ag/AgCl electrodes with a patch-clamp amplifier (Axopatch 200B, Axon Instruments). The amplified signal was low-pass-filtered at 5 kHz (or 10 kHz) and sampled at 250 kHz with a Digidata 1550 digitizer (Axon Instruments). The recorded ionic current traces are analyzed using MATLAB (The MathWorks, Inc., MA) to capture the current blockage events and their durations.

Analytical Model: The model described was formulated and solved using Mathematica. The mathematical expression for τ_{cmp} and τ_2 was generated and then used to find the best fit to the entire data (τ_{cmp} and τ_2 vs voltage) set in the Mathematica software.

2.10 Appendices

Figure 2.21. Histogram of the life-time of the complex for 16kD PSS and 1.6kD PLL 23.3 μ M at (a) 140mV for 3079 events (b) 160mV for 2104 events (c) 180mV for 4955 events (d) 200mV for 2413 events (e) 220mV for 3144 events (f) 240mV for 7036 events, At 140mV different concentration of PLL (g) 1.5 μ M PLL 1809 events (h) 7.5 μ M PLL for 1170 events (i) 50 μ M PLL for 1251 events, and for different molecular weight of NaPSS for (j) 5.18kD PSS at 140mV for 3416 events (k) 33.4 kD PSS 140mV 1065 events (l) 126.7 kD PSS at 140mV for 466 events. The black solid curve represents fit of the histogram with double exponential probability density.

Figure 2.22. Histogram of the life-time of the complex for 16kD PSS and 3.3kD PLL (23.3 μ M) at (a) 140mV for 1195 events (b) 160mV for 1208 events (c) 180mV for 1116 events (d) 200mV for 1200 events, and for 4.9kD PLL (23.3 μ M) at (e) 140mV for 920 events (f) 160mV for 801 events (g) 180mV for 1001 events (h) 200mV for 1026 events. The black solid curve represents fit of the histogram with double exponential probability density function.

Figure 2.23. Histogram of the life-time of the complex for 5.18kD PSS and 1.6kD PLL (23.3 μ M) at (a) 160mV for 2783 events (b) 180mV for 2242 events (c) 200mV for 1185 events (d) 220mV for 1468 events (e) 240mV for 1153 events. For 33.4kD NaPSS and 1.6kD PLL (23.3 μ M) (f) 160mV for 579 events. (g) 180mV for 613 events (h) 200mV for

764 events **(i)** 220mV for 1045 events **(j)** 240mV for 316 events. For 126.7kD NaPSS and 1.6kD PLL (23.3 μ M) **(k)** 160mV for 266 events **(l)** 180mV for 386 events **(m)** 200mV for 402 events **(n)** 220mV for 177 events.

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CHAPTER 3

IDENTIFICATION OF INTERACTIONS IN TWO DIFFERENT POLYCATION-POLYANION COMPLEXES VIA SINGLE NANOPORE.

3.1 Introduction

To understand the behavior of a polyelectrolyte complex in an electric field, in chapter 2 we have shown that two isolated oppositely charged macromolecules can be driven by an electric field to form a polyelectrolyte complex inside a nanopore. The lifetime of the complex inside the nanopore has enabled us to gain insight about the dynamics of complex pair in an electric field.

In this chapter, we use this new approach of voltage-driven polyelectrolyte complexation to determine whether and how intermolecular interactions in a complex pair of negatively charged ssDNA with positively charged poly-L-lysine (PLL), differ from that of interactions in complex pair of negatively charged polystyrene sulfonate (PSS) with positively charged PLL. Although, PSS has been celebrated as a model system to understand the behavior of negatively charged bio-polymers in electrolytic solutions due to its geometric and electrical resemblance to ssDNA¹⁻⁶, the location of the charge on the DNA backbone and associated local dielectric constant may interfere, (as discussed in chapter 1, section 1.3) making the interactions of these two negatively charged polymer (ssDNA and PLL) with positively charged polymer different.

A schematic of our experimental setup is shown in Figure 1(a) with a sketch of the chemical structure of PSS, PLL and ssDNA respectively. We form a phospholipid bilayer which creates two compartments, *cis/trans*, in our experimental chamber. To create a channel between the chambers, we embed a protein nanopore α -hemolysin (α -HL) in the membrane. By loading polyanion (PSS or ssDNA) in the *cis*, polycation (PLL) in the *trans*

Figure 3.1. (a) Experimental setup: A general method for observing interaction between an isolated polyanion (red) and a polycation (blue), sketch of the chemical structures of two different polyanion employed: poly-styrene-sulfonate and ssDNA, and polycation employed (poly-lysine) in this study. (b) The ionic current signature obtained from the interaction in PSS-PLL complex and in ssDNA-PLL complex. These two signatures demonstrate that regardless of the electrical and geometric similarity, interactions in two different macromolecular pairs are different. The ionic current signature is obtained at 120mV in 1MKCl and at 7.5pH.

chambers, and, by applying a voltage, we bias the dynamics such that the oppositely charged polymers may meet inside the pore. This process can be monitored by measuring the ionic current across the pore.

Previously we have established the ability of α HL pore, to detect the signature of complexation between PSS and PLL (Chapter 2). We take advantage of this capability to differentiate the signature of the interaction in ssDNA-PLL with its synthetic analog PSS-PLL. We performed all experiments in 1M KCl, at 7.5 pH and at the room temperature of

20⁰C. In contrast to the complexation of PSS-PLL in the pore, for the complexation of ssDNA-PLL, we observed a unique type of event where ionic current undergoes a reversible transition between the two states (Figure 1(b)). Suggesting, the effect of local dielectric heterogeneity due to the specificity of the macromolecules and there is weaker interaction in ssDNA-PLL complex pair compared to the PSS-PLL complex. In the rest of the chapter by quickly reviewing the translocation of only ssDNA and PLL, we will analyze the reversible conductance state due to the complexation of ssDNA and PLL in detail.

3.2 Translocation of ssDNA Through the Nanopore

Before studying the interaction between ssDNA and PLL using α HL pore, we performed two control experiments: (i) translocation of only ssDNA and (ii) translocation of only PLL. At an applied voltage of 120 mV during ssDNA (*cis*) translocation, open pore current (120pA) is blocked to less than 20pA and the most probable translocation time for ssDNA is 0.17 ms, which agrees with the earlier reports⁷ for the same sequence of ssDNA (Figure 3.3 (a)). We also observed some events where the current was blocked for a longer time and the blocked current was near 20pA. For PLL induced blockage, a typical observation is shown (Figure 3(b)), most of the events correspond to nearly 50 % blockage of open pore current, with (Figure 3.2 (b) and (b)). However, most of the durations were short (less than 1 ms). We also observed some events which last for a longer time (For PLL3.3kD and PLL4.9kD) where current is blocked to around 35pA and which might be due to the binding of PLL with the β -barrel of the pore.

Figure 3.2. Representative ionic current trace at 120mV, 7.5pH, in 1M KCl, when (a) only 50 μ l of 15-20 μ M ssDNA was placed in *cis* compartment (b) only 50 μ M of PLL₂₀ (3.3 kD) was placed in *trans* compartment.

Figure 3.3. (a) Histogram displaying dwell time of ssDNA during its translocation through the pore at 120 mV, 1M KCl and 7.5pH. (b) Histogram displaying dwell time of poly-L-lysine (PLL_{3.3kD}) for its interaction with the pore at 120 mV, 7.5 pH and 1M KCl.

3.3 Observation of ssDNA-PLL Complexation Inside the Nanopore

To detect the interaction between ssDNA and PLL, ssDNA was added to the *cis* chamber, PLL was added to the *trans* chamber, and an electrical potential of 120mV to 180mV was applied such that the two molecules may meet inside the pore. During this process, we observed different types of transient current blockages. In search for the signature of interaction between ssDNA and PLL, we observed a type of event where the open pore current blocked to the level where the ionic current is identical to ssDNA translocation (20pA), and then further blocked reversibly to the level where ionic current nearly vanishes as shown in Figure 3.1(b) and Figure 3.4.

Figure 3.4. A representative current trace for the ssDNA-PLL complexation events for PLL_{3.3kD} at 120mV.

3.4 Comparison of Conductance State of the Pore in ssDNA-PLL and PSS-PLL Complexes

In the chapter 2 we learnt that when a PLL molecule meets a translocating PSS inside the pore, the ionic current nearly vanishes. However, the current does not go back to the level of PSS translocation. In other words, in PSS-PLL complexation events, conductance state of the pore is irreversible, whereas in ssDNA-PLL, the ionic current undergoes a transition

between two reversible states. The reversible transition between two conductance states may be due to the binding/unbinding of PLL with the translocating ssDNA. The reversible nature of binding of PLL with ssDNA may be due to the location of charges on ssDNA, which is protected, causing weaker electrostatic interaction (Figure 1(a)). In contrast, the charges in PSS are relatively exposed to the PLL, which makes the interaction stronger and produces an irreversible conductance state which involving the sliding of PSS over PLL (chapter 2).

We also observed that there is a distribution of the number of events undergoing reversible transition between the two states. A representation and histogram for 120mV is shown in Figure 3.5,

Figure 3.5. Representation of number n^* of *on/off* events in each ssDNA-PLL complexation events and histogram of the n^* for PLL_{3.3kD} at 120mV.

two distinct current levels indicating the two reversible states. The first level has the same conductance as that of the ssDNA passing through the pore. In the second level, the pore becomes blocked completely as indicated by an ionic current that nearly vanishes.

3.5 Association/Dissociation of PLL with Translocating ssDNA

We hypothesized that the first ssDNA gets captured and while trying to navigate through the pore, PLL gets captured and binds with ssDNA which leads to a complete blockage of the pore. PLL then dissociates leaving only ssDNA in the pore and then again binds with ssDNA. This reversible process continues to occur until ssDNA is pulled out of the pore completely. In the following sections, we analyze these reversible transitions between the two states as a binding/unbinding of PLL with translocating ssDNA. We describe the state of the pore when ssDNA is passing through the pore as “on” state and when ssDNA bind PLL as “off” state, we define a two-state model corresponding to the binding/unbinding of PLL with ssDNA (Figure 3.6). To validate our hypothesis, we first constructed the histograms for *on* state lifetime (τ_{on}) and *off* state lifetime (τ_{off}) at various voltages and concentrations of PLL.

Figure 3.6. Hypothesis of observed reversible conductance state as a binding/unbinding of PLL molecule with translocating ssDNA.

3.6 Only One Mode in Association

The histogram of τ_{on} is shown in Figure 3.7 (b). The lifetime of the on state can be described by an exponential distribution function (can be derived by following section 2.7 of chapter 2). Indeed, the histogram follow the single exponential probability density function. This

suggest that there is only one time constant in the distribution of τ_{on} , indicating that there is only one mode in the association of PLL with translocating ssDNA. This mainly occurs due to the electrostatic interaction between ssDNA and PLL, and the applied bias voltage. In other words, only non-covalent electrostatic forces drive the association of ssDNA and PLL molecules. For 50 μ M of PLL(3.3kD) and at 120mV the time constant is 68.49ms. The single-exponential fit to the histogram agrees with the existing binding/unbinding models for the bimolecular interactions^{8,9,10}, affirming our two state-model for the process and suggests that, under the conditions examined here, there exist only one mode for the association of ssDNA and PLL.

3.7 Two Modes in Dissociation

The histogram of τ_{off} can be described by an exponential distribution (following section 2.7 of chapter 2). A single exponential probability density function does not give a perfect fit with the histograms. However, the histogram gives very good fit with a double exponential probability density function (Figure 3.7 (c)). This suggests that there are two time constants in the distribution of τ_{off} , indicating that there are two modes in the association of PLL with translocating ssDNA. This means that apart from the electrostatic attractive forces, there can be other forces which hold PLL to bind with translocating ssDNA. At 120mV and for 3.3kD PLL the observed time constants are 1ms and 5.8ms. The 1ms time constant has a contribution of 37% and whereas the time constant 5.8 ms has a contribution of 63% in the distribution of τ_{off} . The two observed modes in the distribution of τ_{off} are likely due to the fact that ssDNA enters the pore either with the direction of 3' terminal or with the direction of 5' terminal. This could cause the PLL to interact with the

terminal end differently and therefore dissociation times are different for the case when PLL interacts with ssDNA in either of the mentioned directions.

Figure 3.7. (a) A representation of *on* and *off* conductance states corresponding to binding/unbinding of PLL with translocating ssDNA. (b) Histogram for the lifetime of *on* state and its fit with a single exponential probability density function. (c) Histogram of lifetime of *off* state and its fit with a double exponential probability density function. The histogram displayed here is for 50 μ M of 3.3kD PLL₂₀ and at 120mV.

3.8 Rate Constant for the Association of ssDNA and PLL

By measuring τ_{on} at various concentrations of PLL for different voltages, we also analyzed the rate constants for the association of ssDNA-PLL complex. As expected from our two-state model, the plot of $1/\tau_{on}$ is a straight line with respect to concentration of PLL, the slope of which yields the rate constant k_{on} for an association between ssDNA and PLL (Figure 3.8 (a)). At 120mV for PLL₂₀ (3.3kD) the rate constant is $0.279 \pm 0.067 s^{-1} \mu M^{-1}$,

Figure 3.8. (a) The rate constant k_{on} for the association of PLL and translocating ssDNA is obtained from the slope of linear fit to $1/\tau_{on}$ vs $[PLL]$. (b) The rate constant k_{on} for the association of between PLL and only αHL pore is obtained from the slope of event frequency due to the current blockage during the translocation of only PLL vs $[PLL]$. (c) The dependence of association rate constant k_{on} with voltage, suggests that an electric field kinetically favor the association. The data displayed here is for (a), (b), and (c) are for 3.3kD PLL₂₀. (d) $1/\tau_{on}$ vs $[PLL]$ for 1.6kD PLL₁₀ at 120mV. (e) $1/\tau_{on}$ vs $[PLL]$ for 4.9kD PLL₃₀ at 120mV. (f) $1/\tau_{on}$ vs $[PDL]$ for 6.3kD PDL₃₀ (poly-D-lysine) at 120mV.

which is roughly a factor of 4.5 larger as compared to the rate constant for the capture of PLL ($0.279 \pm 0.067 \text{ s}^{-1} \mu\text{M}^{-1}$) into αHL pore (Figure 3.8 (b)). This observation, further supports our hypothesis of binding/unbinding of PLL with translocating ssDNA (Figure 3.6), and discards the possibility of binding/unbinding of same PLL molecules with ssDNA, as this would require having very high rate constant for the association of PLL with the translocating ssDNA as compared to the association of PLL with only αHL pore. The association rate constant k_{on} increases with applied transmembrane voltage (Figure 3.8 (c)), suggesting that with an increase in the applied voltage, kinetically favors the

association of ssDNA and PLL. A higher voltage can induce more induced electric field generating stronger force on the charged PLL molecules, therefore it gets associated with translocating ssDNA at a faster rate. These results agree with the existing association models for the bimolecular interactions based on single-channel electrical recording^(8,9,10,11). The single rate constant k_{on} confirm our two state-model for the process and suggest that, under the conditions examined here, there exist only one mode for the association of PLL with translocating ssDNA.

We have also investigated the effect of PLL length on the kinetics of association of ssDNA and PLL. The association rate constant k_{on} depends weakly on the length of PLL (Figure 3.8 (d), and (e)). At a voltage of 120 mV, for PLL₁₀ (Mw = 1.6kD) the value of k_{on} is $0.493 \pm 0.081 \text{ s}^{-1} \mu\text{M}^{-1}$. The values are $0.279 \pm 0.067 \text{ s}^{-1} \mu\text{M}^{-1}$ and $0.373 \pm 0.066 \text{ s}^{-1} \mu\text{M}^{-1}$ for PLL₂₀ (Mw = 3.3kD) and PLL₃₀ (Mw = 4.9kD) respectively. The PLL with higher molecular weight or length diffuses faster, therefore one will expect to have higher association rate constant for the shorter PLL, if the association is diffusion limited. On the other hand, longer PLL contains higher number of charges, and if one assumes that the interaction between ssDNA and PLL is purely electrostatic, a longer PLL may have higher attraction with ssDNA. Therefore, longer PLL is likely to have a higher association rate constant if association is attraction limited. The observed k_{on} with respect to PLL length may have both effect leading to non-monotonicity in k_{on} .

To test the chirality response of the interaction between ssDNA and poly-lysine, we performed some measurements with poly-D-lysine (PDL₃₀). We did not observe any significant difference in the ionic current signature of interaction of ssDNA-PLL compared

to that of ssDNA-PDL. At 120 mV the observed value of k_{on} for PDL30 is $0.536 \pm 0.085 \text{ s}^{-1} \mu\text{M}^{-1}$, which is 1.436 times higher than that of PLL₃₀ (Figure 3.8 (f))

3.9 Rate Constants for the Dissociation of ssDNA and PLL

We measured τ_{off} at various concentrations of PLL and found that both the modes observed in the distribution (Figure 3.7 (b)) are approximately independent with respect to the [PLL]. This yields two rate constants k_{off1} and k_{off2} for the dissociation of ssDNA-PLL, which is evaluated as the average of $1/\tau_{off}$ at different PLL concentrations. The two rate constants k_{off1} and k_{off2} obtained are $862.07 \pm 65.16 \text{ s}^{-1}$ and $142.266 \pm 9.34 \text{ s}^{-1}$ respectively at 120mV and for 3.3kD PLL₂₀ (Figure 3.9 (a)). The observed rate constants k_{off1} and k_{off2} increases with the applied voltage PLL₂₀ (Figure 3.9 (b)). This indicates that the electric potential gradient favors the dissociation of PLL from a translocating ssDNA. The value of k_{off1} and k_{off2} increases with decreasing the molecular weight of PLL (Table 1.). For example, at 120mV, the rates k_{off1} and k_{off2} are $1923.07 \pm 79.3 \text{ s}^{-1}$ and $338.983 \pm 33.45 \text{ s}^{-1}$ respectively for PLL₁₀, where the rates are

Figure 3.9. (a) The two-rate constants k_{off1} and k_{off2} for the dissociation of PLL with translocating ssDNA is obtained from the average of $1/\tau_{off}$ values for different [PLL]. (b) Dependence of rate constant k_{off1} and k_{off2} on the voltage for PLL₂₀. The solid line represents an exponential fit of the data.

$852.07 \pm 65.16 \text{ s}^{-1}$ and $142.266 \pm 9.34 \text{ s}^{-1}$ respectively for PLL₂₀. This can be possible since the higher molecular weight of PLL causes stronger electrostatic attraction with a translocating ssDNA, which tries to hold PLL with ssDNA. Therefore, there is a higher dissociation barrier and the rate is lower. If the dissociation is diffusion limited, then one would expect to have faster dissociation rate constant for a shorter PLL. If the dissociation is against the electrostatic interaction between ssDNA, then also one would expect to have a higher dissociation rate constant for shorter a PLL, because PLL may get arrested by ssDNA due to stronger electrostatic attraction. Therefore, the observed values of k_{off1} and k_{off2} aligns with our intuition.

$k_{\text{off}} (\text{s}^{-1})$	PLL ₁₀	PLL ₂₀	PLL ₃₀	PDL ₃₀
k_{off1}	1923.08 ± 79.3	852.07 ± 65.16	288.18 ± 20.24	568.18 ± 35.34
k_{off2}	338.98 ± 33.45	142.27 ± 9.34	30.73 ± 5.39	50.761 ± 9.89

Table 3.1. Rate constants for the dissociation for different length of PLL and PDL at 120mV.

Another interesting observation is that the rate constants k_{off1} and k_{off2} are higher for poly-L-lysine compared to that of the poly-D-lysine. For example, at 120mv, the rates k_{off1} and k_{off2} are $288.18 \pm 20.24 \text{ s}^{-1}$ and $30.73 \pm 5.39 \text{ s}^{-1}$ respectively for poly-L-lysine, where the rates are $568.18 \pm 35.34 \text{ s}^{-1}$ and $50.761 \pm 9.89 \text{ s}^{-1}$. This significant effect of chirality on the rate constants of dissociation indicates that the orientation of the PLL molecule also plays a role in the ssDNA-PLL binding.

3.10 Apparent Equilibrium Dissociation Constants for PLL Binding/Unbinding with ssDNA

The histograms displaying the dwell time for the ssDNA occupied state of α HL (τ_{on}) could be fitted by single-exponential distributions for the data obtained within an applied voltage range. (Fig. 3.7(b)). These data confirm that, under the wide range of conditions examined here, there exists only a single mode for the association of PLL with translocating ssDNA, which is accessible from the *trans* side of the bilayer. The histograms displaying the dwell time for the ssDNA-PLL binding (τ_{off}) could be fitted by double-exponential distributions for the data obtained for applied voltage range (Fig 3.7 (b)). This can be due to the fact that ssDNA may enter from the 3' terminal or 5' terminal; Therefore, depending upon the direction of of ssDNA the interaction strength of PLL may be different. For example, the 5' terminal end of ssDNA has 10 A bases (Adenine), whereas the 3' terminal of ssDNA has 10 C bases (cytosine),

Scheme 1. Binding/unbinding equilibrium scheme for ssDNA-PLL interaction.

therefore, the PLL binding strength may be different and therefore it is possible that is why we are observing two modes in the dwell time distribution of binding of PLL with translocating ssDNA.

Therefore, the kinetics of the interaction between PLL and ssDNA should obey the simple kinetic given in Scheme1. Since, there are two modes in the dwell time distribution of τ_{off} corresponding to two rate constants, where one is k_{off1} and other is k_{off2} , we define two apparent equilibrium dissociation binding constants $K_{d1}^{app} (k_{off1}/ k_{on})$ and $K_{d2}^{app} (k_{off2}/ k_{on})$,

both arising due to the sequence and orientation specific binding of PLL with the translocating ssDNA. The equilibrium dissociation constants were calculated for different voltages for PLL₂₀ and at a fixed voltage for PLL₁₀, PLL₃₀, and PDL₃₀ respectively. For PLL₂₀, the value of K_{d1} decreases from 3.05mM to 1.18 mM by changing the applied voltage from 120 mV to 160 mV, but it rises to 1.56 mM for 180mV (Figure 3.10). The values of K_{d1} has also has a similar dependence with respect to voltage. It decreases first till 160mV and then rises again at 180mV. This non-monotonic response of the equilibrium dissociation constant with respect to the applied voltage may arise due to competition between an increase in the association rate constant (k_{on}) and dissociation rate constants (k_{off1} and k_{off2}). The equilibrium dissociation constant K_{d1}^{app} and K_{d2}^{app} decreases with increasing the PLL length (Table 2.). At 120 mV for PLL₁₀ the observed value of K_{d1}^{app} and K_{d2}^{app} are 3.90 ± 0.64 mM , and 0.70 ± 0.13 mM respectively. The values are 3.05 ± 0.77 mM and 1.44 ± 0.25 mM for PLL₂₀ respectively. For PLL₃₀ the values of K_{d1}^{app} and K_{d2}^{app} further decreases and are 0.77 ± 0.14 mM and 0.08 ± 0.004 Mm, respectively. These observations manifest the role of number of charges present in the system and align with the intuition that a longer PLL will face difficulty to dissociate because of a stronger electrostatic interaction. These observations indicate that the PLL acts as a multivalent cation in the system. Furthermore, we observed that for the poly-D-lysine (PDL₃₀) K_{d1}^{app} is slightly higher compared to the PLL₃₀, however the value of K_{d2}^{app} are approximately similar.

Figure 3.10. Apparent equilibrium dissociation constant K_{d1}^{app} and K_{d2}^{app} for 3.3kD PLL₂₀ as a function of voltage.

3.11 Discussions

Although, the reversible two-state model of ssDNA -PLL binding/unbinding is able to quantify the interaction between ssDNA and PLL, it does not capture the following molecular details. We do not know whether one PLL associates with the ssDNA in each binding/unbinding events or whether it is multiple PLL molecules associate with ssDNA. In either case, the two-state model holds but the molecular mechanism might be different. The dwell time of the individual binding event decreases with voltage. One can relate this observation to association of the same or different PLL molecule.

$K_d^{app}(mM)$	PLL ₁₀	PLL ₂₀	PLL ₃₀	PDL ₃₀
K_{d1}^{app}	3.90 ± 0.64	3.05 ± 0.77	0.77 ± 0.14	1.06 ± 0.18
K_{d2}^{app}	0.70 ± 0.13	1.44 ± 0.25	0.08 ± 0.004	0.09 ± 0.005

Table 3.2. Apparent equilibrium dissociation constants for different molecular weight of PLL and PDL at 120mV.

If one assumes that, the same PLL molecule is associating with the ssDNA, then possible mechanism could be that the bound part of the ssDNA-PLL complex is pulled out of the pore and dissociates in the trans compartment. Then the same PLL molecule which has just dissociated with ssDNA, again recombines because of the presence of applied electric potential. This agrees with the decrease in dwell time of binding event with respect to the voltage and increase in the association rate constant with respect to voltage. An increase in the k_{on} with [PLL] can be due to the intermolecular repulsion between PLL molecules, pushing it to bind with ssDNA. Whereas, if one assumes that the different PLL molecule is associating with ssDNA in each binding event, then the possible mechanism would be, PLL binds with ssDNA near the trans exit of the β -barrel and then it dissociates and then different PLL molecules come and bind with ssDNA. In agreement with the observed experimental results, one would expect to have a smaller dwell time of binding event with respect to the voltage, the association rate constant k_{on} increases with the concentration of [PLL] and voltage. However, at 120mV for PLL₂₀ (3.3kD) the rate constant is 0.279 ± 0.067 s⁻¹ μ M⁻¹, which is roughly a factor of 4.5 larger compared to the rate constant for the capture PLL (0.279 ± 0.067 s⁻¹ μ M⁻¹) into α HL pore (Figure 3.8 (b)). This supports our hypothesis of binding/unbinding of PLL with translocating ssDNA (Figure 3.6), and discards the possibility of binding/unbinding of same PLL molecules with ssDNA, as this would require

having very high rate constant for association of PLL with translocating ssDNA, compared to the association of PLL with only α HL pore. This is a limitation of our experiments, we are not able to directly distinguish between same vs different PLL bound to single ssDNA.

3.11.1 Two Dissociation Constants vs Sequence Specific Interactions

It could be possible that the observed two-time constants in the distribution of the binding life time of ssDNA-PLL, are due to the difference in the binding strength of PLL with translocating ssDNA, when (i) ssDNA enters the pore from the 5' terminal, and (ii) ssDNA enter the pore from 3' terminal.

Figure 3.11. Schematics of sequence of ssDNA used in the experiments, with 5' terminal mostly consisting of Adenine bases and whereas 3' terminal consisting of cytosine, the molecular structures of Adenine, Cytosine, and Lysine are also presented to illustrate the importance of the relative strength of interaction in lysine-adenine pair compared to that of lysine-cytosine pair.

The ssDNA used in the experiments has 92 bases, with 21 adenine bases at its 5' terminal and 10 cytosine bases at its 3' terminal. Therefore, if the binding strength of lysine is different with the adenine bases as compared to the binding strength of lysine with the cytosine base, then the rate constant for the dissociation of PLL may differ, depending on the interacting with either the 5' terminal of ssDNA or the 3' terminal. The higher the binding strength a higher barrier will be required for the dissociation of PLL molecules from translocating ssDNA.

Several studies have been performed to understand the sequence-specific interactions of proteins with DNA¹²⁻¹³. There has been an active interest to address the biological role of histones. Several studies have been carried out to explore if certain histone fractions can interact preferentially with regions of DNA, that possess a special base sequence. In some studies, it was found that precipitates formed by the reaction of DNA with histones or poly-lysine could be re-dissolved in NaCl solutions of concentrations 1-2M, and under these conditions, guanine-cytosine (G-C) rich DNA was eluted more readily than adenine-thymine (A-T) rich DNA^{14, 15, 16}. In another study, it was found that in 1M NaCl, poly-lysine exhibited an almost preferential precipitation with A-T- rich DNA, whereas poly-arginine showed preferential selectivity with G-C rich DNA¹².

If one extrapolates these findings in the literature of preferential binding of PLL with A-T rich DNA, then it may be concluded that lysine may have a higher strength of interaction with adenine as compared to the interaction with cytosine. This insinuates, that if the above mentioned extrapolated conclusion is true then we could assign the faster dissociation rate constant (k_{off1}) to the dissociation of PLL with 3' terminal of ssDNA, and the slower dissociation rate constant (k_{off2}) to the dissociation of PLL with 5' terminal of ssDNA.

However, it could also be possible that the observed two rate constants are different may be due the interaction of PLL with only the A and C bases of ssDNA. Therefore, to get further insight we should perform experiments with poly[dA], poly[dC], poly[dG] and poly[dT]. Investigation with different polybases may give us valuable insight about the interaction of lysine with specific nucleic acids.

An interesting fact observed in the ssDNA-PLL complexation is that the rate constants for the dissociation becomes almost half by doubling the length of PLL from 10 to 20 lysine residues (Table 1). This becomes roughly one sixth upon tripling the length of PLL from 10 to 30 lysine residues, however the rates are one third for poly-D-lysine. If there are only electrostatic interactions between ssDNA-PLL then one would expect to have a similar decrease in the rates for both poly-L-lysine and poly-D-lysine. This indicates that there is a non-linear dependence of k_{off1} and k_{off2} with PLL length, which may be due to the incorporation of other attractive forces such as hydrogen bonding, interaction of positive charges of lysine with aromatic ring of the Adenine or Cytosine bases via cation- π interactions. Hydrogen bonding and cation- π interactions have been proposed to play a significant role in the DNA-Protein interactions^{17,18,19}. The molecular recognition of ATP in the ATP-binding proteins is also a subject of great importance for understanding the enzymatic mechanism for designing new materials for future medicine¹⁷. Next, we will try to enhance our understanding of observed rate constants for the dissociation based on the chemistry of Adenine-Lysine pair and Cytosine-Lysine pair.

3.11.2 Chemistry of Adenine-Lysine and Cytosine-Lysine

The positive charge on the lysine can interact with the aromatic ring of the Adenine and Cytosine via cation- π interactions. However, in Adenine the π electron cloud is more

because of the higher number of π electrons (10), compared to that in cytosine (6 π electrons). Therefore, in terms of cation- π interactions, Adenine-Lysine seems to have a higher interaction strength compared to the Cytosine-Lysine. On the other-hand, Adenine molecules have a capacity to form five hydrogen bonds, acting as a hydrogen bond donor at its N6 position and hydrogen bond acceptor at N1, N3, and N7 positions. Thus, this can make four stable recognition of lysine molecules via formation of stable six-member hydrogen bonded ring (Figure 3.12). In cytosine there are two hydrogen bond donors at, the N4 position, which is the same as Adenine. But, it also has two nitrogen atoms and one oxygen atom, which can accept hydrogen bonds. However, the possibility of formation of

Figure 3.12. (a) Representative intermolecular interactions between adenine and positively charged lysine residue. Image is reproduced from references. (b) Schematic of intermolecular hydrogen bonding between N6 of adenine and carbonyl group of lysine. This type of bond can also be formed in N4 of cytosine and carbonyl of lysine. Other possible combination for the formation of six-member hydrogen bonded rings in adenine-

lysine pair making it stronger compared to the cytosine-lysine pair. **(c)** N7, N9 of adenine and terminal amine group of lysine **(d)** N1, N3 of adenine and terminal amine group of lysine, and **(e)** N3, N9 of adenine and terminal amine group of lysine.

-six-member ring via hydrogen bonding in adenine-lysine, makes it stronger pair compared to cytosine-lysine. Therefore, it is possible that because of the chemistries involved, the adenine-lysine pair has stronger strength of interaction than that of cytosine-lysine pair, making two modes in the dissociation of PLL with translocating ssDNA. Therefore, k_{off1} could be due to the dissociation of PLL with the cytosine rich region of ssDNA and k_{off2} could be due to the dissociation of PLL with Adenine rich ssDNA.

But how does the chirality influence the dissociation rate constants? There can be a possibility that chirality of lysine can influence the local orientation of relative to adenine and cytosine, which can affect the specific interactions. More experimental and theoretical efforts are required to resolve this puzzle.

3.11.3 Comparison with Other Nanopore Based Binding/Unbinding Models

Kinetic parameters for the interaction of individual molecules with the α -HL pore have been reported. The studies have been performed for a fundamental understanding of channel blockers such as β -CD⁸⁻¹¹, PEG²⁰, peptide²¹. For example, kinetic parameter for the interaction of β -CD with α -HL pore has been studied extensively⁸⁻¹¹. Several studies have focused on covalently attaching single molecules within α -HL pore and driving another charged molecule into the pore to study the inter-molecular interaction^{22, 23, 24}. In another study an electrical force is applied to individual ssDNA-protein complex to pull it apart and the rate constant for association and dissociation is determined²⁵. However, the DNA-protein complex was made in a bulk solution and was then electrophoretically captured by the pore, which did not give the signature of the process of assembly of DNA

and protein. In the present study, we used electrical force to observe the signature of assembly between the two macromolecules.

3.11.4 Binding/unbinding Events of a Characteristic of Molecular AND Logic Gate

The reversible conductance state of α -HL pore because of binding/unbinding of PLL with translocating ssDNA can be represented as a logical operation. Let us consider a binary variable S1, representing presence of ssDNA in the pore and assume that S1 is 0 if the DNA is absent in the pore and is 1 if the DNA is present in the pore. Similarly consider a variable S2, which is 0 if PLL is absent and is 1 if PLL present. Now, let us consider another variable as S, which is basically 0 if any of the DNA and PLL is absence and 1 if both are present. Thus, S is a function of S1 and S2. If any of S1 and S2 is 0 or both are 0, then S is 0. This is basically characteristic of AND gate. This describes the binding/unbinding events of PLL with ssDNA, as a potential for performing basic logical operations.

3.12 Outlook

We have demonstrated that despite electrical and geometrical similarities between PSS and ssDNA, the α HL pore is able to identify, and distinguish between the interactions in ssDNA-PLL and the PSS-PLL complexes. In contrast to the PSS-PLL complex in the pore, PLL undergoes a reversible binding/unbinding with translocating ssDNA, indicated by the signature of a reversible conductance of the pore transitioning between the two states. By hypothesizing a two-state model of binding/unbinding of ssDNA-PLL, we have estimated the association and dissociation rate constants, which can be tuned by changing the applied voltage. The distribution of dwell time of ssDNA-PLL binding events suggests that the dissociation of PLL with ssDNA occurs via two rate constants, which can be due to the sequence specific-interaction of lysine with adenine and cytosine rich region of ssDNA.

We have also obtained an apparent equilibrium dissociation constant (mM) for the binding/unbinding of PLL with translocating ssDNA. An investigation of the effect of PLL length on binding constants indicates that the PLL acts as a multivalent cation in the system. We also find differences in dissociation rate constants with respect to the chirality of polylysine. Our results demonstrate the power of nanopore technology in identifying and distinguishing between the interaction in two different macromolecular complexes. Our research also opens a new doors to explore the sequence specific interactions between different nucleic acid bases and amino acid residues, which is extremely important for the fundamental understanding of DNA-Histone interactions. It could also be possible to use our general idea of voltage-driven polyelectrolyte complexation to explore the effect of DNA and Histone modifications on their binding strength, which can give us a valuable insight about the organization and packaging in the nucleosome.

3.13 Materials/Methods

Formation of perpendicular bilayer: We used 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DphPC, purchased from Avanti Polar Lipid.) to form perpendicular phospholipid bilayer across an aperture (diameter, 50 μm) in a Teflon film separating the two compartments (1 ml each *cis/trans*) of the polytetrafluoroethylene (PTFE) flow cell apparatus (purchased from Nanopore Solutions, Portugal).

Buffer solution: 1M potassium chloride (KCl) solutions in 10 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethane sulfonic acid (HEPES) in deionized water (18 M Ωcm) were prepared for the measurements. The pH of the buffer solution was adjusted to 7.5 by adding potassium hydroxide (KOH) or hydrochloric acid (HCl).

α HL pore: Wild-type α HL monomers, α -toxin from staphylococcus aureus were purchased from EMD Millipore (MA). A stock solution of α HL were prepared by adding 50:50 (v/v) of water and glycerol mixture to make a final concentration of 0.5 mg/ml. The resulting solution was further diluted by 1M KCl buffer solution to make a final concentration of 0.5 μ g/ml. A portion of the resulting α HL protein solution (2 μ l) was added to the *cis* compartment of a bilayer apparatus to form a single-channel. Once the single pore is formed, the *cis* chambers is flushed with 1M KCl solution to avoid the formation of multiple channels.

ssDNA: The sequence of 92-nt DNA was selected to have a minimum secondary structure ⁽⁷⁾.5'AAAAAAAAAAAAAAAAAAAAAAAAATTCCCCCCCCCCCCCCCCCCCCCTTAAA AAAAAAATTCCCCCCCCCCTTAAAAAAAAAATTCCCCCCCCCCC-3'. The sample of ssDNA was purchased from integrated DNA technology via custom oligo services (IDT USA). After the formation of single α HL pore, the *trans* chambers were replaced with the KCl buffer solutions containing desired concentration of poly-lysine and then 50 μ l of 15-20 μ M of ssDNA were added to *cis* side.

Poly-lysine: The poly-L-lysine (PLL) and poly-D-lysine (PDL) was purchased from Alamanda polymers with average molecular weights of 1.6 (PLL₁₀), 3.3 (PLL₂₀), 4.9 kD (PLL₃₀), and 6.3 kD (PDL₃₀). After the insertion of a single α HL pore from the *cis* compartment, the solutions of *trans* chambers were replaced with the KCl buffer solutions containing desired concentration of poly-lysine.

Electrical measurements and data analysis: Ionic currents were measured by using Ag/AgCl electrodes with a patch-clamp amplifier (Axopatch 200B, Axon Instruments).

The amplified signal was low-pass-filtered at 5 kHz (or 10 kHz) and sampled at 250 kHz with a Digidata 1550 digitizer (Axon Instruments). The recorded ionic current traces are analyzed using MATLAB (The MathWorks, Inc., MA) to capture the current blockage events and their durations.

3.14 References.

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CHAPTER 4

ADDITIONAL

In this chapter we will discuss about some of the additional experimental investigations to understand the forces on single macromolecule during its transport through the nanopore. First, we will discuss about the role of identity of an electrolyte on the translocation behavior, then we will also discuss about the long term ionic current blockage detected by the pore during its interaction with β -crystallin proteins. In the end we will comment on the behavior of polymer translocation under an oscillating electric field and in a crowding environment.

4.1 Effect of Specific Identity of Electrolyte on the Polyelectrolyte Translocation Through the Nanopore.

4.1.1 Introduction

In single-molecule electrophysiology experiments, there are four major components: charged polymer, ions, water and the pore. The polymer reaches to the pore based on the drift-diffusion mechanism and its translocation is driven by an external electric potential gradient exerting an electrophoretic force on a charged polymer. The force experienced by the polymer depends upon net effective charges on individual monomer. Although each monomer carries a charge equal to the elementary electronic charge, when a counter-ion binds to the monomer it can neutralize the charge. Therefore, the electrophoretic force on the polymer is likely to depend on the interaction of counterions with the polymer. In addition to that if a counterion interacts strongly with the charged polymer, it can reduce the hydrodynamics radius of the polymer and therefore can increase the diffusion, which can enhance the capture of the polymer.

Interaction of a polyelectrolyte chain with counterion may be different in bulk solution and inside a confined nanopore environment. Before thinking about the how polymer and counterion interactions can affect the translocation through the nanopore, we will now turn our attention to the basics on ions dissolved in water.

4.1.2 Ionic Conductivity of Bulk Solution vs Nanopore

In a bulk solution the diffusion of ions is mainly determined by their hydration radius, is the reason for following trend of ionic conductivity of aqueous solutions $\text{LiCl} > \text{NaCl} > \text{KCl} > \text{RbCl} > \text{CsCl}$. This is because smaller ions (Li^+ , Na^+) have a greater charge density at the surface compared to larger ones; they therefore form more links with the water molecules and their hydration shells are larger. Therefore, in a bulk solution movement or mobility or diffusion of ions depends on the hydration radius or number of water molecules surrounding them.

Salt (1M)	Conductivity (mS/cm)
LiCl	73.2 ± 0.7
NaCl	86.2 ± 1.2
KCl	111 ± 2.1
Gdm-HCl	84.6 ± 0.6

Table 4.1.1. Specific conductivity of electrolyte solutions at 7.5pH, containing 5mM HEPES buffer measured at room temperature.

Now, let's consider ions and water inside the nanopore. The ionic current across the nanopore has the same trend as ionic conductivity of the bulk solution: $\text{LiCl} > \text{NaCl} > \text{KCl}$ (Table 4.1.1 and Figure 4.1.1). The ratio of ionic current for the different electrolyte is not

Figure 4.1.1. The ionic current vs voltage curves, measured through the α HL pore for different electrolyte solutions. The pH of the solution is fixed at 7.5 and each electrolyte contain 5mM HEPES buffer.

the same as the ratio of ionic conductivity of the bulk solution, suggests that although the amount of water molecule surrounded by an ion inside the nanopore may be different from the bulk solution. However, the observed ionic current trend across nanopore indicate that Li^+ carrier more water molecules compare to Na^+ and K^+ .

Now let's think about a charged polymer in a bulk aqueous electrolyte solution. (i) One possibility is that the counterions bind to the polymer in an un-hydrate state. (ii) Another possibility is that the counterions bind to the polymer a hydrated state. If the possibility is (i) then by assuming that the binding between counter-ion and polymer is only electrostatic in nature, the hydrodynamic radius of the polymer should be independent of the identity of cations. It can be thought on this way: polymer and ions are not collecting water molecules. If the possibility is (ii) then since, the counter-ions are binding to the polymer in a hydrated state, the hydrodynamics radius of the charged polymer should depend primarily on the identity of electrolyte. One would expect that the hydrodynamic radius will be higher in the presence of an electrolyte and thus having smaller cation size.

Similarly, another question arises when the charged polymer is translocating through the nanopore, it binds the counter-ions in a hydrated state or in an un-hydrated state. One way to resolve this puzzle is to observe the ionic current blockade during the translocation of a charged polymer with respect to various identity of electrolytes. In this chapter we will investigate the ionic current blockage produced during the translocation of a negatively charged polymer poly-styrene-sulfonate (PSS), in different electrolyte solutions LiCl, NaCl, KCl and Gdm-HCl. This chapter specifically throws light on the following issues:

- (i) Efficiency of translocation with respect to identity of the electrolyte solution.
- (ii) Role of water molecules in ionic current blockade during the translocation of polymer.
- (iii) Translocation kinetics versus the identity of electrolyte.

We will also compare the translocation behavior of ssDNA and PSS with respect to the electrolyte solution.

4.1.3 Efficiency of Translocation vs Identity of Electrolyte

To investigate the efficiency of translocation, we analyze the threshold voltages for polymer capture and for successful translocation. When a negatively charged polymer is placed in a *cis* compartment and an electric field is applied, it undergoes drift-diffusion motion and as a result it can be captured by the pore, and then depending on the voltage it

can translocate or retract back to donor compartment (Chapter 1).

Figure 4.1.2 Three types of events observed during the passage of individual PSS through the nanopore. Event1 is unsuccessful, Event 12, and Event2 are successful translocation events.

However, the polymer must overcome the free energy barrier to get captured inside the pore, as it must pay the price in the form of loss of conformational entropy by aligning one end of the chain inside the pore. Therefore, not at every voltage polymer gets captured by the pore, there exist a threshold voltage above which it experiences strong enough force to get captured. This voltage is called the threshold voltage for the polymer capture which also depends on the strength of the pore-polymer interaction, size and charge on the polymer. The threshold voltage for the capture is regarded as the minimum voltage required to observe the event type 1. Given a polymer captured inside the pore, it may translocate and retract back to the donor compartment. Right after the capture in relatively spacious vestibule of the pore, it again aligns its monomers in the narrowest constriction of the α HL for the successful translocation. If the polymer undergoes a successful translocation, the ionic current is further blocked to level 2 (Chapter 2, section 2.2). Threshold voltage for the successful translocation is regarded as the minimum voltage for the observation of event type 12 or event2 (event 12 and event2 has durations proportional to the molecular weight of PSS and inversely proportional to the voltage).

Threshold voltages for the polymer capture and successful PSS translocation is provided in Table 4.1.2. We can see that the observed threshold voltage for the capture of the polymers are 30mV, 60mV, 80mV, and 20mV for LiCl, NaCl, KCl and Gdm-HCl respectively. This observation suggests identity of the electrolyte can increase or decrease the free energy barrier for capture the polymer inside the pore. For Gdm-HCl and LiCl this efficient capture may be regarded as the lower barrier required to capture the polymer, compared to NaCl and KCl. There can be two explanations for this: First, Li^+ and Gdm^+ can have a greater extent of binding with sulfonate back-bone of PSS and therefore more

counterion condensation may lead to decrease in the effective hydrodynamic radius of the polymer and which increases the diffusion coefficient, facilitating the capture.

Second explanation can be based on the idea the Li^+ and Gdm^+ may have a greater number of water molecules in the hydration shell, therefore, making water molecules less available for the polymer, which pushes the polymer inside the pore. The 50mv difference is roughly equivalent to $2k_B T$ at the room temperature, one can imagine that there can be $2k_B T$ decrease in the energy barrier for the polymer capture from 1M KCl to LiCl.

Electrolyte (1M)	Threshold Voltage (Capture)	Threshold Voltage (Translocation)
LiCl	30mV	80mV
NaCl	60mV	110mV
KCl	80mV	120mV
Gdm-HCl	20mV	60mV

Table 4.1.2. Threshold voltages for the PSS capture inside the pore and successful PSS translocation. Translocations more efficient in LiCl and GdmHCl compare to NaCl and KCl.

The threshold voltage for successful PSS translocation also find the same trend as the threshold voltage for polymer capture inside the pore. The observed differences in the threshold voltages for KCl and LiCl are 40mv, 60mv for the KCl and Gdm-HCl, respectively. This observation indicates that the interaction of Li^+ and Gdm^+ is stronger which facilitates the translocation of PSS through the nanopore. The cations Li^+ and Gdm^+ can bind strongly with the interior surfaces of the protein nanopore, compare to the Na^+ and K^+ (exit ring of the β -barrel contains negative charges), this may lead to a net

electrostatic interaction with the pore and hence cause an efficient translocation compared to the case of the NaCl and KCl, where negative charges at exit ring of the β -barrel may be more visible to the translocating negatively charged PSS.

Another interesting observation is the percentage of successful translocation with respect to the identity of the electrolyte solution. We define the percentage of successful translocation as the ratio of number of events of type 12 and event type 2 to the total number of observed events multiplied by hundred. This percentage is plotted as a function of voltage for different identity of electrolyte solution in Figure 4.1.3 (a). In 1M KCl the probability for the success of translocation increases from 4% to 80% by changing the voltage from 120mV to 240mV. However, this probability is 85% event at 100mV for Gdm-HCl (Figure 4.1.3(a)). Moreover, for all the identity of electrolytes the % successful translocation behaves sigmoidal with the voltage. Another, interesting we see if that for fixed 1M KCl, additional amount of Gdm-HCl increases the % of success for the translocation (Figure 4.1.3 (b)).

Figure 4.1.3 (a) Percentage of successful translocation of PSS with voltage in different electrolyte solutions. Dotted curves represent sigmoidal function. (b) Percentage of successful translocation as a function of Gdm-HCl concentration in a fixed 1M KCl solution at different voltages.

4.1.4 Ionic Current Blockade vs Identity of Electrolyte

The presence of polymer inside the pore blocks the ionic current. The extent of ionic current blockade depends on the amount of space occupied by the polymer during its translocation or presence in the pore. In the experiments with different identity of electrolytes, we observed that the current is blocked to a higher level with Gdm-HCl, LiCl, compared to the NaCl, and KCl. The trend for the ionic current blockade and its duration is: Gdm-HCl > LiCl > NaCl > KCl. The slow duration for Gdm-HCl and LiCl can be attributed to the stronger interaction of Li^+ and Gdm^+ cations with negative charges present on the sulfonate backbone and thus the polymer experiences a weaker electrophoretic force. This trend in the duration of blockage or translocation times is similar to one recently reported transport of dsDNA and ssDNA with sub 10nm solid state nanopores¹. However, in that particular study the extent of ionic current blockade was found to be same in different electrolyte solution. In this study, where the pore diameter is 1.5 nm we found a greater extent of ionic current blockade with LiCl and Gdm-HCl. This strongly indicates towards the role of water molecules in the ionic current blockage and dynamics of the polymer during its transport through the pore.

Figure 4.1.4 Event diagrams representing the extent of ionic current blockade and its duration for each type of event at 140mV in (a) 1M KCl (b) 1M NaCl (c) 1M LiCl (d) 1M Gdm-HCl. The red dots are corresponding to event 122, blue is event 121, cyan is event 1, and black is event 2. We can see from this diagram that in the events the ionic current is more blocked and blocked for longer time in Gdm-HCl, and LiCl compare to KCl and NaCl.

We did not observe any significant difference in the translocation behavior of ssDNA with respect to the identity of electrolyte solution from KCl to Gdm-HCl (Figure 4.1.5). It may be due to the fact that the charges on ssDNA is less exposed to the counterions and thus the extent of counterions bounded is lower in ssDNA. Therefore, there is no significant difference in the electrophoretic force on ssDNA with respect to identity of electrolyte.

Figure. 4.1.5. Histograms of translocation time of ssDNA at 120mV in different electrolyte condition.

4.1.5 Conclusion

Data presented in this section clearly demonstrates that the specific ions can significantly increase and decrease the efficiency of polymer translocation. The threshold voltages for the capture and successful translocation are found to be higher for LiCl and Gdm-HCl compared to NaCl and KCl. The trend for the extent of ionic current blockage and its

duration suggests that Gdm-HCl may be utilized for slowing down the translocation of PSS. However, no significant difference is found in the translocation rate of ssDNA if the electrolyte is changed from KCl to Gdm-HCl.

4.2 Protein Translocation

4.2.1 Introduction

Unfolding-derived aggregation of proteins is a major cause of diseases. For example, Alzheimer, Huntington, type II diabetes, and cataract.^{2,3} Age-related cataract resulting from aggregation of the lens crystallin is responsible for 48% of world blindness and affects 20.5 million Americans age 40 and over⁴. The general concept is that cataracts form due to the aggregation of lens proteins. These aggregations are due to the deleterious effects of exposure to radiation, chemical reactions, and noncovalent forces⁵. The proteins of the lens become insoluble upon aggregation which leads to scattering of incident light and obstructing it from reaching the retina⁴. However, the molecular mechanism of cataract formation is not fully understood⁵. The fundamental question is what kind of inter and intra molecular interactions are involved in different proteins in the lens of the eye.

About 90% of the total protein content in the lens is crystallin proteins. These crystallin proteins are found at high concentrations of 200–450 mg/ml⁶. There are two main classes of crystallins : (i) α -crystallins which are responsible for the remarkable stability of crystallins and the chaperone activity in the lens which prevent aggregation for a long period of time and (ii) β - and γ -crystallins which are primarily structural⁷.

The β -crystallins comprise a complex group of heteropolymers which are assembled from 6 primary gene products, the acidic (β A1, β A2, β A3) and the basic (β B1, β B2, β B3)

polypeptides and average molar mass of about 25 kDa. β A1 and β A3 are identical, except for their N-terminal, and appear to be generated by alternative translation of the same gene^{8,9}. The β -crystallins are capable of forming dimers as well as homo- and hetero-oligomers, the unaggregated γ -crystallins are monomers in the eye¹⁰. The human γ -crystallin family consists of five members, the γ A-, γ B-, γ C-, γ D-, and γ S-crystallins, which are highly similar in sequence and molar mass of about 21 kDa.

Protein unfolding, because of oxidative or UV-induced damage is one of the proposed mechanism for cataract formation^{11,12}. Unfolded conformations of protein can lead to aberrant intermolecular interactions and resulting in aggregations. Another mechanism is α -crystallin prevents the β - and γ -crystallins from aggregation by binding denatured proteins¹³. In case of excessive prevention of α -crystallin, the population of partially unfolded β - and γ -crystallins can rise to a threshold level where aggregation begins^{13,14}. Leading to cataracts condition. Insoluble aggregates of proteins can be solubilized in the presence of denaturing agent 6M guanidine hydrochloride and the reducing agent 1M dithiothreitol (DTT), which suggests that both noncovalent interactions and disulfide bonds are responsible¹⁵. Recently, it was observed by light scattering that in-vitro aggregation of γ -crystallins is electrostatically driven². Another mechanism is the fluctuations in the protein concentration which lead to scattered light. The lens proteins have liquid-like order², in absence of aggregation and fluctuations in concentration are avoided by the protein-protein interactions, instead of the initially hypothesized regular array of proteins¹⁶. Deaggregation of γ - and β - crystallin aggregates by chaperone activity of α -crystallins has been well understood¹⁷⁻²³. Specificity of crystallin molecules for aggregation and deaggregation have also been studied^{24,27}. Folding/unfolding experiments of γ D-crystallin has been also studied which have shown the existence of a

partially unfolded intermediate with C-terminal domain (C-td) mostly folded and N-terminal domain (N-td) unfolded ⁽²⁸⁾.

Despite the huge progress briefly mentioned above, the nature of intra and intermolecular forces among crystallin proteins still remains elusive from a full understanding. The primary objective of this research is to explore the nanopore technology as a complementary to study the unfolding kinetics and state of β , γ –crystallin proteins in very dilute in buffer solutions under varying conditions.

4.2.2 Materials and Methods:

β_L -crystallins was purchased from Enzo Life Sciences. All the rest of the materials and methods are same as described in Appendix A. 0.5 μ M β_L -crystallins was used in each experiment. For the *trans* side measurements after forming lipid bilayer, 0.5 μ M of β_L -crystallin (1 ml approximately) was added to *trans* chamber and then insertion of the pore process was carried out. For the *cis* side measurements, solution on *cis* side was flushed with 0.5 μ M of β_L -crystallin after formation of the pore.

4.2.3 Results

We measured blockages produced by β_L -crystallin. Different salt concentrations were used to measure the blockages. In 1 M KCl at 140mV, we found that most of the blockages were short, spike-like and shallow (Fig. 25). Therefore, we increased the salt concentration and performed the experiments in 2M, 3M and 4M KCl concentration. The frequency of blockages first increases with respect to salt concentration and then decreases at 4M KCl. Moreover, in 3M KCl the frequency of blockages or blockade rate increases exponentially with voltage but remains constant in 4M KCl. There were deeper blockages events at higher

Figure 4.2.1 Ionic current blockages produced due to the interaction of β -crystallin protein with α HL nanopore at 140 mV (7.5pH) in (a) 1M KCl (b) 2M KCl (c) 3M KCl (d) 4M KCl. (e) The event frequency as a function of voltage for 3M KCl (f) as a function of salt concentration at 140 mV.

salt concentration compared to lower salt concentration. Deeper blockages events are very large in case of 3M KCl (some events also show permanent blockage). We also observed blockages at negative and positive voltages when β_L -crystallin was on cis/trans side, respectively. Our intuition is that high salt perturbs the native structure of this crystallin protein, due to which there are partially unfolded conformations of polypeptides, which enter the nanopore and creates deeper and long blockages.

Figure 4.2.2 Event diagram representing ionic current blockages produced and their durations due to interaction of β -crystallin protein with α HL nanopore at 140 mV (7.5pH) in different salt concentration conditions.

We also investigated the effect of denaturing agents such as Gdm-HCl and Urea to see whether unfolded kinetics can be observed in nanopore measurements. The addition of urea did not affect the dynamics and were unable to probe the kinetics of unfolding. Also, in 1M KCl the event frequency and the duration of events remains unchanged with the addition of urea.

Figure 4.2.3. Event frequency of ionic current blockade when $0.5\mu\text{M}$ β_{L} -Crystallin protein was placed in trans compartment, as a function of voltage for three different incubation time of sample in 1M Gdm-HCl solution.

duration of events. We do observe some increase in the event frequency upon adding Gdm-HCl in 1M KCl, however no significant effect on event durations, suggesting that increase in the event frequency may be due more collision events

of protein molecule with the nanopore. Moreover, we also observed that the event frequency dependent upon incubation time of the sample in Gdm-HCl

4.2.4 Stability of α HL upon Denaturation with Urea.

Before investigating the idea of detection of unfolding intermediate induced by denaturing agent Urea, we also investigated the stability of α HL protein nanopore. In a study²⁹, it has been claimed that urea can cause loss of structural asymmetry of α HL nanopore. The α HL protein nanopore is stable on lipid bilayer even at 7.2 M concentration compared to the bulk urea solution (maximum in 4.66M urea we can form the pore upon incubation α HL monomer in the urea). However, in another study³⁰ the proposed idea of loss of conformational symmetry of α HL pore is claimed to be erroneous.

In this study we form the pore even in 7M urea solution. We did not observe any signature of loss of conformational asymmetry and we think that vestibule of the pore remains stable even at 7M concentration of Urea. We instead find some interesting if the urea is placed on the *cis* or *trans* compartment. (Figure 4.2.4 (a), (b), (c), and (d)). The ionic current through the pore decreases with increasing the concentration of urea for all the cases with respect to placement of urea in the compartments. However, the behavior of decrease in the current is similar for the only *cis* and only *trans* side compare to the case when urea is present on both side. The current symmetry is more when urea is present only on *cis* side.

Figure 4.2.4. Open pore current through α HL nanopore as a function of [Urea]. **(a)** when urea is present on both cis/trans side. **(b)** When Urea is present on *cis* side. **(c)** When Urea is present only on *trans* side. **(d)** Normalized current asymmetry $(1 - \frac{I_-}{I_+})$ % as a function of [Urea].

4.2.5 Conclusion

High salt perturbs the native structure of crystallin protein, which causes partially unfolded populations of the proteins, this partially unfolded populations can be detected by long blockage of ionic current during its interaction with α HL nanopore.

4.3 Polymer Translocation Under Oscillating Electric Field.

4.3.1 Introduction

Aim of the work in this section was to match the translocation time with the frequency of the applied oscillating electric field. We were interested to know what happens to the translocating molecule when the force due to the electric field oscillates at a fixed frequency. We applied a sinusoidal electric potential gradient across the membrane and observed the ionic current when 8 μ M amount of PSS (16kD) molecules were placed in the *cis* compartment.

4.3.2 Results

We find that at the lower frequencies we could detect the events which corresponds to the translocation of PSS molecules. At 1Hz frequency we find events which are more frequent when the current is at the peak (Figure 4.3.1). At higher frequencies the signal due to the translocation is harder to detect. This may be due the fact that measured current has contribution from resistance of the pore and capacitance of the membrane. Current due the resistance of the pore is simply the ratio of applied voltage to the resistance (V/R). However, current due to capacitance is proportional to derivative of applied oscillating voltage and therefore resultant current amplitude increases with increasing the frequency.

4.3.3 Conclusion

The capacitive nature of lipid-bilayer also contributes in the measured current at oscillating electric field and hence causes difficulties in detecting the translocation events at higher frequencies. Because of this difficulty we could not observe the resonance of the applied frequency if the oscillating electric field with the translocation of charged polymer.

Figure. 4.3.1 Representative ionic current signature through the α HL nanopore when $8\mu\text{M}$ NaPSS (16 kD) were placed in the cis compartment and an oscillating electric potential gradient (Base 140mV, varying amplitude) of different frequency was applied. At higher frequencies the signal due to the polymer translocation is difficult to detect due to the capacitive response of the membrane.

4.4 Polymer Translocation in Crowding Environment.

4.4.1 Introduction

Recently it has been proposed that if a mixture of two polymers (one is large and another small) is brought in thermodynamic equilibrium with the nanopore such that only

the smaller molecule can penetrate through the pore, then big polymer can push small polymer inside the pore³¹. This idea is also validated by the experimental investigation with the biological pores³². To extend this idea we investigated whether a translocation of negatively charged polymer (PSS) can be slowed down or fasten by introducing crowded solution of PEG in cis chamber. To test it, we measured the translocation rate of PSS (33.4 kD) at different concentration of PEG (20 kD).

4.4.2 Results

As shown in Figure 4.4.1 the most probable translocation time does not have any significant difference with respect to the amount of PEG introduced in chamber. This may be due to the fact that driving force due to electric field is already very high and therefore forced due to the osmotic stress of the PEG may not be significant enough to increase or decrease the translocation rate of the PSS.

4.4.3 Conclusion

Amount of force induced by crowding agent on the translocating polymer is not sensitive enough to either decrease or increase the electric field driven translocation rate of negatively charged PSS.

Figure. 4.4.1 Translocation time as a function of [PEG] in *cis* chamber for 33.4kD PSS.

4.5 References

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CHAPTER 5

CONCLUSIONS AND FUTURE SCOPE

In this thesis, we have explored using nanopores as tool to investigate macromolecular assembly and transport at single-molecule resolution. First, we have shown that by generating electrical forces on individual charged macromolecules, we can manipulate the motion of two oppositely charged macromolecule so that they can meet and assemble in the α HL protein nanopore. This complexation of the two polymers produces a distinguishable ionic current signature, which is different from those due to the passage of the individual molecules through the nanopore. Furthermore, this ionic current signature allows us to detect the interactions between the two macromolecules and thus, probe the nature of the complex pair. We measured the lifetime of the complex inside the pore, which depends upon the molecular weights of the polycation and polyanion.

After showing that we can isolate a complex pair in the nanopore, we studied the effects of molecular identity (of both the small ions and polyions) and applied field on translocation and complexation dynamics. The following sections outline the main conclusions drawn from the studies performed in this thesis and suggest directions for future research on each topic.

5.1 Complexation between PSS and PLL

The distributions of the measured complex lifetimes under various experimental conditions follow double-exponential form and thus it can be concluded that two distinct processes occur while the complex is inside the pore. The time scales of the two processes were obtained by fitting our collected data, and we found that one of the processes occurs an order of magnitude faster than the other. Both time constants are roughly proportional to

the square of molecular weight of PSS, the polycation, supporting a common “sliding” mechanism occurring during both processes. The faster and slower processes’ time constants are shown to be weakly dependent on voltage, however both time constants seem to agree with our crude theoretical model; and supporting that the faster may be due to complex pair translocation whereas the slower one may be due to the complex pair dissociation, with both process involving sliding of PSS over PLL. Because faster time process requires less energy barrier compare to slower as it require more barrier due to the dissociation. The relative populations of both type of events are weakly voltage dependent but both have significant contributions under almost all studied voltages.

Based on these observations, it can be concluded that the physical nature and strength of the interactions between two oppositely charged macromolecules in a bulk electrolyte solution is fundamentally different from the confined nanopore environment. In the nanopore the physical effects are very local and time dependent, and a global conceptual model is not applicable. For example, in a bulk solution the size of the charged polymer depends upon amount of salt and it can be like gaussian in high salt or approximately rod like in low salt limits.

5.2 Future Scope for the PSS-PLL Complexation

- (1) One possible future direction is to investigate the effects of temperature on the slower and faster modes of the complex lifetime distribution. If the faster mode is due to complex pair translocation, then by decreasing the temperature the faster process may slow down and its relative fraction would also be influenced. Thus, the effect of temperature may give us deeper insight about the physical nature of the fast and slow processes in the complexation dynamics.

- (2) Exploration of voltage-driven complexation with respect to different nanopores architectures will allow us to probe different local environments on the behavior of polyelectrolyte complex. While different protein nanopores will elucidate the effects of various charged patterned environments, solid state nanopores allow us to systematically probe the effects of nanopore diameter on the dynamics of the complex. Solid state nanopores also allow us to apply higher voltages, which might allow for the possible direct detection of dissociation of the complex.
- (3) Exploration of complex life-time with respect to different chiral systems such poly-D-lysine, poly-L/D-arginine will allow us to probe the local orientation of monomer on complex pair dynamics.
- (4) Another possible future experiment is to explore the effects of different electrolyte solutions, which may lead us to know the local role of ions and water in the complexation process. Different ions, may have different hydration shells and hence different numbers of water molecules bound to it.

5.3 Macromolecular Identity vs. Interactions in Complexes

The ionic current signature obtained in the complexation of ssDNA and PLL molecules, suggest there is a weak interaction between ssDNA and PLL when compared to the interaction in a PSS–PLL complex. In contrast to the irreversible conductance state in the PSS–PLL complexation measurement, during ssDNA–PLL complexation we observed a reversible conductance state, which enabled us to measure the rate constants for association and dissociation of PLL with translocating ssDNA and hence apparent equilibrium binding constants. The weaker interactions in ssDNA-PLL may be due to nature of the local

dielectric constant; the negative charges on PSS are on a side group and are thus relatively exposed, whereas in ssDNA the charges are on the backbone and are more hidden. Due to this, the local dielectric constant near the charges is lower in PSS compared to ssDNA and so the electrostatic interactions in the PSS–PLL complex are stronger than in the ssDNA–PLL complex. This conclusion can be further manifested by comparing the translocation time of ssDNA and PSS. Under identical conditions, the same number of monomer of PSS translocate through the α HL pore in 1 ms, whereas ssDNA takes only 0.2 ms for translocation. This is because the charge is exposed in PSS, causing more counter ion condensation and reducing the electrophoretic force on it. On the other hand, in ssDNA charge is relatively hidden, causing less counterion condensation and a higher electrophoretic force, therefore it moves faster through the pore.

Another interesting conclusion we have is that, the binding life-time of ssDNA-PLL tend to follow double exponential probability distribution, suggesting that there are two modes in the dissociation of PLL with translocating ssDNA occurring with two different rate constants. We assign the slower rate constants to be dissociation of PLL with adenine rich region in the ssDNA and whereas faster rate constant to be the dissociation of PLL with cytosine rich region of the ssDNA.

The following future studies are proposed based on the conclusions we have.

5.4 Future Scope for ssDNA-PLL Complexation

- (1) Effect of temperature on ssDNA-PLL interaction may allow us to determine the, temperature effect on association/dissociation rate constant and equilibrium constant. Which may enable us to quantify the energy barriers involved in the

process. Apart from this investigations in other different macromolecular complexes such as ssDNA - polyarginine, polyglutamic acid - polylysine, polyasparticacid - polylysine may allow us to understand the physical nature and strength of the interactions in molecular complexes.

- (2) Exploration of life-time of the binding/unbinding states with respect to different sequence of ssDNA may allow us to check our assigned dissociation rate constants with respect to strength of interactions of lysine with adenine and cytosine. For example, investigations with polyd[A], polyd[T], polyd[C], and polyd[G], with poly-L/D-lysine and poly-D/L-arginine may allow us to probe the nature of interactions in DNA bases and positively charged amino acid residues.
- (3) Another extremely interesting possible future work to use our general idea of voltage-driven polyelectrolyte complexation to explore the effect of DNA and Histone modifications on their binding strength and which can give us valuable insight about the organization and packaging in the nucleosome. Understanding DNA and Histone modification are active area of research in the epigenetics.

5.5 Role of Water in Macromolecular Transport through the Nanopore

We investigated PSS transport through the nanopore in different electrolyte solutions. These experiments found that shows that during the translocation of PSS molecules, the ionic current is more blocked when the cations in electrolyte has bigger hydration shell; bound water molecules occupy more volume in the pore when cation's hydration shell is in association with translocating PSS molecule. These results leave many open questions and provide opportunities for the following future explorations.

5.5.1 Future scope

- (1) How much water molecules are carried by an ion during its transport through a nanopore?
- (2) Does water have a unique role in transporting the molecules between the compartments?
- (3) Increasing salt concentration vs increasing unavailable water? Several experimental investigations have been carried out to control the polymer transport by creating a gradient in salt concentration or controllably varying the salt concentration^{1,2,3}. These experiments open questions, since by changing the salt concentration we also change the number of water molecules which do not participate in hydration. Does this effect also contribute to the transport behavior of macromolecules? In various cases—either polycation, polyanion or neutral polymer—it has been observed that by increasing the salt concentration that the frequency of polymer transport also increases^{4,5}. KCl enhancement of PEG partitioning inside the pore has been interpreted based on the idea that the PEG interacts with the K^+ ions and making it behave like a positively charged polymer⁴. However, we observed KCl enhancement in the capture of proteins, DNA, and synthetic polyelectrolytes. These results are not fully understood and require future work. In simple words, we should devise experiments, and computations to determine if water molecules with small ions can push the big polymer inside the pore? The idea of polymers pushing a polymer have been validated in the recent experiments for three different biological pore⁶. The investigation of idea that small ions in collaboration with water molecules can push a big polymer could potentially be crucial in the fundamental

understanding of the macromolecular transport in confined and crowded cellular environment.

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APPENDIX A

**PROTOCOL FOR FORMATION OF PERPENDICULAR LIPID-
BILAYER AND SINGLE-CHANNEL ELECTRICAL RECORDING.**

Materials

- (a) Polytetrafluoroethylene (PTFE) flow cell from Nanopore Solutions (Portugal).
- (b) Required concentration of (typically 1M) potassium chloride (KCl) solutions in 10 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES) in deionized water (18 M Ω cm). Adjust the pH of above buffer solution by adding potassium hydroxide (KOH) or hydrochloric acid (HCl).
- (c) 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DphPC) from Avanti Polar Lipid (AL). Add 495 μ L of pentane to 5mg DphPC powder sample from Avanti and store it into a freezer below -20⁰C.
- (d) α -toxin from staphylococcusaureus from EMD Millipore (MA). To make its stock solution add 50:50 (v/v) of water and glycerol mixture to make final concentration of 0.5 mg/ml store this solution in a freezer below -20⁰C.
- (e) Silver/silver chloride (Ag/AgCl) electrodes connected to Axopatch 200B integrated patch clamp amplifier (Axon Instruments, CA). pClamp software (Molecular Devices, LLC, MA)) for recording the ionic currents.
- (f) Sodium salt of poly (styrene sulfonate) (NaPSS) (typically 16 Kg/mol) from Scientific Polymer Products, Inc. (NY).

Methods

Preparation of perpendicular lipid bilayer.

Assemble two PTFE chambers (each having volume of 1.2 ml) with two O-rings and a piece of 12.5 μM thick PTFE film between them (Hansatech Instruments Ltd, UK), such that the only connection between two chambers is a 50-100 μM wide aperture positioned at the center of PTFE film. For the ease of making lipid bilayer and low noise, it is good if film aperture is around 50-60 μM wide. Using glass pipette, paint the small amount of 10 % (v/v) of hexadecane in pentane on the both sides of PTFE aperture. Wait around 1 min or dry it such that pentane can completely evaporate. Small amount of the hexadecane on the aperture increases the lipid bilayer stability. Then, fill the both chambers by 10mM HEPES buffer containing 1M KCl just above the aperture level. Add a drop of DphPC lipid solution in pentane at the surfaces of buffer solution in both chambers. Wait a minute to evaporate pentane from the aqueous surfaces. Lower and risen the aqueous surfaces of buffer solution using micropipette to form a suspended lipid bilayer. Now, dipped the electrodes (which are connected to Axopatch instrument) in both chambers with trans side is at positive potential and cis side is grounded.

Check the stability of lipid bilayer (seal test) by applying square wave voltage with 5mV

of amplitudes and 60 Hz frequency across cis and trans chamber using patch clamp amplifier. When a lipid bilayer formed, the square wave changes to Gaussian

Figure A1. Flow cell, containing two chambers (upper one is *trans* and lower one is *cis*). Both chambers are filled with electrolyte solution and connected with electrode such that trans is at positive potential and *cis* is grounded.

Figure A2. Montal-Muller technique for formation of perpendicular lipid bilayer membranes on a small hole on a PTFE thin film partitioning two chambers.

shape peak and no passage of ionic current occurs upon a constant voltage applied (voltage clamp mode). Formation of proper lipid bilayer can be checked by “zapping” on the patch clamp, which deliver high and momentary voltage to the electrode and eliminates an appropriately formed lipid bilayer. If zapping does not remove the bilayer, which means that excessive lipid exists on the aperture, then we repeat the pipetting in and out process until we get proper stable bilayer. Usually, if height of Gaussian shape peaks goes to 10,000 pA, and zapping eliminates the formed lipid bilayer than it means we are getting stable enough membrane.

Formation of alpha-hemolysin pore.

Once a stable lipid bilayer formed, add 2-10 μL of 0.5 $\mu\text{g/ml}$ of α -hemolysin toxin solution (in a KCl/HEPES buffer) near the PTFE aperture (cis side) and monitor the ionic current under 120 mV of constant applied voltage in the voltage clamp mode. Wait till, current rises instantaneously to around 115-120 pA (in room temperature in 1M KCl, 7.5 pH) as shown in fig 3. Rinse the cis chamber immediately with excess amount (typically 200 ml) of fresh buffer solution using syringes connected the PTFE flow cell to avoid formation of multiple pores. After formation of single pore, add desired concentration of

NaPSS (typically 0.5- 8 μM) on cis side to see the translocation event. Turn on the temperature control and set it to the 30°C, wait until open pore current reaches to 135-140 pA (typically takes 45-60 minutes), such that temperature inside the flow cell reaches to 30°C.

Figure A3. Increase in ionic current when single pore formed.

For single molecule complexation of NaPSS and PLL

For complexation experiments between NaPSS and PLL in the alpha-hemolysin pore, every step is same, but we need to put desired concentration of PLL (1.5 – 25 μM) on trans chamber before proceeding for insertion of pore in lipid bilayer. Once, pore is formed then put desired concentration of NaPSS to see NaPSS translocation and complexation events. In our previous experiments we have used PLL (Alamanda polymers) having degree of polymerization ranging from 10, 20 and 30.

See Also

(1) Formation of Bimolecular Membranes from Lipid Monolayers and a Study of Their Electrical Properties, M. Montal and P. Mueller, 3561-3566, **12**, Proc. Nat. Acad. Sci. USA, 1972.

(2) Protein reconstitution into freestanding planar lipid membranes for electrophysiological characterization, Thomas Gutschmann, Thomas Heimburg, Ulrich Keyser, Kozhinjampara R Mahendran and Mathias Winterhalter, 188-198, **10**, Nature Protocol 2015.

APPENDIX B

PROTOCOL FOR THE FABRICATION OF SOLID-STATE NANOPORE

VIA DIELECTRIC BREAKDOWN METHOD.

Materials

- (a) SiN chip having 10-30 nm diameter window (nanopore solution).
- (b) Cassettes to load the chip (nanopore solution).
- (c) Flow cell to assemble cassettes across two reservoirs (nanopore solution).
- (d) Piranha solution.

Below is the complete procedure for mounting the chips in the cassettes and begging an experiment.

Main Principles

The idea with the cassette is that we need to mount the chip once and once only. We should only remove the chip from the cassette if we no longer want to use it, if it is broken or if it is blocked with something that we cannot remove. Otherwise we should not remove the chip ever (exception: if for some reason we wish to treat the chip with acetone or hexane, then we will need to remove it from the cassette first. Acetone causes the sealing rings to expand enormously - sufficient to break the cassette. Hexane seems to cause a degree of swelling too). We must properly wet the chip and remove bubbles before beginning any new treatment or beginning an experiment. If we properly wet the chip before beginning Piranha treatment, then we will find that the Piranha treatment is much more successful. While treating with Piranha it is advisable to stand the cassette on its side (so that the cones are horizontal, and the chip is held vertically within the cassette). Stir/move/rotate the

Figure A4. Key principle to mount the chip into the cassettes and assembling into flow cell.

cassette gently to try to move any bubbles that may be trapped within the cassette. Piranha produces a lot of bubbles. Sometimes these get trapped in the cassette (within the cones or against the chip). Whenever we do Piranha treatment (or anything else that may introduce bubbles) it is advisable to repeat the wetting procedure with ethanol and then water or buffer. If we leave the chip to dry out at any point (not recommended) then we will need to repeat the wetting procedure. If we wish to remove the cassette from the flow cell, then flushing the wells with degassed water several times is recommended. To dismantle the flow cell and remove the cassette. Do not dry the cassette. Place it carefully into a beaker of degassed water and check that there are no bubbles trapped in the cassette. Once the chip is mounted and properly wet you should not need to repeat the wetting procedure. If the chip is treated with anything that introduces bubbles or is dried out for any reason then we will need to repeat the ethanol and buffer or water procedure. Piranha solution is highly recommended to clean the nanopore.

Notes on Piranha Solution

Piranha solution is a mixture of concentrated sulphuric acid (I use 95-97%) and 30% hydrogen peroxide solution. I use a 3:1 ratio of sulphuric acid to hydrogen peroxide. We perform piranha treatment on chips that are mounted in the cassettes. Treatment of the chips by themselves is risky as the treatment generates a lot of bubbles that stick to the chip and cause it to flip in solution. Restraining the chip in the cassette prevents breaking of the

chips during treatment. We use a small beaker that the cassette just fits inside (25ml DURAN beaker). We mix the Piranha solution and then add enough Piranha to cover the entire cassette. We heat the Piranha to ~80C and leave the chips to treat for 10 minutes. Be very careful! Piranha solution is very unpleasant and you should use metal tweezers to handle anything in Piranha solution. When we remove the cassette after the incubation, hold it directly above the beaker to allow any excess solution to "drip" off back into the beaker and then place the whole cassette into a beaker of pure water. Piranha solution produces a lot of bubbles, these need to be removed before beginning an experiment. Gentle stirring or turning of the cassette with tweezers during the 10 minutes helps to remove any bubbles that build up. If the nanopore becomes blocked during an experiment, then use the Piranha treatment to remove the blockage.

Full Experimental Procedure

I recommend Piranha cleaning before starting any experiment, below is my complete procedure for beginning an experiment. Initial mounting of the chip in the cassette: Wetting of the chips is difficult. The surface tension/viscosity of water means that it is difficult to get into the window on the chip. Ethanol has a low viscosity and excellent for wetting of the window and for removal of bubbles. This ethanol is then replaced with water or buffer. When handling the O-rings (especially the small ones), We find it is best to use soft (plastic) tweezers or tools. Repeated use of metal tools will eventually damage them. Assemble the cassette with the chip inside and ensure that there is compression in the O-rings (do not try to tighten too much - Teflon is soft and you will damage the screws or the cassette). Take a pipette and pipette ~60ul of 100% ethanol into the cone on one side of the cassette. Make sure that you do not touch the chip itself with the pipette. Pipette up and

down gently to remove any air bubbles (it really helps to do this under a bright light so that you can clearly see any bubbles). Remove most of the excess ethanol with the pipette (but not all of the ethanol!). Repeat this procedure with a second application of ethanol Repeat this procedure twice with water instead of the ethanol. Remove most of the excess. Turn the cassette over and repeat the ethanol and water steps for the other side. Take extra care with the side that contains the window on the chip. We often need to pipette quite carefully to ensure that there is no bubble trapped in the window.

We now have a cassette that contains a wet chip with a small amount of water associated with each face of the chip. Surface tension is sufficient to keep the water in place and the cassette can be handled without the water being displaced. These steps ensure that the chip is properly wet. If we are unable to measure a current when you apply a voltage, the most likely explanation is that there is an air bubble trapped in the window of the chip or in the opening of the cassette (less likely).

Piranha treatment

Place the cassette into piranha solution and leave on a hotplate (bubbling gently) for ~10 minutes. I find that gently stirring or turning the cassette with tweezers helps to remove any bubbles that build up. I do this in a small beaker with about 15ml of Piranha solution - sufficient to cover the entire cassette. Remove the cassette and rinse carefully with water (best to place it into a beaker of water). Leave in a beaker of water until ready to use.

Beginning an experiment

Remove the cassette from water and carefully dry the faces of the cassette (blot with paper). Remove most of the excess water from the cone on the cassette. Take a pipette and pipette ~60ul of 100% ethanol into the cone on one side of the cassette. Make sure that you do not

touch the chip itself with the pipette. Pipette up and down gently to remove any air bubbles. Remove most of the excess ethanol with the pipette. Repeat this procedure with a second application of ethanol. Remove the excess ethanol and add ~60ul of buffer to each side. Remove the excess and repeat. Again, remove the excess. Repeat for the other side of the cassette. Mount the cassette in the flow cell, add buffer and begin. This looks like a lot, but with practice the ethanol/water, ethanol/buffer steps take about 1 minute and give a much higher chance of successfully mounting the chip and wetting the pore - the success rate of mounting pores is >95%.

Figure A5. Dielectric breakdown method to fabricate the solid-state nanopore. Once the chip is successfully mounted in the cassettes and assembled in the flow cell, upon wetting of the chip a 12V DC pulse is applied across two reservoirs so that dielectric breakdown occurs which will lead to a leakage current³.

In general, only treat the entire cassette and chip in Piranha when I mount it for the first time. For subsequent cleaning steps you will find that removing most of the buffer/solution from the wells of the flow cell and pipetting fresh Piranha solution directly into the flow cell and leaving for 10 minutes is sufficient to remove most blockages. After 10 minutes, carefully remove and dispose of the Piranha solution, rinse with water. Dismantle the flow cell and repeat the ethanol and buffer wetting procedure. Remount the cassette, add buffer

and begin again. This is quicker, safer and uses less Piranha than the more thorough cleaning.

Pore Diameter vs Conductance across the Membrane

For the cylindrical pore, a mathematical model proposed^{1,2}, and was used to connect the observed conductance across the pore with the pore diameter. The relation is given by,

$$G = \sigma \left[\frac{4l}{\pi d^2} + \frac{1}{d} \right]^{-1} , ; \quad (1)$$

Where G, is the conductance across the pore (1/V), σ is the conductivity of bulk solution, d is the diameter of the pore and l is the length of the pore. The conductance across the 20nm thick SiN membrane is given Figure A6. The predicted diameter for this observed conductance is 15nm.

Figure A6. Current vs voltage curve across 20 nm thick SiN membrane, measured in 1M KCl, 5mM HEPES, at 7.5 pH. The predicted (by equation 1) diameter for observed conductance is 15nm.

Figure A7. A comparison for ionic current traces for DNA translocation in reference (4) and with our pore, measured at 120mV in 3M LiCl, 5mM HEPES, at 7.5 pH.

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APPENDIX C

ELECTROSTATIC ENVIRONMENT OF α -HEMOLYSIN NANOPORE

The α -HL protein pore is a heptameric assembly of α -HL molecules with a mushroom shape (Figure A8(a)). In the single-molecule translocation experiments, a single α -HL pore is incorporated into a membrane separating the donor (*cis*) and receiver (*trans*) compartments, both of which contain a buffered electrolyte solution. The configuration of the α -HL pore^{1,2} consists of a vestibule on the *cis* side and a transmembrane β -barrel on the *trans* side. The length of the pore is about 10nm. The opening of the vestibule at the *cis* side is roughly 2.9 nm and the diameter of the vestibule's cavity is roughly 4.1nm. The average internal diameter of the β -barrel is roughly 2nm. The two domains of the pore's lumen are separated by a constriction of 1.5nm. The inner surface of the pore is richly decorated with charged and hydrophobic amino acid residues. For the concentration of 1M monovalent electrolytes, the PNP results³ for the variation of the electric potential from the *cis* side (0mV) to *trans* side (120mV) are presented in Figure A8(b). The pore axis is along the x-axis and the y-axis is normal to the pore axis. The steep gradient in the electric potential is only across the pore, and the electric fields in the *cis* and *trans* chambers are very weak for the electrolyte concentrations used in these calculations. The sharp peaks in Figure A8(b) correspond to the locations of fixed charges of the amino acid residues.

In formulating the free energy landscape for a polymer translocation process through narrow channel in section 2.7 and 2.8, it is assumed that the externally applied electric potential, sourced from the electrodes located far from the channel in each of *cis*

Figure A8. (a) The space-filling model of the α HL protein pore on pH = 7.5. Different shades denote the heterogeneities in charge and hydrophobicity. Blue: positively charged residues. Red: negatively charged residues. Green: hydrophobic residues. Gray: hydrophilic residues. (From Wong, C.T.A., and Muthukumar, M., *J. Chem. Phys.* 2010.) (b) The variation of the electric potential along the axis of the α HL protein pore. (From Kong, C.Y. and Muthukumar, M., *J. Am. Chem. Soc.* 2005.) and *trans* side, changes only inside the channel, linearly along the translocation axis, and that it remains constant in bulk solutions both in *cis* and *trans* even near the channel

entrance and the exit as illustrated in Figure A9. With this model, in each stage, the free energy of a polymer chain translocating through a narrow channel can be formulated by

considering interaction between the polymer segment and channel wall, electrostatic energy gain from the electric field, and conformational entropic penalty.

Figure A9. Illustration of a model for the electric field-driven single file polymer translocation through a narrow cylindrical channel.

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