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DETECTION OF NUCLEOTIDES IN HYDRATED SS-DNA VIA 2-D H-BN
NANOPORE WITH IONIC-LIQUID/SALT-WATER INTERFACE

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DETECTION OF NUCLEOTIDES IN HYDRATED SS-DNA VIA 2-D H-BN
NANOPORE WITH IONIC-LIQUID/SALT-WATER INTERFACE

A Dissertation Presented to the Graduate Faculty of the
Lyle School of Engineering
Southern Methodist University

in

Partial Fulfillment of the Requirements

for the degree of

Doctor of Philosophy

with a

Major in Applied Science

by

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Dec 19, 2020

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Oct 31, 2020

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Advisor: Dr. Min Jun Kim, Committee Chair

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Nanopore sensor, which generally includes two types of platforms: biological nanopore and solid-state nanopore (SSN), has become one of the leading technologies for sensing single molecules. There has been higher attraction to the nanopore research because it is a low cost, time efficient, high throughput, label-free tool for the advanced studies of single molecule analysis. As it showed many successes in detection of various biomolecules, the focus has been overwhelmingly been on sensing and analyzing DNA molecules, *i.e.*, DNA sequencing, due to its importance in human health and well-being. Because of such huge interests, there was the international Human Genome Project between 1990-2003 for thirteen years sponsored by NIH, for decoding human DNA sequence. The project was successfully finished, but it cost roughly \$5 billion with enormous human resources for over a decade. Such huge cost was mainly caused by the limitations in sequencing technologies, *e.g.* short-reading length, low-throughput, complicated process, *etc.* As a comparatively new technology overcoming such drawbacks, nanopore sequencing has been receiving strong attention as a next-generation sequencer for reducing the general cost of DNA sequencing.

Application of nanopore for DNA sequencing has been successful using biological channel protein, *i.e.*, biological nanopore, which is evident through its commercially available devices. The initial demonstration for the use of biological nanopore was not much different from the case of SSN; it was limited to a strand-level detection, permitting one pulse per translocating strand due to the fast translocation speed and low electrical sensitivity. De-

spite these hurdles, the biological nanopore could dramatically increase its resolution with the assistance of genetic and protein engineering, and it succeeded in reading the sequence of the DNA. Although there has been an impressive advance in biological nanopore application for DNA sequencing, a few fundamental limitations still exist, *e.g.* low base-calling accuracy, low reading stability, and the weak structure of the protein channel with the lipid membrane. Thus, it is worth putting more research efforts on solid-state nanopores due to their characteristic strengths over the biological nanopore, *e.g.* full-synthetic body without biological components, compatibility to semiconductor based mass-production facility, superior material stability for longer usage, *etc.* Thus, as biological nanopore has been through, SSN requires such leap for stretching towards DNA sequencing, as shown by biological counterpart. During the research for the Ph.D. degree, I have focused on the development of solid-state nanopore to improve its spatial and temporal resolutions to get a step forward to the realization of DNA sequencing using solid-state nanopore.

All the basic and practical knowledges were described in the first chapter 1 to 3 that I have acquired in the laboratory based on the experiences and literature in detail. In Chapter 1, the basic nanopore device platform is introduced, which are generally used for the nano-particle analysis, including DNA sample. In chapter 2, the basic nanopore fabrication procedures are introduced, including 2-D h-BN nanopore fabrication and its characterizations. In chapter 3, the nanopore sensing experiments are introduced, including the general experimental preparation and the electrical nanopore characterizations.

Chapter 4 includes the main researches of the nucleotide detection using $\text{TiO}_2/\text{h-BN}$ nanopore with $[\text{bmim}][\text{PF}_6]/\text{KCl}$ viscosity/ionic gradient interface. The data analyses are presented with their theoretical studies. Also, Computational studies using COMSOL Multiphysics simulations were included to support the data analyses. I tried to include further studies in the chapter 5, wherein possible additional experiments were described to improve the current experimental platform. Chapter 6 concludes the dissertation with the summery of the studies executed herein.

TABLE OF CONTENTS

LIST OF FIGURES	ix
LIST OF TABLES	xviii
CHAPTER	
1. INTRODUCTION	1
1.1. Nanopore Sensor	1
1.1.1. General Components of Nanopore Sensor	1
1.1.2. General Solid-State Nanopore Fabrication Methods	6
1.1.3. Materials for Solid-State Nanopore Chips	11
1.1.4. Experimental Noise	12
1.2. Nanopore DNA Sequencing	14
1.2.1. Biological Nanopore Sequencing	14
1.2.2. Solid-State Nanopore Sequencing	15
2. FABRICATION OF NANOPORE DEVICE	17
2.1. Fabrication of Si_xN_y Supporting Window	18
2.1.1. Deposition of Si_xN_y Membrane	18
2.1.2. Photolithography and Dry Etching	18
2.1.3. Wet Etching	20
2.1.4. TEM Pore Fabrication	21
2.2. h-BN Transfer	22
2.2.1. h-BN Preparation	23
2.2.2. h-BN Transfer	24
2.2.3. ALD TiO_2 Deposition	25
2.3. h-BN Characterization	26

2.3.1.	Atomic Structure of h-BN	27
2.3.2.	SAED Pattern	27
2.3.3.	Atomic Distance Calculation	28
2.3.4.	Contact Angle Measurement.....	29
2.4.	TEM Nanopore Fabrication	30
2.4.1.	TEM Beam Alignment	31
2.4.2.	TEM Nanopore Drilling	32
3.	NANOPORE EXPERIMENTS	36
3.1.	Experiment Preparation	36
3.2.	Nanopore Characterization	38
3.2.1.	Nanopore Noise	39
3.2.2.	Baseline Stability	40
4.	DETECTION OF NUCLEOTIDES USING SOLID-STATE NANOPORE	42
4.1.	Purpose and Strategy	42
4.1.1.	Purpose.....	42
4.1.2.	Strategy	42
4.2.	Methods	44
4.2.1.	h-BN Nanopore Fabrication	45
4.2.2.	Experimental Preparation	49
4.2.3.	Data Acquisition and Analysis.....	52
4.2.4.	COMSOL Simulation	52
4.3.	Results	54
4.3.1.	Viscous/Ionic Gradient Interface.....	56
4.3.2.	DNA Translocation Events	57
4.3.3.	Data Analysis.....	62

4.3.3.1.	Translocation Time (Δt) and Peak Amplitude (ΔI).....	62
4.3.3.2.	Base-Translocation Analysis.....	66
4.3.3.3.	Translocation Speed Analysis.....	72
4.3.3.4.	Nanopore Sensitivity and Hydrated ssDNA	72
4.3.3.5.	COMSOL Multiphysics for Hydrated ssDNA Translocation	75
4.3.4.	Discussion	77
5.	FUTURE STUDIES.....	83
5.1.	Detection of Nucleotides in Longer ssDNA	83
5.1.1.	Denaturation and Linearization of DNA	84
5.1.2.	Thermal Gradient-Assisted Electrophoresis	85
5.2.	Discriminating the Resistive Pulses of Each Type of Nucleotides	86
5.2.1.	Optimization of Nanopore size.....	87
5.2.2.	Optimization of Molecular Conductivity of ssDNA with Different Lengths	88
6.	SUMMARY AND CONCLUSION	90
6.1.	Summary	90
6.1.1.	2-D h-BN Nanopore.....	90
6.1.2.	Viscosity/Ionic Gradient Interface	90
6.1.3.	High-Conductive Hydrated ssDNA.....	91
6.2.	Conclusion	91
	REFERENCES	92

LIST OF FIGURES

Figure		Page
1.1	Schematic figure for DNA translocation through a solid-state nanopore. The inset figure is a TEM micrograph of h-BN nanopore.....	2
1.2	(a) Representative 200 ms current trace of 92-nt DNA translocation. The dots at the tip of each peak are marked by custom-built automatic analyzing software (MATLAB). (b) Each event is characterized by current drop (ΔI), translocation time (Δt), and inter-event time (δt).....	2
1.3	Scatter plot of peak amplitude <i>vs.</i> translocation time for 92-nt DNA events at 0.5 V (red) and 0.8 V (blue).	3
1.4	The nanopore chip (purple in Figure (a)) was assembled in the flow cell (not to scale). After the injection of the solution into the chambers (blue in Figure (a)), the Ag/AgCl reference electrodes were inserted to the channels. Through the electrodes, the Axopatch 200B (Figure (b)) from Molecular Devices clamped the voltage and measured the current. The amplified current signal was digitized by a digitizer (DAQ board (BNC-2110 from National Instruments, shown in Figure (c)) or Digidata from Molecular Devices) and then processed by a software (custom-made LabVIEW program or clampex program from Molecular Devices (Figure (d))).....	4
1.5	Schematic image of nanopore fabrication using Ga^+ FIB	7
1.6	(a) SEM image of the 200 nm thick Si_xN_y free-standing window of $50 \times 50 \mu m^2$. (b) SEM image of a FIB drilled Si_xN_y nanopore of 250 nm in diameter.	7
1.7	(a) TEM image of the Si_xN_y membrane with a ~ 8 nm nanopore drilled by electron beam of TEM at 200 kV. (b) TEM image of the Si_xN_y membrane with a ~ 30 nm nanopore drilled by electron beam of TEM at 200 kV.	9
1.8	An example of CAD design for the chip loading tip to TEM sample holder.	9
1.9	Schematic image of h-BN membrane structure with ssDNA	10
1.10	(a) TEM micrograph of TiO_2 /h-BN membrane on ~ 30 nm Si_xN_y window. (b) TEM micrograph of ~ 3 nm TiO_2 /h-BN nanopore on TiO_2 /h-BN membrane.	12

1.11	(a) External set-up for nanopore platform. (b) Flowcells wired to the head-stage pre-amplifier in the Faraday cage.	14
2.1	Schematic figure of ssDNA translocation event through the 2-D h-BN nanopore	17
2.2	(a) Schematic image of the cross-section of the (100) Si etched with KOH. (b) The optical view of the (100) Si after the backside KOH wet-etching. ...	19
2.3	(a) An example of CAD design for nanopore chip photolithography. (b) Schematic figure explaining the principle of photolithography.	20
2.4	(a) KOH Si wet-etching set-up. (b) 4 inch Si_xN_y coated Si wafer after the wet-etching process. (c) Backside view of the nanopore chip after the wet-etching process.	21
2.5	TEM micrograph of ~ 40 nm nanopore on 50 nm thick Si_xN_y membrane	22
2.6	h-BN transferred Si_xN_y chips on a silicon boat right before the furnace step. The rectangular h-BN on the chip is visible by the light reflection.	25
2.7	(a) TEM micrograph of the case of unsuccessful ALD TiO_2 coating with a bad image contrast. (b) TEM micrograph of the case of successful TiO_2 coating with a better image contrast.	26
2.8	(a) TEM micrograph of h-BN membrane. (b) Zoomed-in TEM micrograph of h-BN.	27
2.9	SAED pattern of (a) CVD-grown h-BN single layer, (b) a few h-BN layers with different directions (no stacking direction), (c) h-BN multi-layers with different directions (no stacking direction)	28
2.10	Measurement of atomic distance using image contrast of TEM image. (a) TEM micrograph of h-BN where to take the image contrast. (b) The image contrast measurement, showing the strong and weak values.	29
2.11	Contact angle measurements for each step of building $\text{TiO}_2/\text{h-BN}/\text{Si}_x\text{N}_y$ (UV- O_3) using a goniometer. Each contact angle was acquired by averaging the the measurements from twelve $1\ \mu\text{L}$ water droplets. Each case represented (a) Si_xN_y (39.2°), (b) $\text{h-BN}/\text{Si}_x\text{N}_y$ (45.3°), (c) Si_xN_y with UV- O_3 (9.3°), (d) $\text{h-BN}/\text{Si}_x\text{N}_y$ with UV- O_3 (24.3°), (e) $\text{TiO}_2/\text{Si}_x\text{N}_y$ (48.5°), (f) $\text{TiO}_2/\text{h-BN}/\text{Si}_x\text{N}_y$ (62.0°), (g) $\text{TiO}_2/\text{Si}_x\text{N}_y$ with UV- O_3 (7.5°), and (h) $\text{TiO}_2/\text{h-BN}/\text{Si}_x\text{N}_y$ with UV- O_3 (18.1°).	30
2.12	General procedure of TEM alignment.	32
2.13	(a) TEM micrograph of $\text{TiO}_2/\text{h-BN}$ membrane in focus with (b) its FFT image. (c) TEM micrograph of $\text{TiO}_2/\text{h-BN}$ membrane out of focus with (d) its FFT image.	33

2.14	TiO ₂ /h-BN membrane (a) before drilling the nanopore, (b) after drilling the nanopore with larger diameter of ~ 7 nm. TiO ₂ /h-BN membrane (c) before drilling the nanopore, (d) after drilling the nanopore with ~ 3 nm in diameter.	35
3.1	An example of power spectral density (PSD). Red and blue graphs represent the PSD of Si _x N _y and TiO ₂ /h-BN nanopore, respectively. The Si _x N _y nanopore had 200 nm thickness and 250 nm in diameter. The TiO ₂ /h-BN had ~ 1 nm thickness and ~ 4 nm in diameter. The PSDs were acquired using 4 s current baselines at 0 V. The data were recorded at 200 kHz frequency and filtered with 10 kHz low-pass Bessel filter.	40
4.1	3-D atomic structure of (a) ssDNA with h-BN nanopore and (b) the components of ssDNA that include the phosphate backbone (dashed red circle), ribose sugar (dashed black circle), and nucleobase (dashed blue circle). There are four types of nucleobases, <i>i.e.</i> , adenine, guanine, thymine, and cytosine. Adenine and guanine are purine bases with two-ring structure, and thymine and cytosine are pyrimidine bases with one-ring structure. The images were drawn using 3-D illustration software of visual molecular dynamics ver. 1.9.3.	43
4.2	TiO ₂ /h-BN nanopore fabrication flow chart. (a) Si _x N _y /SU-8 chips were supplied by Protochips, Inc. (a-1) and (a-2) show the top- and sidecut-view of the chip, respectively. The 50 nm thick Si _x N _y membrane (purple) and the 5 μ m thick SU-8 insulation layer (green) were built on the 300 μ m Si wafer (gray). Si _x N _y membrane had a 50 $\times$ 50 μ m ² window on the silicon wafer, and the SU-8 had a 30 $\times$ 30 μ m ² square cut on the Si _x N _y membrane. (b) ~ 40 nm hole was drilled on the Si _x N _y free-standing membrane with 200 kV electron beam of TEM. (c-1) CVD-grown single-layer h-BN was purchased from Graphene Laboratories, Inc. (c-2) The backside of h-BN was etched with the reactive ion etcher (RIE). (c-3) PMMA layer was coated on h-BN using a spin coater. (c-4) The Cu foil was etched with 0.5 M ammonium persulfate. (c) PMMA-coated single layer h-BN was transferred on the top of Si _x N _y . (d) The PMMA layer was removed with acetone and the following furnace baking at 350° with Ar gas flow. (e) The 2nd layer of h-BN was transferred on to the 1st layer h-BN using the same steps outlined in (c-d). (f) ~ 0.5 nm thick TiO ₂ layer was deposited using ALD (Savannah S100, Veeco/cnt). (g) 3-4 nm pore was made by exposing the focused electron beam of TEM at 200 kV.	47

4.3	(a) TEM micrograph of TiO ₂ /h-BN bilayer nanopore (JEM-2100F TEM, JEOL Ltd.). Dashed magenta circle indicates the location of the nanopore. (b) TEM micrograph showing the hexagonal structure of boron nitride (h-BN) (JEM-ARM 200F, JEOL Ltd.). The image was processed using an average background subtraction filter (ABSF). (c) TEM image of h-BN monolayer before processing the average background subtraction filter (ABSF) (JEM-ARM 200F, JEOL, Ltd.). (d) SAED pattern of crystalized structure of h-BN.(e) The fast-Fourier transform (FFT) of the Figure (c). The dots are clearly matching the SAED pattern in Figure (d).	48
4.4	(a-1, b-1, c-1) TEM micrographs of the nanopores and (a-2, b-2, c-2) I-V curves with the linear fittings for 10-nt, 50-nt, and 92-nt samples, respectively. The initial pore conductance for each nanopore was (a-2) 9.1 nS for 10-nt, (b-2) 23.1 nS for 50-nt, and (c-2) 14.2 nS for 92-nt. The pore conductances (G) of each nanopore were derived from the slopes of the linear fittings.	50
4.5	The power spectral density (PSD) at different biases for each nanopore, (a) 10-nt, (b) 50-nt, and (c) 92-nt. The PSD graphs were taken from 1.0 s baselines at different levels of biases from 0 V to 0.8 V. The nanopores showed higher stability at low bias and they became noisier as the bias got higher.	51
4.6	Detection of base-translocations using a custom-built analyzing program in MATLAB scripts. The baseline (orange line) was calculated for every certain length of the data. (a) Peak amplitude (ΔI) was measured by the difference from the baseline to the minimum point of the peak. (b) Strand-translocation time (Δt) was measured by the period between the maximum points of two consecutive entrance current bursts. (c) base-translocation time (Δt_{base}) was calculated by the full-width at the half maximum (FWHM) of the peak. (d) Inter-base-translocation time (Δt_{base}) was measured by the period between the minimum points of two consecutive base-peaks. (e) Number of base-translocations per strand (n_{base}) was counted by the number of base-translocations between the two consecutive entrance/exit current bursts.	53

- 4.7 (a) Schematic figure for $\text{TiO}_2/\text{h-BN}$ nanopore sensor with $[\text{bmim}][\text{PF}_6]/\text{KCl}$ interface. (b) Zoomed-in view of the nanopore sensor, where ssDNA is driven by the diffusion ($r > r^*$) and electrophoresis ($r < r^*$) from the high viscous ionic-liquid $[\text{bmim}][\text{PF}_6]$ (*cis*) to the 2.0 M KCl aqueous solution (pH 8.0) (*trans*). (c-e) Results of COMSOL simulation for (c) electrical potential field of the axisymmetric nanopore system, (d) ionic concentration distribution of each ion and (e) electric field magnitude along the z-axis at the center of nanopore. (f-h) Representative current traces of (f) 10-nt, (g) 50-nt, and (h) 92-nt ssDNA translocations. They have the same scale bar for the time x-axis. 58
- 4.8 (a) Molecular structure of $[\text{bmim}][\text{PF}_6]$. (b) Immiscibility of $[\text{bmim}][\text{PF}_6]$ and H_2O . $[\text{bmim}][\text{PF}_6]$ and H_2O built a boundary layer in the 15 ml glass vial, because the solubility of H_2O to $[\text{bmim}][\text{PF}_6]$ is $\sim 2\text{-}3\%$, *i.e.*, $\sim 30\ \mu\text{l}$ water in 1 ml $[\text{bmim}][\text{PF}_6]$. The liquid below was 3 ml $[\text{bmim}][\text{PF}_6]$, and the above was 3 ml pure water. 59
- 4.9 COMSOL simulation results for $[\text{bmim}][\text{PF}_6]/2.0\ \text{M KCl}$ interfacial system, showing the ionic concentrations of (a) K^+ , (b) Cl^- , (c) $[\text{bmim}]^+$, and (d) $[\text{PF}_6]^-$ and ionic fluxes of (e) K^+ , (f) Cl^- , (g) $[\text{bmim}]^+$, and (h) $[\text{PF}_6]^-$. The intensity plots are the time-independent 2-D finite element simulations (COMSOL Multiphysics ver. 5.4) using Poisson-Nernst-Planck-Stokes system. The geometry consists of a *cis* reservoir ($40\ \text{nm} \times 50\ \text{nm}$) defined as the ionic-liquid $[\text{bmim}][\text{PF}_6]$ with a viscosity of $0.382\ \text{Pa}\cdot\text{s}$, while a *trans* reservoir ($40\ \text{nm} \times 50\ \text{nm}$) was defined as an aqueous 2.0 M KCl solution. The reservoirs were separated by a 1 nm thick layer ($0.5\ \text{nm TiO}_2/0.5\ \text{nm h-BN}$) with a 2.0 nm radius nanopore. An electrophoretic bias of $+0.8\ \text{V}$ was applied to the bottom of the simulation box, while its top was grounded. $-0.02\ \text{C}\cdot\text{m}^{-2}$ surface charge density was assigned to the surface of the TiO_2 , and $0\ \text{C}\cdot\text{m}^{-2}$ on h-BN. The boundary conditions were set as open boundaries for the laminar flow, an electric potential and ground at the bottom and top of the geometry for the electrostatics, and no charges on the sides, and initial concentrations on the boundaries of 4.8 M $[\text{bmim}][\text{PF}_6]$ for the *cis* side and 2.0 M KCl for the *trans* side for the transport of diluted species. 60

4.10	Base-translocations of 10-nt ssDNA vs. strand-translocations of 50-nt and 92-nt ssDNAs. Schematic figures for translocations of 10-nt, 50-nt, and 92-nt are shown in the first row (a-1, b-1, and c-1). Dashed red lines at the nanopore represent the [bmim][PF ₆]/KCl interface. Translocation events of 10-nt have a steady interface, but those of 50-nt and 92-nt had unsteady interfaces due to the radius of gyration (r_g) larger than the nanopore. The second row (a-2, b-2, and c-2) shows 20 ms of translocation events for 10-nt, 50-nt and 92-nt, respectively. The third row (a-3, b-3, and c-3) shows 5 ms of raw data for 10-nt, 50-nt, and 92-nt, respectively, which are the zoomed-in views of the dashed red boxes on the second row. The inter-base-translocation time (δt_{base}) is marked on (a-3).....	63
4.11	Figures (a-1, b-1, c-1) show the real-time currents of each nanopore loaded with the samples (10-nt, 50-nt, and 92-nt, respectively) at various biases. Figures (a-2, b-2, c-2) show the zoomed-in views of the translocation events for each sample (10-nt, 50-nt, and 92-nt) at higher voltages ($\Delta V > 0.8$ V). Figure (a-2) shows the zoomed-in view of the dashed red box in (a-1), presenting the 10-nt translocation events at 0.8 V. Figure (b-2) shows the 50-nt translocation events at 1.0 V. (c-2) shows the zoomed-in view of the dashed red box in (c-1), presenting the 92-nt translocation events at 1.0 V. (d-1) The initial entrance current burst and (d-2) the final exit current burst of 10-nt translocation train.	64
4.12	(a-e) The representative 0.5 s current traces of 10-nt translocation events through the TiO ₂ /h-BN nanopore with [bmim][PF ₆]/KCl 2.0 M viscosity/ionic gradient interface.	65
4.13	Scatter plots of peak amplitude (ΔI) vs. translocation time (Δt_{base} or Δt) with the corresponding histograms. The mean ($\bar{X}$) and the standard deviation (σ) of ΔI were (a) $\bar{X} = 1.4517$ nA, $\sigma = 0.2863$ nA (10-nt, $n = 22,145$), (b) $\bar{X} = 1.5799$ nA, $\sigma = 0.9554$ nA (50-nt, $n = 11,014$), (c) $\bar{X} = 0.3407$ nA, $\sigma = 0.0540$ nA (92-nt, $n = 10,598$). The mean ($\bar{X}$) and the standard deviation (σ) of translocation time (Δt_{base} or Δt) were (a) $\bar{X} = 0.1220$ ms, $\sigma = 0.0207$ ms (10-nt), (b) $\bar{X} = 0.0969$ ms, $\sigma = 0.0453$ ms (50-nt), and (c) $\bar{X} = 0.3385$ ms, $\sigma = 0.0948$ ms (92-nt).	66
4.14	Histograms for (a) inter-base time of 10-nt ($n = 19,624$, $\bar{X} = 1.4354$ ms, $\sigma = 0.1168$ ms), (b) translocation time of 10-nt ($n = 2,830$, $\bar{X} = 12.5512$ ms, $\sigma = 14.8852$), (c) translocation speed of 10-nt ($n = 2,440$, $\bar{X} = 1.8408$ ms·nt ⁻¹ , $\sigma = 0.4954$ ms·nt ⁻¹), and (d) normalized peak amplitude ($\Delta I/I_0$) for 10-nt (light gray, $n = 22,145$, $\bar{X} = 0.0616$, $\sigma = 0.0093$) and 92-nt (dark gray, $n = 10,598$, $\bar{X} = 0.0234$, $\sigma = 0.0037$). All histograms are fitted by the normal distributions.	68

- 4.15 (a) The histogram of the number of the base-translocations per strand (n_{base}) of 10-nt with normal distribution ($n = 2,440$, 8.6 ± 19.4 bases per strand). (b) An example of skipping small current bursts (dashed green circles), compared to the normal current bursts (dashed black circle). Three small current bursts were skipped and four strands were counted to be one strand. The red numbers in the parentheses were the number of base-translocation per strand (n_{base}) if the current bursts were detected by the custom-built analysis program written in MATLAB scripts. (c) A schematic figure in the case of the longer number of base-translocation per strand ($n_{base} > 10$). The distance between the two molecules are too close to discern one from the other by the system. (d) A schematic figure in the case of the normal detection of two ssDNA molecules when there is an enough spacing between the molecules. 69
- 4.16 The representative current traces of the loose-translocations events of 10-nt ssDNA through the $\text{TiO}_2/\text{h-BN}$ nanopore with $[\text{bmim}][\text{PF}_6]/\text{KCl}$ 2.0 M viscosity/ionic gradient interface. Figure (a-2) is the zoomed in image of the dashed red box in Figure (a-1), presenting the method how the inter-DNA time (δt_{DNA}), translocation time (Δt), inter-base translocation time (δt_{base}), base-translocation time (Δt_{base}) were analyzed using the custom-built MATLAB program. 70
- 4.17 The histograms of the loose-translocations, *i.e.*, translocations with longer inter-DNA duration, of 10-nt ssDNA. (a) The histogram of the number of base-translocations per strand (n_{base}) with normal distribution ($n = 292$ strands, $\bar{X} = 7.4 \pm 15.4$ bases per strand). (b) The histogram of the inter-base translocation time (δt_{base}) with normal distribution ($n = 2,185$ bases, $\bar{X} = 1.6 \pm 0.2$ ms). (c) The histogram of the translocation time (Δt) ($n = 292$ strands, $\bar{X} = 10.8493 \pm 20.9516$ ms). (d) The histogram of translocation speed with normal distribution ($n = 292$ strands, $\bar{X} = 1.5491 \pm 0.7844$ ms $\cdot$ nt $^{-1}$). 71
- 4.18 (a) The histogram of 50-nt peak amplitudes (ΔI) with the two Gaussian fittings ($\bar{X}_1 = 1105.24 \pm 560.4$ pA, $\bar{X}_2 = 3477.75 \pm 997.48$ pA, total $n = 11,014$). (b) The schematic drawing for the shorter (case 1) and larger (case 2) peak amplitudes. Due to the larger size of nanopore device (~ 5 nm in diameter), there could be the translocations of folded ssDNA. 74

- 4.19 The representative current traces of the larger and the smaller translocation base-peaks of 10-nt ssDNA through TiO₂/h-BN nanopore with [bmim][PF₆]/KCl interface. Figure (a) shows the current trace with the larger and the smaller base-peaks together. Figure (b) and (c) show the smaller and the larger base-peaks, respectively. (d) The histograms in probability density function to compare the peak amplitudes of the normal, smaller, and larger base-peaks in a graph with the normal distributions. (Normal base-peaks have a distribution of 1.4517 ± 0.4572 nA ($n = 22,109$), smaller base-peaks 0.4387 ± 0.4329 nA ($n = 2,143$), and larger base-peaks 2.5796 ± 0.9185 nA ($n = 1,033$)). 76
- 4.20 Results of computational simulations using COMSOL Multiphysics. Time-independent static simulations depending on the locations of the ssDNA along the z-axis were studied individually. The electric flux ($\Phi / \text{V}\cdot\text{m}$) was derived at the center area of the nanopore for (a) H₂O-ssDNA and (b) IL-ssDNA. The inset figures are the geometry of ssDNA located at 5 nm from the nanopore in the middle of the translocation. [bmim][PF₆], H₂O, TiO₂, and h-BN are colored in yellow, light blue, gray, and white, respectively. The dashed magenta line indicates the region where the electric flux was calculated. The specific information for the simulation was described in Figure 4-21 in detail. 79
- 4.21 (a) The geometry of COMSOL Multiphysics simulations for ssDNA translocations. The basic physics for solving the system were the same with the case without ssDNA structure in Figure 4-9. The 30 nm $\times$ 50 nm chamber was used for each side. The length of ssDNA was 10 nm with ten nucleotides of 0.125 nm thickness with rounded corners, and the distance between each nucleotide was 1 nm. TiO₂/h-BN nanopore was built on 1 nm thick membrane, *i.e.*, 0.5 nm thick TiO₂ and 0.5 nm thick h-BN. The material property of TiO₂ was given to the *cis* side of the membrane, *i.e.*, $\epsilon_{\text{TiO}_2} = 8$, $\rho_{s,\text{TiO}_2} = -0.02 \text{ C}\cdot\text{m}^{-2}$, and h-BN to the *trans* side, *i.e.*, $\epsilon_{\text{hBN}} = 4.6$, $\rho_{s,\text{hBN}} = 0 \text{ C}\cdot\text{m}^{-2}$. The surface charge density of SixNy was also given to the right wall of the *trans* chamber (red line) to immitate the SixNy membrane, *i.e.*, $\rho_{s,\text{SiN}} = -0.08 \text{ C}\cdot\text{m}^{-2}$. (b) Water-bound ssDNA, *i.e.*, H₂O-ssDNA, in light blue color. (c) [bmim][PF₆]-bound ssDNA, *i.e.*, IL-ssDNA, in yellow color. (d) The region of interest for the electric flux at the center area of the nanopore (dashed magenta line). The graph (Figure 4-20(a)) shows the electric flux depending on the locations of ssDNA, moved by 0.5 nm along the z-axis for each simulation (e). 80

4.22	The representative current traces of 92-nt translocation events at the applied biases of (a) 0.6 V, (b) 0.7 V, and (c) 0.8 V. (d) The histograms in probability distribution function for peak amplitudes (ΔI) at 0.6 V (green), 0.7 V (yellow), and 0.8 V (red) with normal distribution fittings ($\overline{X_{0.6V}} = 0.2139 \pm 0.0428$ nA (n = 3,037), $\overline{X_{0.7V}} = 0.2522 \pm 0.0546$ nA (n = 3,167), and $\overline{X_{0.8V}} = 0.3195 \pm 0.0966$ nA (n = 2,975)). There was a positive correlation between the bias and the peak amplitudes (ΔI). (e) The histograms in probability distribution function for translocation time (Δt) at 0.6 V (green), 0.7 V (yellow), and 0.8 V (red) with normal distribution fittings ($\overline{X_{0.6V}} = 0.3032 \pm 0.2240$ ms, $\overline{X_{0.7V}} = 0.3074 \pm 0.1850$ ms, and $\overline{X_{0.8V}} = 0.3237 \pm 0.2324$ ms). There was no specific correlation between the bias and the translocation time (Δt).	82
5.1	Schematic figure of heat control system with 2-D nanopore platform.	85
5.2	Schematic figure of temperature control system for heating the <i>cis</i> chamber and cooling the <i>trans</i> chamber with 2-D nanopore platform.	86
5.3	(a) STEM micrograph of h-BN membrane. The inset image is the SAED pattern of transferred h-BN membrane. (b) Zoomed-in STEM micrograph with the schematic nanopore fabrication procedure using an atomic-size STEM probe (0.05-0.2 nm).	88

LIST OF TABLES

Table	Page
4.1 The variables for COMSOL Multiphysics simulations	55
4.2 The material properties for COMSOL Multiphysics simulations	56
4.3 The derivation of the normalized electric flux ($\overline{\Delta\Phi/\Phi_0}$)	78

This is dedicated to my beloved wife, Abigail,
my warm-hearted parents, S. M. Lee and H. S. Song,
and the Lord before whom I stand.

Chapter 1

INTRODUCTION

1.1. Nanopore Sensor

The history of nanopore sensor goes back to 1953 when Wallace H. Coulter (1913-1998) invented the Coulter counter, which has been used for counting and sizing the red blood cells of the blood sample in almost every hospital around the world. Nanopore sensor is basically $\sim 1,000$ times smaller version of the Coulter counter to analyze the nano-scale single molecules instead of the micron-scale cells. It measures the electrical resistive pulses to the open pore current while the molecules translocate the nanopore (Figure 1-1) [1–4]. By analyzing the shape of the pulse, a multitude of information about the molecule could be derived; for example, the size from the peak amplitude (ΔI), the length from the translocation time (Δt), the event frequency from the inter-translocation time (δt), *etc.* (Figure 1-2, 1-3).

1.1.1. General Components of Nanopore Sensor

Nanopore sensor platform is made up of several components, including nanopore chip, flowcells, ionic fluids, electrodes, Faraday cage, amplifier, digitizer, and computer with a controlling software (Figure 1-4). There are two types of nanopore chips; biological nanopore and solid-state nanopore. Biological nanopores are made up of biological channel protein which is inserted in bilipid membrane layer. Since there are only a few types of biological nanopores available, the applicable samples are also restricted by their sizes and shapes. Solid-state nanopores are different from biological nanopores, and they were manually built by silicon-based manufacturing processes, including CVD, photolithography, wet- and dry-etching, *etc.* The nanoscale membranes are grown on silicon wafer, and the membrane and the wafer are etched by dry- or wet-etchants, with the help of soft-lithography methods.

Figure 1.1. Schematic figure for DNA translocation through a solid-state nanopore. The inset figure is a TEM micrograph of h-BN nanopore.

Figure 1.2. (a) Representative 200 ms current trace of 92-nt DNA translocation. The dots at the tip of each peak are marked by custom-built automatic analyzing software (MATLAB). (b) Each event is characterized by current drop (ΔI), translocation time (Δt), and inter-event time (δt).

Figure 1.3. Scatter plot of peak amplitude *vs.* translocation time for 92-nt DNA events at 0.5 V (red) and 0.8 V (blue).

Based on the experimentalist's interested analyte, the size and shape of the nanopore could be custom-made, which is the fundamental difference, and also the strength of the solid-state nanopore.

There are many types of flowcells available in the field of nanopore research. Traditionally, poly(dimethylsiloxane) (PDMS) has been widely used for a microfluidic channel [5] and nanopore research due to its advantages in clear vision (refractive index (n) = 1.43) [6], well-established fabrication protocols using soft-lithography, and good electrical stability with low dielectric constant ($\epsilon_0 \approx 2.3$) [7]. Polycarbonate is another type of material for the flowcells with nanopore chips [8]. It has similar material properties to PDMS, but with much higher mechanical stability (Young's modulus (E) = 2.0-2.4 GPa) than PDMS. Due to its strong mechanical property, CNC machine could be utilized for the accurate structure by an automated motorized platform. Polytetrafluoroethylene (PTFE or Teflon) has also been very popular for the flowcells, since it has mechanical and electrical stabilities as good as polycarbonate, with much better chemical stability, by which it could be even cleaned using Piranha solution (Conc. H_2SO_4 : H_2O_2 = 3 : 1). Since the flowcells with the *cis* and *trans* chambers should be cleaned thoroughly before running any type of nanopore experiment, it

Figure 1.4. The nanopore chip (purple in Figure (a)) was assembled in the flow cell (not to scale). After the injection of the solution into the chambers (blue in Figure (a)), the Ag/AgCl reference electrodes were inserted to the channels. Through the electrodes, the Axopatch 200B (Figure (b)) from Molecular Devices clamped the voltage and measured the current. The amplified current signal was digitized by a digitizer (DAQ board (BNC-2110 from National Instruments, shown in Figure (c)) or Digidata from Molecular Devices) and then processed by a software (custom-made LabVIEW program or clampex program from Molecular Devices (Figure (d))).

has been used by many nanopore researchers. However, Teflon is not transparent as PDMS or polycarbonate due to its high crystallinity, it could not be the best choice for some experiments requires the visibility of the filled-in solutions.

For the electrodes, silver (Ag)/silver chloride (AgCl) has been widely used in the nanopore research. The electrodes are connected to each chamber of each flowcell; the electrode connected to the *cis* chamber is used for the voltage application, and the electrode connected to the *trans* chamber is for the reference. There is a reversible electrochemical reaction occurs at the electrode. When the electrode applies positive voltage (anode), oxidation reaction occurs by $Ag(s) + Cl^- \rightarrow AgCl(s) + e^-$, when the electron migrates to the electrometer. When the electrode applies negative voltage (cathode), reduction reaction occurs by $AgCl(s) + e^- \rightarrow Ag(s) + Cl^-$, releasing chloride ion from the electrode and using an electron from the circuit. To prepare the electrodes, it is recommendable to use 15% sodium hypochlorite solution for building AgCl cloth on Ag wire, by dipping the Ag wire (0.25 mm in diameter) in the solution for an overnight. Instead of the sodium hypochlorite solution, liquid bleach (Clorox regular) could be also used to prepare the electrode. When the Ag/AgCl is ready, the wire becomes to have dark brown color. The end part of the wire should be ground by a sand paper to expose the inside Ag part for the soldering to the electrometer, *i.e.*, amplifier. We should take care not to touch or damage the electrode part which is going to be dipped in the flowcell chambers.

Faraday cage is a one of the critical components to enable the sensitive nanopore measuring tool. The cage should have a thick Cu wall (~ 3 mm thick) to protect the flowcells from the external electrical noise. There should be no empty holes except for the necessary wires for the electrical measurements. It is also recommendable to have a vibration isolation table for the Faraday cage stage. The electrical measurements could be affected by the mechanical movement of the system by any small vibrations or unnecessary movement.

The assembly of the nanopore chip and the flowcells depends on the shape of the flowcells. Generally, the two flowcells locate on both sides of the nanopore chip, and each flowcell has each chamber for the *cis* and the *trans* sides. To protect the chip from the mechanical

force at the contact with the flowcells, PDMS gaskets locate between the flowcell and the chip at both sides. To avoid any unnecessary electrical noise, the sandwich structure of flow cell/PDMS gasket/nanopore device/PDMS gasket/flowcell should be strongly fastened using stainless bolts and nuts at the four corners of the square.

1.1.2. General Solid-State Nanopore Fabrication Methods

There are a few most famous solid-state nanopore fabrication methods. Since the shape and structure of the nanopore are not fixed but always custom-made according to the purpose of the experimentalist, there could be no limitation in the methods. Here I want to briefly describe the popular ways to make the nanopore depending on each purpose.

Focused ion beam (FIB) machine has long been used for fabrication of the solid-state nanopore. Ions with the energy of several thousands electron volts hit on a target location of the membrane, then it experiences atomic-scale erosion process. This is called as ion-beam sculpturing or sputtering [9]. There are a few types of FIB depending on the type of ion sources, such as liquid metal ion source (LMIS) (Ga^+ ion) and gas field-ionization source (GFIS) (Ne ion, He ion). LMIS source usually utilize a nA-level ion beam, and GFIS uses a pA-level ion beam, by which LMIS Ga^+ beam could build larger size of nanopore (*e.g.*, $>\sim 100$ nm on $>\sim 100$ nm thick Si_xN_y membrane), and GFIS Ne or He beam could be used for smaller nanopores (*e.g.*, ~ 1 nm-100 nm on 100 nm thick Si_xN_y membrane). Unfortunately, GFIS He or Ne FIB is not yet commonly available, since it is a comparatively new technique with extremely high price, so LMIS Ga^+ FIB is currently the main type of accessible FIB in most cases (Figure 1-5). For the successful nanopore fabrication, the FIB should be working together with scanning electron microscopy (SEM) for the imaging. FIB itself can take images of the sample, but it also damages the sample in a short time during the operation. SEM, on the other hand, has extremely gentle beam with a pA-level current, so it does not damage the sample in a short time. Thus, with the application of SEM, the ion milling process could be checked for the best result. To take an image using SEM, the sample should be a conductive material. If not, the scanning electrons exposed on the sample are

bounced off the surface and distort the images, making it hard to take the real image of the nanopore device. Due to this limitation, the electrical property of the membrane material affects to the quality of the image and the final product. For example, since silicon dioxide (SiO_2) has extremely low electrical conductivity ($\sim 10\text{-}12 \text{ S} \cdot \text{cm}^{-1}$), *i.e.*, insulator, the SEM image becomes distorted during the operation. To overcome this, the surface of the material could be coated with a conductive material (*e.g.* $\sim 5 \text{ nm}$ Au thin layer) using a sputter coater and be removed using a wet- or dry-etchant after the fabrication.

Figure 1.5. Schematic image of nanopore fabrication using Ga^+ FIB

Figure 1.6. (a) SEM image of the 200 nm thick Si_xN_y free-standing window of $50 \times 50 \mu\text{m}^2$. (b) SEM image of a FIB drilled Si_xN_y nanopore of 250 nm in diameter.

Transmission electron microscopy (TEM) is another popular method to make a nanopore. TEM has been originally used for imaging atomic structure of the materials of thin samples (typically thinner than ~ 15 nm for the high resolution). It utilizes hundreds of thousands of voltage to accelerate the electron beam, usually in the range of 80-300 kV. When the electron beam is converged to a point, the spot size becomes less than 2 nm in diameter when there occurs a three-pointed star-like electron beam shape, and the electron beam intensity goes $\sim 1 \cdot 10^8 \text{ A} \cdot \text{m}^{-2}$ [10, 11]. The duration for the nanopore fabrication varies depending on the electrical and mechanical properties of the membrane. If the electrical conductivity is small, the drilling efficiency drops and the drilling time becomes longer. For example, since SiO_2 is a non-conductive material, it takes much longer time than that for the silicon nitride membrane (dielectric constant, $\epsilon_0=7.5$), or it cannot even make a hole if the thickness is over a certain level (*e.g.* $> \sim 10$ nm thick SiO_2 or $> \sim 100$ nm thick Si_xN_y (Figure 1-7)). Lastly, the chip loading tip is required to load the chip to the TEM holder, which is one of the most sensitive parts of the TEM. In most cases, TEM does not provide such chip loading tip with the microscope, because TEM sample is usually much smaller than the chip dimension. Thus, the researchers need to build their own chip loading tip to load it to the TEM sample holder. Based on the experiences, the TEM specimen holder of JEOL allows a good amount of space to build the loading tip enough for the nanopore device, but that of Hitachi does not have enough area for loading a chip (*e.g.* $5 \times 5 \text{ mm}^2$). The chip loading tip should be made up of sturdy material (*e.g.* Al, Cu) which could stably fix the chip at one position without any movement during the operation (Figure 1-8).

Recently, 2-D materials, such as graphene, MoS_2 , or h-BN, have been tested to build nanopore sensors with higher spatial resolution (Figure 1-9). Since such materials are much thinner than other amorphous materials like Si_xN_y or SiO_2 , the pore fabrication becomes much more sensitive to the beam exposure. Thus, a heavy training is required for the TEM user to expose the focused electron beam on the membrane for the right amount of time. For such purpose, scanning transmission electron microscopy (STEM) could be a better choice than the conventional TEM, which uses scanning electron probe to expose the electron beam

by pixel-by-pixel regulation [8, 12]. The STEM probe has atomic sized tip (0.05-0.2 nm), which expose electron beam at the interested region to make an exact sized nanopore with the sub-nanometer accuracy. At the same time, STEM machine could take an extremely-high resolution image of the sample without damaging the 2-D materials.

Figure 1.9. Schematic image of h-BN membrane structure with ssDNA

Finally, another popular method for the nanopore fabrication is the controlled-dielectric breakdown (CDB) method [13]. It uses the dielectric current across the dielectric membrane under a high voltage ($\sim 5-15$ V). When a high voltage voltage is applied across the thin membrane in a conductive ionic solution, a leakage current occurs in the membrane, and a redox reaction occurs on the membrane surface which builds up free charges on the membrane and the current leakage becomes stronger in a positive feedback process. The material could be any types of dielectric materials including Si_xN_y , SiO_2 [14], or even graphene [15], h-BN [16]. The nanopore could be controlled by enlarging it atom-by-atom using the automated feedback control system, which can stop the bias as soon as the nanopore conductance reaches to a preset value. Also, there is no risk of contamination or the variation of the nanopore, since the experimentalist could execute the test as soon as the pore is made, using the same flowcells. Even though the convenience of CDB, it has a few drawbacks to consider: First, the experimentalist does not know the location on the membrane where the nanopore was made, or the shape how it was made. Second, the nanopore cannot be made on a thick membrane.

For example, based on the experience, if the thickness of Si_xN_y is thicker than 100 nm, the nanopore fabrication becomes almost impossible. Third, the membrane could get damages on many locations of the membrane except the nanopore region. Fourth, since the dielectric breakdown method basically applies the strong electric field on the whole membrane, the thinning process also occurs at the same time all over the membrane. Theoretically, this process could make more than one pore on the membrane at the same time, even though it is a rare case.

1.1.3. Materials for Solid-State Nanopore Chips

Materials for the nanopore chip are limitless because there are so many types of thin membranes available. In this sub-section 1.1.3., I would like to describe the popular materials for building solid-state nanopores. SiO_2 nanopore was the first nanopore made by TEM in 1998 [11]. SiO_2 is the major component of glass and one of the most common materials in the world. Since SiO_2 has a superior electrical stability and widely used for the semiconductor industry, the material was adopted before other materials. SiO_2 could be evenly grown on the silicon wafer by wet-oxidation process. However, the low electrical capacitance made it hard to make a nanopore on the SiO_2 membrane using TEM. Also, the weak mechanical properties of SiO_2 caused the nanopore becoming fragile, which basically requires to change the nanopore for every experiment.

To make a nanopore with higher spatial resolution, 2-D materials has been used, such as graphene, MoS_2 , and h-BN. Since these materials have good mechanical properties, many researchers have been trying to build and test the 2-D nanopores using the materials. Graphene is famous for its metallic conductivity with no band-gap, but such characteristics could cause higher electrical noise and stronger pore-to-analyte interaction causing more noise [17]. MoS_2 is a semiconductor with a low band-gap (~ 1.88 eV) [18]. and a few researches has proven that it has higher stability and sensitivity than graphene as a nanopore sensor [19]. h-BN is also a type of semiconductor like MoS_2 , but it has much wider band-gap (~ 5.9 eV) [20] and dielectric strength ($4.4 \text{ MV} \cdot \text{cm}^{-1}$) [21]. Based on the higher electrical stability, h-BN

nanopore has higher SNR than other 2-D materials (Figure 1-10). There are two types of transfer method in large; wet-transfer and dry-transfer methods. Wet-transfer method is using a liquid solution to etch, clean, or float the materials during the transfer procedure; thus, there is higher chance to be contaminated by any molecules dissolved in the solutions, except it occurs in the extremely clean environment. The PMMA transfer method is one of the popular wet-transfer method, in which thin PMMA layer is used as a scaffold to hold 2-D materials, and then removed after the transfer [22]. The limitation of this method is that the PMMA layer is not fully removable from 2-D materials even after using chemicals or furnace at high temperature, which could change the properties of the materials. Dry-transfer method, on the other hand, uses dry polymer stamp to transfer 2-D membrane from the source (e.g., graphene, MoS₂, or h-BN block) to the target place; thus, it could yield a cleaner product without any residues. The limitation of this method is that the transferred membrane is usually not continuous but in a small scale ($\sim 100\ \mu\text{m}$) [23]. Since both wet and dry transfer methods have their strengths and drawbacks, the nanopores could be built according to the design of the research.

Figure 1.10. (a) TEM micrograph of TiO₂/h-BN membrane on $\sim 30\ \text{nm}$ Si_xN_y window. (b) TEM micrograph of $\sim 3\ \text{nm}$ TiO₂/h-BN nanopore on TiO₂/h-BN membrane.

1.1.4. Experimental Noise

The noise of the nanopore sensor could be coming from the various sources, but it could include intrinsic noise and external noise in large. The intrinsic noise is basically originated from the nanopore and analyte interaction which could cause undesirable signals from the unexpected chemical reaction or electrical interactions. The intrinsic noise could be overcome or improved by changing the pH of the solution, the material of the membrane, the external bias, or the conformational structures of the analytes. First, pH of the medium affects the surface charges of the analyte and the membrane, which increases or decreases the analyte-to-pore interactions, the event frequency, and the translocation time. There is a characteristic value of isoelectric point (pI), at which pH the material (membrane or analyte) will have no charge on the surface. If the pH is higher, the material will have negative charge, and vice versa. Since the analyte and the material experience different ionic distribution on the surfaces, there will be electrostatic interactions causing variations in the dwell time. Also, electrical properties of material could cause undesirable electrical signals. The capacitance of the material decides how much electrons it could accept. If the capacitance of the material becomes higher, there will be more electrons resides on the membrane, which could cause dielectric loss, increasing the noise at high-frequency (>20 kHz) [24]. Since the data acquisition normally occurs at 100k -200kHz, the high-frequency noise occupies the major part of the noise. On the other hand, the low-frequency ($1/f$) noise could be explained by the Hooge's relation,

$$\frac{S_I}{I^2} = \frac{A}{f} = \frac{\alpha}{(N_c f)} \quad \text{Equation 1-1}$$

, where S_I , A , f , α , N_c are current power spectral density, noise power, frequency, Hooge's parameter, number of charge carrier, respectively, wherein The noise decreases as the charge carrier increases. In other words, as the ionic concentration of medium increases, the electric noise decreases. Thus, since the intrinsic noise comes from the system itself, it cannot be effectively decreased unless changing the condition of the components. On the other hand, the external noise is originated from any electromagnetic waves, heat, or vibrations from

the out of system. To protect from the undesirable sources of noise, the thick Cu Faraday cage is necessary (section 1.2.), made of at least 3 mm thick Cu plate without unnecessary exposures to the outside (Figure 1-11). Double Faraday cage is also recommendable. The electric wires should be untangled without touching each other. To protect the system from the heat, the headstage pre-amplifier could be put on the heat sink inside the Faraday cage. Also, to protect it from the vibrations, the Faraday cage could be installed on the vibration isolation table, floating the table on the air cushion.

Figure 1.11. (a) External set-up for nanopore platform. (b) Flowcells wired to the headstage pre-amplifier in the Faraday cage.

1.2. Nanopore DNA Sequencing

Nanopore sensor has been applied to analyze various types of organic and inorganic particles and molecules. In the case of biological nanopore, DNA is the major analyte, because of the small size of the pore. On the other hand, solid-state nanopores could analyze any type of sample from the single molecule to the biological cells, because the nanopore fabrication and the geometry of solid-state nanopore chip depend on the type of sample.

1.2.1. Biological Nanopore Sequencing

The sequencing effort using nanopore has been started by using biological nanopore. The nanopore is originated from the channel proteins of the cell membrane, thus people named it as biological nanopore. There are only a few of biological nanopores available, *e.g.* α -Hemolysin (~ 1.4 nm), MspA (~ 1.2 nm), and phi29 (~ 3.6 nm). Since they have fixed size of diameter, any other types of sample with larger dimension cannot be analyzed using biological nanopores. Because of this limitation, the type of sample has been heavily focused on DNA due to its similar size compatibility; ssDNA is ~ 1.2 nm, dsDNA is ~ 2 nm in width. In the first demonstration using biological nanopores for DNA detection, the resolution was not good to tell the difference between the nucleotides or to even detect nucleobases. With the assistance of genetic/protein engineering, the biological nanopore became to have an advanced shape by assembling the channel protein with an adopter protein at the entrance, which slowed the translocation speed. Also, the amino acid components of the narrowest constriction part of the nanopore were replaced with other types of amino acids for increasing the electrochemical sensitivity of the nanopore. As a result, the nanopore sensor successfully detected the nucleotides, which is evident by the commercially available product [25]. Despite such success, there has been a few limitations which includes the low base-calling accuracy (~ 90 -95%), the low stability with high failure rate, and the low mechanical stability of the lipid bilayer membrane.

1.2.2. Solid-State Nanopore Sequencing

Biomolecules including various DNAs and proteins have been detected by solid-state nanopores [26–30]. Since the molecular-level detection has been successful, the detection of the building units of the biopolymers is the next goal, *i.e.*, the nucleotides of DNA or amino acids of proteins. There are four types of nucleotides called Adenine (A), Thymine (T), Cytosine (C), and Guanine (G). Adenine and thymine are called as pyrimidine, since they are made up of single ring structure. Guanine and cytosine are called as purine, since they are made up of double ring structure. Because of this property, purine nucleotides have slightly

larger sizes than pyrimidine nucleotides, possibly allowing different peak amplitudes for each type of base. Another component that affects the resistive pulses is the electrochemical interactions between DNA and nanopore. The charge of each nucleotide is decided by its acid dissociation constant (pK_a) and the pH of medium. Interestingly, the pK_a of A and C are located between 3.6-4.3, and the pK_a of G and T are between 9.2-9.6 [31]. This tells us that the nucleotides have different charges depending on the general pH range; A and C will be negatively charged and G and T positively charged at pH 6-8. Such difference builds a unique dipole-dipole interaction between each nucleotide and nanopore, which eventually affects the dwell time and the peak amplitude of each nucleotide. For maximizing the electrical signals, the charge of the nanopore and each nucleotide should be optimized to have the distinct interactions with unique changes in free-energy $\Delta G = -RT \ln(K)$.

Chapter 2

FABRICATION OF NANOPORE DEVICE

Preparing nanopore is the first step of the experiment. Since our research is targeting to detect nucleotides of single-stranded DNA, we had to go beyond what the conventional nanopore has been done using amorphous materials, such as silicon dioxide (SiO_2) or silicon nitride (Si_xN_y). We adopted 2-D h-BN nanopore instead of conventional amorphous materials because of its ultrathin atomic thickness (~ 0.5 nm) with higher electrical stability (Figure 2-1). The general nanopore fabrication is briefly reviewed in the sections 1.1.2. (general solid-state nanopore fabrication method) and 1.1.3. (materials for solid-state nanopore chip) for the introduction.

Figure 2.1. Schematic figure of ssDNA translocation event through the 2-D h-BN nanopore

There are various types of materials used for the nanopore fabrication, from amorphous to crystallized 2-D materials. 2-D material has atomically thin thickness, which showed

much higher resolution than amorphous materials. Thus 2-D materials are the most popular materials for detecting DNA. The 2-D materials, such as graphene, MoS₂, or h-BN, have to be transferred on a strong supporting part, for which Si_xN_y has been widely used, due to its strong mechanical property (~ 166 GPa). The 2-D h-BN nanopore chip preparation procedure includes a few parts of 1) supporting window preparation, 2) 2-D membrane transfer, 3) h-BN characterization, and 4) TEM nanopore fabrication.

2.1. Fabrication of Si_xN_y Supporting Window

2.1.1. Deposition of Si_xN_y Membrane

To build a h-BN membrane, a supporting window should be first prepared. In our case, we prepared Si_xN_y circular window of ~ 40 nm in diameter. The first step is to deposit Si_xN_y on silicon wafer. A 4 inch silicon wafer was used for preparing enough number of chips (over 200 $5\text{mm} \times 5\text{mm}$ chips per 4 inch wafer) at the same time. The wafer was polished on both sides for the flat deposition of the Si_xN_y on the top of the silicon wafer. Also, the silicon wafer should have (100) plane on the surface for the right wet etching angle in the later step.

The 50 nm thick Si_xN_y film was deposited on both sides of the polished silicon wafer with (100) plane, using low pressure chemical vapor deposition (LPCVD). LPCVD is a type of chemical vapor deposition (CVD) which uses heat to initiate the chemical reactions of precursor gases to uniformly grow solid materials on the solid surface. LPCVD machine is managed in the extremely clean environment in a cleanroom and usually run by specially trained personnel.

2.1.2. Photolithography and Dry Etching

Certain part of the Si_xN_y on the backside of the chip should be opened for wet-etching the silicon part. Since the wet-etching has a slope of $\sim 54.7^\circ$, the backside opening should be much larger than the targeting dimension of Si_xN_y membrane. The dimension of the

backside opening can be calculated according the equation,

$$b = \frac{l + 2h \cot 54.7}{\sin \delta + \cos \delta} \quad \text{Equation 2-1}$$

, where b , l , h , δ are the backside dimension, the frontside targeting dimension, the thickness of wafer (etch depth), and the misalignment angle, respectively (Figure 2-2) [32]. The alignment is extremely important, since half degree of misalignment causes about 13% larger sized targeting dimension. The (100) 4 inch circular wafer has a flat part on the wafer, by which the wafer could be aligned in the process of photo-lithography. If the wafer is not aligned there will be significant variations in the size of free-standing membrane on the frontside.

Figure 2.2. (a) Schematic image of the cross-section of the (100) Si etched with KOH. (b) The optical view of the (100) Si after the backside KOH wet-etching.

The photomask was designed using CAD and printed in a film (Figure 2-3). For the best quality, a chrome mask is recommended for the photomask, but it has a higher price than the film mask. If the mask is for a permanent design, chrome mask should be used, since it could be used semi-permanently. Since the positive photoresist was used for the soft-lithography, the mask was needed to be a dark field, which allowed the light to pass only where the photoresist was to be removed. Then the positive photoresist (S1813) was coated on the backside of the wafer using a spin coater. The thickness of S1813 depending on the speed of spin coater is broadly shared. In our case, we used 3,000 RPM for 60 sec

and 1,000 RPM for 60 sec in sequence, which gave $\sim 1.5 \mu\text{m}$ thick S1813. The wafer with photoresist should be baked on the 115°C hot plate for 2 min to harden the photoresist. $6.5 \text{ mW} \cdot \text{cm}^{-2}$ at 365 nm (i-line) was exposed for ~ 17 sec on the wafer with mask at $110 \text{ mJ} \cdot \text{cm}^{-2}$, and the wafer was developed using a developer (MF-319) for 1 min. The exposed Si_xN_y was removed by reactive ion etcher (RIE) using oxygen plasma, for which SF_6 gas was used at 180 mT, 200 W, 13 sccm. The Si_xN_y etching rate with SF_6 was $\sim 2 \text{ nm} \cdot \text{s}^{-1}$ which took ~ 25 s for etching 50 nm Si_xN_y . Then, the S1813 layer was removed using S1813 ripper (AZ 400T) at 100°C for 5 min. The AZ 400T was cleaned at acetone, IPA, DI water in sequence, each for 1 min. The DI water was dried with N_2 blowing gas.

Figure 2.3. (a) An example of CAD design for nanopore chip photolithography. (b) Schematic figure explaining the principle of photolithography.

2.1.3. Wet Etching

After the backside Si_xN_y was etched, the silicon was wet-etched by the KOH etchant. 20% KOH etchant was used at 85°C with a magnetic bar at 300 RPM at the base hood. The wafer was placed in a specialized KOH wafer chuck and dipped in the solution (Figure 2-4(a)). The concentration and the temperature of KOH could be increased or decreased to control the etching rate; higher concentration of KOH reduces the etching rate, and lower concentration of KOH increases the etching rate. In the case of 20% KOH etchant, the etching rate was $\sim 112 \mu\text{m} \cdot \text{hr}^{-1}$, and it took ~ 4.5 hr to etch the $525 \mu\text{m}$ thick silicon wafer

(Figure 2-4(b)). The etching status of the wafer should be checked every 10 min using optical microscopy when the estimated time is reached (Figure 2-4(c)). It would be much easier and more accurate to use a hot plate with an automatic feedback temperature control system. If the hot plate does not have the temperature control system, the experimentalist should reside with the wet-etching set-up, checking the temperature until the etching is done. TMAH is also very well-known for the silicon etchant, and it could be a better choice than KOH, since it does not crystalize the salt, which can prevent undesirable contaminations on the surface of the membrane, especially during checking the etching status under optical microscopy. After it reached the right size of Si_xN_y window ($\sim 50 \times 50 \mu\text{m}^2$), the wafer with the chuck was dipped in the ample clean DI water for an overnight to slowly remove the KOH salt by diffusion without damaging the membrane. Then, the wafer was taken out of the chuck and gently dried using a gentle N_2 blowing gas.

Figure 2.4. (a) KOH Si wet-etching set-up. (b) 4 inch Si_xN_y coated Si wafer after the wet-etching process. (c) Backside view of the nanopore chip after the wet-etching process.

2.1.4. TEM Pore Fabrication

The wafer had the dicing lines by the photo-lithography process. The wafer was manually divided into chips following the dicing line, and each chip had the size $5 \times 5 \text{ mm}^2$ for each chip. Also, a professional dicing company can divide the wafer into the chips instead the manual process. To make a Si_xN_y circular hole, the chip was loaded in a TEM sample holder

using a specialized custom-made chip loading tip. The tip was made up of copper and could stably hold the chip in the TEM holder without a noticeable movement. The TEM beam should be well aligned before drilling the hole, by which the beam could be focused into a small spot (~ 2 nm). When the chip is loaded, the $50 \times 50 \mu\text{m}^2$ square window was visible on the phosphorescent screen, which was the 50 nm thick Si_xN_y membrane. A circular hole of ~ 40 nm hole was drilled on the middle of the hole (Figure 2-5). The hole, *i.e.*, the Si_xN_y supporting window, should not be too small, since it makes it harder to find it again, when a nanopore is drilled on the h-BN membrane in the later step. On the other hand, a larger hole lowers the stability of the h-BN membrane during the transfer process. We found ~ 40 nm hole was the most adequate size for the successful transfer. After drilling a hole on the membrane, the chip should be stored in a vacuum chamber for avoiding undesirable contamination or oxidation of the membrane and the nanopore.

Figure 2.5. TEM micrograph of ~ 40 nm nanopore on 50 nm thick Si_xN_y membrane

2.2. h-BN Transfer

After Si_xN_y supporting window was prepared, the h-BN layer was transferred on the top of the surface. To make the membrane stabler for longer usage of the nanopore, we built h-BN bilayer membrane with atomically thin TiO_2 film on it. The h-BN transfer

procedure is complicated and usually takes a long period (~ 2 -3 days for single layer transfer), thorough scheduling for using the TEM machine and the transfer bench is critical. Also, each transfer step requires experiences and training to achieve good membrane handling skills. The transfer procedure includes 1) h-BN preparation, 2) h-BN transfer, and 3) TiO_2 film using atomic layer deposition tool.

2.2.1. h-BN Preparation

Polymethyl methacrylate (PMMA) transfer method was used to transfer h-BN layer to the solid surface. This transfer method has been widely used in 2-D material research field (please check the Ch.1 section 1.1.3.) [23]. First, We purchased a CVD-grown single layer h-BN from a manufacturing company named Graphene supermarket. H-BN layers were grown on both sides of 25 μm thick copper foil. Since we needed a single-hBN layer, the h-BN on the bottom side of the copper film was dry-etched using RIE. To avoid any damage on h-BN on the other side of the copper layer, the copper film was taped on the slide glass during the etching process, exposing the bottom h-BN surface upward. The h-BN etching condition was at 100 W, 10 sccm O_2 , for 2 min. Then, the h-BN on the top surface was coated with PMMA using a spin-coater; the h-BN/copper film was taped on another slide glass, exposing the top h-BN surface upward to coat PMMA on the surface. The PMMA solution should be prepared at least a day before this step, since PMMA does not dissolve in the solvent fast. The solution was 20 mg (960k MW PMMA)/mL 1,2-dichlorobenzene solution, which was prepared by dissolving the PMMA in the solvent on a hot plate at 60 $^\circ\text{C}$ for an overnight in the chemical fume hood. The molecular weight of PMMA is critical, because the long and heavy PMMA molecule increases the resiliency of the PMMA film which plays a crucial role in holding h-BN during the transfer step. If the molecular weight of PMMA is lower, then the PMMA/h-BN piece will be torn apart during the wet-etching step. The PMMA solution was spin-coated on h-BN layer twice each at 3,000 RPM for 60 s and 1,000 RPM for 60 s for the cleaner removal of PMMA in the later step [33]. Also, if the PMMA layer is not thick enough, the PMMA cannot support h-BN during the transfer step later. The PMMA film

should be baked at 100 °C for 2 min to harden the PMMA layer after each coating step, then the PMMA layer became sturdy which can hold h-BN single layer during the transfer process afterwards.

2.2.2. h-BN Transfer

$3 \times 3 \text{ mm}^2$ h-BN piece was cut from the foil using a sharp and strong surgical blade. Since the used one does not have a good sharpness, it is recommended to use a new blade. We made ~ 5 nanopore devices in one batch, 5 pieces of h-BN was prepared at the same time. The cut pieces are floated on 17.5% nitric acid in a glass dish for 1 min to remove the oxide layer on the Cu foil. The adequate dish size is ~ 6 cm in diameter dish with ~ 1 cm in height, and each piece of h-BN should be processed in each dish. If the pieces are floating in a small dish together, a strong interaction occurs between each h-BN piece, which disturbs to treat each piece with accuracy. After rinsing it with ultra-pure water for over 1 min, the Cu layer was etched by floating it on 0.5 M ammonium persulfate solution overnight. The etching rate was very fast and the $25 \text{ }\mu\text{m}$ copper foil could be theoretically etched in ~ 4 hr. However, the etchant does not react with h-BN or PMMA at all, the etching time could be longer for the perfect etching. When the Cu foil was clearly removed, the piece became transparent. The h-BN piece was cleaned with ultrapure water by floating it for 3 min. After etching Cu foil, the PMMA/h-BN piece became very flexible, so we cannot use tweezer to transfer it. So, we used a piece of a polished clean silicon wafer ($\sim 10 \text{ mm} \times \sim 60 \text{ mm}$) as a spoon to move it from a dish to another dish. It is extremely important to keep the wafer spoon in a clean state always to avoid any type of contamination. The h-BN piece was cleaned once more with warm ultrapure water in the same way to effectively remove left salts on the piece. The piece of PMMA/h-BN was transferred on the top of the prepared chip with the $\sim 40 \text{ nm}$ Si_xN_y hole. In this step, a self-closing tweezer was used to hold the chip at the corner, and gently pushed it to the floating h-BN to transfer on the surface. In most cases, an electrical interaction or repulsion occurred between the h-BN and the chip, so I should have gently pushed the floating piece to the edge of the solution at the dish wall to stably transfer it.

The transferred film was gently dried in the air by holding the chip above a hot plate at 60 °C. The drying should be done very gently with a weak air convection because it can easily tear the membrane. After the chip was allowed to cool down, it was submerged in acetone for 4 hr to remove the PMMA layer. The dissolving rate of PMMA in acetone is very fast, but we waited for 4 hr to make sure to remove most of the PMMA on the surface. The chip was then placed in a furnace with a quartz tube (MTI Corp.) and baked under vacuum with an Ar flow at 100 sccm at 350 °C for 2 hr to remove the remaining residual PMMA on the h-BN and to stably anneal the h-BN layer on the Si_xN_y membrane (Figure 2-6). For a bilayer h-BN preparation, the second layer of h-BN was transferred on top of the first h-BN film following the same procedure. Because it takes at least 2-3 days to transfer single layer h-BN, the 1st and the 2nd transfer should be scheduled in a time efficient way. The reason for the usage of bilayer h-BN was to increase the mechanical strength of the membrane to stand the nanopore drilling process using TEM by the strong electron beam exposure. The h-BN could get damaged less than a second under the strong electron beam of TEM.

Figure 2.6. h-BN transferred Si_xN_y chips on a silicon boat right before the furnace step. The rectangular h-BN on the chip is visible by the light reflection.

2.2.3. ALD TiO_2 Deposition

A ~ 0.5 nm TiO_2 film was deposited on top of the bilayer h-BN film using a thermal atomic layer deposition (ALD) tool (Savannah S100, Veeco/cnt) by running 12 cycles at a growth rate of $\sim 0.4 \text{ \AA} \cdot \text{cycle}^{-1}$ with the precursor tetrakis(dimethylamino)titanium (IV). The TiO_2 film provided mechanical strength to the h-BN membrane to withstand the electron

beam exposure in the TEM chamber, and it also allowed a good image contrast to tell the nanopore formation during the drilling procedure (Figure 2-7). The good image contrast was very important, since the image taking process should be done in a few second (less than 10 s) to avoid serious damages of the nanopore and the membrane. If there is no TiO_2 layer, the bilayer h-BN cannot stand the electron beam for ~ 5 s at 200 kV. The ALD (Savannah S100) took less than 2 hr in total, including warming-up, deposition, and cooling-down process. It is recommended to drill a nanopore in a few hours after the ALD step. If not, the chip should be stored in a vacuum chamber until the TEM drilling step.

Figure 2.7. (a) TEM micrograph of the case of unsuccessful ALD TiO_2 coating with a bad image contrast. (b) TEM micrograph of the case of successful TiO_2 coating with a better image contrast.

2.3. h-BN Characterization

In this sub-section, I would like to share the result of the h-BN transfer, and how we confirmed that the material we transferred was h-BN. We mainly used TEM tool to confirm the structural properties of h-BN, by 1) the atomic structure of h-BN, 2) the characteristic SAED pattern of the hexagonal structure of h-BN, 3) the characteristic distance between the atoms of h-BN from the image contrast, and 4) the contact angle measurement.

2.3.1. Atomic Structure of h-BN

h-BN is known for its crystalized hexagonal structure, like a graphene (Figure 2-8). In the case of graphene, it is only made up of carbon, so there is no angle at all between the carbon atoms. In the case of h-BN, boron and nitride atoms have slightly different size variation, which causes a slight angle between the atoms. Even though such difference, h-BN is a flat, crystalized 2-D material like graphene. Transmission electron microscopy (TEM) image of h-BN monolayer was taken to confirm the material to show its hexagonal structure (Figure 2-8). In a large view, the structure was not clearly visible (Figure 2-8(a)), but only showed a stripe like pattern. When it was zoomed-in, the TEM image showed a clear hexagonal structure (Figure 2-8(b)). One thing we need to be careful during the TEM imaging is that, there could be attractions of carbon residues to the imaging spot due to the weak energy of the electron beam [34]. Because of this, the sample should be thoroughly cleaned under the TEM ultraviolet ozone cleaner before loading the sample.

Figure 2.8. (a) TEM micrograph of h-BN membrane. (b) Zoomed-in TEM micrograph of h-BN.

2.3.2. SAED Pattern

Another simpler way to confirm the material is to take a selected area electron diffraction pattern (SAED). The SAED pattern was acquired at the same time when the h-BN atomic structure was taken. This pattern can prove the crystalized structure of h-BN by showing its characteristic spot locations. In the case of h-BN, it has a hexagonal structure which creates a SAED pattern of six-edged star shape (Figure 2-9(a)) [35]. Because of such structure, the SAED pattern becomes very similar to that of graphene. The SAED pattern showed the same pattern to the fast-Fourier transform of the image, which also proved the single crystallinity of h-BN. If there were more layers than single layer h-BN, the SAED pattern was not to be such a clear star pattern but many dots on a concentric circle (Figure 2-9(b), (c)).

Figure 2.9. SAED pattern of (a) CVD-grown h-BN single layer, (b) a few h-BN layers with different directions (no stacking direction), (c) h-BN multi-layers with different directions (no stacking direction)

2.3.3. Atomic Distance Calculation

Using the TEM image of h-BN atomic structure, the distance between the atoms was calculated using the contrast calculation. The color intensity variation in 2.5 nm distance in the image was calculated (Figure 2-10(a)), showing that there were ten valleys observed (Figure 2-10(b)); the high color intensity was when there was an atom, and the low color intensity was when there was no atom at the center of the hexagonal atomic pattern. This tells us that the distance between a center to another center was 0.25 nm, which matched

the real distance of the atomic structure.

Figure 2.10. Measurement of atomic distance using image contrast of TEM image. (a) TEM micrograph of h-BN where to take the image contrast. (b) The image contrast measurement, showing the strong and weak values.

2.3.4. Contact Angle Measurement

As one of the characterization methods, the contact angle measurement was executed for each step of fabrication process. This is important not only for characterizing h-BN, but also finding the right condition for the experiment. Since the good surface wettability is very important for the nanopore experiment, we tried to improve it by treating the sample with ultraviolet-ozone (UV-O₃) on both sides of the chip. A 50 nm Si_xN_y film was deposited on 300 nm thick SiO₂ film by CVD using a plasma-enhanced chemical vapor deposition (PECVD) tool (Plasma-Therm 790, Plasma-Therm LLC.). CVD-grown h-BN monolayer was transferred onto these substrates using the well-established PMMA method [23], cleaned in acetone, and annealed under Ar flow at 350 °C as described in the nanopore fabrication section (Figure). The h-BN layer transfer, TiO₂ deposition, or ultraviolet-ozone (UV-O₃) surface treatment were applied to the wafer according to each case of contact angle measurements (Figure). The contact angles were measured using a goniometer (ramé-hart Instrument Co.) by gauging both right and left contact angles of twelve 1 μL water droplets. These values were

then averaged and reported in Figure 2-11. Bare Si_xN_y was found to be more hydrophilic (smaller contact angle) than $\text{h-BN/Si}_x\text{N}_y$ (Figure (a, b)), and UV-O_3 treatment increased the hydrophilicity in both cases (Figure (c, d)). TiO_2 coating on Si_xN_y ($\text{TiO}_2/\text{Si}_x\text{N}_y$) and $\text{h-BN/Si}_x\text{N}_y$ ($\text{TiO}_2/\text{h-BN/Si}_x\text{N}_y$) made their surfaces less hydrophilic (Figure (e, f)) compared to Si_xN_y and $\text{h-BN/Si}_x\text{N}_y$ without TiO_2 coating (Figure (a, b)). Interestingly, the contact angle of the UV-O_3 treated $\text{TiO}_2/\text{h-BN/Si}_x\text{N}_y$ dramatically dropped from 62° (Figure (f)) to 18.1° (Figure (h)) and that of $\text{TiO}_2/\text{Si}_x\text{N}_y$ dropped from 48.5° (Figure (e)) to 7.5° (Figure (g)) [36,37]. Since h-BN is extremely resilient to oxidation [38], the UV-O_3 treatment could be repeated without causing noticeable defects in its lattice.

Figure 2.11. Contact angle measurements for each step of building $\text{TiO}_2/\text{h-BN/Si}_x\text{N}_y$ (UV-O_3) using a goniometer. Each contact angle was acquired by averaging the the measurements from twelve $1\ \mu\text{L}$ water droplets. Each case represented (a) Si_xN_y (39.2°), (b) $\text{h-BN/Si}_x\text{N}_y$ (45.3°), (c) Si_xN_y with UV-O_3 (9.3°), (d) $\text{h-BN/Si}_x\text{N}_y$ with UV-O_3 (24.3°), (e) $\text{TiO}_2/\text{Si}_x\text{N}_y$ (48.5°), (f) $\text{TiO}_2/\text{h-BN/Si}_x\text{N}_y$ (62.0°), (g) $\text{TiO}_2/\text{Si}_x\text{N}_y$ with UV-O_3 (7.5°), and (h) $\text{TiO}_2/\text{h-BN/Si}_x\text{N}_y$ with UV-O_3 (18.1°).

2.4. TEM Nanopore Fabrication

After $\text{TiO}_2/\text{h-BN}$ free-standing membrane was prepared, a nanopore ($\sim 4\ \text{nm}$) was drilled on the membrane using focused electron beam. TEM machine is usually run by specially trained personnel, but I could get a chance to learn it for using it directly. Since the machine

is very sensitive and the beam is easily unstabilized by the vacuum status, it is important to check the beam and vacuum stability before the experiment. If the beam or vacuum is not stable, it shows a noticeable amount of beam shaking which cannot be focused on a single point. The vacuum stability is affected by 1) how well it is baked by the last user, 2) how well it is cooled down by the nitrogen liquid before using the beam, and 3) how well the sample is cleaned before loading into the vacuum chamber. Sometimes, the TEM should rest for more than a week to get the vacuum back to the normal level. This section includes 1) TEM beam alignment and 2) h-BN nanopore drilling process.

2.4.1. TEM Beam Alignment

To use TEM for drilling a nanopore, a chip loading tip was custom-built. We used JEM-2100F (JEOL), which TEM holder has a tip that could be replaced by the custom-built tip. The tip was made with copper to hold the chip stably without causing noticeable movement during the procedure in the TEM vacuum chamber. Before loading the sample, the TEM beam was aligned using a mock chip. Since the beam could be misaligned by the previous user with different samples, this step could prevent any unexpected risks to the sample. The alignment procedure is very practical steps which should be learnt by using the machine; therefore, only the rough procedure is written here (Figure 2-12). After loading the mock sample and opening the electron beam, condenser lens aperture was inserted. The z-height of the beam was first adjusted to find a focus on the sample surface. Then, the beam was aligned by moving the brightness shift, by confirming that the size was increased and decreased concentrically. If it did not work, the aperture position was needed to be adjusted. Then, the electron gun was aligned by matching the beam at the same point, by centering the beam using gun deflector at the spot 1, and also by centering the beam using condenser lens aperture at the spot 5. By repeatedly matching the spot 1 and spot 5, the gun shift became aligned. Then, gun tilt was aligned by moving the gun tilt position in the anode wobbler mode, in which the beam automatically expanded and shrank repeatedly. By moving the gun tilt, the beam expanded and shrank concentrically at the same point. The tilts in the

x and y directions were also adjusted by finding the center at the wobbler mode. Then, the voltage was centered using the HT wobbler by moving the beam until it stopped shifting. The condenser astigmatism was corrected by changing the beam shape into a triangle shape. Finally, the objective lens astigmatism was corrected using the live fast-Fourier transformed (FFT) image making a circular image (Figure 2-13). It became much more straight forward when we use it in practice.

Figure 2.12. General procedure of TEM alignment

2.4.2. TEM Nanopore Drilling

The mock sample was replaced with the real sample chip. Since the beam was just aligned, the slight height adjustment and the condenser lens astigmatism correction were enough to start the drilling. The ~ 40 nm Si_xN_y hole was found as soon as possible, since even short e-beam exposure could damage the membrane. If the hole was not visible well, the low-magnification mode ($\times \sim 1\text{k}$) was used to check any contrast with brighter area, and the area was examined in the high-magnification mode ($\times \sim 10\text{k}$). First, the $\text{TiO}_2/\text{h-BN}$ membrane was checked if it is intact or not. The membrane could be damaged during the transfer step due to its ultrathin thickness. Near the hole area, the beam was focused into a point at high magnification ($\times \sim 300\text{k}$ - 500k), and lens alignment was checked once more

Figure 2.13. (a) TEM micrograph of $\text{TiO}_2/\text{h-BN}$ membrane in focus with (b) its FFT image. (c) TEM micrograph of $\text{TiO}_2/\text{h-BN}$ membrane out of focus with (d) its FFT image.

along with the condenser and objective lens astigmatism correction. Then, the beam was expanded, and the sample was put at the center of the phosphorescence screen. By focusing the beam at the center, the drilling begins; the drilling time was ~ 2 s, and it stopped by enlarging the beam size. The nanopore should be imaged as soon as possible before there would be any damages. The size of the nanopore could be controlled by using different beam accelerating voltage (80 kV – 200 kV), size of condenser aperture (aperture 1 - 3), spot size (1 - 5), or duration of beam exposure. In this case, we used 200 kV, aperture 2, spot size 1, for 2 s exposure (Figure 2-14). Like the process of beam alignment, the nanopore drilling and imaging procedure heavily depends on the experimentalist's skill, and it requires heavy training. The prepared chip should be used for the nanopore measurement experiment in a short time, or preserved in a vacuum chamber under a clean environment. Since the conventional gel-pak case is not clean, the final nanopore chips should be preserved in a new petri-dish or a custom-built box.

Figure 2.14. $\text{TiO}_2/\text{h-BN}$ membrane (a) before drilling the nanopore, (b) after drilling the nanopore with larger diameter of ~ 7 nm. $\text{TiO}_2/\text{h-BN}$ membrane (c) before drilling the nanopore, (d) after drilling the nanopore with ~ 3 nm in diameter.

Chapter 3

NANOPORE EXPERIMENTS

The chapter describes about general procedures for the nanopore experiments to understand the DNA translocation experiment in the next chapter. This chapter includes 1) experiment preparation, 2) basic characterization, and 3) nanopore noise measurement.

3.1. Experiment Preparation

The nanopore platform includes a few components: nanopore chips, medium (filling solution), samples, and external nanopore set-up. The nanopore chip fabrication method was earlier described in the chapter 2 of nanopore fabrication. Before using the fabricated chip, it was subjected to the ultraviolet ozone cleaner on both sides of the chip each for 20 min, to make the surface super-hydrophilic. The effect of ultraviolet ozone treatment was specifically studied in the section 2.3.4. The ultraviolet light made the TiO_2 surface extremely hydrophilic, by which it facilitated the translocation events of the analyte molecules during the experiment. This treatment should be done right before the experiment to achieve the best result for the experiment. If the chip is not made up of h-BN but simple Si_xN_y or other amorphous material, RCA cleaning could be used to clean the contamination on the surface and to make the surface much more hydrophilic by slightly oxidizing the surface. The cleaner was made up of DI water, NH_4OH (29%), H_2O_2 (30%) in 5:1:1 ratio. The temperature was set at 80°C on a hot plate, and the chip was dipped in the solution for 10 min.

In general, the medium is made up of salt aqueous solution, because water is the best solution for the salts, such as NaCl , KCl , or LiCl , *etc.* Depending on the type of salt, the ionic characteristic conductivity changes. For example, K^+ has its molar conductivity of $\sim 73.5 \text{ S}\cdot\text{cm}^2$ at the room temperature, but Li^+ has $\sim 38 \text{ S}\cdot\text{cm}^2$, which means K^+ move about twice faster than Li^+ . Stronger salt with higher conductivity can give stronger signals at the

same concentration. To achieve similar signal using weaker salt, higher concentration should be used. This gives an interesting effect on the analyte; for example, by using much higher LiCl 4M solution, the translocation speed of molecule became ~ 10 times slower than 1M KCl solution [39]. Thus, the type and the concentration of salt directly affect the nanopore experiment and the translocation events.

For slowing down the translocation time of analyte, many different types of liquids were used instead of the simple salt water solution; for example, polyethylene glycol (PEG) is a hydrophilic liquid which molecular weight depends on the length of the polymer. The viscosity of PEG 400 is ~ 120 mPa·s which is ~ 120 times higher than water (~ 1 mPa·s). Because of this property, the translocation speed decreased to $20\text{-}100 \mu\text{s} \cdot \text{nt}^{-1}$ [40]. In the same context, an ionic-liquid has been used to slow down the translocation speed of DNA [41]. [bmim][PF₆], a type of ionic-liquid, is a salt itself existing as a liquid at the room temperature. It is a high-viscous non-aqueous solvent with a non-flammability, high ionic conductivity, and high thermal and chemical stability [42]. Because of the high viscosity (~ 380 mPa·s) [43], the translocation speed of DNA was efficiently decreased like the case of PEG [44]. Since the viscosity of [bmim][PF₆] was much higher than water ([bmim][PF₆]: ~ 380 mPa·s and water: ~ 1 mPa·s) [43, 45], the velocity of the analyte decreased in the *cis* chamber accordingly.

Interestingly, viscosity gradient system has been used to slow down the translocation speed, in which different viscous solutions were used for each chamber [41, 46]. *Feng et al.* successfully demonstrated the viscous gradient system in their experiments using MoS₂ nanopore, in which the ionic-liquid [bmim][PF₆] was used for the *cis* chamber, and 2.0 M KCl aqueous solution for the *trans* chamber. They showed the translocation speed of 30-nt single-stranded DNA was slowed down to $\sim 3\text{-}5 \mu\text{s} \cdot \text{nt}^{-1}$, and $\sim 0.1 \text{ ms} \cdot \text{nt}^{-1}$ for single nucleotide molecules (dAMP, dTMP, dCMP, dGMP). Since such slow translocation speed was very promising result, we adopted and redesigned their platform to further slow down the translocation speed of single stranded DNA, which is shared in the next sub-chapter 3.2.

After the medium is chosen, sample concentration is considered to yield desirable translo-

cation frequency from the experiment. The concentration has a positive correlation with the translocation frequency at the given voltage bias. For the stable data acquisition, $\sim 10\text{-}100$ events $\cdot\text{min}^{-1}$ has been good for most type of the samples, such as polystyrene, liposome, DNA, or Protein. If the frequency goes over 1000 events $\cdot\text{min}^{-1}$, the pore could get mechanically affected by the restless electrical read-out, which can cause early termination of the experiment due to the irreversible pore damage. In the case of DNA, broad concentrations has been used by many researchers, ranging from $\sim 1\text{-}1000$ $\mu\text{g} \cdot \text{mL}^{-1}$ [41, 47, 48]. This could be because the translocation frequency is closely related to many factors: the nanopore wettability, the sample hydrophilicity, the nanopore geometry, and the surface charge of the nanopore and the sample, *etc.* To theoretically understand the translocation frequency, Kramers' theory should be used, *i.e.*, $J = \dot{\omega} \exp\left(\frac{q\Delta V - U}{\kappa_B T}\right)$, where $\dot{\omega}$, q , U , ΔV were translocation attempt rate ($\dot{\omega}$), effective charge of ssDNA end segment (q), energy barrier (U), applied voltage (ΔV), respectively [49]. The translocation frequency is proportional to the translocation attempt rate ($\dot{\omega}$) that has a positive relation with the sample concentration, and the exponential term that includes the external energy term ($q\Delta V$) and the energy barrier (U). Since the energy barrier of nanopore experiment represents the translocation barrier of the analyte through the nanopore, it is closely related to the size and flexibility of the analyte, the dimension and wettability of the nanopore, *etc.* From time to time, we could observe almost no events, even though the sample solution had DNA molecules with high concentration. In that case, the wetting status of the nanopore should be improved by treating the ultraviolet-ozone (UV-O₃) for a longer time up to ~ 30 min.

3.2. Nanopore Characterization

Before the DNA translocation experiments, the nanopore should be first characterized to check 1) nanopore conductance, 2) nanopore noise level, and 3) baseline stability. The nanopore conductance (G) can be derived by an equation, $G_0 = \sigma[\frac{l_{eff}}{\pi D_0^2} + \frac{1}{D_0}]^{-1}$, where σ , l_{eff} and D_0 are the conductivity of the salt medium, the effective thickness of nanopore, and the diameter of nanopore, respectively [50]. Assuming that we know the nanopore size from

the TEM image, the nanopore conductance should match to the real conductance by the Ohm's law, $G_0 = I_0 V$. If the nanopore conductance is smaller to the expected value, there is a high chance that the nanopore is contaminated or unwetted by the water molecules. If the conductance is larger, the nanopore could be damaged during the TEM drilling process or the cleaning step. In the case of the small nanopore (2-10 nm in diameter), the effective diameter should be well considered because there is an electrical double layer (EDL) on the nanopore surface. For example, if we use 1 M KCl, the EDL thickness could become near 1 nm [51], by which the 2 nm nanopore could be almost not conductible for ssDNA molecules which has ~ 1 nm in diameter. Thus, by considering the EDL, the equation based conductance could be larger than the one from the experiment.

3.2.1. Nanopore Noise

Nanopore noise is one of the most practical issue that we need to consider before running the experiment. It mainly depends on the membrane material, so there should be an expected range of noise level. The simplest way to see the noise level is to check the peak-to-peak distant in a certain time resolution at the certain data recording frequency with a certain frequency filter (Figure 3-1). For example, the SiO_2 nanopore has lower noise level than the Si_xN_y nanopore, and the peak-to-peak noise level were ~ 200 pA (Si_xN_y) and ~ 50 pA (SiO_2) when they were recorded at 200 kHz filtered by 10 kHz low-pass Bessel filter. This method works handy to briefly check the noise during the experiment. The Clampex software (Molecular Devices) has the function to measure the noise amplitude, by which we can calculate it reliably. The RMS noise could be simply calculated by dividing the peak-to-peak noise with 6 for about 99.73% of the noise.

Another way to check the noise is to use the power spectral density graph depending on the frequency [52]. This analysis could be easily done by the Clampex software (the same software for the recording the data) using a certain length (~ 1 s) of baseline at the certain voltage bias (Figure 3-1). Figure 3-1 (a) and (b) are representing the power spectral density graph of Si_xN_y and $\text{TiO}_2/\text{h-BN}$ nanopore, respectively. The power spectral densities were

measured using 4 s current baseline traces at 0 V application, which were recorded at 200 kHz frequency and filtered with 10 kHz low-pass Bessel filter. The Si_xN_y material is known for its low electrical noise, but the $\text{TiO}_2/\text{h-BN}$ nanopore have even better electrical stability, showing lower noise at both low (<1 kHz) and high (>1 kHz) frequencies. For a thorough presentation, the power spectral density could be measured at different biases showing its noise variations according to the external biases. This method is a more standardized method than the peak-to-peak measuring method, which fits to the publication level data analysis. If the power spectral density graph is different from the reference data, the nanopore could have defects or contaminations on the membrane. Therefore, the manufacturing method or cleaning process should be carefully chosen for the best device with higher SNR.

Figure 3.1. An example of power spectral density (PSD). Red and blue graphs represent the PSD of Si_xN_y and $\text{TiO}_2/\text{h-BN}$ nanopore, respectively. The Si_xN_y nanopore had 200 nm thickness and 250 nm in diameter. The $\text{TiO}_2/\text{h-BN}$ had ~ 1 nm thickness and ~ 4 nm in diameter. The PSDs were acquired using 4 s current baselines at 0 V. The data were recorded at 200 kHz frequency and filtered with 10 kHz low-pass Bessel filter.

3.2.2. Baseline Stability

Nanopore sensor is normally required to have a good electrical stability for experimental data acquisition for a long time. The stable baseline can prove that the nanopore device is durable and feasible for the long-recording experiment. In most cases, the baselines were measured at different biases from 0 V to 800 mV by increasing 200 mV each for 30 s to 1 min; the protocol is not fixed, but it is important to show a good baseline stability. In most cases, the baseline becomes unstabler as the applied bias becomes stronger, by which the experimentalist could decide the proper range of data acquisition using the given nanopore.

Chapter 4

DETECTION OF NUCLEOTIDES USING SOLID-STATE NANOPORE

4.1. Purpose and Strategy

4.1.1. Purpose

The universal red cell counter, Coulter counter, has been widely used in almost every hospital for counting and sizing of red blood cells since its invention in 1953 by Wallace H. Coulter. Nanopore sensor is basically $\sim 1,000\times$ smaller version of Coulter counter to assay the nano-scale single molecules instead of the micron-scale cells, by analyzing the electrical resistive pulses from the translocations of analytes through the nano-scale nanopore. Among the various biomolecules, DNA has been doubtless receiving the greatest attention by the nanopore researches due to its importance in human health and well-being (Figure 4-1(a-b)). Nanopores, with their great potential, have been making significant progresses in reading DNA sequence. The application of biological nanopores (the channel proteins from the cell membrane) succeeded in reading DNA sequence, proving the feasibility of nanopore technology as the next-generation sequencing technology. Despite such success, a few fundamental limitations still exist, *i.e.*, low base-calling accuracy ($\sim 90\%$), low reading stability with high failure rate, and the weak mechanical structures of the channel protein and the bilipid membrane. Thus, it is worth putting more research efforts on solid-state nanopores (SSNs) due to their characteristic strengths over the biological nanopores, *i.e.*, full-synthetic body without biological components, compatibility to the mass-production facility with the cutting-edge 1-nm lithography machines, and superior material stability for longer usage.

Figure 4.1. 3-D atomic structure of (a) ssDNA with h-BN nanopore and (b) the components of ssDNA that include the phosphate backbone (dashed red circle), ribose sugar (dashed black circle), and nucleobase (dashed blue circle). There are four types of nucleobases, *i.e.*, adenine, guanine, thymine, and cytosine. Adenine and guanine are purine bases with two-ring structure, and thymine and cytosine are pyrimidine bases with one-ring structure. The images were drawn using 3-D illustration software of visual molecular dynamics ver. 1.9.3.

4.1.2. Strategy

Numerous approaches have been attempted to decrease the translocation speed of ssDNA, such as lowering temperature [53], decreasing size of the nanopore [54], increasing viscosity of the medium [41, 55], lowering bias [37], *etc.* Among them, the viscosity gradient system, which was first demonstrated by *feng et al.* [56], presented the slowest translocation speed of ssDNA ($\sim 5 \mu\text{s} \cdot \text{nt}^{-1}$ for 30-nt and $\sim 0.1 - 0.5 \text{ ms} \cdot \text{nt}^{-1}$ for single nucleotide molecules), for which a 2-D MoS₂ nanopore was used with an ionic gradient where the *cis* and *trans* chambers were filled with different liquids, *i.e.*, a high viscous ionic-liquid (IL) of 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim][PF₆]) for the *cis* side and a low viscous 2.0 M KCl aqueous solution for the *trans* side [41, 56]. In this study, the viscosity gradient system was improved upon in the following ways: First, ssDNA solubility in [bmim][PF₆] was dramatically increased by simply adding 3% water molecules to the ionic-liquid. Second, the added water molecules increased the molecular conductivity by forming H₂O-bound ssDNA, hence the electrical signals of nucleotides were efficiently amplified. Third, h-BN was used to fabricate a 2-D nanopore, which has higher electrical stability than other 2-D materials ($\sim 5.9 \text{ eV}$ bandgap [21] and ~ 3.5 dielectric constant [57]). With the improved platform, the continuous detection of the nucleotides in ssDNA was observed, for which the novel term, base-translocation, is presented here to call the resistive pulses of nucleotide translocations. Various statistical analyzes for each ssDNA sample are presented for a comparative study, and a physical model is provided by COMSOL simulations to explain the base-translocation phenomenon using the SSN with H₂O-bound ssDNA. The goal of this study is not to sequence DNA, but to showcase a new base-translocation phenomenon, which could potentially drive SSN research in the direction towards sequencing.

4.2. Methods

This sub-chapter introduces the specific methods how the experiment was prepared for the experiment. It includes 1) nanopore fabrication, 2) experimental preparation, 3) data acquisition and analysis, 4) COMSOL simulations. The nanopore chip fabrication part is

described in the chapter 2 with more details.

4.2.1. h-BN Nanopore Fabrication

$\times 4 \text{ mm}^2$ Si_xN_y /SU-8 low-noise chips, provided by Protochips, Inc., had a $50 \text{ }\mu\text{m}$ (w) $\times 50 \text{ }\mu\text{m}$ (l) $\times 50 \text{ nm}$ (h, thickness) Si_xN_y window and a $5 \text{ }\mu\text{m}$ thick SU-8 layer coated on the top of the chip with a $30 \times 30 \text{ }\mu\text{m}^2$ square-cut in the middle, exposing the Si_xN_y window (Figure 4-2(a, a-1, a-2)). A circular hole of $\sim 40 \text{ nm}$ in diameter was drilled in the middle of the free-standing Si_xN_y window by converging a 200 kV electron beam of transmission electron microscopy (TEM) (JEM 2100F, JEOL Ltd.) (Figure 4-2(b)) [3, 17]. Monolayer h-BN (Graphene Laboratories, Inc.) was transferred on the top of Si_xN_y hole using the well-established poly-methyl methacrylate (PMMA) transfer method (Figure 4-2(c)) [23]. Because the purchased monolayer h-BN was grown on both sides of Cu foil by chemical vapor deposition (CVD) (Figure 4-2(c-1)), the h-BN layer on one side of the foil was removed by treatment via oxygen plasma in a reactive ion etcher (PX-250, Nordson March) at 100 W, 50 mTorr for 30 s (Figure 4-2(c-2)). The h-BN layer on the other side was then coated with PMMA layer by spreading a PMMA solution ($20 \text{ mg}\cdot\text{ml}^{-1}$ in 1,2-dichlorobenzene, 960k MW, Sigma Aldrich) using a spin coater. The solution was spin-coated on h-BN layer twice each at 3,000 RPM for 60 s and 1,000 RPM for 60 s for cleaner removal of PMMA layer [33] (Figure 4-2(c-3)). The coated PMMA layer was baked at 100°C for 2 min to solidify it after each coating step. A $3 \times 3 \text{ mm}^2$ h-BN piece was cut from the foil using a sharp razor blade and floated on 17.5% nitric acid for 1 min to remove an oxide layer on the Cu foil. After rinsing it with ultra-pure water for 1 min, the Cu layer was etched by floating it on 0.5 M ammonium persulfate solution overnight (Figure 4-2(c-4)). When the Cu foil was clearly removed, the piece became transparent. After rinsing it with ultrapure water for 10 min, the floating piece of PMMA/h-BN was transferred on the top of the prepared chip with the $\sim 40 \text{ nm}$ Si_xN_y hole (Figure 4-2(c)). The SU-8 layered structure on Si_xN_y film helped to hold and settle the PMMA/h-BN layer on the Si_xN_y hole. The transferred film was dried in the air by holding the chip above a hot plate.

After the chip was allowed to cool down, it was submerged in acetone for 3 hr to remove the PMMA layer. The chip was then baked in a furnace with a quartz tube (MTI Corp.) under vacuum with an Ar flow at 100 sccm at 350 °C for 2 hr to remove residual PMMA on the h-BN and to anneal the h-BN layer on Si_xN_y membrane (Figure 4-2(d)). Because h-BN monolayer membrane could be easily damaged during the PMMA cleaning step and the nanopore drilling procedure by the dose of the exposed electron beam, *i.e.*, pinhole or larger pore formation, bilayer h-BN devices were used for the experiment [58]. The second layer of h-BN was transferred on the top of the first layer following the same procedure in Figure 4-2(c-e). Since the bilayer h-BN was fabricated by the manual PMMA transfer method, there was no specific direction dependency in the bilayer stacking structure. ~ 0.5 nm thick TiO_2 layer was deposited on top of the bilayer h-BN film using an atomic layer deposition (ALD) tool with a precursor tetrakis(dimethylamino)titanium (IV) (Savannah S100, Veeco/cnt), by running 12 cycles at a growth rate of $\sim 0.4 \text{ \AA} \cdot \text{cycle}^{-1}$ (Figure S2(f)). The TiO_2 film provided additional mechanical strength to the h-BN membrane to withstand the electron beam exposure in the TEM chamber, as well as a good image contrast while taking a TEM image of the nanopore. Also, TiO_2 made the nanopore super-hydrophilic with the ultraviolet-ozone (UV- O_3) treatment [30, 59], which could increase the hydrophilic interaction with the ssDNA [60, 61]. A ~ 2 s exposure of a focused electron beam at 200 kV was the best for fabricating $\sim 3\text{-}4$ nm diameter nanopore (Figure 4-2(g), Figure 4-3(a)).

Transmission electron microscopy images of the h-BN atomic structure were acquired using JEM-ARM 200F (JEOL Ltd.). As shown in Figure 4-3(b-c), the hexagonal structure of h-BN was clearly observed before coating the TiO_2 film. A selected area electron diffraction (SAED) pattern was obtained to identify the hexagonal structure of boron nitride (Figure 4-3(d)). The pattern in a fast-Fourier transform (FFT) image (Figure 4-3(e)) matched the SAED pattern in Figure 4-3(d), confirming the hexagonal structure of h-BN. TEM micrographs of the nanopores utilized for each ssDNA sample are shown in Figure 4-4(a-1, b-1, c-1) with the current-voltage (I-V) characteristic curves (Figure 4-4(a-2, b-2, c-2)). The electrical noise for each $\text{TiO}_2/\text{h-BN}$ nanopore was benchmarked by the power spectral

Figure 4.2. $\text{TiO}_2/\text{h-BN}$ nanopore fabrication flow chart. (a) $\text{Si}_x\text{N}_y/\text{SU-8}$ chips were supplied by Protochips, Inc. (a-1) and (a-2) show the top- and sidecut-view of the chip, respectively. The 50 nm thick Si_xN_y membrane (purple) and the 5 μm thick SU-8 insulation layer (green) were built on the 300 μm Si wafer (gray). Si_xN_y membrane had a $50 \times 50 \mu\text{m}^2$ window on the silicon wafer, and the SU-8 had a $30 \times 30 \mu\text{m}^2$ square cut on the Si_xN_y membrane. (b) ~ 40 nm hole was drilled on the Si_xN_y free-standing membrane with 200 kV electron beam of TEM. (c-1) CVD-grown single-layer h-BN was purchased from Graphene Laboratories, Inc. (c-2) The backside of h-BN was etched with the reactive ion etcher (RIE). (c-3) PMMA layer was coated on h-BN using a spin coater. (c-4) The Cu foil was etched with 0.5 M ammonium persulfate. (c) PMMA-coated single layer h-BN was transferred on the top of Si_xN_y . (d) The PMMA layer was removed with acetone and the following furnace baking at 350° with Ar gas flow. (e) The 2nd layer of h-BN was transferred on to the 1st layer h-BN using the same steps outlined in (c-d). (f) ~ 0.5 nm thick TiO_2 layer was deposited using ALD (Savannah S100, Veeco/cnt). (g) 3-4 nm pore was made by exposing the focused electron beam of TEM at 200 kV.

Figure 4.3. (a) TEM micrograph of TiO₂/h-BN bilayer nanopore (JEM-2100F TEM, JEOL Ltd.). Dashed magenta circle indicates the location of the nanopore. (b) TEM micrograph showing the hexagonal structure of boron nitride (h-BN) (JEM-ARM 200F, JEOL Ltd.). The image was processed using an average background subtraction filter (ABSF). (c) TEM image of h-BN monolayer before processing the average background subtraction filter (ABSF) (JEM-ARM 200F, JEOL, Ltd.). (d) SAED pattern of crystalized structure of h-BN.(e) The fast-Fourier transform (FFT) of the Figure (c). The dots are clearly matching the SAED pattern in Figure (d).

density (PSD), shown in Figure 4-5(a-c). The PSD for each nanopore was taken using 1.0 s of each baseline at different levels of biases from 0 V to 0.8 V at 200 kHz. It was recommendable to use one nanopore chip to acquire data for the three types of samples because it could provide the consistent experimental condition for the comparative studies. However, [bmim][PF₆] solution on the used chip surface could not be completely cleaned up because it has a poor solubility to the weak solvents, *i.e.*, water, IPA, or acetone, or the strong solvents could damage the ultra-thin membrane in a short time. As a result, three nanopore devices were used for each type of ssDNA sample in this research.

4.2.2. Experimental Preparation

Preliminary experimental work completed within 24 hours prior to experimentation with nanopore included preparing a set of Ag/AgCl electrodes by dipping silver wire segments in 15% sodium hypochlorite solution (Sigma Aldrich) overnight. The electrodes were to be connected to the *cis* and *trans* chambers to apply bias voltage and record ionic current through the nanopore. Three types of ssDNA samples were ordered for 100 μ mol dried pellets from a manufacturer (Integrated DNA Technologies Inc.), which had different lengths and nucleobase compositions: ATG(TCC)₂A (10-nt, 2,962.96 Da), (CCCCCAAAAA)₅ (50-nt, 14,997.75 Da), and (AAGCT)₁₈AA (92-nt, 28,449.35 Da). 1 ml of [bmim][PF₆] (Fisher Scientific) and 30 μ l of distilled and deionized water (18.0 M Ω ·cm) were added together to dissolve the dried ssDNA pellet in the tube, in which the 3% of water was about the maximum soluble water in [bmim][PF₆] [62]. Since [bmim][PF₆] could not dissolve the ssDNA pellet alone, the addition of 3% of water was necessary to make the ssDNA solution uniform. To actively dissolve ssDNA pellet, additional mechanical forces, *i.e.*, shaking, vortexing, and pipetting, were applied to the test tube with ssDNA pellet, which made the solution a milky white color due to the mixture of [bmim][PF₆] and H₂O. After a few hours, the solution became back to *transparent* with the uniform solution of hydrated ssDNA. Based on the literature [63,64], the full hydration of a nucleotide requires $\sim$ 10 H₂O, *i.e.*, 6 to phosphate, 2 to pyrimidine (A, T), and 3 to purine (C, G). The added water molecules have an additional

Figure 4.4. (a-1, b-1, c-1) TEM micrographs of the nanopores and (a-2, b-2, c-2) I-V curves with the linear fittings for 10-nt, 50-nt, and 92-nt samples, respectively. The initial pore conductance for each nanopore was (a-2) 9.1 nS for 10-nt, (b-2) 23.1 nS for 50-nt, and (c-2) 14.2 nS for 92-nt. The pore conductances (G) of each nanopore were derived from the slopes of the linear fittings.

Figure 4.5. The power spectral density (PSD) at different biases for each nanopore, (a) 10-nt, (b) 50-nt, and (c) 92-nt. The PSD graphs were taken from 1.0 s baselines at different levels of biases from 0 V to 0.8 V. The nanopores showed higher stability at low bias and they became noisier as the bias got higher.

role to increase the molecular conductivity of ssDNA and to locally amplify resistive pulses from nucleotides, which is discussed in the result section in detail. The following final concentrations were dissolved in the [bmim][PF₆]/H₂O solution: 1 μ M (= 2.9630 μ g·ml⁻¹) for 10-nt, 1 μ M (= 14.9978 μ g·ml⁻¹) for 50-nt, and 10 μ M (= 284.4935 μ g·ml⁻¹) for 92-nt. 2.0 M KCl aqueous solution for the *trans* chamber was buffered with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Both sides of the nanopore device were subjected to ultraviolet-ozone (UV-O₃) treatment (UV Ozone ProCleaner Plus, Bioforce Nanosciences, Inc.) for 20 min per side to make the surface of TiO₂ more hydrophilic by forming a Ti-OH⁻ layer on its surface [38, 39] (Figure S6). The *cis* chamber was first loaded with [bmim][PF₆] without ssDNA to check any possible contaminants in the solution, nanopore wettability, and its ionic conductance. Afterwards, the content in the *cis* chamber was exchanged with the prepared ssDNA solution.

4.2.3. Data Acquisition and Analysis

Electrical signals were recorded using a software (Clampex, ver. 10.7, Molecular Devices) with an amplifier (Axopatch 200B amplifier, Molecular Devices). The data were recorded at a sampling frequency of 200 kHz, filtered with the inbuilt 10 kHz low-pass Bessel filter, and digitized using a digitizer (Digidata 1440A, Molecular Devices). The recorded resistive pulses were analyzed by custom-built scripts written in MATLAB (ver. 2016, MathWorks, Inc.) with a moving average method [27, 65]. Events were marked when a peak amplitude went over a threshold (ΔI_{thresh}) with respect to a mean baseline, *i.e.*, ΔI_{thresh} were 0.8 nA for 10-nt, 1.0 nA for 50-nt and 0.18 nA for 92-nt. Various features were extracted from the data including peak amplitude (ΔI), translocation time (Δt), base-translocation time (Δt_{base}), inter-base-translocation time (δt_{base}), and number of base-translocations per strand (n_{base}). The methods for analyzing such variables were described in Figure 4-5 in detail.

Figure 4.6. Detection of base-translocations using a custom-built analyzing program in MATLAB scripts. The baseline (orange line) was calculated for every certain length of the data. (a) Peak amplitude (ΔI) was measured by the difference from the baseline to the minimum point of the peak. (b) Strand-translocation time (Δt) was measured by the period between the maximum points of two consecutive entrance current bursts. (c) base-translocation time (Δt_{base}) was calculated by the full-width at the half maximum (FWHM) of the peak. (d) Inter-base-translocation time (δt_{base}) was measured by the period between the minimum points of two consecutive base-peaks. (e) Number of base-translocations per strand (n_{base}) was counted by the number of base-translocations between the two consecutive entrance/exit current bursts.

4.2.4. COMSOL Simulation

To understand the physics of the nanopore model, COMSOL Multiphysics (ver. 5.4, COMSOL, Inc.) was used to solve: 1) the interplay of the fluid flow, 2) the electrostatics, and 3) the transport of ionic species using Poisson-Nernst-Planck-Stokes system of equations. A 2-D axisymmetric system was chosen for building the geometry. The specific procedures for building the geometry and physics are described as follow. First, a 2-D axisymmetric system was chosen. The following physics was then added to the model: Transport of Diluted Species, Electrostatics, and Laminar flow. The defined parameters are listed at the end of the caption. A variable was created for the space charge ($\text{C}\cdot\text{m}^{-3}$) of [bmim][PF₆] and KCl domains. Four materials were added to the model: water, [bmim][PF₆], TiO₂, and h-BN. Their characteristic properties of h-BN and [bmim][PF₆] were manually added, and the properties of TiO₂ and water from the material library of COMSOL. The material properties of h-BN, TiO₂ and [bmim][PF₆] are also shared at the end of the caption. In Electrostatics, a ground was created and assigned at the end of the *cis* chamber. Also, an electric potential was created and assigned at the end of the *trans* chamber. A surface charge density was created and assigned to the TiO₂ and h-BN surface boundaries. A space charge density was created and assigned to the domains of [bmim][PF₆] and KCl. In the model of Transport of Diluted Chemical Species, ‘Convection’ and ‘Migration in Electric Field’ were selected for the transport mechanisms. The diffusion coefficient and the concentration for each ions, K⁺, Cl[−], [bmim]⁺, and [PF₆][−], were created and assigned respectively. In Laminar Flow, two open boundaries were created corresponding to the edges of the simulation box for [bmim][PF₆] and KCl. A volume force was created by ‘space charge’ $\times$ ‘electric field’, and assigned to [bmim][PF₆] and KCl domains. Finally, a ‘Physics Controlled Mesh’ was chosen with the size ‘Finer’, and a stationary study was created to simultaneously solve the multiphysics problem. The table for the used variables for the COMSOL Multiphysics simulation was recorded at Table 4-1, and the material properties of h-BN and [bmim][PF₆] was added to Table 4-2.

Table 4.1. The variables for COMSOL Multiphysics simulations

Description	Expression
Reference concentration	2 [mol·dm ⁻³]
K ⁺ diffusion constant	1.96e-9 [m ² · s ⁻¹]
Cl ⁻ diffusion constant	2.03e-9 [m ² · s ⁻¹]
Electrophoretic bias	0.8 [V]
Temperature	298.15 [K]
Concentration of species 1 K ⁺	2 [mol·l ⁻¹]
Concentration of species 2 Cl ⁻	2 [mol·l ⁻¹]
Valence of K ⁺	1
Valence of Cl ⁻	-1
Mobility of K ⁺	DK/(R_const*T)
Mobility of Cl ⁻	DCl/(R_const*T)
Surface charge density of h-BN	-0.020 [C·m ⁻²]
[bmim] ⁻ diffusion constant	4.35e-12 [m ² · s ⁻¹]
[PF ₆] ⁺ diffusion constant	4.70e-12 [m ² · s ⁻¹]
[bmim][PF ₆] conductivity	0.18 [S·m ⁻¹]
Surface charge of [bmim][PF ₆]	325 [mC·m ⁻²]
Concentration of species 3 [bmim] ⁻	4.8 [mol·l ⁻¹]
Concentration of species 4 [PF ₆] ⁺	4.8 [mol·l ⁻¹]
Valence of [bmim] ⁻	1
Valence of [PF ₆] ⁺	-1
Mobility of [bmim] ⁻	DBmim/(R_const*T)
Mobility of [PF ₆] ⁺	DPF6/(R_const*T)
Viscosity of [bmim][PF ₆]	0.382 [Pa·s]
Density of [bmim][PF ₆]	1.362 [g·cm ⁻³]
Relative permittivity of [bmim][PF ₆]	11.8

Table 4.2. The material properties for COMSOL Multiphysics simulations

Description	h-BN	TiO ₂	[bmim][PF ₆]
Relative permittivity	4.6	8	1
Surface charge density	0 [C·m ⁻²]	-0.02 [C·m ⁻²]	NA
Dynamic viscosity	NA	NA	0.382 [Pa·s]

4.3. Results

In the results section, the experimental data from wet-lab and dry-lab are introduced with the data analyzes. The section includes 1) viscous/ionic gradient interface, 2) DNA translocations, and 3) data analysis.

4.3.1. Viscous/Ionic Gradient Interface

The UV-O₃ treated TiO₂/h-BN nanopore chip was loaded in a set of Teflon flowcells to build the [bmim][PF₆]/2.0 M KCl (pH 8.0) interfacial system at the nanopore, wherein the viscosity/ionic gradient interface was earlier demonstrated by *Feng et al.* using a MoS₂ nanopore [41]. Since the aqueous solubility of [bmim][PF₆] is known to be low ($\sim 2\text{-}3\%$ [62]), the interface built an immiscible layer at the nanopore (Figure 4-7(a, b), Figure 4-8(a, b)), resulting 1) viscosity gradient and 2) ionic gradient with 3) a liquid-junction potential at the nanopore [46, 66]. Due to the high viscosity of [bmim][PF₆] in the *cis* chamber, the access velocity of ssDNA became slower than the case of the conventional aqueous system. According to the Stokes-Einstein relation [51], *i.e.*,

$$\vec{v} = \frac{q}{6\pi\eta R_h} \vec{E} \quad \text{Equation 4-1}$$

, where $\vec{v}$, q , η , R_h , $\vec{E}$ are drift velocity, molecular charge, viscosity, hydrodynamic radius, and electric field, respectively, the velocity is inversely related to the solution viscosity. Since the viscosity of [bmim][PF₆] is much higher than water ([bmim][PF₆]: ~ 350 mPa·s [67] and

water: ~ 1 mPa·s), the velocity of ssDNA decrease accordingly. There was a phase shift at the [bmim][PF₆]/H₂O (*cis/trans*) interface, which built a significant ionic gradient that caused the junction potential, *i.e.*, ~ 0.6 V on the *cis* side ([bmim][PF₆]) and ~ 0 V on the *trans* side (2.0 M KCl) [46]. From the Boltzmann equation, the junction potential ($\Delta\phi_{trans}^{cis}$) could be expressed as,

$$\Delta\phi_{trans}^{cis} = \frac{RT}{2F} \ln \left[\frac{P_{cations}}{P_{anions}} \right] \quad \text{Equation 4-2}$$

, where $P_{cations}$ and P_{anions} are the partition coefficients for cations ([bmim]⁺, K⁺) and anions ([PF₆]⁻, Cl⁻), respectively [68]. The viscosity/ionic gradient interface with the junction potential was the characteristic difference from the conventional water mediated nanopore system. To understand how the ionic interface could further slow down the translocation speed, computational simulations (COMSOL Multiphysics) were executed to calculate 1) the electrical potential field, 2) the ionic concentrations and fluxes, and 3) the electric field using a time-independent finite element method (Figure 4-7(c-e), Figure 4-9(a-h)). Different from a conventional aqueous system, there were abrupt changes in ionic concentrations at the nanopore; these were evident where the concentrations of [bmim]⁺ and [PF₆]⁻ steeply dropped (Figure 4-7(d)) and the magnitude of the electric field sharply decreased across the *cis/trans* interface (Figure 4-7(e)). The electric field on the *cis* side of the nanopore was $1.93 \cdot 10^8$ V·m⁻¹ which was $\sim 4\times$ higher than that on the *trans* side of the nanopore ($0.51 \cdot 10^8$ V·m⁻¹) (Figure 4-7(e)). Since the drift velocity ($\vec{v}$) is proportional to the electric field ($\vec{E}$) (Equation (1)), a steep drop of the electric field magnitude across the nanopore could effectively reduce the translocation speed of ssDNA. Further simulation results including each ionic concentration and flux are described in Figure 4-9 with its associated details.

4.3.2. DNA Translocation Events

Three types of ssDNA samples with different lengths were used, *i.e.*, ATG(TCC)₂A (10-nt), (CCCCCAAAAA)₅ (50-nt), and (AAGCT)₁₈AA (92-nt); the sample preparation steps were described in the methods section in detail. It is known that the radius of gyration (r_g) of ssDNA proportionally increases with the length of ssDNA, *i.e.*, ~ 10 Å for 10-nt, ~ 35 Å

Figure 4.7. (a) Schematic figure for TiO₂/h-BN nanopore sensor with [bmim][PF₆]/KCl interface. (b) Zoomed-in view of the nanopore sensor, where ssDNA is driven by the diffusion ($r > r^*$) and electrophoresis ($r < r^*$) from the high viscous ionic-liquid [bmim][PF₆] (*cis*) to the 2.0 M KCl aqueous solution (pH 8.0) (*trans*). (c-e) Results of COMSOL simulation for (c) electrical potential field of the axisymmetric nanopore system, (d) ionic concentration distribution of each ion and (e) electric field magnitude along the z-axis at the center of nanopore. (f-h) Representative current traces of (f) 10-nt, (g) 50-nt, and (h) 92-nt ssDNA translocations. They have the same scale bar for the time x-axis.

Figure 4.8. (a) Molecular structure of [bmim][PF₆]. (b) Immiscibility of [bmim][PF₆] and H₂O. [bmim][PF₆] and H₂O built a boundary layer in the 15 ml glass vial, because the solubility of H₂O to [bmim][PF₆] is $\sim 2\text{-}3\%$, *i.e.*, $\sim 30 \mu\text{l}$ water in 1 ml [bmim][PF₆]. The liquid below was 3 ml [bmim][PF₆], and the above was 3 ml pure water.

for 50-nt, and $\sim 50 \text{ \AA}$ for 92-nt [69, 70]. Since the radius of gyration and the persistence length have a correlation, *i.e.*,

$$r_g = \left(\frac{l_c \cdot l_p}{3} \right)^{0.5} \quad \text{Equation 4-3}$$

, where r_g , l_c , l_p are radius of gyration, contour length, and persistent length, respectively [71], the persistent length (l_p) could be derived from the radius of gyrations (r_g), *i.e.*, $\sim 0.47 \text{ nm}$ (10-nt), $\sim 0.86 \text{ nm}$ (50-nt), and $\sim 1.29 \text{ nm}$ (92-nt), respectively (Figure 4-10(a-1, b-1, c-1)). This informs that, as the length of ssDNA increased, the flexibility of ssDNA decreased, which could increase the threading barrier across the nanopore. According to the Kramers theory, *i.e.*,

$$j = \dot{\omega} \exp \left(\frac{q\Delta V - U}{\kappa_B T} \right) \quad \text{Equation 4-4}$$

, where $\dot{\omega}$, q , U , ΔV were translocation attempt rate ($\dot{\omega}$), effective charge of ssDNA end segment (q), energy barrier (U), applied voltage (ΔV), respectively [49], the translocation frequency is proportional to the translocation attempt rate ($\dot{\omega}$) that has a positive relation with the sample concentration. The desirable translocation frequency (j) could be achieved by preparing ssDNA samples as follow, *i.e.*, 95 s^{-1} ($1 \mu\text{M}$ 10-nt at 0.8 V , $n = 3,450$), 663 s^{-1} ($1 \mu\text{M}$ 50-nt at 1.0 V , $n = 11,014$), and 136 s^{-1} ($10 \mu\text{M}$ 92-nt at 0.8 V , $n = 10,599$) (Figure

Figure 4.9. COMSOL simulation results for [bmim][PF₆]/2.0 M KCl interfacial system, showing the ionic concentrations of (a) K⁺, (b) Cl⁻, (c) [bmim]⁺, and (d) [PF₆]⁻ and ionic fluxes of (e) K⁺, (f) Cl⁻, (g) [bmim]⁺, and (h) [PF₆]⁻. The intensity plots are the time-independent 2-D finite element simulations (COMSOL Multiphysics ver. 5.4) using Poisson-Nernst-Planck-Stokes system. The geometry consists of a *cis* reservoir (40 nm × 50 nm) defined as the ionic-liquid [bmim][PF₆] with a viscosity of 0.382 Pa·s, while a *trans* reservoir (40 nm × 50 nm) was defined as an aqueous 2.0 M KCl solution. The reservoirs were separated by a 1 nm thick layer (0.5 nm TiO₂/0.5 nm h-BN) with a 2.0 nm radius nanopore. An electrophoretic bias of +0.8 V was applied to the bottom of the simulation box, while its top was grounded. -0.02 C·m⁻² surface charge density was assigned to the surface of the TiO₂, and 0 C·m⁻² on h-BN. The boundary conditions were set as open boundaries for the laminar flow, an electric potential and ground at the bottom and top of the geometry for the electrostatics, and no charges on the sides, and initial concentrations on the boundaries of 4.8 M [bmim][PF₆] for the *cis* side and 2.0 M KCl for the *trans* side for the transport of diluted species.

4-7(f-h)). Interestingly, stable translocation events were observed at higher bias ($\Delta V > +0.8$ V) for the three types of samples, but the translocation events were not consistent at lower bias ($\Delta V < +0.8$ V) (Figure 4-11(a-b)). This phenomenon does not commonly occur with the water-mediated nanopore system, wherein the stable translocation occurs from the lower voltage, showing characteristic translocation time and peak amplitudes [72]. However, the nanopore platform with the viscosity/ionic gradient did not follow it, probably because of its unique attribute of the strong junction potential at the [bmim][PF₆]/H₂O liquid interface (~ 0.6 V) [46]. *Shankla et al.* [46] showed that the external bias lowered the junction potential; the junction potential decreased to ~ 0.4 V at the external bias of 0.2 V. This tells us that the high external bias ($\Delta V > +0.8$ V) might have weakened the junction potential, *i.e.*, close to zero, at the nanopore, when the ssDNA could start to translocate the nanopore. Based on the open-pore current (Figure 4-10), the effective diameter of nanopore was calculated using the conductance equation [50],

$$G_0 = \sigma \left[\frac{4l_{eff}}{\pi D_0^2} + \frac{1}{D_0} \right]^{-1} \quad \text{Equation 4-5}$$

, where σ , l_{eff} , D_0 are the conductivity of the salt medium (2.0 M KCl, 22 S·m⁻¹), the effective thickness of nanopore membrane (1 nm), and the effective diameter of nanopore, respectively. By this Equation (5), the diameter of each chip was derived as 2.03 nm (chip 1 for 10-nt, $I_0 = 22$ nA at 0.8 V), 5.11 nm (chip 2 for 50-nt, $I_0 = 90$ nA at 1.0 V), and 1.52 nm (chip 3 for 92-nt, $I_0 = 14.5$ nA at 0.8 V). The chip 1 and 3 were remained to have a good effective diameter of nanopore for base-detection (~ 1.5 -2 nm), but the nanopore on chip 2 became much larger than the other two (~ 5 nm), possibly due to the damage occurred during the nanopore drilling process using TEM. Because of the nanopore variation, the event frequency of 50-nt became much higher (663 s⁻¹) than that of 10-nt (95 s⁻¹) and 92-nt (136 s⁻¹). Due to the bigger size of chip 2, the 50-nt data was not adequate for the comparative analysis with the data from 10-nt and 92-nt or for the base-detection. As shown in Figure 4-10(a) and Figure 4-11(d), there was a brief surge of ionic conductance ($\sim 1.3 \times$ of baseline

value) at the entrance/exit of 10-nt ssDNA, followed by multiple resistive pulses by the nucleobase translocations. The abrupt conductance surge at the entrance/exit could be due to the brief ionic crowding effect near the translocating strand [44]. As far as our knowledge goes, this is the first time to continuously detect nucleobases using SSN; and therefore, more of the representative current traces with base-peaks are shared in Figure 4-12(a-e). Unlike the case of 10-nt ssDNA, both cases of 50-nt and 92-nt ssDNA showed neither base-translocations nor entrance/exit current bursts, but only conventional translocation peaks, *i.e.*, one pulse per translocating strand (Figure 4-10(b, c)). In the case of 50-nt, its nanopore device had the diameter of ~ 5 nm, which was not adequate for presenting base-translocations. On the other hand, the nanopore device of 92-nt did not show the base-translocations, although its effective diameter was ~ 1.5 nm. This could be explained by the mechanical interaction of the large size of the 92-nt (r_g : ~ 5 nm [69, 70]) and the lower flexibility with higher persistence length (l_p : ~ 1.29 nm, (Equation (4-3))). The threading process of 92-nt translocations could break or weaken the [bmim][PF₆]/KCl interface, hence the translocation speed became faster than the temporal resolution required for sensing nucleobases. The shapes of resistive pulses were spike-like with shorter translocation time for 50-nt (Figure 4-10(b-2, b-3)) and square-like with longer translocation time for 92-nt (Figure 4-10(c-2, c-3)). These shapes were expected because 1) the nanopore device for 50-nt had $\sim 3\times$ larger diameter and 2) the length of 92-nt ssDNA was $\sim 2\times$ longer than 50-nt, taking a longer time for threading the nanopore [73].

4.3.3. Data Analysis

4.3.3.1. Translocation Time (Δt) and Peak Amplitude (ΔI)

Figure 4-13 shows scatter plots of the peak amplitude (ΔI) versus i) the base-translocation time (Δt_{base}) for 10-nt (Figure 4-13(a)) and ii) the strand-translocation time (Δt) for 50-nt and 92-nt (Figure 4-13(b, c)). The mean peak amplitudes ($\overline{\Delta I}$) and the mean base-translocation time ($\overline{\Delta t_{base}}$) of 10-nt ($n = 22,145$) were analyzed to be 1.4517 ± 0.4573 nA and 0.1220 ± 0.0417 ms (Figure 4-13(a)). In the case of 50-nt ($n = 11,014$), $\overline{\Delta I}$ and $\overline{\Delta t}$ were

Figure 4.10. Base-translocations of 10-nt ssDNA vs. strand-translocations of 50-nt and 92-nt ssDNAs. Schematic figures for translocations of 10-nt, 50-nt, and 92-nt are shown in the first row (a-1, b-1, and c-1). Dashed red lines at the nanopore represent the [bmim][PF₆]/KCl interface. Translocation events of 10-nt have a steady interface, but those of 50-nt and 92-nt had unsteady interfaces due to the radius of gyration (r_g) larger than the nanopore. The second row (a-2, b-2, and c-2) shows 20 ms of translocation events for 10-nt, 50-nt and 92-nt, respectively. The third row (a-3, b-3, and c-3) shows 5 ms of raw data for 10-nt, 50-nt, and 92-nt, respectively, which are the zoomed-in views of the dashed red boxes on the second row. The inter-base-translocation time (δt_{base}) is marked on (a-3).

Figure 4.11. Figures (a-1, b-1, c-1) show the real-time currents of each nanopore loaded with the samples (10-nt, 50-nt, and 92-nt, respectively) at various biases. Figures (a-2, b-2, c-2) show the zoomed-in views of the translocation events for each sample (10-nt, 50-nt, and 92-nt) at higher voltages ($\Delta V > 0.8$ V). Figure (a-2) shows the zoomed-in view of the dashed red box in (a-1), presenting the 10-nt translocation events at 0.8 V. Figure (b-2) shows the 50-nt translocation events at 1.0 V. (c-2) shows the zoomed-in view of the dashed red box in (c-1), presenting the 92-nt translocation events at 1.0 V. (d-1) The initial entrance current burst and (d-2) the final exit current burst of 10-nt translocation train.

Figure 4.12. (a-e) The representative 0.5 s current traces of 10-nt translocation events through the $\text{TiO}_2/\text{h-BN}$ nanopore with $[\text{bmim}][\text{PF}_6]/\text{KCl}$ 2.0 M viscosity/ionic gradient interface.

1.5787 ± 1.9108 nA and 0.0969 ± 0.0906 ms (Figure 4-13(b)), and for 92-nt ($n = 10,598$), $\overline{\Delta I}$ and $\overline{\Delta t}$ were 0.3407 ± 0.1080 nA and 0.3385 ± 0.1896 ms, respectively (Figure 4-13(c)).

Figure 4.13. Scatter plots of peak amplitude (ΔI) vs. translocation time (Δt_{base} or Δt) with the corresponding histograms. The mean ($\overline{X}$) and the standard deviation (σ) of ΔI were (a) $\overline{X} = 1.4517$ nA, $\sigma = 0.2863$ nA (10-nt, $n = 22,145$), (b) $\overline{X} = 1.5799$ nA, $\sigma = 0.9554$ nA (50-nt, $n = 11,014$), (c) $\overline{X} = 0.3407$ nA, $\sigma = 0.0540$ nA (92-nt, $n = 10,598$). The mean ($\overline{X}$) and the standard deviation (σ) of translocation time (Δt_{base} or Δt) were (a) $\overline{X} = 0.1220$ ms, $\sigma = 0.0207$ ms (10-nt), (b) $\overline{X} = 0.0969$ ms, $\sigma = 0.0453$ ms (50-nt), and (c) $\overline{X} = 0.3385$ ms, $\sigma = 0.0948$ ms (92-nt).

4.3.3.2. Base-Translocation Analysis

To characterize the base-translocations of 10-nt, the periods between two consecutive base-translocation peaks, *i.e.*, inter-base-translocation time (δt_{base}), were analyzed, and the sample mean ($\overline{X}$) was found to be 1.4354 ± 0.2335 ms ($n = 19,624$) (Figure 4-14(a)). The distribution was heavily centered near the mean, which could be due to the uniformly distributed nucleotides in ssDNA, *i.e.*, ~ 0.63 nm between each base [46]. Interestingly, the inter-base-translocation time (δt_{base}) of 10-nt was densely centered at $\sim 1.43 \pm 0.23$ ms, which was $\sim 12\times$ of base-translocation time (Δt_{base}), *i.e.*, $\sim 0.12 \pm 0.04$ ms. This tells us that the 10-nt molecule translocated nanopore in a go(base)-stop(inter-base)-go(base) fashion [60,74], spending much longer time at the inter-base region, *i.e.*, phosphate group, than at the base of ssDNA. The strand-translocation time (Δt) of 10-nt was analyzed by calculating the dura-

tion between the two consecutive current bursts (Figure 4-14(b)), which resulted in 12.5512 ± 29.7705 ms. The translocation speed of each 10-nt was derived from dividing the translocation time by the number of nucleotides in each strand, which was found to be 1.8408 ± 0.9908 ms·nt⁻¹ (Figure 4-14(c)). The number of base-translocations per strand (n_{base}) of 10-nt was also calculated by counting the base-translocation peaks between two adjacent entrance/exit current bursts (Figure 4-15(a)). The sample mean ($\bar{X}$) of the distribution of n_{base} was 8.6622 ± 19.3326 (2σ) ($n = 2,440$ strands) which was close to the target number of 10. The unfavorable standard deviation (σ) could be because of a few reasons: First, according to the DNA manufacturer (Integrated DNA Technologies, Inc.), the manufacturing accuracy was limited to $\sim 93\%$ for 10-nt with the uncertainty of $\sim 7\%$, comprising of shorter fragments. Second, the sample preparation steps for dissolving ssDNA with [bmim][PF₆], such as shaking, vortexing, or pipetting, could mechanically sheer the sample, making it shorter than the targeting length (Figure 4-15(a)). Third, the custom-built data analyzing program (MATLAB) could not distinguish two or more consecutive ssDNA molecules entering the nanopore, if there was little spacing between the molecules, surpassing the temporal resolution needed to distinguish the first from the second molecule (Figure 4-15(a-d)). During the data acquisition, a noticeable difference in the inter-DNA time (δt_{DNA}) was observed (Figure 4-16(a-c)). The ssDNA molecules loosely translocated with longer δt_{DNA} when the ssDNA translocation events started to be detected, *i.e.*, loose-translocations, and after a while, the events began to show the regular translocations with shorter δt_{DNA} , *i.e.*, tight-translocations. The loose-translocations showed $\sim 13\times$ longer δt_{DNA} ($\bar{X} = 0.4442$ ms) than the tight-translocations ($\bar{X} = 0.0336$ ms), telling the distance between ssDNA molecules near the nanopore was also longer as much. Because of the longer δt_{DNA} , the peak analyzer (MATLAB) could detect with higher accuracy, showing more favorable distribution of n_{base} ($\bar{X} = 7.4486 \pm 15.3914$ (2σ), Figure S14(a)) than the tight-translocations ($\bar{X} = 8.6622 \pm 19.3326$ (2σ), Figure 4-15(a)). The data analyzes for the long-translocation were shown in Figure S14(b-d) for the inter-base duration (Δt_{base}), the translocation time (Δt), and the translocation speed of the loose-translocations, respectively.

Figure 4.14. Histograms for (a) inter-base time of 10-nt ($n = 19,624$, $\bar{X} = 1.4354 \text{ ms}$, $\sigma = 0.1168 \text{ ms}$), (b) translocation time of 10-nt ($n = 2,830$, $\bar{X} = 12.5512 \text{ ms}$, $\sigma = 14.8852$), (c) translocation speed of 10-nt ($n = 2,440$, $\bar{X} = 1.8408 \text{ ms} \cdot \text{nt}^{-1}$, $\sigma = 0.4954 \text{ ms} \cdot \text{nt}^{-1}$), and (d) normalized peak amplitude ($\Delta I / I_0$) for 10-nt (light gray, $n = 22,145$, $\bar{X} = 0.0616$, $\sigma = 0.0093$) and 92-nt (dark gray, $n = 10,598$, $\bar{X} = 0.0234$, $\sigma = 0.0037$). All histograms are fitted by the normal distributions.

Figure 4.15. (a) The histogram of the number of the base-translocations per strand (n_{base}) of 10-nt with normal distribution ($n = 2,440$, 8.6 ± 19.4 bases per strand). (b) An example of skipping small current bursts (dashed green circles), compared to the normal current bursts (dashed black circle). Three small current bursts were skipped and four strands were counted to be one strand. The red numbers in the parentheses were the number of base-translocation per strand (n_{base}) if the current bursts were detected by the custom-built analysis program written in MATLAB scripts. (c) A schematic figure in the case of the longer number of base-translocation per strand ($n_{base} > 10$). The distance between the two molecules are too close to discern one from the other by the system. (d) A schematic figure in the case of the normal detection of two ssDNA molecules when there is an enough spacing between the molecules.

Figure 4.16. The representative current traces of the loos-translocations events of 10-nt ssDNA through the $\text{TiO}_2/\text{h-BN}$ nanopore with $[\text{bmim}][\text{PF}_6]/\text{KCl}$ 2.0 M viscosity/ionic gradient interface. Figure (a-2) is the zoomed in image of the dashed red box in Figure (a-1), presenting the method how the inter-DNA time (δt_{DNA}), translocation time (Δt), inter-base translocation time (δt_{base}), base-translocation time (Δt_{base}) were analyzed using the custom-built MATLAB program.

Figure 4.17. The histograms of the loose-translocations, *i.e.*, translocations with longer inter-DNA duration, of 10-nt ssDNA. (a) The histogram of the number of base-translocations per strand (n_{base}) with normal distribution ($n = 292$ strands, $\bar{X} = 7.4 \pm 15.4$ bases per strand). (b) The histogram of the inter-base translocation time (δt_{base}) with normal distribution ($n = 2,185$ bases, $\bar{X} = 1.6 \pm 0.2$ ms). (c) The histogram of the translocation time (Δt) ($n = 292$ strands, $\bar{X} = 10.8493 \pm 20.9516$ ms). (d) The histogram of translocation speed with normal distribution ($n = 292$ strands, $\bar{X} = 1.5491 \pm 0.7844$ $\text{ms} \cdot \text{nt}^{-1}$).

4.3.3.3. Translocation Speed Analysis

In the cases of 50-nt and 92-nt, the translocation speeds were simply derived from dividing the mean translocation time ($\overline{\Delta t}$) by the number of nucleotides (50 or 92), which resulted in $1.938 \pm 1.812 \mu\text{s}\cdot\text{nt}^{-1}$ for 50-nt and $3.6902 \pm 2.0608 \mu\text{s}\cdot\text{nt}^{-1}$ for 92-nt, thus presenting $\sim 500\text{--}1000\times$ faster than the case of 10-nt ssDNA. Interestingly, 50-nt and 92-nt samples showed very close values to the translocation speed suggested by *Feng et al.* [41] ($\sim 3\text{--}5 \mu\text{s}\cdot\text{nt}^{-1}$ for 30-nt ssDNA), where a similar ionic gradient was used. Also, the translocation speed of nucleobase in 10-nt ssDNA, *i.e.*, ~ 0.12 ms for nucleobase (Figure 4-13(a)), was close to the translocation speed of single nucleotide molecules (deoxynucleotide monophosphate such as dAMP, dGMP, dCMP, dTMP) also suggested by *Feng et al.* [41] ($\sim 0.1\text{--}0.5 \text{ ms}\cdot\text{nt}^{-1}$). Both 10-nt ssDNA used in this study and the single nucleotide molecules by *Feng et al.* [41] had the radius of gyration (r_g) of ~ 1 nm [70], by which the nanopore device with [bmim][PF₆]/KCl interface could successfully reduce the translocation speed without breaking the viscous and ionic interface.

4.3.3.4. Nanopore Sensitivity and Hydrated ssDNA

To compare the peak amplitudes, the normalized peak amplitude ($\Delta I/I_0$) was derived from 10-nt and 92-nt sample (Figure 4-14(d)) [27, 28, 75]. The mean ($\overline{\Delta I/I_0}$) for each 10-nt and 92-nt was 0.0616 ± 0.0186 and 0.0234 ± 0.0074 , respectively, presenting that the mean ($\overline{\Delta I/I_0}$) of 10-nt was $\sim 3\times$ higher than that of 92-nt (Figure 4-14(d)). Thus, the sensitivity for detecting 10-nt ssDNA was much higher than that of 92-nt ssDNA. In the case of 50-nt data with larger nanopore (~ 5 nm), two groups of different mean amplitudes were observed in the histogram, which could be fitted by the two Gaussian distributions that are in the blue and the red lines of Figure 4-18(a), respectively [73]. The smaller peak amplitude ($\overline{X} = 1105.24 \pm 560.4$ pA), which is dominant in the population, could be representing for the group of linear translocation without folding, and the larger peak amplitude ($\overline{X} = 3477.75 \pm 997.48$ pA) for those of translocating in a folded form (Figure 4-18(b)). It was found by comparing the sample preparation method with that of *Feng et al.* [41], that the

reason for the higher sensitivity of the nanopore device for 10-nt than that for 92-nt could be originated from the different molecular conductivity of ssDNA. The research by *Feng et al.* [41] used the ionic-liquid [bmim][PF₆] alone to dissolve dried ssDNA pellet and produced [bmim][PF₆]-bound ssDNA (IL-ssDNA) [46, 76], whereas, in this study, 3% of water was added to [bmim][PF₆] liquid to facilitate the dissolution of ssDNA pellet, producing water-bound ssDNA (H₂O-ssDNA) [77]. As a result, the water molecules bound to ssDNA increased the molecular conductivity of ssDNA and amplified the electrical signals from nucleotides in consequence [78–80]. In Figure 4-14(d), 10-nt ssDNA could be more hydrated than 92-nt due to its simpler conformational structure of 10-nt ssDNA, which allowed 3× higher sensitivity than the cases of 92-nt events. The physics of this could be understood by the Ohm's law, *i.e.*,

$$\vec{J} = \sigma \vec{E} \quad \text{Equation 4-6}$$

, where $\vec{J}$, σ , $\vec{E}$ are current density, electrical conductivity, and electric field, respectively. Assuming the electrical conductivity doesn't change over time, the time derivative terms could be derived by differentiating the equation with time, *i.e.*,

$$\frac{d\vec{J}}{dt} = \sigma \frac{d\vec{E}}{dt} \quad \text{Equation 4-7}$$

, where the time derivative of the current density $\left(\frac{d\vec{J}}{dt}\right)$ represents the sensitivity of the nanopore platform which is directly related to the time derivative of the electric field $\left(\frac{d\vec{E}}{dt}\right)$. The electrical conductivity (σ) works as a coefficient that amplifies the time derivative of the electric field, *i.e.*, electrical sensitivity of the nanopore. Based on the literature [64, 66], ssDNA molecular conductivity is exponentially related to its hydration ratio of ssDNA, and the relationship could be derived as,

$$\sigma_{ssDNA} \cong 0.0025 \cdot 1.15^H \quad \text{Equation 4-8}$$

, where σ_{ssDNA} and H are the molecular conductivity of ssDNA (nS) and the hydration ratio (%) of ssDNA molecule [79]. If ssDNA is hydrated by 50%, the molecular conductivity is increased by $\sim 1,000\times$ compared to the non-hydrated ssDNA. This could explain how water molecules bound to ssDNA play a critical role to amplify the electrical signals from nucleotides during the translocation event.

Figure 4.18. (a) The histogram of 50-nt peak amplitudes (ΔI) with the two Gaussian fittings ($\overline{X}_1 = 1105.24 \pm 560.4$ pA, $\overline{X}_2 = 3477.75 \pm 997.48$ pA, total $n = 11,014$). (b) The schematic drawing for the shorter (case 1) and larger (case 2) peak amplitudes. Due to the larger size of nanopore device (~ 5 nm in diameter), there could be the translocations of folded ssDNA.

The hydration dependency of the electrical signals of ssDNA translocation events was experimentally observed. In the most time of the data acquisition for 10-nt translocation events, the base-peaks showed very uniform amplitudes (1.4517 ± 0.5726 nA, Figure 4-14(a)), but infrequently, there were peak amplitudes evidently larger or smaller than the normal-sized base-peaks (Figure 4-19(a-c)). In the case of the current traces for larger base-peaks (Figure 4-19(c-1, c-2)), there were clear inter-DNA durations (δt_{DNA}) with the baseline recoveries before and after each translocation, which further support the base-translocation phenomenon of 10-nt ssDNA. The mean peak amplitude ($\overline{\Delta I}$) of smaller base-peaks was 0.4387 ± 0.4329 nA ($n = 2,143$) (Figure 4-19(d)), which was $\sim 70\%$ smaller than the normal base-peaks (1.4517 ± 0.5762 nA). On the other hand, the mean peak amplitude ($\overline{\Delta I}$) of

larger base-peaks was 2.4397 ± 1.3976 (Figure 4-19(d)), which was $\sim 70\%$ larger than the normal case. Based on the literature [63, 64], the different number of H_2O molecules were bound to the different parts of the nucleotide, *i.e.*, phosphate backbone and nucleobases (Figure 4-1(a)), as well as to the different types of nucleobases, *i.e.*, purines (C, G) and pyrimidines (A, T) (Figure S1(b)), as described earlier in the methods section. As a result, the differently hydrated ssDNA, *i.e.*, more- or less-hydrated ssDNA, could show different signal amplitudes, *i.e.*, larger or smaller base-peaks, while increasing or decreasing the SNR with evident differences.

4.3.3.5. COMSOL Multiphysics for Hydrated ssDNA Translocation

COMSOL Multiphysics was used to further prove the effect of water molecules bound to ssDNA during the translocation (Figure 4-20, Figure 4-21). Multiple static simulations were executed to see how the electric field changes depended on the location of 10-nt ssDNA during the translocation event. The basic physics to solve the system remained the same as the case without ssDNA structure (Figure 4-9), and geometries were newly added or designed for chambers, nanopore, and ssDNA (Figure 4-21(a)). To compare the translocation events of H_2O -ssDNA (Figure 4-21(b)) and IL-ssDNA (Figure 4-21(c)), the material property of ssDNA structure was assigned according to its surface-bound molecule (H_2O or $[\text{bmim}][\text{PF}_6]$). The electric flux ($\Phi / \text{V}\cdot\text{m}$) at the center area of the nanopore was calculated by COMSOL to track the electric field variations depending on the location of ssDNA (Figure 4-21(d)). The position of ssDNA structure was moved by 0.5 nm following the center axis of the nanopore for each simulation to measure the electric flux (Figure 4-21(e), Table 4-3). The simulation result for the case of H_2O -ssDNA showed a similar translocation phenomenon to the case of the experiment (Figure 4-10(a), Figure 4-20(a)). There was a brief current surge at the entrance of the molecule, followed by base-translocations during the translocation, and the final current burst flagging the exit of ssDNA. On the contrary, the simulation result for the case of IL-ssDNA did not show any base-translocations or current bursts (Figure 4-20(b)). To prove the feasibility of the COMSOL simulation, we compared both cases of the base-

Figure 4.19. The representative current traces of the larger and the smaller translocation base-peaks of 10-nt ssDNA through $\text{TiO}_2/\text{h-BN}$ nanopore with $[\text{bmim}][\text{PF}_6]/\text{KCl}$ interface. Figure (a) shows the current trace with the larger and the smaller base-peaks together. Figure (b) and (c) show the smaller and the larger base-peaks, respectively. (d) The histograms in probability density function to compare the peak amplitudes of the normal, smaller, and larger base-peaks in a graph with the normal distributions. (Normal base-peaks have a distribution of 1.4517 ± 0.4572 nA ($n = 22,109$), smaller base-peaks 0.4387 ± 0.4329 nA ($n = 2,143$), and larger base-peaks 2.5796 ± 0.9185 nA ($n = 1,033$)).

translocations from the simulation and the experiment using the measure of sensitivity in non-dimensionalized form. By the normalization of Equation (6), the non-dimensionalized form is derived, *i.e.*,

$$\frac{\Delta \vec{J}}{\vec{J}_0} = \frac{\Delta \vec{E}}{\vec{E}_0} \quad \text{Equation 4-9}$$

, where $\vec{J}_0$ and $\vec{E}_0$ are the baselines of current density and electric field, respectively. By surface integral of Equation (4-6), the electric flux term could be derived, *i.e.*,

$$\frac{\Delta I}{I_0} = \frac{\Delta \Phi}{\Phi_0} \quad \text{Equation 4-10}$$

, where I_0 and Φ_0 are the baselines of current and electric flux, respectively. The mean normalized electric flux ($\overline{\Delta \Phi / \Phi_0}$) of the base-translocations from the simulation was calculated to be 0.0618 (Figure 4-20(a), Table 4-3) which was almost the same value as the mean normalized peak amplitude ($\overline{\Delta I / I_0}$) from the experiment (0.0616 in Figure 4-14(d)), thus corresponding to Equation (4-10). This implied that the simulation was reliable and produced a sound result, thus indicating that the water molecules bound to ssDNA played a pivotal role in yielding the clear resistive pulses from the nucleobases using the SSN in the experiment.

4.3.4. Discussion

In the case of 92-nt, the effective nanopore size was ideal (~ 1.5 nm), but the nanopore platform still lost its high sensitivity and low translocation speed. This could be explained by the two reasons: 1) the less hydration ratio of 92-nt and 2) the loss of the strong viscosity/ionic gradient interface. The right amount water for dissolving ssDNA should be added according to the amount of ssDNA dissolved in the solution. Based on the research of *Bastos et al.* [81], the H₂O:nt binding ratio was ~ 0.434 g·H₂O (~ 0.024 mol) / g·ssDNA (~ 0.002 mol of nt), *i.e.*, 12 (H₂O):1(nt). Applying this ratio to the ssDNA sample preparation, the utilized ssDNA samples of 10-nt ($2.9630 \mu\text{g}\cdot\text{ml}^{-1}$), 50-nt ($14.9978 \mu\text{g}\cdot\text{ml}^{-1}$), and 92-nt ($284.4935 \mu\text{g}\cdot\text{ml}^{-1}$) required $1.2859 \mu\text{g}\cdot\text{H}_2\text{O}$, $6.5090 \mu\text{g}\cdot\text{H}_2\text{O}$, and $123.4702 \mu\text{g}\cdot\text{H}_2\text{O}$, respectively in this

Table 4.3. The derivation of the normalized electric flux ($\overline{\Delta\Phi/\Phi_0}$)

Loc. (nm)	Electric flux ($\Phi/V \cdot m$)	base	$\Delta\Phi(V \cdot m)$	$\Delta\Phi/\Phi_0$	$\overline{\Delta\Phi/\Phi_0}$
0	1.3738E-10				
0.5	1.3739E-10				
1	9.4749E-11	First	3.4480E-12	3.5112E-2	6.1832E-2
1.5	9.8198E-11				
2	9.0092E-11	Second	7.4649E-12	7.6519E-2	
2.5	9.6916E-11				
3	9.3155E-11	Third	5.3270E-12	5.4090E-2	
3.5	1.0005E-10				
4	9.4788E-11	Fourth	6.3260E-12	6.2562E-2	
4.5	1.0218E-10				
5	9.6140E-11	Fifth	6.5650E-12	6.3920E-2	
5.5	1.0323E-10				
6	9.6827E-11	Sixth	6.6179E-12	6.3976E-2	
6.5	1.0365E-10				
7	9.7077E-11	Seventh	6.5570E-12	6.32701E-2	
7.5	1.0361E-10				
8	9.6688E-11	Eighth	6.5069E-12	6.3055E-2	
8.5	1.0278E-10				
9	9.6498E-11	Ninth	7.2510E-12	6.9889E-2	
9.5	1.0472E-10				
10	1.0136E-10	Tenth	7.1550E-12	6.5929E-2	
10.5	1.1233E-10

Figure 4.20. Results of computational simulations using COMSOL Multiphysics. Time-independent static simulations depending on the locations of the ssDNA along the z-axis were studied individually. The electric flux ($\Phi / \text{V}\cdot\text{m}$) was derived at the center area of the nanopore for (a) H_2O -ssDNA and (b) IL-ssDNA. The inset figures are the geometry of ssDNA located at 5 nm from the nanopore in the middle of the translocation. $[\text{bmim}][\text{PF}_6]$, H_2O , TiO_2 , and h-BN are colored in yellow, light blue, gray, and white, respectively. The dashed magenta line indicates the region where the electric flux was calculated. The specific information for the simulation was described in Figure 4-21 in detail.

study. This informs that $30 \mu\text{l}\cdot\text{H}_2\text{O}$ could be enough for hydrating 10-nt and 50-nt but not for 92-nt; as a result, the SNR of 92-nt peaks significantly decreased as well (Figure 4-14(d)). Also, the fast translocation speed of 92-nt was because of the loss of the slowing down effect of the viscosity/ionic gradient interface; the mechanical interactions between the large and stiff 92-nt ssDNA ($r_g = \sim 5 \text{ nm}$, $l_p = \sim 1.29 \text{ nm}$) and the small nanopore ($D_0 = \sim 1.5 \text{ nm}$) interrupted the viscosity/ionic gradient interface during the threading process. It was earlier explained that the reason for the absence of stable translocation events of 10-nt and 50-nt at lower voltage ($\Delta V < +0.8 \text{ V}$) could be because of the junction potential ($\sim 0.6 \text{ V}$) at the interface (Figure 4-11). On the other hand, in the case of 92-nt, the junction potential was weakened due to the loss of the strong viscosity/ionic gradient interface, which allowed the translocation events at lower biases (0.6 V, 0.7 V, and 0.8 V) as shown in Figure 4-22(a-c), respectively. As expected, the peak amplitudes were proportionally increased with the applied biases, showing that the mean current amplitudes were $0.2139 \pm 0.0428 \text{ nA}$ for 0.6 V

Figure 4.21. (a) The geometry of COMSOL Multiphysics simulations for ssDNA translocations. The basic physics for solving the system were the same with the case without ssDNA structure in Figure 4-9. The 30 nm $\times$ 50 nm chamber was used for each side. The length of ssDNA was 10 nm with ten nucleotides of 0.125 nm thickness with rounded corners, and the distance between each nucleotide was 1 nm. TiO₂/h-BN nanopore was built on 1 nm thick membrane, *i.e.*, 0.5 nm thick TiO₂ and 0.5 nm thick h-BN. The material property of TiO₂ was given to the *cis* side of the membrane, *i.e.*, $\epsilon_{\text{TiO}_2} = 8$, $\rho_{s,\text{TiO}_2} = -0.02 \text{ C}\cdot\text{m}^{-2}$, and h-BN to the *trans* side, *i.e.*, $\epsilon_{\text{hBN}} = 4.6$, $\rho_{s,\text{hBN}} = 0 \text{ C}\cdot\text{m}^{-2}$. The surface charge density of SixNy was also given to the right wall of the *trans* chamber (red line) to immitate the SixNy membrane, *i.e.*, $\rho_{s,\text{SiN}} = -0.08 \text{ C}\cdot\text{m}^{-2}$. (b) Water-bound ssDNA, *i.e.*, H₂O-ssDNA, in light blue color. (c) [bmim][PF₆]-bound ssDNA, *i.e.*, IL-ssDNA, in yellow color. (d) The region of interest for the electric flux at the center area of the nanopore (dashed magenta line). The graph (Figure 4-20(a)) shows the electric flux depending on the locations of ssDNA, moved by 0.5 nm along the z-axis for each simulation (e).

($n = 3,037$), 0.2522 ± 0.0546 nA for 0.7 V ($n = 3,167$), and 0.3195 ± 0.0966 nA for 0.8 V ($n = 2,975$), respectively (Figure 4-22(d)). However, the translocation time did not show the conventional negative correlation with the applied bias, but no certain trend was observed, presenting 0.3032 ± 0.2240 ms for 0.6 V, 0.3074 ± 0.1850 ms for 0.7 V, and 0.3237 ± 0.2324 ms for 0.8 V, respectively (Figure 4-22(e)). We assume that this phenomenon could be due to the possible electroosmotic flow from the *trans* to *cis* chambers that might have balanced against the electrophoresis, but further nanofluidic research is required to understand the unusual translocation behavior of the viscosity/ionic gradient system. Like the case of 92-nt, the nanopore platform for shorter ssDNA, *e.g.* 10-nt, could also lose its function to detect nucleobases if the viscosity/ionic gradient interface becomes weaker as the finite width of the interface gets thicker due to the continuous ionic transportations across the interface by the electrophoresis. Thus, the base detection phenomenon could be affected by the duration and extent of the applied bias and the integrity of the interface.

Figure 4.22. The representative current traces of 92-nt translocation events at the applied biases of (a) 0.6 V, (b) 0.7 V, and (c) 0.8 V. (d) The histograms in probability distribution function for peak amplitudes (ΔI) at 0.6 V (green), 0.7 V (yellow), and 0.8 V (red) with normal distribution fittings ($\overline{X_{0.6V}} = 0.2139 \pm 0.0428$ nA ($n = 3,037$), $\overline{X_{0.7V}} = 0.2522 \pm 0.0546$ nA ($n = 3,167$), and $\overline{X_{0.8V}} = 0.3195 \pm 0.0966$ nA ($n = 2,975$)). There was a positive correlation between the bias and the peak amplitudes (ΔI). (e) The histograms in probability distribution function for translocation time (Δt) at 0.6 V (green), 0.7 V (yellow), and 0.8 V (red) with normal distribution fittings ($\overline{X_{0.6V}} = 0.3032 \pm 0.2240$ ms, $\overline{X_{0.7V}} = 0.3074 \pm 0.1850$ ms, and $\overline{X_{0.8V}} = 0.3237 \pm 0.2324$ ms). There was no specific correlation between the bias and the translocation time (Δt).

Chapter 5

FUTURE STUDIES

We have now demonstrated the feasibility of 2-D h-BN nanopore for detecting nucleotides in 10-nt ssDNA, opening a new possibility of SSN as the future DNA sequencer. However, two critical limitations were found along with the success: 1) the nucleotides in longer ssDNA (92-nt) molecules were not detectible and 2) the resistive pulses from four types of nucleotides were not distinguishable from each other. In this project, we will develop an h-BN nanopore-based DNA sensor that overcomes the limitations of previous platform, for which two specific aims are described: First aim is to detect nucleotides in long ssDNA (100-nt or longer) by (a) denaturing and linearizing ssDNA molecules by applying their melting temperature, and (b) slowing down the translocation speed with lower electrical potential and thermal gradient at the nanopore. Second aim is to distinguish four types of nucleotides with their respective resistive pulses by (a) utilizing smaller nanopores fabricated using STEM machine with higher accuracy, and (b) increasing the molecular conductivity through optimizing the hydration ratio of ssDNA. The proposed research focuses on demonstrating the real-time nucleotides detection in longer ssDNA with higher base-calling accuracy using 2-D h-BN SSN, for a great step towards DNA sequencing. The success of the proposed platform could answer the critical questions about the molecular behaviors under the complicated ionic/viscous/thermal/voltage gradient environment of the nanopore system.

5.1. Detection of Nucleotides in Longer ssDNA

Nucleotide detection of 10-nt was successful with the current viscous/ionic gradient nanopore platform using h-BN nanopore, but longer ssDNA (92-nt) did not allow any signals from nucleotides, due to its larger size than the nanopore, *i.e.*, radius of gyration (r_g) of 92-nt = ~ 5 nm [70, 82]. In this proposed research, the nucleotides in longer ssDNA will

be detected 1) by thermally engineering the molecular structures of DNA through denaturation and linearization and 2) by further slowing down the translocation speed through the temperature-gradient assisted low-voltage driven translocation.

5.1.1. Denaturation and Linearization of DNA

It is well-studied that the ssDNA denatures when it reaches to a certain temperature, *i.e.*, melting temperature (T_m), when half of the DNA molecules in solution are denaturated, existing as ssDNA in the solution [83–85]. This tells that, if the system has its temperature over T_m , the hybridized DNA strands in the solution begins to be denaturated to be single-stranded DNA without binding each other or by themselves, hence the translocation energy could be lowered and the event frequency, *i.e.*, translocation rate ($\dot{J}$), could be increased for the longer DNA. The translocation rate ($\dot{J}$ increases with lower energy barrier (U), according to the Kramers theory, *i.e.*, $\dot{J} = \omega \exp\left(\frac{q\Delta V - U}{\kappa_B T}\right)$ (section 4.3.1.) [86]. This strategy is feasible with solid-state nanopores because they have higher thermal stability than the protein-based biological nanopores with bilipid membrane which could experience permanent structural deformation by the excessive thermal energy of T_m [87, 88]. The T_m of DNA in water solution is well-studied and its information is widely shared,[3-5] but there has been no specific research was done for finding the T_m of DNA in [bmim][PF₆]. In this proposed research, the T_m of DNA in [bmim][PF₆] will be first discovered using spectrophotometer [89, 90], checking the temperature effect on the absorbance of DNA at 260 nm. Then, a temperature controller will be built for the system to keep the temperature steady over the acquired T_m during the experiment; *e.g.*, an electric heat pad can be installed on the bottom of the Teflon flowcell blocks inside the Faraday cage, with the thermal sensor connected to the *cis* chamber for the automated feedback control system (Figure 5-1). Since the thermal conductivity of Teflon is comparatively low ($0.25 \text{ W}\cdot\text{m}^{-1} \cdot \text{K}^{-1}$), the thickness of the flowcell bottom should be thin enough to effectively deliver the heat energy to the chambers. We expect the denaturated, linearized, untwisted longer ssDNA (100-nt or longer) will show similar translocation behavior as the 10-nt translocation signals. The heating inte-

grated electrical measurement can be utilized to examine the temperature dependency of the molecular translocation behavior, which is directly related to the conformational structures, *i.e.*, denaturation and linearization, of translocating DNA at the given temperature.

Figure 5.1. Schematic figure of heat control system with 2-D nanopore platform.

5.1.2. Thermal Gradient-Assisted Electrophoresis

The major limitation of the current nanopore platform to detect nucleotides in longer ssDNA (92-nt) is the fast translocation speed by the strong electrophoresis at the high external bias. As planned in Aim 1(a), the thermal energy will be applied to the *cis* chamber (Figure 5-1). In this Aim 1(b), while increasing thermal energy at the end of the *cis* chamber, the temperature of the *trans* chamber will be lowered to increase the thermal gradient (∇T) across the chamber and the nanopore, which gives two significant benefits: First, ssDNA molecules could be captured more efficiently by thermal diffusion with the application of lower electric potential. Second, the lower electrical potential can significantly decrease the strength of electrophoresis during the translocation due to the lower voltage gradient (∇V) as described in the equation, $\vec{u}_V = -\mu \nabla V$, where μ is the electrical mobility. If there is no thermal gradient in the chamber, the ssDNA is only driven by diffusion in the bulk. With the thermal gradient (∇T), ssDNA molecules in the bulk are thermally diffused to the capture zone, *i.e.*, the region in the critical radius from the nanopore, by the thermal diffusion, as described in the equation, $\vec{u}_T = -D_T \nabla T$, where D_T is thermodiffusion coefficient. For the

experiment, the heating(*cis*)/cooling(*trans*) system will be installed by attaching thermo-electric pad (Peltier device) on the side wall of each chamber, while the thermal controller automatically controls the temperature of each chamber by the feedback control system (Figure 5-2). The side wall of each chamber should be thin enough to effectively diffuse or absorb heat energy. Because of the principle of Peltier device, it absorbs and releases heat on each side of the device (top surface: hot, bottom surface: cold), there should be a heat sink on the *trans* side to cool down the accumulated heat. In this proposed research, the temperature gradient dependency of the translocation frequency and the dwell time will be systematically investigated for searching the optimal value of thermal gradient for reducing the translocation speed of longer ssDNA molecules.

Figure 5.2. Schematic figure of temperature control system for heating the *cis* chamber and cooling the *trans* chamber with 2-D nanopore platform.

5.2. Discriminating the Resistive Pulses of Each Type of Nucleotides

The nucleotides in ssDNA were successfully detected by the viscous/ionic gradient system with hydrated ssDNA, but the type of nucleotides (A, T, C, G) were not distinctive enough to find recognizable differences. About a decade ago, similar limitation was also observed for the biological nanopores, that the four types of nucleotides could not be resolved using a wild-type α -HL biological nanopore [91]. With the assistance of genetic/protein engineering, a novel biological nanopore of mutant α -HL with adapter protein could successfully discrim-

inate the four types of nucleotides by reducing the dimension of nanopore even smaller. In the same manner, the limitation of base-calling accuracy of SSN could be resolved 1) by fabricating and utilizing a smaller nanopore (~ 1.5 nm), slightly larger than the width of the nucleotides (~ 1.2 nm), and 2) by increasing the molecular conductivity for raising the sensitivity of the nanopore platform.

5.2.1. Optimization of Nanopore size

In the successful case of biological nanopore using heptameric protein α -HL, the narrowest constriction of the nanopore channel is ~ 1.4 nm which is almost the same as or slightly larger than the width of the nucleotide (~ 1.2 nm) [92, 93]. Following this example, the diameter of 2-D nanopore could be made smaller under 2 nm using a gentler method of scanning transmission electron microscopy (STEM) [8, 12]. STEM has originally been used to take an image of ultra-high atomic resolution without damaging the sample material by the scanning method (Figure 5-3(a)). The inset figure is a selected area diffraction pattern (SAED) of h-BN, which six-pointed star shape proves h-BN. In the case of conventional TEM, the electron beam is exposed on the whole sample at the same time, like an opening/closing a shutter of a camera, which could damage the ultrathin 2-D membrane in a short time (less than a sec). Different from the conventional TEM, STEM uses electron probes (0.05-0.2 nm) [94, 95] to scan the sample pixel-by-pixel in the specific region of interest where the nanopore would locate, hence it will make a precise ~ 1.5 nm nanopore in diameter without damaging the membrane by avoiding the undesirable exposure (Figure 5-3(b)). Theoretically, the signal sensitivity could be expressed as the normalized peak amplitude ($\frac{\Delta I}{I_0}$) [53], which is the same as the normalized pore conductance variation ($\frac{\Delta G}{G_0}$) at the same bias, according to the Ohm's law, *i.e.*, $G_0 = I_0/V$, where I_0 and G_0 are open pore current and open pore conductance, respectively. The nanopore conductance (G_0) is theoretically expressed as, $G_0 = \sigma \left[\frac{4l_{eff}}{\pi D_0^2} + \frac{1}{D_0} \right]^{-1}$ (Equation 4-5) [50]. In the case of 2-D nanopore, the effective thickness (l_{eff}) becomes less than 1 nm, the conductance equation could become simpler as, $G_0 \approx \sigma D_0$. Thus, the nanopore sensitivity is simplified to $\frac{\Delta I}{I_0} \approx \frac{\Delta D}{D_0}$, by which the

sensitivity of 1.5 nm STEM nanopore is increased by $\sim 3\times$ of that of 4.5 nm TEM nanopore. In this proposed research, the SNR of each nucleotide will be amplified by fabricating smaller nanopore using the cutting-edge STEM machine, yielding significantly larger SNR than the previous nanopore made by conventional TEM.

Figure 5.3. (a) STEM micrograph of h-BN membrane. The inset image is the SAED pattern of transferred h-BN membrane. (b) Zoomed-in STEM micrograph with the schematic nanopore fabrication procedure using an atomic-size STEM probe (0.05-0.2 nm).

5.2.2. Optimization of Molecular Conductivity of ssDNA with Different Lengths

Another way to improve the base-calling accuracy is to increase the molecular conductivity of ssDNA through optimizing the [bmim][PF₆] to water (H₂O) ratio for preparing uniform ssDNA solution [79]. We have shown that 3% of H₂O added to [bmim][PF₆] was the key recipe to increase the SNR of nucleotides in ssDNA by increasing the molecular conductivity of translocating ssDNA. According to the Ohm's law, $\vec{J} = \sigma \vec{E}$, the peak amplitude (ΔI) is proportionally related to the molecular conductivity (σ). The electrical conductivity of DNA has been studied by many researchers [78, 80, 96], showing that the electrical conductivity of ssDNA is exponentially related to the number of water molecules per nucleotides [78, 79]. Since the hydration of DNA in the [bmim][PF₆]/H₂O mixed solution has not yet studied, the

spectrophotometry will be used to find the optimized concentration of ssDNA by checking the maximum intensity at the characteristic wavelength [97]. According to the literature [62], the maximum soluble H₂O in [bmim][PF₆] is ~3%, which tells that the maximum amount of H₂O in the solution is fixed, hence the amount of dissolved ssDNA could be decided by the H₂O:nt binding ratio. Based on the literature [63, 64, 98], the full hydration of a nucleotide requires ~10 H₂O, *i.e.*, 6 to phosphate, 2 to pyrimidine (A, T), and 3 to purine (C, G). The H₂O:nt binding ratio was ~0.434 g H₂O (0.024 mol) / g ssDNA (~0.002 mol of base) [81], which molecular ratio was about 12(H₂O):1(nt). This ratio is limiting the maximum dissolvable concentration of ssDNA by the nucleotide composition of ssDNA, hence the ssDNA solution should have a unique molar concentration in accordance with its characteristic composition. In this proposed research, the different solutions of H₂O-to-ssDNA ratio will be systematically studied to optimize the sample preparation protocol for yielding the highest SNR with the distinctive nucleotide signals.

Chapter 6

SUMMARY AND CONCLUSION

6.1. Summary

The detection of nucleotides in ssDNA using SSN was possible by an ensemble of a few strategies; 1) ultrathin and electrically stable 2-D h-BN nanopore, 2) viscosity/ionic gradient interface, and 3) high-conductive hydrated ssDNA molecules. The research demonstrated our capacity to perform the proposed research.

6.1.1. 2-D h-BN Nanopore

The spatial resolution of nanopore should cover the sub-nanometer range to detect the nucleotides which dimension is ~ 1.2 nm in width and ~ 0.63 nm for in-between distance [46]. Thus, 2-D materials have recently been used for the nanopore fabrication and the nucleotide detection, including graphene, MoS_2 , or h-BN. Among these 2-D materials, h-BN has a superior electrical stability with larger band gap (~ 5.9 eV) [21] and lower dielectric constant ($\epsilon_r = \sim 3.5$) [57]. As a result, the signal-to-noise ratio (SNR) of translocation signals increased by avoiding undesirable electric signals except the resistive electrical pulses by the volume exclusion of the ionic current.

6.1.2. Viscosity/Ionic Gradient Interface

Since the viscosity of $[\text{bmim}][\text{PF}_6]$ in the *cis* chamber is much higher than water ($[\text{bmim}][\text{PF}_6]$: ~ 200 - 380 mPa·s and water: ~ 1 mPa·s), the access velocity of the molecule becomes much slower than the case of general aqueous solution. During threading the nanopore, the charge density of the medium abruptly changes across the *cis* and the *trans* side of the nanopore; as a result, the translocation speed further reduced to detect the signals of nucleotides. With

this slow translocation speed, the increased sensitivity was the key technique to detect the signals from nucleotides with the help of water molecules bound to ssDNA.

6.1.3. High-Conductive Hydrated ssDNA

The sensitivity of the platform could be efficiently improved by increasing the molecular conductivity of ssDNA, which could amplify the current variation (ΔI), according to the Ohm's law, *i.e.*, $\vec{J} = \sigma \vec{E}$, where $\vec{J}$, σ , $\vec{E}$ are current density, electrical conductivity, and electric field, respectively. The molecular conductivity works as an amplifying factor to increase the electrical signal during translocation event. By increasing the molecular conductivity of ssDNA, the electrical signal from each nucleotide could be efficiently amplified to be detected by the nanopore platform. Computational simulations using COMSOL Multiphysics were executed to see how the electric flux (Φ) changes at the nanopore depending on the location of H₂O-bound ssDNA and [bmim][PF₆]-bound ssDNA for the comparative study.

6.2. Conclusion

We anticipate these findings could provide vital information for ssDNA studies using SSN devices, and insights found and discussed herein could be extended to existing techniques, such as 2-D nanopores, viscosity gradient systems, or molecular conductivity engineering, to further increase the temporal/spatial resolution and the sensitivity of SSN-based measurements. Since the base-translocations were only observed with the short 10-nt ssDNA, this study provides prospects of extending this phenomenon to longer strands essential for SSN-based sequencing efforts. Also, a computational study of molecular dynamics simulation is necessary to further demonstrate the critical role of water molecules in the nucleotide detection by showing the time-dependent molecular-level dynamics of the H₂O-bound ssDNA translocation through a 2-D nanopore with [bmim][PF₆]/KCl interfacial system.

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