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SOLID STATE MICRO AND NANOPORE SENSORS FOR SINGLE ENTITY
DETECTION

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Abstract

A general description of the work presented in this thesis can be divided into three areas of interest: micropore fabrication, nanopore modification, and their applications. Following the introduction, the first part of the thesis is related to the novel, reliable, cost-effective, potable, mass-productive, robust, and ease of use micropore flowcell that works based on the RPS technique. Based on our first goal, which was finding an alternate materials and processes that would shorten production times while lowering costs and improving signal quality, the polyimide film was used as a substrate to create precise pores by femtosecond laser, and the resulting current blockades of different sizes of the nanoparticles were recorded. Based on the results, the device can differentiate the particles based on their size by changing the current level. The experimental and theoretical investigation, scanning electron microscopy, and focus ion beam were performed to explain the micropore's performance comprehensively. The second goal of the project was design and fabrication of a leak-free, easy-to-assemble, portable, and small polymethyl methacrylate (PMMA) flowcell for nanopore experiments. Here, ion current rectification was studied in our nanodevice. We showed a self-assembly-based, controllable, and monitorable in situ monolayer Poly(L-lysine)-g-poly (ethylene glycol) (PLL-g-PEG) coating method under voltage-driven electrolyte flow and electrostatic interaction between nanopore walls and PLL backbones. Using our designed nanopore flowcell and in situ monolayer PLL-g-PEG functionalized 20 ± 4 nm SiN nanopores, we observed non-sticky α -1 anti-trypsin (AAT) protein translocation. In addition, we could show the enhancement of translocation events through this non-sticky nanopore, and also, estimate the volume of the translocated protein. In this study, by comparing the AAT protein translocation results from functionalized and non-functionalized nanopore we demonstrated the 105 times dwell time reduction (31 to 0.59ms), 25% amplitude enhancement (0.24 to 0.3 nA), and 15 times event's number increase (1 to 15events/s) after functionalization in 1×PBS at physiological pH. Also, as a result of non-sticky translocations, the volume of AAT protein was measured which was comparable to the calculated AAT protein hydrodynamic volume computed as Mass/density and previous reports.

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List of Acronyms

AAT	-	α_1 -antitrypsin
AFM	-	Atomic force microscopes
BLM	-	Bi-layer membrane
BSA	-	Bovine serum albumin
CBD	-	Controlled dielectric breakdown.
CD	-	Circular dichroism
ch#-	-	Input pins
CTCs	-	Circulating tumour cells
DLS	-	Dynamic light scattering
DRIE	-	Deep reactive ion etching
DUT-	-	Device under test
EDL	-	Electrical double layer
EIB	-	Electron-ion beam
EMI	-	Electro-magnetic interference
eNPR	-	Elements Nanopore Reader
EOF	-	Electroosmotic flow
EPF	-	Electrophoretic force
FIB	-	Focused-ion beam
fs	-	Femtosecond
GND	-	Ground
HIM	-	Helium ion microscopes
ICR	-	Ion current rectification
IgG	-	Immunoglobulin G
IPA	-	Isopropyl alcohol
IR	-	Infrared spectroscopy
LBL	-	Layer-by-layer
MD	-	Methacrylate polymer

ns - Nanosecond
PAA - Polyacrylic acid
PC - Polycarbonate
PET - Polyethyleneterephthalate
PI - Polyimide
PLL - Poly-L-lysine
PP - Polypropylene
ps - Picosecond
PS - Polystyrene
PSD - Power spectrum density
REF - Reference
RIE - Reactive ion etching
RMS - Root-mean-square
RPS - Resistive pulse sensing
SiNx - Silicon nitride
SIOS - Scanning ion occlusion spectroscopy
SNR - Signal to noise ratio
STM - Scanning tunnelling microscopy
TE - Track etching
TEM - Transmission electron microscopy
VAUM-Vacuum-assisted UV micro-moulding process
VC - Voltage clamp
VDAC - Voltage-dependent anion channels
PAAMA -Polyacrylic acid-maleic acid
PMMA-Poly (methyl methacrylate)
PDMS -Polydimethylsiloxane
RNAP - RNA polymerase
GBMs - Glioblastoma multiforme
LPCVD - Low-pressure chemical vapour deposition

Introduction

It is crucial to detect and distinguish molecules and particles from nano up to micrometre size and concentrations in fluid. As a result, the scientific community has made several attempts over the past decades to develop new materials, processes, and technologies able to meet these new emerging needs. The development of large-area and flexible nanotechnology has created new possibilities for implementing innovative micro and nanopore sensors.

There were two main objectives to my research. The first was to design and mass produce solid-state polymer-based micropores to differentiate microparticles by their size, and the second was to use Silicon-Nitride (SiN) nanopores to free-translocate alpha-1 antitrypsin proteins in fluids and estimate their sizes.

The first aim of the thesis was related to a trustworthy, economical, novel, sturdy, and simple-to-use micropore flowcell that employs the RPS method. Based on our goal, the polyimide film as a substrate was used to create precise pores by a femtosecond laser, and the resulting current blockade of different sizes of the nanoparticles were recorded. In order to produce these micropores cost-effectively, portable, and quickly, we introduced a small solid-state polyimide micropore manufacturing method. A silver-mounted polyimide membrane was used as the substrate, and we chlorinated our electrodes with NaOCl and examined the hydrophilicity change in our membrane. Polystyrene (PS) microparticles were distinguished from 100, 300, and 350 nm particles in fluid by mass producing micropores on polyimide membranes and poly (methyl methacrylate) (PMMA) flowcells using femtosecond lasers. The results demonstrate that by varying the current, micropores could differentiate between the particles according to their sizes. Focus ion beam, scanning electron microscopy, and theoretical and experimental study were used to comprehensively explain the micropore's performance.

Several obstacles need to be overcome to use solid-state nanopores for protein detection, which led us to define the second goal of my PhD. Biomolecules are expensive, so keeping a chamber small is essential. In contrast, flowcell leakage led us to consider and design small, reusable nanopore flowcells. We solved this problem by using piranha solution and water-Isopropyl alcohol (IPA) mixture as a wetting solution for nanopores. We later found that proteins sticking to nanopore sidewalls is the most common problem when studying proteins with nanopores, an issue that has been under investigation for a couple of years now. Although many methods have been developed to minimise the sticking problem. They are generally ununiform, non-controllable, and sometimes clog nanopores. By using the physical

adsorption of Poly(l-lysine)-g-poly (ethylene glycol) (PLL-g-PEG), we developed a monitorable in situ functionalization method that provides a homogeneous surface and a reproducible method. We also successfully translocated and estimated the size of alpha 1 antitrypsin using PLL-g-PEG functionalized SiN nanopores.

The introduction chapter of the thesis provided a brief overview about nanopore and its application in detection, and different types of nanopore were investigated. In the Material and Methods chapter, the study was focused on different techniques for the fabrication of the nano/micropores, their functionalization methods, and their properties and characterization. Also, flowcell design and fabrication was discussed as well. In the third chapter, Result and Discussion, the results obtained during this PhD project are explained and discussed. Finally, the project and its achievements are summarized in the Conclusion's chapter. During the work I faced multiple challenges mentioned at the end of chapter three, together with the approaches used to tackle them.

My PhD project is part of Sentinel, an European project funded by the Horizon 2020 research and innovation initiative of the European Union, supporting 15 PhD students, covering 13 countries and involving of 20 organizations run by 11 supervisors. The goal of the Sentinel Innovative Training Network (ITN) is to educate a new generation of scientists and entrepreneur who are capable of studying electrochemistry at the level of single entities, including single redox-active (macro) molecules, living cells at the nanoscale, and metal, semiconducting, and soft matter nanoparticles. One of Sentinel Network's industrial part is Elements srl. It is an Italian firm that develops and manufactures instrumentation for pico- and nanoscale electrochemistry experiments. Their amplifiers can be used in the fields of electrophysiology and electrochemistry and can acquire and analyse very low ion currents.

This study has been carried out at the Department of Physics and Astronomy of the University of Bologna, Elements srl company, and several international research groups, under the Marie Skłodowska-Curie grant agreement number 812398 (Sentinel)- In particular, I collaborated with Prof. Dr. Wolfgang Schuhmann from the Ruhr University (Germany), Prof. Dr. S.J.G. Lemay(Serge) from University of Twente (Netherlands), Prof. Mingdong Dong from University of Aarhus (Denmark).

1. Micro and Nanopore sensors

Science and medicine have made extraordinary advancements because of the capability to detect single molecules. Richard Feynman's 1959 landmark talk predicted that nanotechnology would one day allow us to manipulate objects atom by atom. As this capability becomes more widely available, not only has it been used to manipulate the properties of materials, but also to gain a better understanding of the molecular interactions within materials. Therefore, in biochemistry, medicine, physics, and biology, one molecule at a time can provide insights into fundamental dynamic processes. In contrast to traditional time-consuming experiments data, single molecular detections allow scientists to examine the behaviour and interactions of individual molecules within a molecular population, as well as the structural and kinetics of the molecules.

The detection of single entities has been improved over the years with the development of technologies such as scanning tunnelling microscopy (STM) and atomic force microscopy. In addition to providing atomic resolution, such instruments can also analyse nanoscale surface properties like chemical, mechanical, and electrical of molecules. Since 1976, optical methods have been widely used to diagnosis the single entity in liquids. Recent development in devices and detection methods have made it possible to design sensing platforms, enabling reliable, and detection to gain eminence. A typical example is detecting ionic currents. Based on Coulter counting, which was used in the 1950s to measure the size of suspended particles in electrolytes (1), in 1970s, Bert Sakmann and Erwin Neher could record the current via individual channel (Figure 1.1). However, the idea of using nanopores only gained traction in the late 1990s, mostly driven by the desire to use them for the sequencing of nucleic acids. As nanopore sensing advances, applications such as DNA sequencing as well as diagnostics, biological monitoring, and the biggest challenge to date, protein sequencing, are also being explored.

1.1. Single entity electrochemistry (SEE)

In electrochemistry, measuring smaller quantities is becoming increasingly common and field of interest. In order to develop electrochemical systems, different factors such as rapid and real-time detection tools (2,3) along with analysing hetero or homogeneity (3–5) are driven this aim. This field of research and these efforts are limited by single-entity electrochemistry, in which only one entity like molecule and nanoparticle may be measured at a time (6–8). Single entity measurements require both spatial or temporal isolation of that entity's response and optimized signal-to-noise for smaller signals to be quantified (9). These challenges have been overcome in numerous recent studies. The versatility of nanopore sensing platforms has made them popular as a low-cost, simple method of single entity detection. The technology does not require expensive optics, is label-free, and provides high throughput. Molecular detection, DNA sequencing, protein structural characterization, and study protein unfolding are just some of the interesting applications for nanopores. As a molecule flows within the nanopore the ionic current changes, thus enabling nanopore detection or resistive-pulse sensing to occur. By applying a potential, a constant ionic current is created, when each charged molecule passes through the hole, causes a conductance reduction. Molecular charge, shape, and size determine the amplitude, frequency, and duration of transient current modulations. Wallace Coulter developed the concept of sensing in the early 1950s; however, in that study, he counted red blood cells by micro-hole (10). Figure 1.1 demonstrates the schematic of Tsutsui et al. setup for single cell analysis.

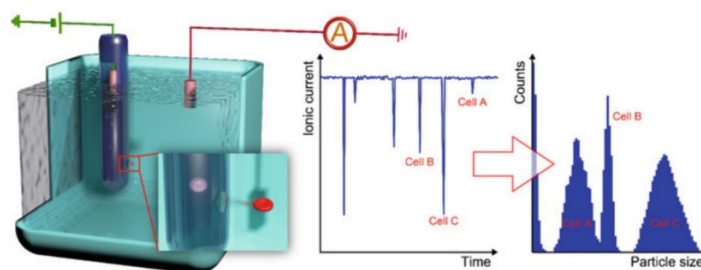


Fig. 1.1. Schematic of a Coulter counter. Figure reprinted with permission from reference (11).

In recent years, nanopore biosensing has gained momentum as part of DNA sequencing and is trending towards disease detection. A nanopore platform can be defined as a versatile system with different kinds of pores, such as biological, synthetic, and combination varieties, each with its unique advantages and characteristics. On the other hand, each variety also

comes with its own set of challenges and limitations that must be overcome by optimizing the methods and modifying the experimental conditions. Despite its nuances, the nanopore biosensing platform is worth exploring because it provides a real-time, cost-effective, and reliable sensing platform. During the last two decades, much effort has been made in over design and theoretical analysis of nanopore sensing performance to detect DNA, RNA, proteins, and chemicals such as drugs and particles (12). The general concept of nanopore structure and function is based on two fluidic reservoirs with electrolyte solutions surrounding the insulating membranes with nanometre-sized pores. During the application of different potential across the dielectric membrane, the ionic-current flows within the pore. Consequently, a biomolecule can be detected from a solution by causing transient current blockades at the pore. In biology, a cell membrane has small nanometre channels and pores, which control the efflux and influx of the molecule. In the 1980s, the idea of nanopore technology was inspired by the research conducted on the performance of the acetylcholine receptors' intrinsic activity and non-specific behaviour of voltage-dependent anion channels (VDAC) in artificial lipid bilayer in response to artificial molecules (13,14). In addition to protein channels, nanopores are also based on solid-state materials and synthetic DNA origami.

1.2. Solid state Nanopore & Biological Nanopore

In the 1980s, biological nanopores were the first to be brought onstage. DNA molecules were the first to be detected by them. There is a wide use of nanopores in the detection of metal ions (1), proteins (2-6), RNA (7), polymers (8,9), DNA-protein (10,15) etc.

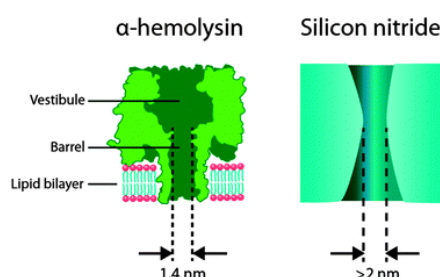


Fig. 1.2. (left) A lipid bilayer membrane that contains a biological nanopore. (right) An example of a solid-state SiN nanopore in cross-section. The schematic is adapted from Reference (16).

It suggests that nanopore sensors, aim to detect small molecular objects, can be combined to form a single sensor that produces a detectable current signature, based on the thickness of the substrate and the size of the access resistance region surrounding the nanopore (13). In

addition, nanopore sensors are easily integrated into portable nano/microchip devices and can be made smaller by miniaturizing them (14). Solid-state nanopores, as next generation of synthetic nanopore, were developed by techniques like drilling, electron beam lithography, laser etching, and electrochemical reactions on SiO₂, SiN_x, graphene, and MoS₂ membranes. However, solid-state nanopores have some restrictions, such as insufficient spatial resolution, which makes them less resilient to chemical and mechanical conditions. As solid-state nanopores are thick, it is possible to pass through them at the same time with dozens of bases (nucleic acids). At present, the thinnest SiN nanopore is 3 nm, not sufficiently thin to differentiate between the four types of bases. It is very important to reduce the thickness of the membrane of solid-state nanopores to amend their spatial resolution. Nanopores made of solid-state materials are commonly made of silicon nitride (Si₃N₄). The fabrication of Si₃N₄ membranes with less than 5nm thickness has not yet been reported, despite a desire to improve spatial resolution with further thickness reduction (17). In order for a nanopore to transfer species, its diameter should be around the same size as the species being transported. As a result of conventional fabrication processes, pores of interest have a larger initial diameter (18–22). To shrink the nanopore diameter, transmission electron microscopes (TEM) or field emission scanning electron microscopes (FESEMs) was used before (23–25). In addition, FIB is used for the sculpting process (26). An comparison of solid-state and biological nanopores can be found in Table 1.1.

Table 1.1. Comparison of biological and solid-state nanopores.

	Types of nanopore	Analysed molecule	Dimension (nm)	ref
Biological Nanopore	ClyA (Modified with aptamer)	dsDNA, protein	3.3	(17,18)
	FhuA (modified with aptamer)	Protein	2.7	(19–22)
	αHL (modified with aptamer)	ssDNA, dsDNA, protein, RNA	1.4	(23–29)
	Aerolysin (aptamer modified)	ssDNA, protein	1.0	(30–35)
Solid-state Nanopore	Al ₂ O ₃ -nanopore	dsDNA, protein, small entities	4≤x≤200	(36–40)
	Si-nanopore	dsDNA, ssDNA, protein, bacteria, virus	5≤x≤3000	(41–44)
	2D-nanopore	dsDNA, protein	0.34≤x≤300	(45–50)
	Nanopipette	Small entities, protein, dsDNA	1≤x≤1000	(51–54)

Biological nanopores are synthesized through biological processes based on solution. Biological pore can be substituted by editing the original DNA sequence, therefore, highly precise implant of functional groups and linkers are possible which lead to accurate control

of the physiochemical environment. Moreover, a low level of noise could also be attributed to the adhesion-resistance properties of lipid bilayers (55) and surface of the proteins (56,57). As an example, with solid-state nanopores, the root-mean-square (RMS) noise is rarely smaller than 1 pA (57,58), whereas biological ones are easy to achieve (59,60). Conversely, biological nanopores can be used only for particles between 1 and 10 nm in size due to their fixed and limited inner diameter of biological nanopore sizes, usually less than 10 nm (61,62). The average range of nanopore diameter is 2 nm and above, and it can be finely controlled as depicted in figure 1.2. Depending on the fabrication process, a nanopore can have the shape of an conical, cylindrical or hourglass. Typical dimensions of cylindrical nanopores are approximately 10 up to 200 nm, which depends on the membrane thickness (16). For translocations and analysis of larger particles the solid state nanopores should be used. The controllability of these techniques is deemed to be rather weak, and they are not easily transferable to a large-scale production. Generally, biological nanopores and biomaterials, such as lipid membranes require special care when stored in aqueous circumstances, and they are typically more fragile than solid-state membranes. However, the common solid state nanopore fabrication techniques including etching and electron beam lithography, are compatible with high-speed parallelized architectures and semiconductor processes (63).

1.3. Principles of sensing with nanopore

The nanopores are formed on membrane materials supported by substrates, with two chambers on either side of the nanopores filled with electrolyte (Figure 1.3). In conventional devices, the substrate and membrane materials are Si and SiN, respectively. The nanopore or nanochannel acts as only connection route between the cis and trans compartments for the biomolecules and ions (64,65). The electrolyte chambers are typically created using flow cells one of them connected to the ground electrode and the other one to the head stage electrode (66). An ionic current signal is produced when ions and biomolecules pass through a nanopore driven by different voltages applied across the membrane (Figure 1.3b) (64).

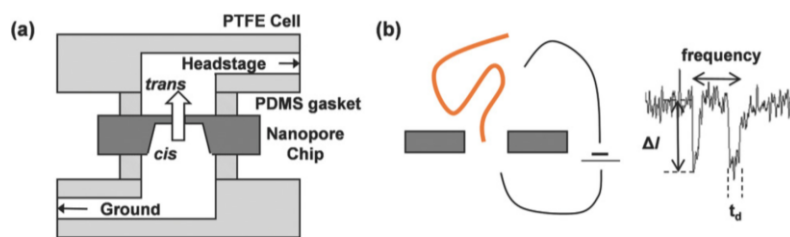


Fig. 1.3. A solid-state nanopore experiment's mechanisms. a) Solid-state nanopore setup. The white arrow indicated the direction of molecules transfer from cis chamber to the trans one. b) Diagram of a DNA translocation through the solid-state nanopore, which leads to ionic current graph. Figure reprinted with permission from reference (64).

Molecular translocation temporarily diminishes ionic current levels by blocking ionic flow through a pore (67,68). There are three primary characteristics of I-t signals: the magnitude of the current dropped (current blockage, ΔI), the frequency of the signal (capture rate, R_c), and the duration of the time (dwell time, t_d). Volume, surface charge, and the molecule number could be achieved by these results (69). Hence, the different signals with different magnitude and dwell time are produced as a result of passing differently sized biomolecules through the nanopore. There are three main strategies for sensing nanopores. Translocation of analytes through nanopores was the focus of early nanopore research. As molecules enter the nanopore, the pore being blocked from ions moving through it which leads to decline in ionic-current. Molecular size or weight of the molecules that are translocated corresponds to the current blockage's amplitude. Recently, solid-state nanopores and protein nanopores both use this method for detection (15). Designing and fabricating appropriate nanopore with high resolution would be one way to develop the solid-state nanopores resolution.

1.4. Nanopore measurement & analysis

The ionic current blockade technique or resistive pulse sensing (RPS) is the concept behind typical nanopore detection. In solid-state nanopore, the electrolyte (NaCl, KCl, LiCl) is divided between two reservoirs and a thin, impermeable membrane, which contains nanopore, is connected them together. The two chambers contain electrodes, such as Ag/AgCl and the constant voltage leads to ionic currents flow through the pores in a steady state. Ionic currents usually decrease when analytes pass through pores due ionic current blockades (70,71). Occasionally, the DNA backbone with negative charge can accelerate the mobilization of counterions near the pore, which enhances ionic current at low salt concentration. Electro-osmotic and electrophoretic forces can be affected by other factors, including pH and salt

concentration. An analysis of the duration, amplitude, and noise of the current pulse can provide valuable information about the analyte such as charge (72), nanopore surface affinity (73), size (74), shape (75), and sequence (76). Moreover, the concentration of the analyte can also be indicated by the modulation frequencies (Figure 1.4).

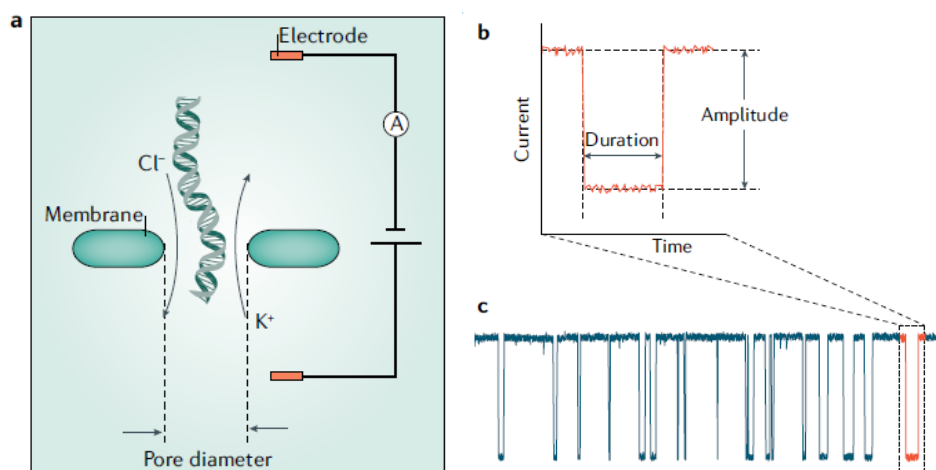


Fig. 1.4. The principles of nanopore diagnosis method. Figure reprinted with permission from reference (77).

Measurement of individual molecules requires confinement in both space and time during the measuring process. In order to achieve the single molecule detection with high sensitivity, the volume of the analyte should be close to size of target (78–80). It is often possible to detect molecules within an identified area with an imaginary volume, in traditional optical methods. On the other hand, molecules are restricted in a real volume in nanopore. Here, in order to accelerate the detection, the nanopore diameter must be similar to the molecules. In addition, to optimize the detection signal, the nanopore thickness should also be the smallest possible (81). One of the most powerful techniques for characterization of single entities and label-free particles is RPS. Since synthetic solid-state nanopores are available in a wide variety sizes and shapes, they are extremely suitable in RPS since they can be controlled in geometry and size and have several desirable features, including reproducibility, chemical and temperature stability (82,83). A number of factors are considered when analysing RPS signals, such as their frequency, amplitude, duration, and shape. Nanopore technology offers some alternative sensing techniques besides the RPS method, which are mentioned in Figure.1.5.

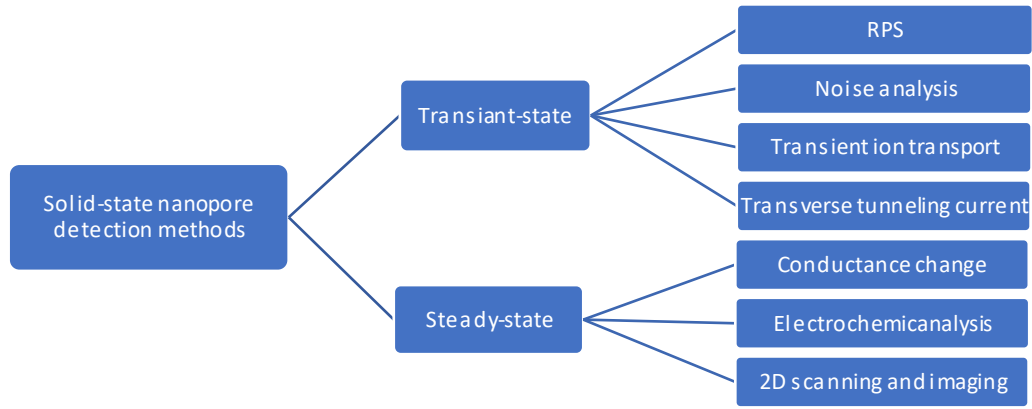


Fig. 1.5. The solid-state nanopore sensing techniques.

1.5. Principles for considering in RPS

For the first time, the concept of resistive-pulse counting was applied for nanoparticle and viruses analysis by DeBlois et al. in 1970s, when he tried to pass the 60 nm viruses and 90 nm polystyrene particles through the submicron pore etched membrane (84). Recently, Crooks et al., used polydimethylsiloxane and Si_3N_4 to fabricate the nanopore and functionalized it with multiwall carbon nanotube. A voltage bias (E_M) was used to drive negatively charged nanoparticles through carbon nanotubes whereas the ionic current was consistently monitored (28-29,53-55). As shown in Fig. 1.4. each current drop in current-time graph represents the particle translocation at different E_M within the nanopore. The amplitude of each pulse can be affected by particle size (ΔI). Therefore, the duration of each pulse can be related to the charge density of particle (Δt), and its frequency to the concentration level of the particles in the solution. Resistive-pulse counting was demonstrated in this experiment, which sparked interest in resistive-pulse analysis of nanoparticles.

1.5.1. Current blockade amplitude & size of particle

After applying a direct current (DC) voltage between two chambers filled with buffer solution, a constant current can be measured. Therefore, by adding the particles to our chamber/chambers, electric field drive nanoparticles into the nanopore. Consequently, each single nanoparticle during translocation occupies part of the nanopore volume which was filled by buffer solution. Generally, $i_0 = E_M / R_0$ where R_0 is the solution resistance in open pore/channel. In the absence of particle in the media, the solution resistance can be

calculated using Equation 1 when there is no surface (or wall)/particle interactions affecting ion distribution.

$$R = \int_0^{l_c} \frac{\kappa}{S} dx + R_{end} \quad (1.1)$$

Where, R_{end} is the spreading resistance at the ends of a nanopore as a result of the slightly obstructed mass transport, and κ is a conductivity of the solution (56). κ is a constant value except when the pore diameter is close to electrical double layer (EDL) thickness. Without surface effects, $\frac{\Delta i}{i_0} = \frac{\Delta R}{R_0}$, which determines the shape and geometry of the particle and nanopore.

$$\frac{\Delta i}{i_0} = S(d_c, d_s) \frac{d_s^3}{(l_c + 0.8d_c)} \quad (1.2)$$

Where S is a correction factor relates to the $\frac{d_s}{d_c}$ ratio (d_s : particle's diameter, d_c : nanopore's diameter). It should be noted that if $\frac{d_s}{d_c} < 0.8$, then $S(d_c, d_s) \sim 1$ (28).

1.5.2. Translocation time & velocity of particle

An inverse relationship exists between translocation time (Δt) and average velocity (v). When particles are not present in the solution, there is no interaction between nanopore wall and particles. In this situation four forces contribute to velocity (v) including a) the electroosmotic force (EOF) and b) the electrophoretic force (EPF). EOF occurs when counterions are carried in the nanopore electrical double layer, the counterions pull the water into the nanopore wall, resulting in a plug flow. EPF arises from external electric field effect on polarized particle; thermal diffusion and applied pressure (61). Electrophoretic velocity is usually separated from the other three components by experimental and mathematical means. Consequently, by applying the Helmholtz-Smoluchowski equation (eq.1.3) and the value of the particle's zeta potential (ζ), the surface charge could be calculated from electrophoretic velocity ($v_{electrophoresis}$):

$$\mu_e = A \frac{\epsilon_r \epsilon_0}{\eta} \zeta = \frac{v_{electrophoresis}}{E} \quad (1.3)$$

where the mobility of the particle is μ_e , gradient of electrical field is E , the solution dielectric constant is ε_r , the viscosity of the solution is η , the permittivity of the vacuum is ε_0 , and the correction factor is A . The amount of A is related to the $\frac{d_s}{\kappa^{-1}}$ (Debye length). Here, when $\frac{d_s}{\kappa^{-1}} \gg 1$, $A = 1$, and if $\frac{d_s}{\kappa^{-1}} \ll 1$, $A = \frac{2}{3}$.

1.5.3. Frequency & concentration of particle

The flow of the particles passing through the nanopore which is equal to velocity multiplied by concentration of nanoparticles could be defined as an event frequency. This expression was derived for cylindrical nanopore by Davenport et al. (60) and its mathematical explanation for conical nanopore was developed by Lan et al. and Roberts et al. between 2011-2012 (62,63). They could confirm that the dominant force in conical nanopore for particle translocation at the opening zone is pressure-force by Hagen-Poiseuille equation. Due to an enhanced nanopore sensing length in cylindrical nanopores, they have a longer translocation time and increased time resolution. In exchange for these benefits, higher sensing volume and an electric field distribution extending over a longer pore result in lowered detection limits and lower event frequency. As explained previously, the nanoparticle is subjected to forces at the entrance to the nanopore, which determine the event frequency. Therefore, the cylindrical nanopore with a high ohmic resistance, exhibits low electric fields due to the voltage drop. On the other hand, due to the fact that most voltage drops in conical nanopore occur near the nanopore aperture rather than along the pore length, this disadvantage is eliminated in conical nanopores (64). Recently, a new method was proposed to increase the event frequency, and electric field at the aperture of conical nanopore and reducing the ohmic resistance by using pores with low aspect ratio: $(\frac{D_{pore}}{L_{pore}})$ (65-66). In addition, increasing the convection velocity (u_{avg}) of particles at the nanopore aperture by introducing pressure-driven flow is another method to decrease the limit of detection which could be confirm via following equation:

$$u_{avg} = \frac{3R\Delta P}{8\eta \cos\theta} \quad (1.4)$$

where R is aperture radius in conical nanopore, applied pressure is presented as ΔP , the solution viscosity is η , and the pore half-cone angle is θ .

Enhancement of forces applying on particles reduces the limit of detection, but at the expense of faster particle translocation. Currently, recording equipment have limited bandwidth, so observing particle translocation is difficult (10-20 kHz). Therefore, translocation events lasting less than 100 μ s are problematic during analyses (62). It is possible to increase the bandwidth (~650 kHz) and decrease the translocation duration till 2 μ s, by using amplifier, metal electrode, and voltage divider to record the variation in voltage along the nanopore (18). It is possible to increase the RPS sensitivity by integration of two-step signal amplification in a circuit and minimizing the stray capacitance (69).

1.5.4. Surface Charge of Nanoparticles

Electrophoretic-based techniques are used to analyse the surface charge or the ζ -potential of nanoparticles. Since nanoparticle motion is complex, researchers conducted experiments to keep electrophoretic motion separate from pressure-driven and electroosmotic flows. For instance, they used Helmholtz-Smoluchowski equation to calculate the ζ -potential of the nanoparticle by applying electrically neutral carbon nanotube and ignoring the electroosmosis force (55). Also, surface charge density can be calculated by using resistive-pulse analysis and Graham's equation when the nanoparticle size is smaller and comparable than the nanopore, under this assumption that the electrophoretic force is the dominate force in the system (97). However, the ζ -potential of the protein can be calculated by considering both electroosmosis and electrophoretic forces with the aid of following equations (98):

$$v_{EO} = -\frac{\epsilon_r \epsilon_0 \zeta_{pore}}{\eta} E \quad (1.5)$$

$$v_{eff} = \frac{\epsilon_r \epsilon_0}{\eta} E (\zeta_{nanoparticle} - \zeta_{pore}) \quad (1.6)$$

Streaming potential is measured at various applied pressures to determine the ζ_{pore} (99). Under complex conditions like presence of all electroosmosis, electrophoresis, and pressure-driven flow forces, the surface charge density can be obtained by using the theoretical model introduced by Trau et al. In this model the all three forces as a function of ζ -potential and nanoparticle size are used to explain the current-time relation (58).

The thickness of the electric-double layer (EDL) generated at the nanopore surface depends on the electrolyte salt concentration. Externally applied electric fields significantly influence the resulting electric field inside the nanopore. As a result of this interaction, ions are not distributed uniformly in the nanopore. In contrast to the fundamental resistive-pulse analysis, at low analyte concentration, Dekker et al. could observe the current increasing during DNA translocation through the SiN nanopore (84,116). According to them, DNA molecules carry counterions, which increase local ion concentrations in nanopores. In another study, the dependence between current blockage ($\Delta i/i_0$) and applied voltage was observed during resistive-pulse analysis of conical nanopore and negatively charged polystyrene nanoparticles at low concentration of salt. A 2% increase of current blockage was observed when the voltage was increased from 0.05 to 0.6 V. In addition, biphasic pulses were recorded in the current-time tracing when nanospheres were driven into the nanopore by pressure and a negative voltage was applied (118).

1.6. Noise Characterization

The following contributions dominate a typical nanopore's power spectrum density (PSD):

$$S(f) = a_0 \frac{(i)^2}{f^\alpha} + a_1 \frac{k_B T}{R_a + R_p} + a_2 k_B T D C f + a_3 \sigma_V^2 C^2 f^2 \quad (1.7)$$

where f is frequency, $R_a + R_p$ is nanopore resistance, i is a DC current, σ_V^2 is the RMS voltage noise, dielectric constant is D , the total capacitance of the system is C , α is a noise exponent around 1 at low frequency, a_i are fitting parameters for each contribution to noise is weighed according to its relative strength (85). An example of the nanopore's power spectrum is represented in Figure 1.6.

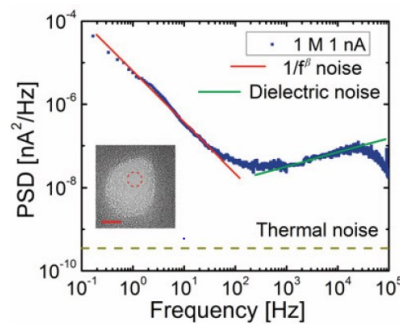


Fig. 1.6. PSD of the typical current noise SiNx nanopore at a 1 nA constant current. Figure reprinted with permission from reference (86).

In the low frequency spectrum, 1/f or flicker noise dominates. Almost any physical system is subject to this type of noise. Although it has been suggested that nanobubbles in the pore itself can contribute to it when this term is predominant (87). A large a_0 magnitude generally makes a pore useless, and therefore it is often possible to determine the usefulness of nanopore by the value of 1/f noise.

As a result of thermal effects, the second term of spectrum, called white thermal noise entirely depends on the electrical resistance of the nanopores. Comparatively, this term is negligible unless the pore is extremely large. White noise can be calculated by using following equation:

$$S_{IT} = a_2 = \frac{4kT}{R} \quad (1.8)$$

where the temperature (Kelvin) is T, the nanopore's resistance is R, and k is the Boltzmann constant.

Two regimes of noise at high frequencies are affected by the device's capacitance. Depending on the membrane and its structure, dielectric noise increases linearly with frequency, whereas capacitive noise scales squarely with frequency. The membrane's parasitic capacitance surrounding the nanopores is an important factor in determining the dielectric noise. However, the noise from the current readout is coupled with the capacitive noise. This last-term noise limits the bandwidth usable by nanopores. The capacitive noise eventually lowers the signal-to-noise ratio to an unusable level despite the amplifier being capable of accurately recording current measurements up to 10 MHz bandwidth (88). Typically, nanopore measurements are carried out with bandwidths of up to 1 MHz (89).

Dielectric and capacitive noises (at high frequencies) are directly related to nanopore parasitic capacitances and instruments. However, a strong correlation between low-frequency noise and many important factors related to the nanopore structure and electrolyte conditions like concentration and pH, both of which can be easily adjusted. By changing nanopore sizes and electrolyte concentrations, a range of current biases were used to investigate low-frequency noise characteristics in Silicon nitride (SiNx) nanopores (90).

Hooge's theory for flicker noise predicts PSD to follow the current square dependence (91,92).

$$S = \frac{a_1}{f^\beta} = \frac{a_H I^2}{N_c f^\beta} \quad (1.9)$$

where the Hooge factor is a_H and N_C is the carriers number. Higher electrolyte concentrations in lower current regions give rise to a more obvious deviation from the flicker noise model. Large pores show a much weaker correlation between the PSD noise and current level than smaller pores.

There is a distribution of time constants between carrier number and mobility, which contributes to flicker noise (92). The conductance of nanopores determines by the number of ions and carriers in the system that can resist thermal fluctuations.

1.7. Rectification Phenomena in Nanopore

Typical ionic diodes have nonlinear I-V curves. These rectifications currents have been observed in nanofluidic systems, especially in quartz nano-pipettes and conical nanopores fabricated by track etching (93,94). Nanopores with charge or/and asymmetric geometry are prone to ionic current rectification as depicted in figure 1.7 (93,95–97). Researchers have studied the rectification in both theoretical and experimental aspects in an attempt to uncover its dependences on a variety of factors, including nanopore geometry (98,99), polarity (100), surface charge (101), electrolyte pH and concentration (102,103), and charge distribution (104). However, we still do not fully understand how the physical process of rectification works, but its theoretical confirmation according to Poisson-Nernst-Planck equations and the distribution of particles are well understood (105–108).

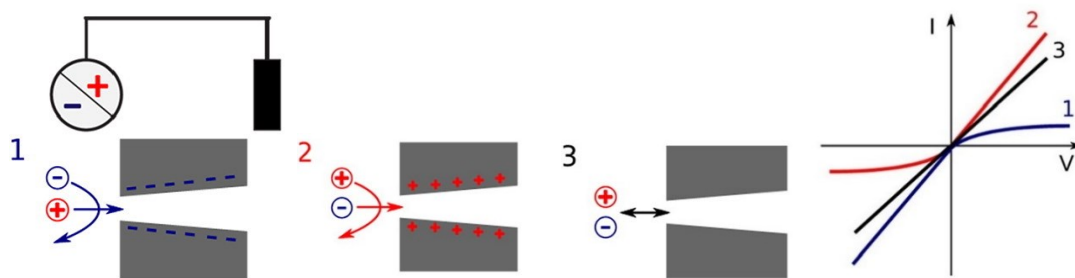


Fig. 1.7. Scheme image of surface charges of asymmetrical nanopores and their corresponding IV curves. Figure reprinted with permission from reference (109).

A nanopore's ability to control the flow of ions is measured by its ionic selectivity (110,111). Nanopores with conical cation-selective tips rectify preferentially from their small (tip) to their large (base) apertures (112). Moreover, ionic-current rectification is inverted as a result of surface charges modification: if polarity is modified, this results in a positive rectification of ionic current. An electrostatic model and a rocking ratchet are used to describe the

mechanisms of the ICR in artificial nanopores (113,114), Woermann model, and Poisson-Nernst-Planck equations explain the ICR through a nanopore, and poly-electrolytic theory used to clarify the electrostatic field (115–117). A rectification model is based on the interaction between ions and surface charges as they pass through the pore. In nanofluidic diodes, cations and anions are attracted from the analyte towards the interior of the pore when opposite polarity potential is applied. This produces a high current when the potential is applied through the pore. Inversed voltage creates a depletion zone within the nanopore, resulting in weak ionic currents when cations and anions head in the direction of the bulk (Figure 1.8) (93).

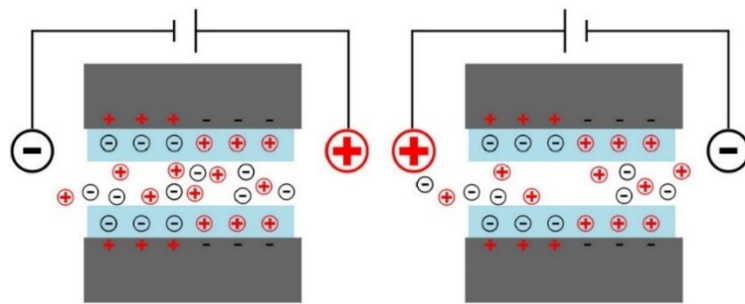


Fig. 1.8. Scheme explanation of the mechanism of diodes during a applied opposite polarity potential. Figure reprinted with permission from (93).

In ion exchange membranes or bipolar semiconductor diodes, those rectification properties are similar (118,119). The rectification factor (R_f) can be used experimentally to determine the amplitude of the rectifications. The definition described below is the current ratio between voltages of opposite polarities, but similar amplitudes.

$$R_f = \pm \frac{I(+V)}{I(-V)} \quad (1.13)$$

where $I(+V)$ is the ionic current level for positive voltage, and $I(-V)$ for a negative one.

1.8. Solid-state nanopore application

Single-molecule measurements are typically performed with nanopores (120–124). Particles and biomolecules passing through nanopores can be analysed and identified by alteration in the ionic current passages along a nanometre-sized pore (figure 4.1). Biomolecules can be measured using nanopores (125–129). As shown in figure 4.1 (c), solid-state nanopores could be optimized to measure proteins, DNA, and microscale bacteria and viruses on a single-unit basis by tuning the pore diameters to the size of their samples (130).

Recently, nanopores have been used to develop peptide and protein sequencers (131). Further, a novel nanopore sensing method for Coronaviruses has been demonstrated using clinical specimens (132). Instrumentation, nanopore devices, and ways of analysing nanopore data that comprise the nanopore platform are represented in figure 1.9(a). By using this platform, ionic current–time profiles can be recorded to analyse the ions, biomolecules, and particles translocation flowing through nanopore. Nanopore devices have been the subject of extensive research; elucidating the mechanisms controlling their electrical properties and developing analytical methods have all contributed to the development of these devices (133–138).

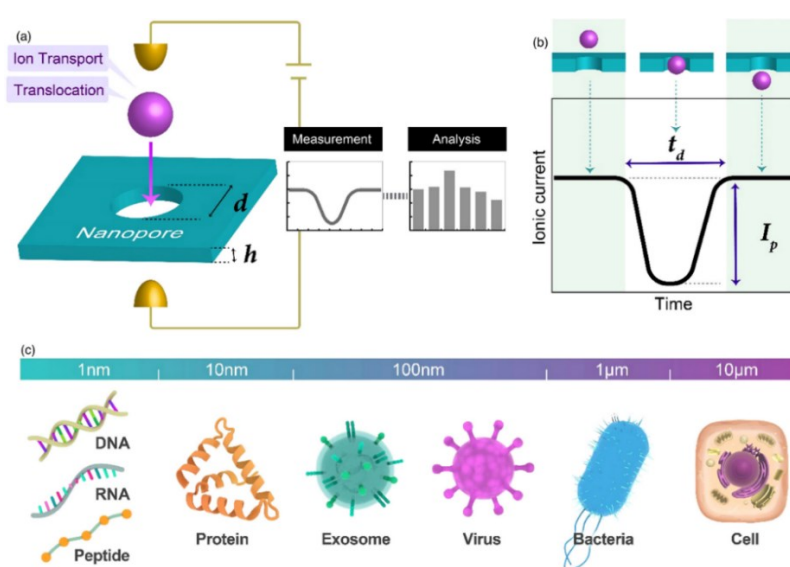


Fig. 1.9. Nanopore system. (a) This system consists of nanopore sensor, measurement equipment, and (b) The nanopore ionic current analytical graph, which changes during passing the biomolecule through the pores. (c) Different size of biomolecule forms few nm to 10 μ m can identify by nanopore. Figure reprinted with permission from reference (130).

A technology pioneered by Coulter quickly became a standard platform for screening biological fluids for particles, notably haematology, which is extensively applied for cell enumeration. Afterwards, de Blois and Wesley adapted this technique to count microorganisms, such as viruses and bacteriophages, in the 1970s (84). RPS-based approaches for particle analysis have regained popularity as a result of this development and the rapid advancement of nanotechnology (139–141). RPS technology provides important data about individual particles in their environment (142). This technology has been applied to study molecule particle concentrations, sizes, charges, and conductivities in various situations (143–145).

1.8.1. Protein detection with nanopore

The cells and tissues of the human body contain proteins. A protein catalyses and controls all the cellular processes essential for the body's proper functioning. An analysis of proteins provides valuable information about most biological processes and cell's phenotype (146). Proteomic data are more complicated to collect than genome data. However, they are a better reflection of cells and tissue's physiological condition (147). Proteins contain information regarding their sequences as well as their spatial structure. Many researchers have realized that with the accumulation of thousands of DNA sequences, they cannot elucidate biological functions merely by having a complete genomic sequence, since DNA sequences cannot be used to deduce structural characteristics such as post-translational modifications and protein subtypes (146). Research on solid-state nanopores is rapidly focusing on proteins, although nucleic acids have been the focus of most recent research. In contrast to nucleic acids, proteins have distinct 3D structures and charge network. Biophysical features of proteins cause them to behave differently from nucleic acids, such as pH sensitivity, structural modifications, translocation specificity, and protein aggregation, which are clearly visible in their event peaks during passing through the nanopore. Also, DNA is inherently negative and is transported by electrophoresis force through the nanopore, whereas proteins are often neutral or weakly charged, so electrophoresis as well as electroosmosis play essential roles in the transport of proteins (148). Despite its advantages, proteomics study faces certain challenges. Amplification of protein cannot be achieved (149), and transcription levels cannot be used as an indirect predictor of protein expression (150,151). Protein secondary structure can be evaluated using various methods, including circular dichroism (CD), nuclear magnetic resonance (NMR), and infrared spectroscopy (IR). However, it is not possible to gather dynamic data about proteins in real-time from these methods. In order to detect interactions and structural information about proteins, ultrasensitive fluorescence microscopes (152) and atomic force microscopes (AFM) (153) are used. There are, however, a number of limitations to these methods. The most common is that they require chemical modification to the targeted molecules, or the use of instruments with a large scale and complexity are often costly and complicated. Moreover, many of these methods may damage the protein's native properties, while complex experimental processes might introduce additional ambiguity.

A uniform, stable, reliable, and robust surface is necessary to detect single biomolecules through nanopores. Nanopores should also be prevented from expanding, shrinking, and absorbing non-specifically. However, inorganic coatings can solve the problem (154) of unmodified SiNx nanopores expanding or shrinking under high salt solutions or polar solvents (155). On the other hand, in rough nanopore walls, biomolecules may adhere and clog because of the hydrophobicity of the nanopores (156–158), thus damaging the device, and disrupting the signals (159). Nevertheless, this drawback can be addressed by coating the vessel with polyethylene glycol and lipid bilayers to prevent fouling (160–162).

A biological nanopore was the first to be used to transport proteins in 1987, when Singer et al. first proposed it. Incorporating translocators into lipid bilayers, a peptide molecule could be transported within a biological nanopore, so, in a conceptual way the translocation of proteins could be detected (163). Moreover, the first protein translocation within a solid-state nanopores report was published by Han et al. in 2006 (164). In this work the single bovine serum albumin (BSA) protein molecule was translocated through 50 nm SiN nanopore with a thickness of 20 nm. A protein's shape or size is another feature that can be resolved by a solid-state nanopore. For example, the denatured state of proteins has been observed in several studies by solid-state nanopores (165,166). Yusko et al. could detect and differentiate between oligomers, spherical, protofibrils, and mature fibre's shapes of A β aggregates in a media by studying their signals during passing through the solid-state nanopore which coated by lipid. As shown in figure 1.10 they demonstrated that different sizes of A β could be discriminated in biological sample (167).

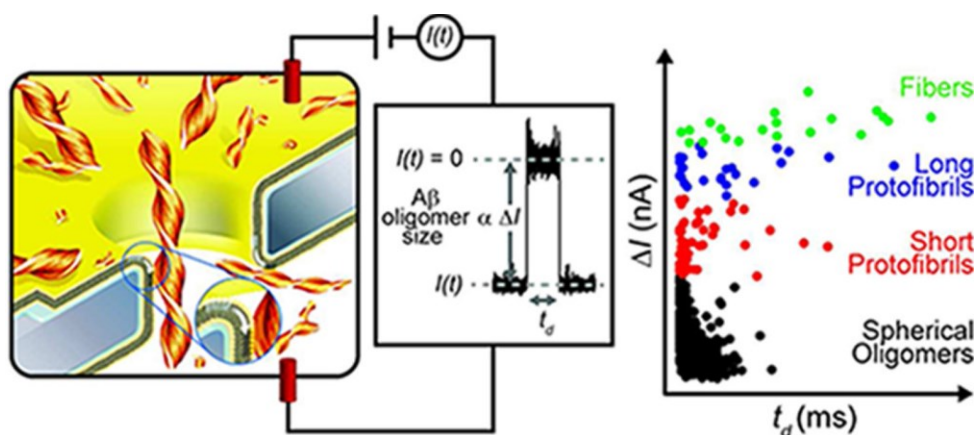


Fig. 1.10. Analyse the conformation of A β with coated solid-state nanopore using a lipid bilayer. Figure reprinted with permission from reference (167).

Many studies have been published since then using solid-state nanopores to examine individual protein molecules (168–171). In organisms, proteins are important biomolecules, and their interactions with other molecules and proteins play a significant role in various biological processes. This interaction causes functional and structural changes that have always been a focus of research. For example, a 15 nm radius and 20 nm thickness of silicon nitride nanopore was used by Kowalczyk et al. to detect protein bounded to DNA (172). In a recent study, Kaur et al. used silicon nitride nanopores to study the binding sites of RNA polymerase (RNAP) on long DNA (173). Five binding sites are found on a DNA, two of which were strong, and three were weak. A subevent occurs when RNAP crosses the pore. As shown in figure 1.11, in order to accurately estimate the binding affinity and location of RNAPs on DNA, they analysed current blockage signals amplitudes, dwell time.

Meanwhile, using antigen–antibody interactions to screen drugs via nanopore is another promising field of research. Despite this, quantity of antibodies binds to the individual virus was determined using a RPS with a nanopore in 2006 (174), but small antibodies or molecules will be difficult to detect. The size or conformation of a protein is another important aspect of studying proteins. In a study by Sha et al., the dynamic behaviour of different shapes of the proteins within the solid-state nanopore was observed when an electric field is generated. Blocking signals of proteins with different sizes and structures was investigated (175).

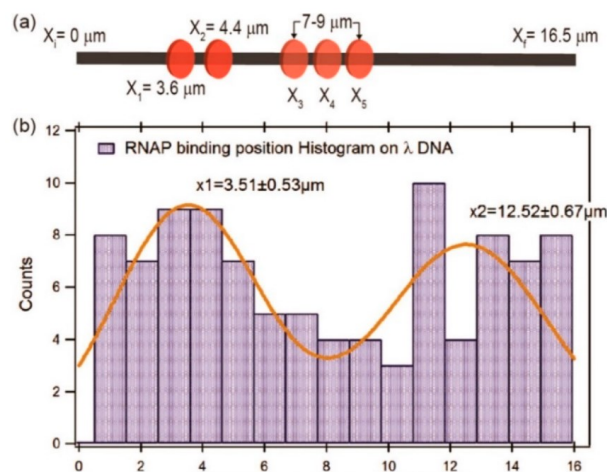


Fig. 1.11. (a) Sketch map of ideal RNAP binding locations; and (b) histogram and Gaussians fitting of measured RNAP binding locations. Figure reprinted with permission from (173).

Using the relative current blockade ($\Delta I_b/I_0$), BSA can be distinguished from the con.A (spherical protein) by its prolate shape. Two distinct levels of ionic current blockade were observed for

the non-spherical configuration BSA at high voltage larger than 300 mV, compared with only one level at low potential (figure 1.12) (175). The properties of proteins are not affected by solid-state nanopores, these studies suggest that it might be possible to characterize proteins in more subtle ways using solid-state nanopores without labelling or modification.

There are commercial products that demonstrate the capability of nanopores to distinguish DNA bases, such as Oxford Nanopore Technologies (176–178). However, protein single-molecule sequencing is still in its infancy.

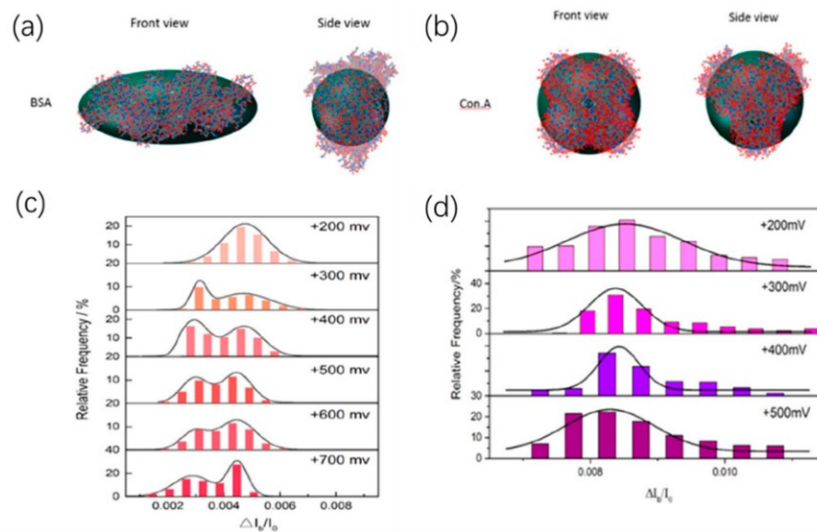


Fig. 1.12. (a) 3D structure of BSA with its non-spherical shape and (b) con.A protein and its spherical shape; and (c) BSA and (d) con.A related current blockade histogram graphs Figure reprinted with permission from reference (175).

A technique for identifying the amino acid sequence of polypeptide chains based on their free N-terminal was developed by Edman et al. in 1949 by electrophoresis or chromatography. Solid-state nanopore sensors are expected to be able to rapidly detect the amino acid sequence of a single protein's primary structure (179). The process, however, is not as straightforward as it appears, and a variety of factors have to be taken into account. First of all, protein sequencers based on nanopores require unfolding structures instead of simple structure of DNA. Furthermore, the surface of proteins is not uniformly charged, and the electrophoretic force is insufficient to control the translocation (180). Additionally, proteins have trouble being amplified, which makes sensitivity challenging. Furthermore, DNA has only four standard bases, while proteins have twenty amino acids which need to be extracted and distinguished. To conclude, solid-state nanopores can apply for characterization of proteins and detect their conformation, providing a base for drug discovery and disease research.

Serpin proteins include serine proteinase inhibitors such as α_1 -antitrypsin (AAT) that participate in fibrinolysis, inflammation, coagulation and complement cascades (181). According to figure 1.13, AAT has the following structural features (182). An increase in the concentration of AAT is linked to neurodegenerative, pulmonary emphysema, liver, and vascular diseases (183–186).

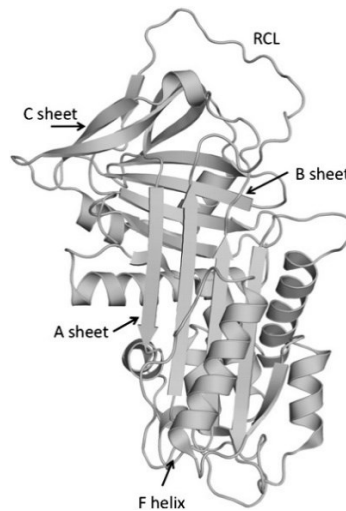


Fig. 1.13. Schematic image of the native α_1 -antitrypsin. Figure reprinted with permission from reference (182).

This protein has the capability of changing shape rapidly and protects the lung against excessive proteinase activity (187). Furthermore, mutations can disrupt the protein's ability to change shape, altering type and rate of changes in conformation, and giving rise to harmful molecules (188). As a result, since lung cancer and Alzheimer's disease change the size, shape, conformation, and number of AAT proteins, we designed and fabricated a functionalized nanopore to non-stickily translocate and analyse this protein.

Measurement system with insufficient temporal resolution limits the ability to detect fast protein translocation, despite numerous studies using solid-state nanopores to characterize single proteins. Therefore, A low-noise nanopore devices and a high-frequency amplifier are necessary for solid-state nanopores to improve and resolve protein translocation events.

1.8.2. Particle detection with nanopore

A resistive pulse counter (Coulter counter) is a traditional method that detects and discriminate the particle size, which is widely used in different field of study such as biomedical science, environmental study, and manufacturing (1). A resistive pulse counter

contain glass membranes (with micrometre/millimetre-sized pores) are separated by two electrolyte solutions. A steady voltage (DC or AC) is applied across the pore, and the created ionic-current is monitored continuously (189). Three factors can determine the baseline current like shape and dimension of the pore, the applied voltage, and the concentration of the electrolyte (190). By entering the particle into the pore, the amount of electrolyte equal to the particle size and volume would be displaced, which leads to increase the resistance and decrease the current. During current-time recordings, pulses are generated by this attenuation transient. Amplitude of electrically insulating particles produce pulses is directly proportional to their volume. Particles mobility and charge can determine translocation time, while event frequency and particle concentration are directly related (191).

In the following, some outstanding studies were reviewed based on particle/protein detection by micropores. For the detection and counting of HEK-293, Carol M. Olmos and his colleagues integrated microfluidic multilayer channels and solid-state micropores obtained through direct etching. It was demonstrated that an integrated microfluidic device with a multilayer microchannel with an individual solid-state micropore is inexpensive for counting cells (figure 1.14) (192). These experiments can be performed in any laboratory with basic chemical capabilities, not just highly equipped laboratories.

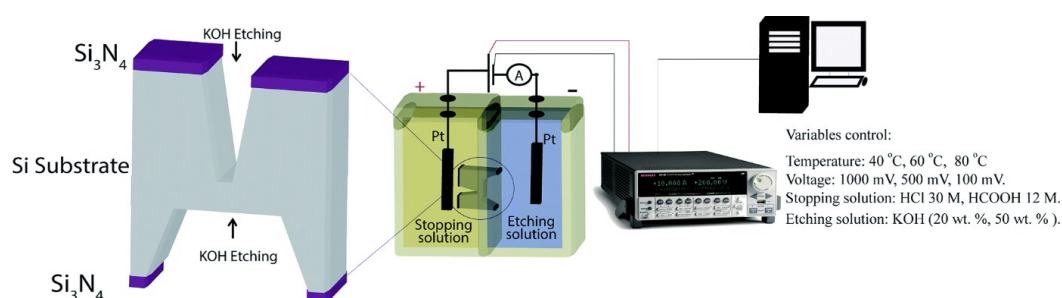


Fig. 1.14. Micropore fabrication via electrochemical technique. Figure reprinted with permission from reference (192).

In another study, Makusu Tsutsui et al. made 30 μm pores using electron beam lithography and dry-etching to measure cross-membrane ionic current to detect *Escherichia coli* and *Bacillus*. As shown in figure 1.15, by studying single resistive pulse spectra at 1 MHz, they were able to discriminate single bacteria shapes and count the number of bacteria (193). Also, researchers proposed drilling solid-state micropores from blood samples using a focused ion beam (FIB).

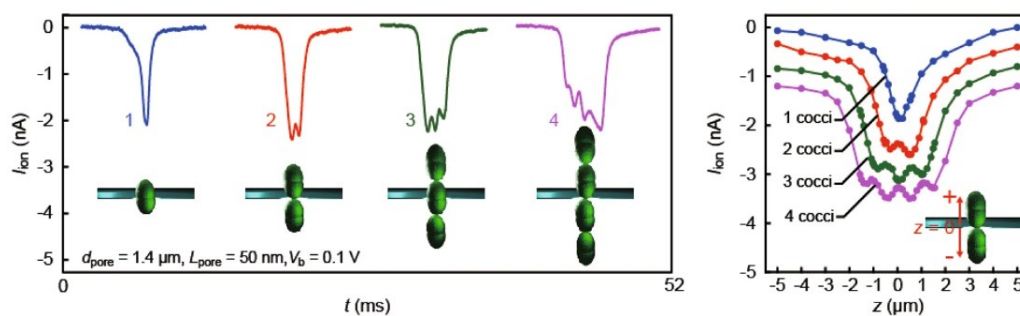


Fig. 1.15. low-aspect-ratio pore ($d_{\text{pore}} = 1.4 \mu\text{m}$ and $L_{\text{pore}} = 50\text{nm}$) sensitivity to single bacterial cell translocation. (left) Corrugated spikes showing up to four peaks at the apex. Insets illustrate the bacterial shape deduced from the spike forms. (right) Finite element analysis of the ionic current profiles during translocation of the beads-chain-like microbes constructed with one (blue), two (red), three (green), and four (purple) cocci. The curves are shifted vertically for the sake of clarity. Figure reprinted with permission from reference (193).

As shown in figure 1.16, different cell populations responded differently when cancer cells were pushed through micropores one by one. Their study showed that the solid-state micropore device is very reliable, cheap, high-performance, and has a detection accuracy of 95% for cancer cells in various samples. It was demonstrated that RPS technology based on solid-state micropores could enumerate circulating tumour cells and characterize specific cells phenotypically (194).

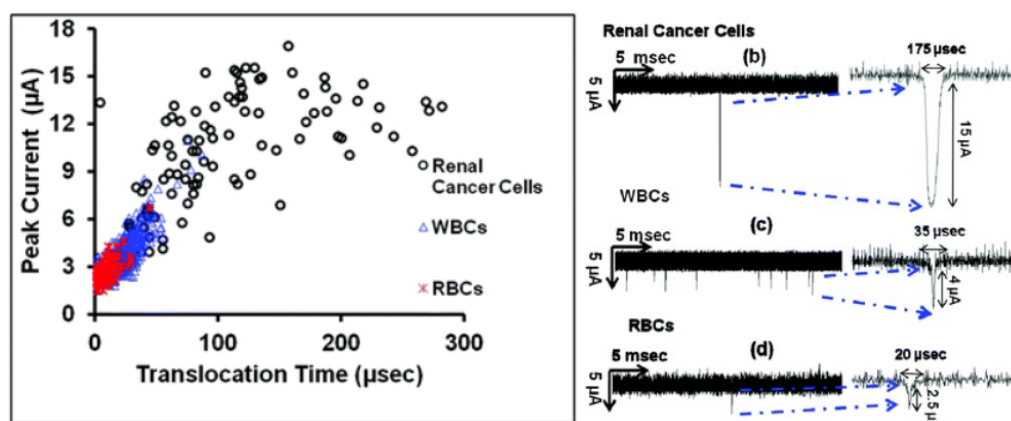


Fig. 1.16. Electrical data and profile of the cells translocation through the $12 \mu\text{m}$ micropore. (a) The plot shows the distribution of the pulses for three types of cells processed separately through the device: renal cancer cells, RBCs, and WBCs. The data from all three runs is shown on a single plot for comparison. The average translocation time for renal cancer cells was significantly higher than those for WBCs and RBCs. The average current blockage amplitude for cancer cells was also significantly higher than those for WBCs and RBCs. (b–d) show the representative electrical profile of each cell type: (b) cancer cells, (c) WBCs and (d) RBCs. Figure reprinted with permission from reference (194).

According to Azhar Ilyas and co-workers, SiN micropores can be used to investigate tissue samples for early cancer detection (figure 1.17). Cancerous cells can be biomechanically

distinguished from normal cells based on their intrinsic properties alone, without the assistance of biomarkers. They also used bladder cancer cells as a model, which did not exhibit the same electrical properties as healthy cells. They were able to detect tumour cells with 75% efficiency using a micropore biosensor (195).

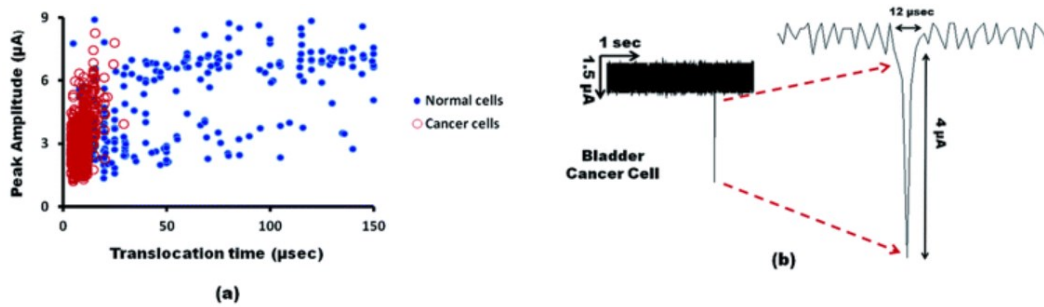


Fig. 1.17. Scatter plot and translocation profile of the cells passing through the micropore. (a) The distribution of Normal and cancer cells translocation events. The plot indicates that cancer cells show distinctively different electrical profiles when compared to their normal counterpart. (b) demonstrate characteristic translocation current traces (translocation time, current peak amplitude) for bladder cancer cells. Figure reprinted with permission from reference (70).

Figure 1.18 illustrates how a 10 µm FIB-milled SiN, which has established criteria for determining the structural properties of individual yeasts *C. albicans*, is electrically addressed in a direct manner using ultra-fast low-concentration electrical signals. *C. albicans* were detected at the lowest concentrations faster than with conventional automated blood culture systems, and their physio-chemical characteristics were also characterized (196).

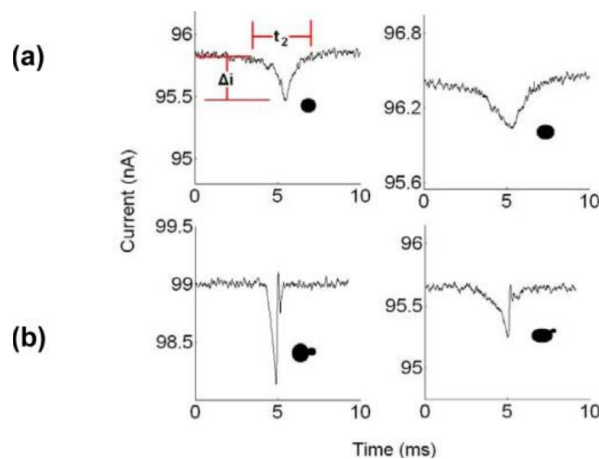


Fig. 1.18. Typical current signatures observed. (a) Two examples of single yeast translocation events recorded using a 10 µm diameter pore. Events are characterized by their individual translocation times, t_2 , and current amplitudes, Δi . (b) Two examples of budding yeast cell translocations. The inset in both (a) and (b) is an illustration of the probable morphology of the translocating yeast cell. Figure reprinted with permission from reference (196).

Tunnelling resistive pulse sensing technology (TRPS) is a technique for measuring the size, concentration and zeta potential of particles as they pass through a size-adjustable nanopore as shown in Figure 1.19.

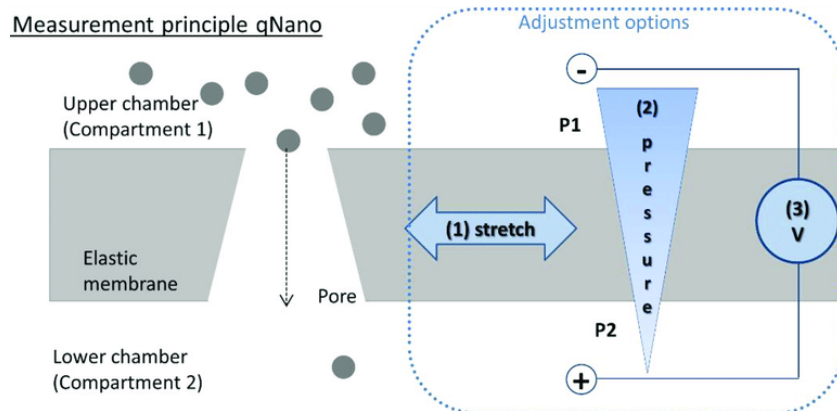


Fig. 1.19. TRPS (Tunnelling Resistive Pulse Sensing) measurement principle. In the upper compartment, suspended particles are translocated through the nanopore, causing the resistive pulse. Adapting the devices to specific samples can be accomplished in three different ways: mechanical pore expansion, pressure difference adaptation, or DC voltage application (right).

In another study, G. Seth Roberts et al. developed resizable elastic nano/micropores to differentiate between different polystyrene (PS) particles sizes by polyurethane membranes and introducing a selective gate. They estimated the sizes of nanoparticles accurately using their flexible micro/nanopore device compared to dynamic light scattering (DLS), as presented in table 1.2 (197).

Table 1.2. Characterization of PS particle diameters.

Particle diameter (nm)	DLS (nm)	Nanopore size estimation (nm)
50	58±3	51±7
80	91±1	82±6
100	120±1	97±3
220	245±7	220±6
400	417±3	404±4

Brain cancers that are most common and most lethal are glioblastoma multiforme (GBMs). A solid-state micropore sensor with a diameter of 20 μm is made out of a thin SiO_2 membrane. Researchers demonstrated that detecting and identifying GBM subtypes from patient samples using the micropore strategy is straightforward, practicable and cheaply. Single-cell heterogeneity of GBM was detected by solid-state micropore characterization (198).

An artificial micropore (2–4 μm) has been created alongside a SiN film by Chenang Lyu et al. Through electroosmotic flow and dielectrophoretic force, they demonstrated that microparticles could be trapped, agglomerated, and repelled around a single micropore without external pumps. Based on all these studies, it is clear that micropore chips have the ability to distinguish the size, shape and confirmation of nano and microparticles. We believe the portable, easy-to-use, low-cost micropore approach is advisable for diagnosis and analyse viruses and bacteria. Our purpose in this order was to develop a fast and mass-production method to manufacture micropore flow cells, as explained in chapter 2 and 3.

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2. Material and methods

In response to biological nanopore limitations and improvement in nanofabrication, solid-state nanopores have been developed by using rigid silicon-based material and polymers, which introduce unique merits to the nanopore, including controllability of pore size, diameter, the stability of the surface morphologies, and chemical and mechanical properties under a different conditions like pH, humidity, temperature, and level of salt. It is crucial to consider all of these factors, especially in a nanopore with a slightly larger diameter and a stable membrane for detecting entities at a molecular level, as it allows the detection of analytes for a longer period of time and generating data with a higher signal to noise ratio (SNR). Nanopores have been identified as an efficient and trustworthy technique for molecular counting over the past 20 years. Transmission electron microscope (TEM) or vacuum-drilling the membrane are commonly used to fabricate the solid-state nanopore, but they are challenging and expensive (1). It takes a great deal of training and specialized equipment to detect single molecules during these tedious, prolonged processes.

In this work, by using a femtosecond laser, we were able to open a micropore in a polyimide (PI) membrane in one step for the first time. Our portable platform, 101 x 44 x 18 mm is built of the inexpensive and readily available polymers poly (methyl methacrylate) (PMMA) and PI, and drilling was performed using a femtosecond laser machine. Nanopore experiments and ionic current were measured by USB-powered patch clamp amplifier. This technology is fast and cost-effective for mass production because the speed is less than a fraction of a second without any consumables. Our objective was to identify alternative materials and methods that would reduce production times, minimise, and enhance signal quality.

In general, solid-state nanopores can be manufactured mainly using three methods which are explained below: transmission electron microscopy (TEM), ion etching, and drilling. Further methodologies are summarized in table 2.1. At the end of this section micropore and flowcell fabrication were discussed as well.

2.1. Material

An important aspect of nanopore or micropores is the selection of the membrane material. For instance, its mechanical characteristics are directly related to the final device quality and ease of fabrication; membrane properties in electrical terms also affect the nanopore behaviour. The primary materials used to fabricate the solid-state nano/micropore being researched are briefly described follow.

Norcada (Canada) supplied Square Frame 20 nm SiN nanopore chips with a 30 nm thick SiN layer, and 60 nm SiO₂ underlayer (part number: NXPR4001X-20nm-AO/O-HR). All Nanopore devices were made with 200 μm thick High Resistivity Silicon frames, and have a 10 μm $\times$ 10 μm window. Phosphate-buffered saline (PBS) tablets (Product number P4417), Methanol (MeOH) anhydrous 99.8%, Sodium hypochlorite (NaOCl) solution (Cas number 7681-52-9), Sulfuric acid (H₂SO₄) 95.0-98.0% (Cas number 7664-93-9), Sodium chloride (NaCl) 99.0% (Cas number 7647-14-5), Tris manufactured by Cytiva (SKU number 17-1321-01), Isopropanol (IPA) 70% in H₂O (Cas number 67-63-0), Silver wire with 0.5 mm diameter and 99.9% trace metals basis (CAS Number: 7440-22-4), Polystyrene particles (100, 300, and 350 nm) (MDL number: MFCD00131491), and Hydrogen peroxide (H₂O₂) 30 % (w/w) in H₂O (Cas number 7722-84-1) supplied by Sigma Aldrich. The SuSoS AG provided the PLL(20 kDa) grafted with PEG(2 kDa) (poly(llysine)-g-poly-(ethylene glycol)). A recombinant Human Serpin A1/ α -1-Antitrypsin Protein (catalog number 1268-PI-010) was purchased from Bio-Techne. Calcium chloride (CaCl₂) powder 97% (Cas: 10043-52-4) were purchased from Fisher Scientific. 12.6 μm thick Polyimide (PI) film (Product number SF335C) was purchased from SHENGYI TECHNOLOGY CO., LTD. Focus ion beam (FIB) and scanning electron microscopy (SEM) images were taken at the CNR (Consiglio Nazionale delle Ricerche) of Modena, Italy.

2.1.1. Si-based materials

Current microfabrication methods make it easy to process solid-state materials because of their mechanical and chemical durability. A common list of materials includes Al₂O₃ (2), Silicon nitride (SiN_x) (3), SiO₂ (4), graphene (5), and SiC (6) which enable to control of the pore fabrication precisely based on required chemical and physical properties. As silicon-based materials enable fast simultaneous fabrication of multiple chips, they are common candidates for nanopore manufacturing. SiN_x, which is deposited by low-pressure chemical vapour

deposition (LPCVD), is the most popular material chosen due to its high dielectric strength and resistivity ($10^{16} \Omega \text{ cm}$ and 10 MV/cm , respectively) (7,8). Using LPCVD SiN_x , low ionic currents can be detected (range of picoamperes) through small nanopores. In 2003, thin SiN_x substrate was drilled with electron and ion beams to produce SiN_x pores (3,9). Also, due to its high thermostability, SiN_x has been employed in a wide variety of industrial purposes compared to novel two-dimensional material like graphene (10). Furthermore, silicon nitride has excellent chemical stability. So, a wide variety of nanopore experiments can be conducted with SiN_x -based nanopores due to this property. Additionally, since SiN_x and Si have similar coefficients of thermal expansion, nitride layers can be deposited at high temperatures on silicon membrane without leaving large residual stresses at room temperature (RT) that could lead to cracks in the unsupported nanopore substrate.

2.1.2. Polymeric-based materials

Materials with adequate resistivity and dielectric strength, including organic polymers, are suitable for manufacturing nanopore substrates. A variety of polymers, such as PI, polyethyleneterephthalate (PET), polycarbonate, are commonly used in this process (11). The production of nanopores is not limited to these two materials; for instance, glass nanopipettes (12), mica devices (13), and PDMS (14) can be used as well. There has been a growing interest in new materials to simplify the manufacturing steps, especially polymeric membrane like Polyethylene terephthalate (PET) or polyimide film (PI), through the use of etching to minimize the usage of semiconductor processing cleanroom nanofabrication (15–17), their chemical structure is represented in Figure 2.1. PI provides better performance than PET because of its excellent thermal stability, resistance to chemicals, electrical and mechanical properties, and high breakdown voltage (18,19). Since PI is an insulator, it can be used to reduce current noise caused by capacitances in devices (20). However, in commercial PI membranes such as Kapton films, current modulation is difficult to detect due to their thickness at the micron level. Therefore, It is imperative to find novel materials and methods that can reduce time of production while cost-effectively enhancing the quality of signals.

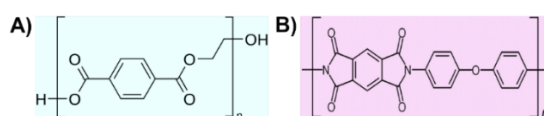


Fig.2.1. Chemical structure of PET (A) and PI (B).

2.2. Nanopore Fabrication Techniques

Nanopore fabrication involves a multi-step, complicated process. The fabrication of nanopores in a cleanroom environment is necessary to prevent them from contamination which causes a reduction in the functionality of the devices (21). The diameter, shape, and optimization can be achieved with a variety of tools by concerning with the thickness and profile of the nanopore. Focused ion beam, electrochemical processing, controlled dielectric breakdown (CBD), laser pulling of glass or quartz pipettes, metal mask, thermal annealing, and chemical etching are such techniques all summarized and explained in details in table 2.1. The following sections describe standard operating procedures for fabrication a SiN on silicon substrate, afterward other techniques of nanopore milling are discussed. An overview of the nanopore fabrication process is represented in Figure 2.2, and schematic image of all nanopore fabrication methods are illustrated in Figure 2.3.

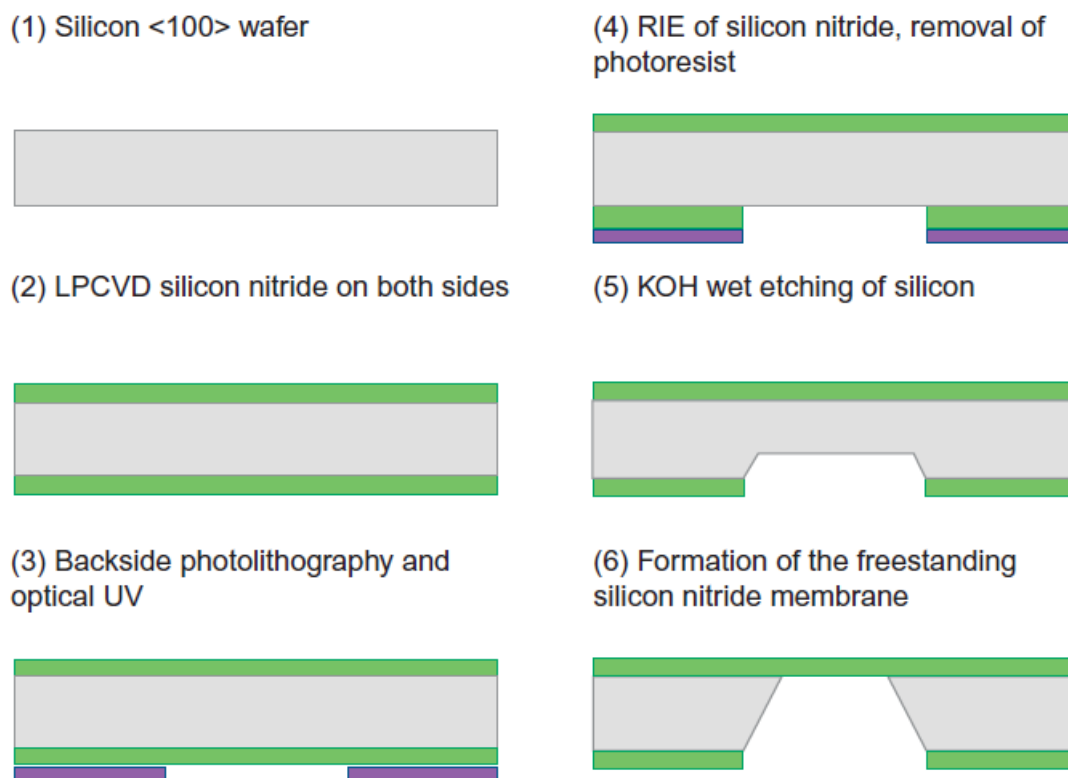


Fig. 2.2. An illustration of the 6 steps involved in fabricating metallic nanopores on a Si wafer substrate is provided. Nanopore milling is performed on individual samples after the chips have been cleaved. (1) Polishing Si wafer. (2) LPCVD deposition of SiN_x on the Si wafer (3) Back side lithography of the back side of the wafer by UV to define the SiN_x etch area. (4) Photoresist removal. (5-6) Chemical etching of Si wafer. Figure reprinted with permission from reference (22).

Table 2.1. Nanopore fabrication methods.

Method	Pore dimension (nm)	Benefits	Drawbacks	Ref.
Focused-ion beam (FIB)	>1.8	Precisely control the shape and size of the pore by adjusting the position, dwell time, and beam spot size. Possibility to monitor the fabrication in real-time	The condition affects the pore shape. Hydrocarbon contamination can affect the hydrophobicity of the pore. Ion beam processing requires high-energy beam tools.	(23–25)
Transmission electron microscopy (TEM)	>0.13			(26–28)
Laser of glass pipettes	>4.7	Cost-effective, fast, utilizing multi-barrel pipettes, it is simple to fabricate closely located groups of multi nanopores	Challenging to fabricate pores less than 10 nm; a glass pipette is required.	(29–32)
Controlled dielectric breakdown (CDB)	>1.1	The in-situ process requires only an I-V reading tool; no pre-treatment like cleaning is needed	Only possible to control the pore location by applying additional techniques like laser etching or AFM tips	(32–37)
Electrochemical process	>8	In-situ process, incorporating a gate electrode into nanopores is possible.	The electrode-integrated membrane must be pre-pored, which results in larger nanopores; optimization of the process is required.	(38,39)
Thermal annealing	>2	Nanopore array fabrication	High temperature is required, long time process, Particles like Au must be dispersion as a local heater	(40–42)
Nanoimprinting	>6	Fast, cost-effective	Only apply on a polymeric material	(43)
Chemical etching	>2	Nanopores can be manufactured in membranes whose thickness ranges from micrometres to nanometres	Long-time process, controlling the number of pores is challenging, sometimes mask is required, precise etching profile is required	(44–49)

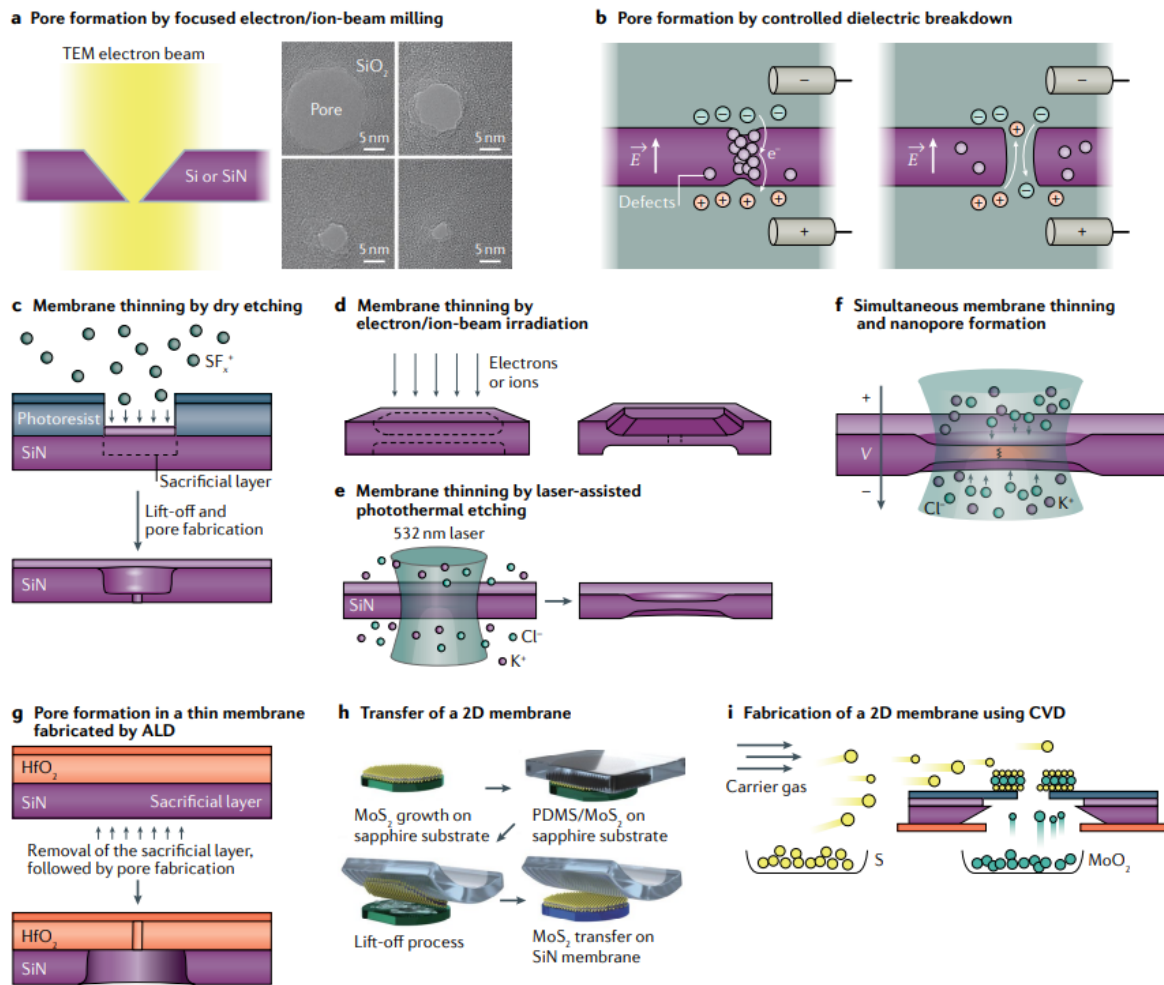


Fig.2.3. Schematic image of different nanopore fabrication methods (50).

2.2.1. Thin film LPCVD deposition of SiN_x

A precondition to fabricate nanopores is freestanding membrane fabrication (51). In this regard, the thin film of SiN_x is deposited by LPCVD on both surfaces of the Si wafer under dichlorosilane (SiCl₂H₂) and ammonia (NH₃) gases at 825°C. The SiN_x deposited film is a production of a reaction between gasses (SiCl₂H₂ and NH₃) (52). To prevent cracking and peeling of the SiN_x films, it is important to leave negligible residual stress in the nitride layer after deposition. LPCVD can be employed to reduce the tensile stress by increasing the silicon content and forming non-stoichiometric silicon nitride (Si_xN_y) films. This phenomenon could be achieved by ratio reduction of NH₃/ SiCl₂H₂ during the LPCVD step. The LPCVD process also permits sub-nanometre resolution control of the nitride film thickness. As a result, signal-to-noise ratios would be increased with thinner membranes. Conversely, decreasing the film thickness makes the device more fragile, hence, device fragility increases as the nanopore

membrane becomes thinner. Thus, it is necessary to find the right compromise between usability and sensitivity (53,54). Generally, an ideal membrane thickness for the membrane is 20 nm, while less thickness provides a fragile device. The photolithography process is used to fabricate multiple membrane templates in Si/SiN_x substrate wafers (55).

2.2.2. Photolithography

The accurately deposited layer of SiN_x on the Si wafer provides the substrates for producing the nanopore. The single nanopore structure is formed by selectively etching away these layers. A uniform photoresist coating is applied to the wafer surface as the first stage of selective etching. Photoresists are necessary to lay down on the wafer surface and create a pattern after exposure to the etchant developer. As a result of these photoresist patterns, SiN_x layers can be selectively processed. It is first necessary to produce shadow masks that correctly expose the photoresist to the incident light to form the patterns. The shadow mask allows certain substrate parts to be shielded from incident light while leaving other parts exposed. Positive and negative photoresists are both types of photoresists. By using the positive, a copy of the shadow mask pattern can be created, whereas by using the negative, the pattern will be reversed (56,57). As a result of using the developer, either the exposed or unexposed parts can be removed (depending on the type of photoresist). Finally, nanopores are milled in SiN_x membranes controlled by shadow mask designs. Trigonometry and the silicon layer thickness can be used to calculate the membrane size (58):

$$membrane\ dimension = Photolithographic\ feature\ size - \sqrt{2(depth\ of\ silicon)} \quad (2.1)$$

2.2.3. Chemical etching

In order to transfer the mask details to the wafer, etching is required after photolithography. A two-step etching process is required to form the nanopore structure. Prior to employing the SiN_x layer in the chemical etching process as a mask, the removal part of SiN_x is determined to remove by reactive ion etching (RIE). The etching gases are applied to the SiN_x/Si wafer during the RIE step. The plasma would be created inside the reactor with the aid of a radio frequency power source (RF) (Figure 2.4). Then the activated ions of the plasma would be accelerated to the nitride layer on the substrate surface as an etching process to etch the membrane (57).

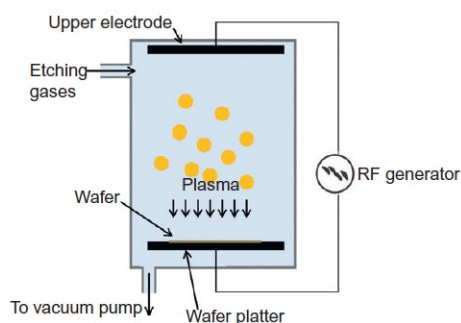


Fig 2.4. Schematic image of the RIE reactor. Figure reprinted with permission from reference (22)

The most common gasses used in this step are pure sulphur hexafluoride (SF_6) or carbon tetrafluoride (CF_4) or a mixture of them with O_2 . All these four compositions can etch the Si and SiN_x (59). Moreover, in order to prevent the substrate from over-extraction and etching, the duration of exposure to plasma must be meticulously controlled. In order to determine the exposure times, etch rates should be measured and used to calculate the depths of etch. In order to control the final etch depth, the reactivity of the plasma could decrease by adding unreactive gases to the mixture of etch gas. This will help to slow down the rate of etching. Following the RIE process, a mask is created by using the SiN_x layer, selective removal of silicon wafers is possible to create the freestanding membranes, which is essential for the fabrication of the nanopore. Here, KOH anisotropic etch should be used to etch the exposed Si wafer (59). The temperature and concentration of the KOH solution play a crucial role in the etching rate. For example, in order to create rough etches, the concentration should be less than 30% (60). Finally, the Si wafer should be washed with deionized water and isopropanol. Milling the nanopore itself is the final process in the nanopore membrane fabrication. High-power electron beams like focused and non-focused ion beams (FIBs), and ion track etching for polymer membranes are the most common approaches in this process. Li et al. pioneered using an ion beam to fabricate the nanopore to study the DNA (61). They created a curve shape cavity by RIE on one side of the substrate; then, they used Ar ion beams to fabricate the nanopore. By ion beam process, the incident ions could sputter or eject the atom on the surface of the membrane if their kinetic energy is higher than the surface binding energy. The cavity formed by RIE will eventually be intercepted by sputtering as material is removed from the SiN_x layer. As a result of this intersection, a nanopore is created. It is necessary to carefully control the sputtering step to achieve a molecular-sized nanopore, which usually involves a feedback system that monitors ions passing within the pore, keeping

the SiN_x from eroding when a sufficient number of monitored ions matches the diameter of the pore dimension. Pores with diameters of 1.5 nm have been successfully fabricated using this method. Li et al. have developed an ion beam technique for creating nanopores that include a FIB. In semiconductor devices, FIBs can be used to image semiconductor material, as well as to make nanopore via drilling, and create different pore sizes by using various materials. The fabrication of nanopores is essentially possible with any ion capable of sputtering material including He⁺, Ga⁺, Ar⁺, Xe⁺, Kr⁺, and Ne⁺ (62). Most nanopore mills use Ga ions, which can implant ions into membranes and consequently modify the surface charge of nanopores.

Gierak et al. pioneered the milling of pores with a FIB at sub-5 nm (63). In this work, by using 20 nm SiC substrate they could create a 5 nm pore via a focused Ga⁺ beam in a 35 keV electric field. They did not use any previous etching step to create the cavity, and the ion beam was strong enough to localize the sputtering and fabricate the nanopore. Following further scanning, the pore shrinks, and its opening becomes more circular because of controlled contraction (Fig. 2.5) (63–65).

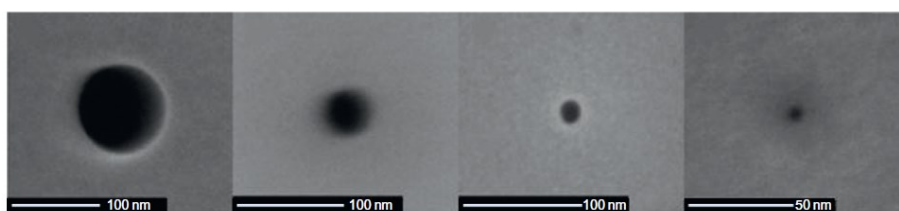


Fig 2.5. Four nanopore sizes were fabricated using FIB on the SiN_x membrane. After milling the nanopore with an ion beam, further scanning the surface by ion beam could create different pore sizes. Figure reprinted with permission from reference (65).

By introducing the Helium ion microscopes (HIM) to the FIB, further, development was achieved in the field of nanopore milling. The source of the HIM uses the tip with atomic size, possibly producing an nm scale pore with a highly FIB (66). In conclusion, using He⁺ leads to sub-nanometre pore precision due to its smaller size compared to other inert gases (67).

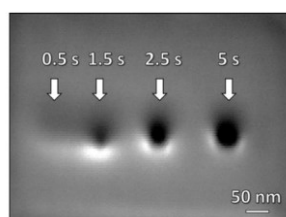


Fig 2.6. Different power of FIB was used to optimize the pore size (68).

Also, when a size of the pore is smaller than the substrate thickness, pore shrinking occurs more easily as FIB drills directly into the surface (69). FIB typically makes pores larger than 15-20 nm. Furthermore, making a thinner membrane directly, lacking ion implanting, and providing higher resolution are the advantages of this technique compared to the traditional ones (70).

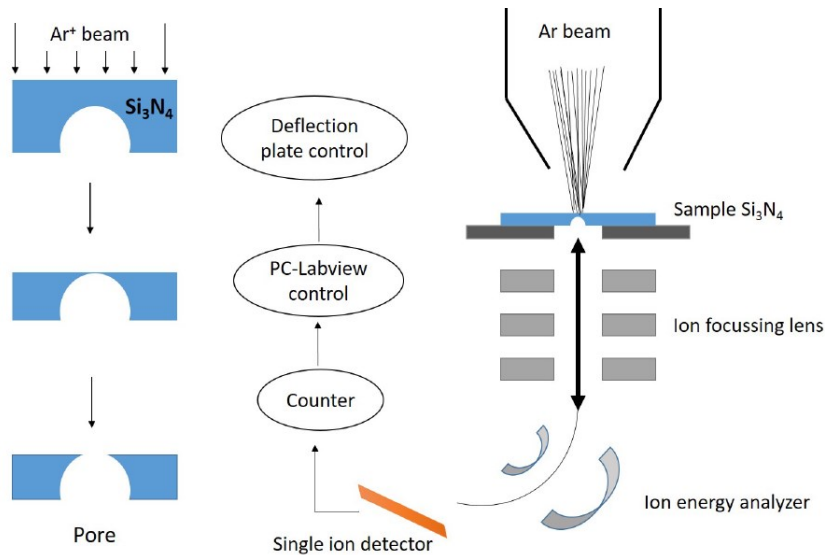


Fig. 2.7. Schematic diagram of Ar^+ focused-ion beam (FIB). Figure reprinted with permission from reference (71).

Based on the recent development, nanopore fabrication and milling can be accomplished with high-energy electron beams rather than ion beams. With the TEM, solid-state nanopores can be produced precisely down to the nanometre level (72). In this process, a SiN_x or SiO_2 material is first used to create the pores up to 50 nm in diameter (73). Pores between 2 and 15 nm are typically made with TEM. An electron beam with an intensity of 10^5 to 10^7 A/m^2 systematically shrinks the layer. A nanometre-per-minute rate of pore shrinkage is observed under these conditions, which makes it possible to control the diameter of nanopores completely. Here, the microscopic image from the surface roughness and resolution determines the nanopores' size. As amorphous SiN_x or SiO_2 is irradiated with electrons, nanopores shrink. Consequently, a reduced surface energy F results in the nanopore shrinking. Pores change a membrane material's free energy, which can be calculated by the following equation.

$$\Delta F = 2\pi\gamma(rh - r^2) \quad (2.2)$$

where h is the membrane thickness, γ the surface tension, and r is the pore radius. It is possible to explain that the size reduction of pores with radius $r < \frac{h}{2}$, reduces their surface energy, no matter what length scale they are applied to (72).

2.3. Micropore Fabrication

A membrane with micrometric pores is fabricated from a polymer, silicon, or glass (74). Several methods can be used to fabricate micropores, depending on their final application and the type of Substrate material selected. A number of manufacturing approaches are available for polymeric substrates, which are biocompatible, relatively wide in the selection, and low cost. Track etching (TE) has recently been the most common technique for commercially producing polymeric substrates (75,76). On many polymers, such as Polycarbonate (PC), Polyethylene terephthalate (PET), Polyimide (PI), and Polypropylene (PP), track etching allows the production of pores with sizes that range from 0.01 μm to 30 μm (75). According to Vona et al., a track-etch polycarbonate substrate with cylindrical pores (8 μm dimension) has been demonstrated (77). Low porosity is attained because of the holes' random positioning, to prevent their overlapping, which is a major disadvantage of this technique. Also, it needs a heavy ion accelerator as well. A phase separation procedure is another method of producing polymeric micromembranes (78). Zhao et al. used this technique to fabricate a microporous silicone rubber membrane with pores ranging between 3 μm to 41 μm (79). Despite the simplicity and low cost of the process, the distribution of pores and their density cannot be controlled. A photolithography-based microfabrication method was improved by Kim et al. to resolve the drawbacks of track etching and phase separation techniques for microfabricating PET substrates that have porosities of 40% and pore sizes varying by less than 5% (80). Membrane thickness was 1 μm , while pores were 0.9 μm in diameter. While this approach can produce roughly the same pore in dimension and the pores are arranged accurately, causing high density of pore and no overlap. Also, soft lithography, combined with chemical etching, has been used to fabricate membranes in polydimethylsiloxane (PDMS) (81). Herein, scientists could fabricate a pore of 10 μm in diameter into a PDMS membrane with a thickness of 10 μm . This technique is able to arrange pores precisely, such as hexagonally or rectangularly, but requires moulds and reagents that are not eco-friendly.

In order to fabricate freestanding polymer membranes, a vacuum-assisted UV micro-moulding process (VAUM) has been developed using a methacrylate polymer (MD) (82). By using this technique, it is possible to drill inexpensive, durable, with a broad range of geometrical properties of 8 to 20 μm pores in 20 to 100 μm thick membrane. The limitation of this technique is that a master mould is required for each membrane design. In addition, a 30 μm thick, uniformly pored silicon substrate constructed using deep reactive ion etching (DRIE) is presented. This study demonstrated a platform for rapidly and efficiently separating circulating tumour cells (CTCs) from blood samples (83). By taking advantage of the differences in deformability and size of normal blood cells and CTCs. By micropore device with densely packed pores, tumour cells are separated quickly from whole blood. The DRIE process produces micropores with high accuracy, depths and uniformity. Although DRIE involves multiple steps, the mask requirement makes reconfiguring the process difficult.

2.3.1. Laser drilling for micropore fabrication

Shallow holes caused by laser-induced surface damage has been extensively studied (84–86), but producing holes with high aspect ratios is also of scientific and technological interest. Previously, it has been demonstrated that redeposited the ablated material on the sides of high aspect ratio holes (87). Laser drilling can also result in the ejection of liquid and the formation of nanoparticles, which significantly affects drilling (88–90). Generally, holes are cleaner when pulse lengths are shorter (84). Moreover, vacuum drilling leads to a faster drilling rate at high fluences (90). In one study, the copper sheet holes with high aspect ratios were studied by A. Weck et al. By systematically varying the number and energy of pulses per pulse; they monitored in situ the hole formation. In addition, they studied how pulse length (femtosecond (fs), picosecond (ps), and nanosecond (ns) pulses) affects the morphology of holes at their entrances and insides as well as their photodiode signal over the range of 150 fs to 7 ns. Based on the results, there appears to be a transition from vaporization to melting at pulse lengths of several picoseconds (1–10 ps). Using 30000 pulses to drill laser holes in the air, the morphology of the walls reveals that ripples can be observed below 10 ps and melting has occurred in greater quantities above 10 ps (91).

In applications ranging from microfluidics (92) to surface texturing (93), femtosecond (fs) lasers have been proven to be versatile and mould-free methods for micrometric device

fabrication accurately. Due to the shorter timescale enabled by femtosecond lasers, the material can be removed from the area that was irradiated by laser ablation and the surrounding environment is damaged by thermal radiation. Here, micro milled structures can be created with a high degree of accuracy and resolution (93) that are almost free from imperfections caused by thermal stress, such as cracks, resolidified melting, burrs, and residual stresses. Material choice is not restricted by this non-contact method. Micropores can be produced more effectively with femtosecond laser processing than by means of other approaches. The polarization level of the beam has a significant impact on the micropore geometry. Drilling thermal effects can also affect micropore geometry as well as beam polarization (94). A high-power femtosecond laser was used to process micropores, which were found to have irregular outlet holes by Lu et al. (95). Also, compared to traditional pulse methods, the manufacturing effect is significantly improved with femtosecond laser bursts mode, according to Ilday and Herman et al. (96,97). As a result, we need to investigate further how the polarization state of femtosecond lasers can be manipulated and improved in order to improve micropore quality. To achieve this aim, we designed and fabricated an innovative, sensitive, portable, and quick micropore-based flowcell to detect nanoparticles in liquids. Due to the micropore-based flowcell simplicity and cost-effectiveness, it can be applied to massive output. By using a femtosecond laser, we were able to open a micropore in a PI membrane in one step for the first time. This technology is cost-effective for mass production due to its high speed (less than a fraction of a second). In section 2.3.2 our method to fabricate the PI micropore is explained, and flowcell design and fabrication is described in section 2.4.2. Also, an explanation about the RPS analysis is given as well.

2.4. Flowcell integration with nano/micropore

A fluidic environment is imperative for biomolecules to function properly, and flowcell devices provide that environment. A flowcell setup can be implemented to pre-process samples, guide flow, and conduct biomolecular analysis in most cases (97–99). Flowcell setups enable the analysis of analytes chemically and biologically at very small volumes due to the development of microfabrication (100,101). Mechanical valves and electrokinetic forces can also be used to operate flowcell setups to allow particle mixing in sealed spaces as required (102–104). PDMS is normally used as a material for flowcell setup by soft lithography methods. A quick and cost-effective prototyping method that allows embedding electrodes,

multilayer stacking, and improved particle flow control within microfluidic channels (105–107). In some studies, nanopores have been incorporated into a hybrid structure primarily composed of a PDMS flowcell setup, with the nanopores situated on a thin Si membrane at its core (14). Additionally, PDMS film molds were 3D printed with a low-cost 3D graphics design, followed by PDMS film molds. For instance, a 30 nm pore created by TEM on a 20 nm SiN membrane thickness on Si substrate with thickness of 200 μm was inserted into the cis (top) chamber of the designed PDMS flowcell. A two-stage plasma bonding process was used to partially attach two PDMS subunits. Here, the PDMS was cast in a special form to guarantee successful sealing. Figure 2.8 (i) illustrates the top and side view of the flowcell setup, and the real image of the setup is presented in figure 2.8 (ii) (108).

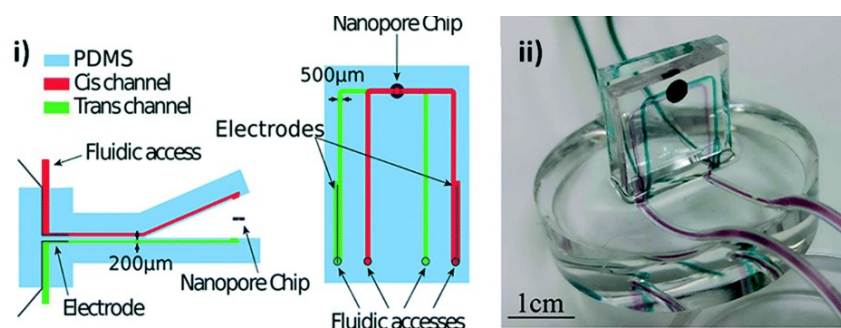


Fig.2.8. The integration of PDMS microfluidic system and nanopore for particles detection. (i) schematic and (ii) real image of the microfluidic. Figure reprinted with permission from reference (108).

2.4.1. Micropore flowcell structure and femtosecond laser drilling

Our portable Micropore flowcell system with 101 x 44 x 18 mm dimension was built by the cheap and readily available PMMA and PI polymers. PMMA was selected to be used for fabrication of the flowcell because of its special mechanical and electrical insulating properties, and its water-proof behaviour (109). As shown in Figure 2.9, flowcell consists of two PMMA compartments. Both chambers at top and bottom of the flow cell made by drilling/engraving cavities on PMMA compartments. As shown in Figure 2.9a, a 4mm diameter hole in 5 mm depth drilled in order to form a top chamber, and as shown in Figure 2.9c, the 0.8 mm arc-shape channel were engraved in bottom PMMA compartments with 1.6 mm depth to make bottom chamber. Bottom chamber's Inlet and outlet holes were created by drilling two small holes into the corners of the PI film and top PMMA compartment (Figures 2.9a and b). As shown in Figure 2.9b, the Silver (Ag) wires were mounted on polyimide (PI)

film as electrodes by Cisel S.R.L in order to apply a voltage across the micropore and allow a fine adjustment of the electric field which is adaptable to the different detection needs. Subsequently, the electrodes mounted PI film were placed and adhered between two PMMA compartments by solvent-resistant glue to prevent leakage of liquid (Figure 2.9d). The double sided tape must be resistant to liquid solutions such as isopropanol, sodium hypochlorite, and water-based buffer solutions. Therefore, double sided tape with a volume resistivity of at least 10^{13} ohm-cm is preferable. There is an assumption that the ionic current will increase significantly due to the leakage of the buffer, but that was not seen in this experiment. In order to eliminate the contaminants before laser drilling, the whole flowcell was soaked in deionised water : ethanol (9 : 1) for up to 5 minutes to remove dust and impurities. The Kirana company used a single 290 femtosecond UV laser pulse (257 nm) 1.5 J/cm^2 to drill a single micropore into the thin PI membrane of any micropore flow cell. Finally, to make quasi-reference electrodes, silver wires were chlorinated by filling both top and bottom chambers with diluted 50% NaOCl/Water for 2.5 minutes. All contaminations and residuals such as NaOCl were removed from the top and bottom chambers by sequentially flushing deionized water for at least five times.

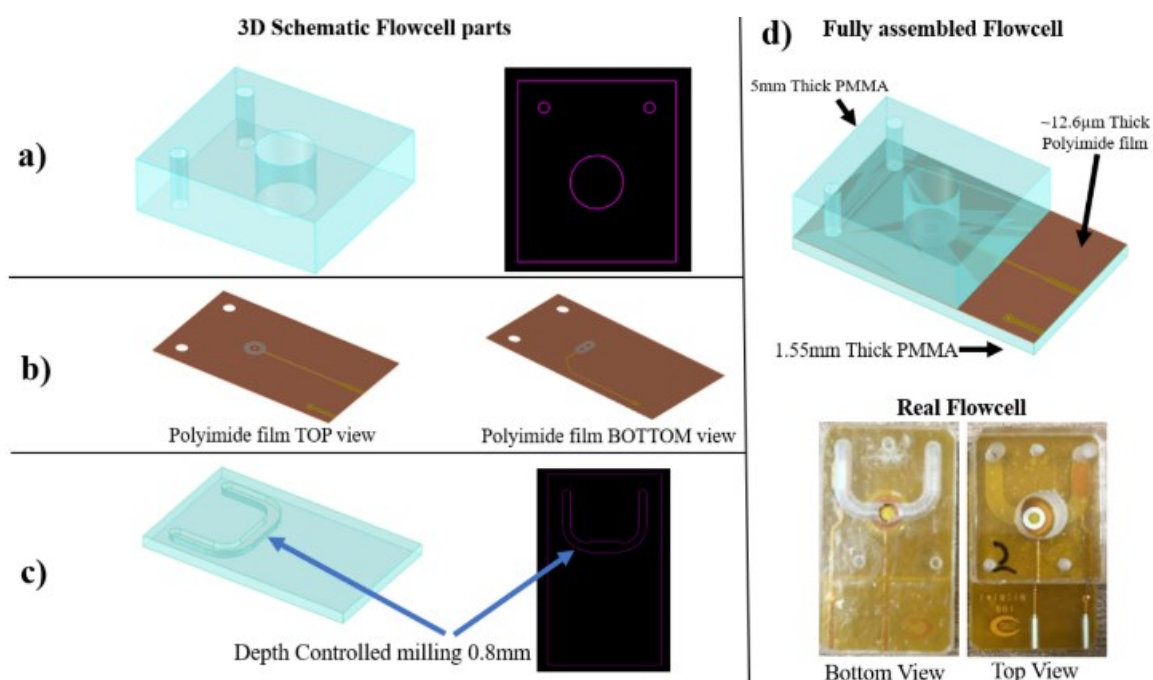


Fig. 2.9. Stack schematics view and real images of the flow cell. Schematic representation of (a) Top PMMA element, (b) PI film including the two Ag electrodes, and (c) Bottom PMMA element. (d) The schematic and real images of the fully assembled flow cell.

2.4.2. Design and structure of nanopore flow cells

The typical chips size is around 4 X 4mm or 5 X 5mm, and this flowcell accommodates both of these sizes. An individual component of the nanopore flowcell is presented in Figure 2.10.

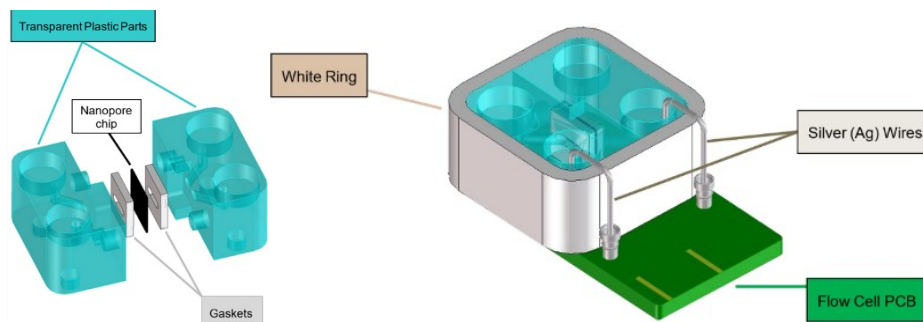


Fig.2.10. schematics of nanopore flowcell. (left) nanopore floccell compartments including PCB, silver wires, PMMA chambers and two gaskets. (right) Assembeld flowcell.

Nanopore chips are available in various sizes and thicknesses. The nanopore chips typically have a thickness of 200 to 500 micrometres. The gaskets have the same length and width but varied in thickness. It is possible to put nanopore chips with different thickness in the flowcell by selecting the right gasket and the right thickness (thicker Chips need thinner gaskets) (Figure 2.11).

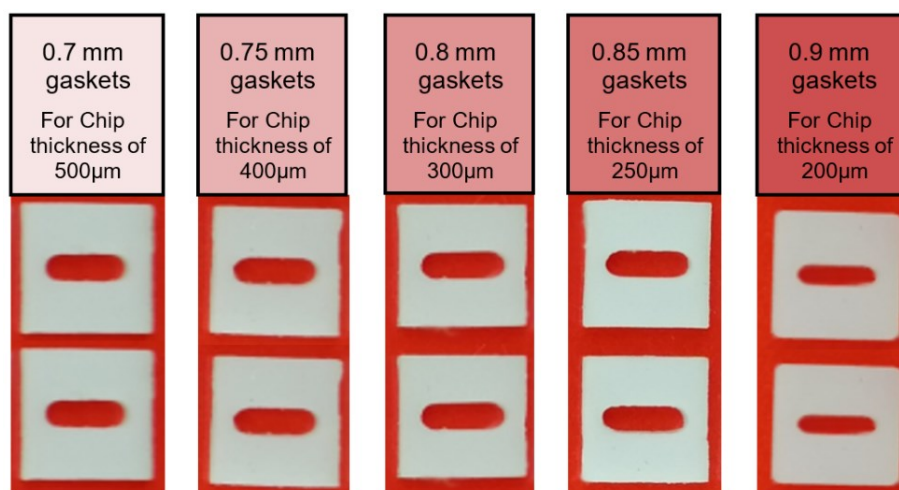


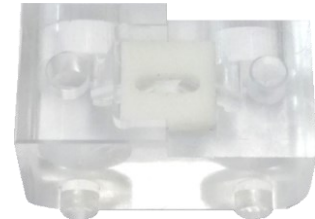
Fig.2.11. Different gaskets thickness.

2.4.3. Flowcell cleaning and assembly process

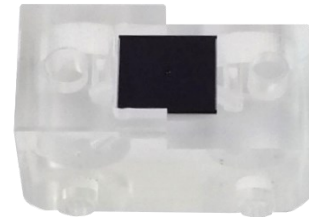
- I. One of the translucent plastic components was selected and set it on a flat surface.



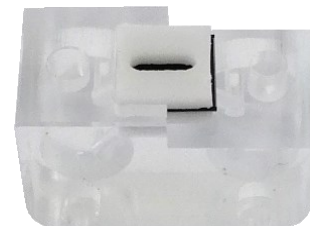
- II. Once the correct gaskets have been chosen (based on the thickness of the nanopore chip), the gaskets were cleaned with isopropanol or ethanol before washing them in with deionized water, then they were dried at room temperature. Next one of them was set on the piece of plastic as depicted.



- III. The nanopore chip was washed with the same procedure and then positioned on top of the gasket.



- IV. After that, the second gasket was placed on top of the nanopore chip.



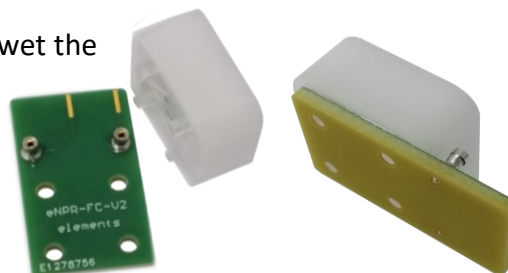
- V. The second transparent plastic piece was inserted to make a sandwich shape.



- VI. The sandwiched flowcell was fixed with the white ring.



- VII. The mixture of the IPA: water (1:1) was used to wet the flowcell and nanopore for 15 minute, then the fixed flowcell was placed on PCB and fixed by four pins.



- VIII. The chlorinated silver wires were assembled on PCB by placing them into the PCB sockets as illustrated.



- IX. Flow cell is now ready to use; it has two chambers, with a capacity of 10 to 60 microliters apiece. Solutions were injected via the openings depicted in the picture using a 10-100-μL pipette tip. (Brown arrows).



- X. The flowcell is inserted into the eNPR reader.
- XI. The plastic components of the flowcell should be cleaned after finishing the experiment.

2.5. Solid-state nanopore functionalization

Many factors influence the translocation process, including electrolyte-pore interface, electroosmotic flow, and receptor interaction, which are significantly influenced by the chemical composition of the nanopore.

Generally, many analytes interact non-specifically with the walls of solid-state nanopores. Due to these interactions, pores become clogged, and molecules cannot be translocated. Proteins are prone to interact with synthetic nanopore walls (110). Hydrophobic interactions (110–113), van der Waals forces (114–116), and electrostatic attraction (117–119) all contribute to these interactions. Figure 2.12 illustrates the coating that can weaken these interactions and promote translocation without disruption (120).

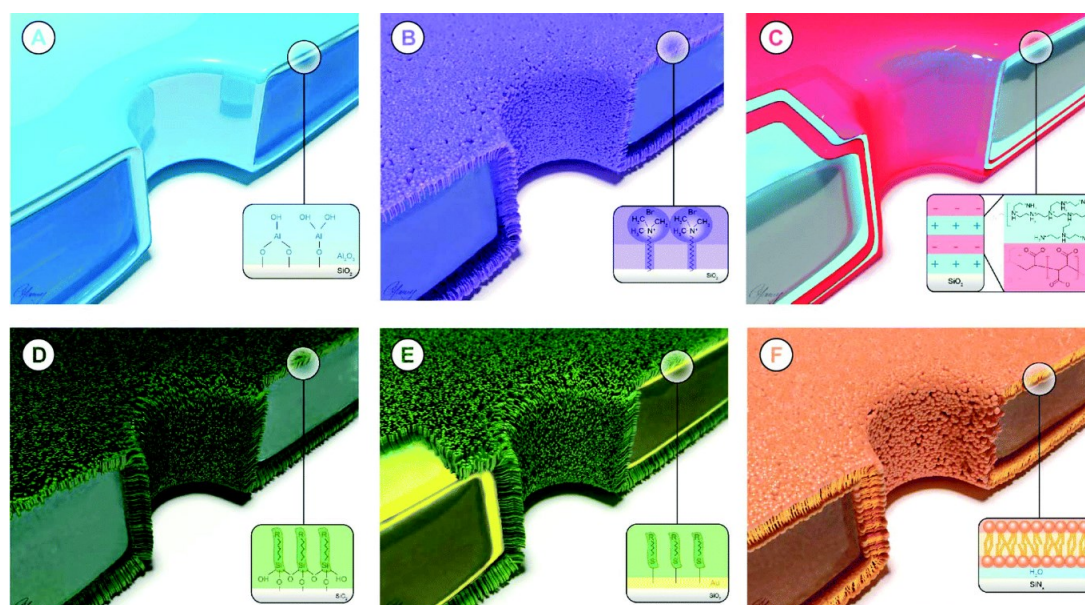


Fig. 2.12. Idealized cartoon representations of the most employed nanopore coatings. A. Cross-section through a SiO₂ membrane with a coating of Al₂O₃ deposited on the membrane and on the walls of the nanopore. B. Coating of a nanopore prepared by physisorption of a surfactant. C. Coating prepared by layer-by-layer self-assembly of negatively and positively charged polymers. D. Coating prepared by silanization. E. Coating of a self-assembled monolayer of alkanethiols on gold. F. Coating of a nanopore with a fluid lipid bilayer. Figure reprinted with permission from reference (121).

Most synthetic nanopore walls have negatively charged surfaces, which lead to two phenomena, ion current rectification (ICR) and electroosmotic flow (EOF), that can be used for nanopore analysis. The accumulation of counter-ions on charged surfaces in contact with electrolytes is referred to as an EDL (122). The applied voltage moves ions electrophoretically along a nanopore in the EDL, causing EOF, in which liquid moves along with the ions (123–

125). Depending on the polarity of charges on the surface of nanopore sidewall and the molecule's net charge, electroosmotic force can be increased or reversed by electrophoretic force inside the nanopore (126,127). In order to analyse and explain the distribution of translocation times from biomolecule or particle motion through nanopores, EOF must be minimized (128–130). Additionally, ICR can be caused by either an asymmetrical geometry of pore or asymmetry in the surface charges distribution across the nanopore (131–133). As a result of the asymmetric ion distribution, current flows preferentially toward one pole of the applied voltage compared to the other one, which leads to non-linear I-V curve (134–137). Sensing applications can be derived from this phenomenon (138–140).

In most cases, ICR should be eliminated when the nanopore benefits from a uniformity of the electric field. Nanopore walls can be charged differently via surface coatings (increasing, reducing, or inverting electron density) (141–144). Nanopore channels can be controlled in terms of EOF velocity by changing the electrolyte solution ionic strength or the charge density on the nanopore wall (145). A variety of coating methods have been developed for addressing issues such as slow "etching" in electrolyte solutions or non-specific interactions between solution and nanopore sidewalls (146,147). It is possible to modify the surface characteristics using a variety of chemical-functionalization techniques. For instance, silane chemistry is widely used to modify the SiN_x, glass-based nanopore, or metal-based nanopore that could be functionalized by vapour deposition (148,149). Deposition of metals (150) and oxides (151,152), as well as organic modifications (153,154), have been reported for nanopore functionalization. Due to a lack of imaging methods, the resulting nanopore structure often increases significantly in thickness.

Further, there is a considerable difference between coating flat surfaces and coating highly concave surfaces in confined volumes. Nanopore's highly confined volume may alter the kinetics of adsorption in addition to the concave surfaces. Additionally, nanopore coatings cannot be probed with characterization methods such as AFM, SEM, TEM.

As part of our nanopore functionalization, we used two methods: in-situ and ex-situ utilizing poly(L-lysine)-g-poly-(ethylene glycol) (PLL-g-PEG) self-assembly. Prior to discuss about our methodology, other approaches were explained in section 2.5.1 and 2.5.2.

2.5.1. Physisorption & Self-assembly

During self-assembly, components spontaneously come together to form an organized structure in a specific, localized way. Molecular self-assembly occurs when molecules are the constitutive components. In this method, a polyelectrolyte solution with an opposite charge of the pore's walls is immersed into the nanopore, which can be done in-situ setup to have a monitorable functionalization process.

The terminal groups of silicon substrate are silane (Si-OH) or siloxane (Si-O-Si) (155,156). Silanol groups are divided into three classes: a) isolated silanols: where Si is bounded to the bulk Si by three bonds; b) bridge silanols: where isolated silanols make hydrogen bond with Si atoms in their neighbour, c) germinal silanols: where Si makes two bonds with Si bulk and has two hydrogen bonds with another Si. Depending on the degree and type of hydroxylation of the Si surface, these types of silanols have different surface densities. For instance, based on infrared investigation, the fully hydroxylated Si with 4.6 hydroxyls/nm² surface density has 25%, 62%, and 13% of isolated, bridge and germinal silanols groups (157,158). According to acid-base titration of mesoporous (159) and planar (160) Si, 80% of the silanols are germinal, and 20% are isolated. It is possible for silane groups to behave as weak bases and acids. Except at very low pH levels, Si is normally negatively charged. The isoelectric point of Si is ~2-3 (161). The isoelectric point of SiN_x nanopores allows them to switch from negative to positive surface charge ($\sim 4.4 \pm 0.3$) (162,163).

Physical adsorption is a straightforward technique that bonds the target molecules to a membrane due to electrostatic forces, van der Waals forces, hydrogen bonding, and hydrophobic interactions. Among the most popular straightforward surface modification methods is physisorption. One of the most widely used approaches for this purpose is to adsorb positively charged poly-L-lysine (PLL) on surfaces with a negative charge. It is reported that for altering the surface charge and engineering the interactions on the surface of pore sidewalls, PLL coatings can prevent molecules from adhering to the pore sidewall (164,165). For example, molecularly specific antibodies were cross-linked with PLL and cysteine residues in an interaction between mycotoxins and the protease thrombin (166,167). According to Schneider et al., non-covalent construction of an amphiphilic molecular monolayer with hydrophilic ends prevented hydrophobic interaction between DNA and graphene walls of

nanopores (164). This modification improved reproducibility for detecting dsDNA (double-strand DNA) and ssDNA (single-strand DNA), highlighting the potential of coated nanopores. On the other side, Umehara et al. (59) studied nanopipette electrodes coated with PLL. Pipettes with uncoated surfaces exhibited ICR, as their conical shapes would indicate, PLL-coated surfaces exhibited enhancement in rectification at the opposite polarity (168). The nanopipette's negative surface charge was inverted due to the PLL coating, which was positively charged. A quartz nanopipette was also coated with PLL for use in signal transduction by an ion "nanogating" (STING) sensor (166,167). Utilizing coated PLL crosslinked to immunoglobulin (IgG) molecules, Actis et al. developed this concept to detect mycotoxin HT-2 (Figure 2.13). The same sensing strategy was used to detect thrombin by immobilizing thrombin aptamers on PLL and polyacrylic acid (PAA) (166).

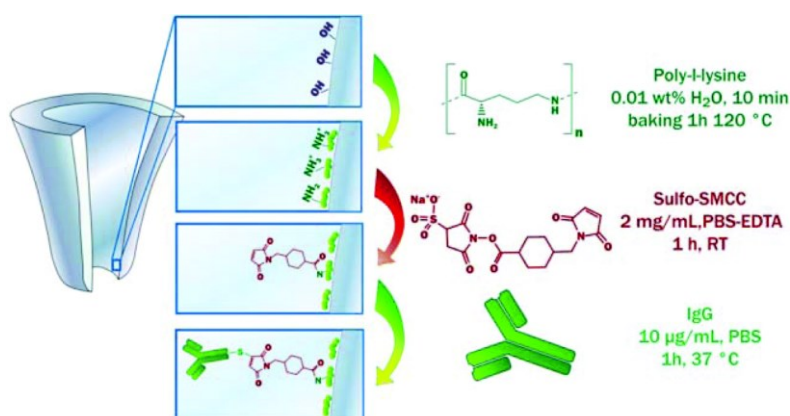


Fig. 2.13. The physisorption modification of quartz nanopipette with PLL and IgG molecules to diagnose the mycotoxin HT-2. Figure reprinted with permission from reference (167).

In layer-by-layer self-assembly (LBL), oppositely charged polyions are alternately deposited to create thin films (169–172). Silicon, metals, and glass have negative charges, which can be utilized to increase the deposition rate by starting with a positively charged layer (172,173). The thickness increase resulting from each bilayer is usually less than 1 nm, LBL self-assembly enables to control the size of a nanopore precisely. LBL coatings are frequently used for controlling the size of nanopore (174), chemistry of the surface can be tailored, or incorporated to other molecules for specific chemical detections. The composition of the layers and deposition techniques may vary (175–180). On track-etched, conically shaped PET nanopores, alternate layers of poly (styrene sulfonate) and PLL were deposited by Lepoitevin et al. (177). As shown in Figure 2.14, using track-etched pores, Zhao et al. designed and

fabricated a nanopore gated by pH variation and responsive to ion concentration variations (178).

Moreover, to functionalize thin polyurethane conical nanopores, Blundell et al. used LBL assembly (176). Here, changing the recording electrolyte's pH and ionic level allowed to control the ionic conductance by the coating across the nanopore. In this work, by incorporating an aptamer into layers of polyacrylic acid-maleic acid (PAAMA) and PEI, the authors demonstrated the ability to detect 5 pM of vascular endothelial growth factor.

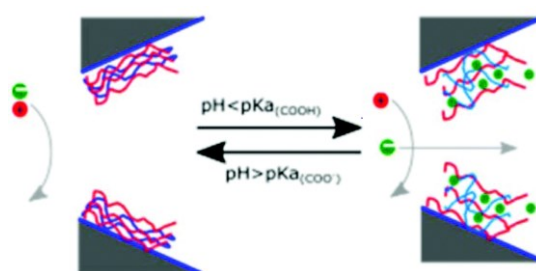


Fig. 2.14. The performance of a ChS and PEI-modified nanopores by LBL self-assembly at different pH level Figure reprinted with permission from reference (178).

2.5.2. Molecular coating approaches

It has long been established that polyelectrolytes can be deposited on flat surfaces (181). Nevertheless, the penetration of the electrolyte into the narrow nanopore with less than 10 nm size via diffusion pathway is complex. As a result, the size of the nanopore aperture, the pH, the ionic concentration, and the molecular weight of the polyelectrolyte are some of the variables that will affect whether electrostatic adsorption has successfully modified the inner surfaces of nanopores. In 2008, the interaction between the inner walls of mesoporous silica with a negative charge and Poly (allylamine hydrochloride) (PAH) and poly-allylamine with a positive charge was studied (182). Wang et al. investigated the attachment of positively charged mesoporous amino-silica with monolayer of polyacrylic acid and PAA with negatively charged(183). Both studies discovered the negative effect of molecular weight and pore size on adsorption of polyelectrolytes on inner walls, indicating either a limitation in the diffusion or adsorption ability of the polyelectrolyte. It was observed that by the salt concentration, the adsorption of PAH to the nanopore would be increased as a result of enhancing the condensation of polyelectrolytes (183). In another study by Brunsen et al., after modification of mesoporous silica film with poly-vinyl-ferrocene, no redox signal was observed as a result

of the difficulty in the diffusion of polymer solution through the nanopore and reaching the ITO as an underlying substrate (184). As a charge inversion event effect, the surface net charge of the nanopore is inverted after polyelectrolyte adsorption (would have the same charge as the adsorbed polyelectrolyte) (185). Therefore, a multilayer of polyelectrolytes could be synthesized by absorbing two polyelectrolytes with opposite charges simultaneously. Decher introduced this layer-by-layer (LBL) self-assembly processor planar membranes (185–187). Following this investigation, an alumina membrane with $R > 30$ nm (R = pore diameter) was studied to identify how positively and negatively charged dendrimers form LBL films with an approximate diameter of 7 nm. Nevertheless, the inner wall of the nanopore would be saturated after the deposition of the first layer if the diameter is 30 nm (188). Nonetheless, when the pore's size is > 30 nm, the researchers observed a film growing linearly, which increase in thickness gradually as the number of the layer increase, to a nano-restrict zone, where the adsorptions steps eventually reach equilibrium to the thickness (Figure 2.15).

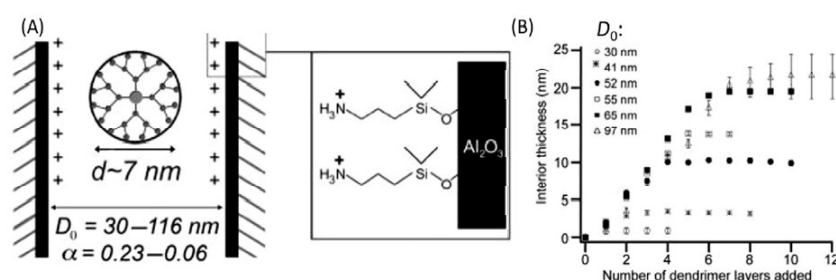


Figure. 2.15. Chemically modification of nanopore with electrostatic layer-by-layer self-assembly method. (A) An aminopropyl-dimethylethoxysilane coated aluminium oxide membrane was modified with positively and negatively charged polyelectrolyte. (B) The number of adsorbed layers affects the thickness of the film, which is highly dependent on the nanopore size Figure reprinted with permission from reference (80).

On the other hand, based on molecular dynamic analysis, preventing nanoparticles from entering the nanopore as a result of adsorbed nanoparticle's repulsive electrostatic interactions at the pore aperture leads to a limiting enhancement in the thickness of the LBL self-assembled layer (189). It was observed that linear polyelectrolytes grow in linear and saturated regimes within track-etched nanochannels, according to Jonas et al. An infiltrating polymer chain is prevented from entering the pore with a polyelectrolyte gel formed when a multilayer polymer is created in the saturated state (190). LBL studies demonstrated that as the pore size of aluminium oxide membranes is reduced, the thickness of PAH/PSS per bilayer decreases (191). Reach saturation level LBL self-assembled nanopore is associated with

eliminating the charge overcompensation effect. For instance, in a conically shaped nanopore with a tip dimension of 36 nm, with increasing layers, the magnitude of inversion of the surface charge almost stops after eight layers of polyelectrolyte (192).

Two molecular coating approaches are shown in the middle panel of figure 2.16: (a) ex-situ assembly, in which the nanopore chip is immersed in the coating solution; b) reaction between nanopore surface and organic molecule via in-situ assembly under solution flow-driven conditions. PLL-g-PEG mixture is injected into nanopore chambers, and the potential is applied through the nanopores for in-situ coating approach. The flow of electrolytes across the nanopore is induced by the electric field, immobilizing molecules by non-specific interaction. Nanopores larger than 10 nm are highly reliable for ex-situ coating; however, small pores tend to clog, possibly because silane molecules accumulate inside. Compared to ex-situ coating, in situ assembly allows them to coat nanopores as small as 5nm without clogging. Nanopores can now be used for biological sensing through in situ coatings; for instance, it is possible to control the surface hydrophobicity, charge, and chemical functionality of inorganic pores by coating them with a variety of organic materials.

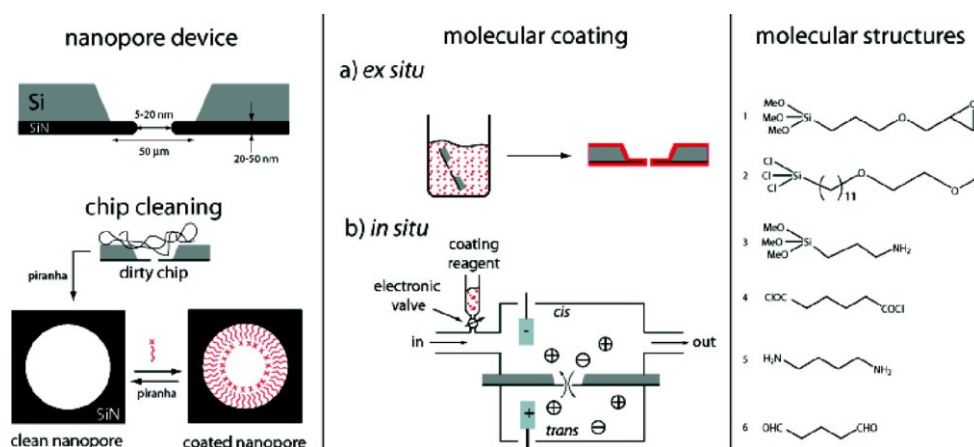


Fig. 2.16. (Left panel) A schematic picture of the nanopore device. Piranha solution is used to clean the nanopore surfaces before coating with organosilanes, as well as to "uncoat" the nanopores. (Middle panel) A depiction of two schemes for coating nanopores. In the *ex-situ* method, the activated nanopore is simply immersed in silane solution, followed by cleaning steps (not shown). In the *in-situ* method, the nanopore device is assembled in a two-chamber cell and a voltage is applied across it, driving supporting electrolyte through the pore during the silane deposition process. (Right panel) Structures of the molecules used for various coatings. Molecules **1–3** are organosilanes, while **4–6** are used in further reactions with functional silane monolayers. Figure reprinted with permission from reference (193).

In this study, we presented two techniques for PLL-g-PEG self-assembly-based in situ and ex-situ functionalization of our nanopore. Here, besides imaging techniques like FIB and FEB, which are costly and complex methods to observe the nanopore functionalizing layer morphology, paying attention on controllable approaches for functionalizing which reach nanometre levels is essential. The schematic image of our designed and fabricated PMMA nanopore flowcell, and its structure and dimension are illustrated in Figure 2.17 (A) and (C), respectively. The poly(l-lysine)-g-poly-(ethylene glycol) (PLL-g-PEG) copolymer chemical structure is represented in Figure 2.17 (D).

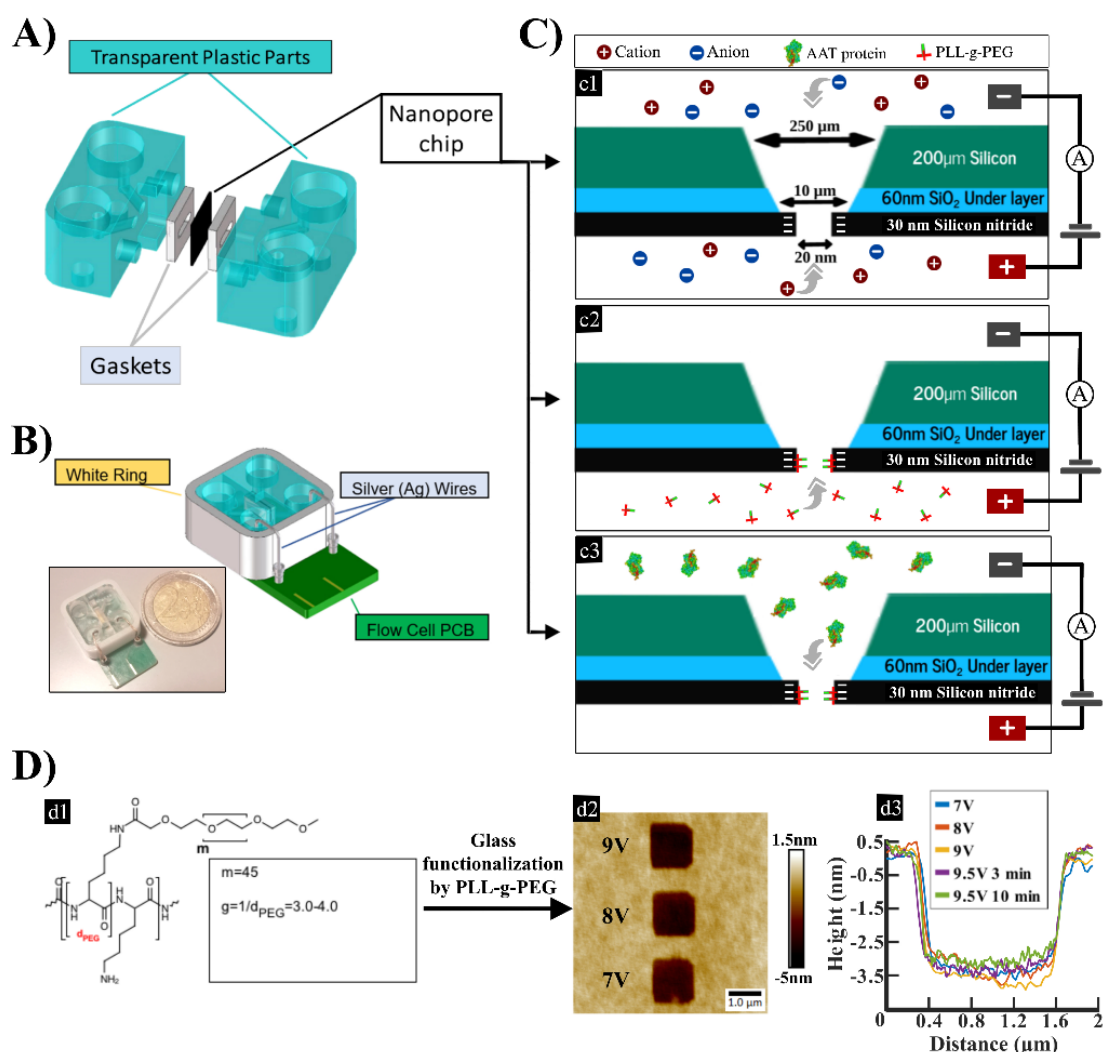


Fig. 2.17. The schematic of the nanopore flow cell pieces (A), and the assembled nanopore flow cell (B), is presented. The inset displays the real size nanopore flowcell. The nanopore fluidic setup and its structure consist of 200 μm thick Silicon, 60 nm thick SiO₂, and 30 nm thick SiN layers (C) is shown and the non-functionalized 20 nm SiN nanopore(c1), *in situ* PLL-g-PEG functionalization (c2), and protein translocation (c3) are explained. PLL-g-PEG chemical structure and thickness measurement on flat glass coverslip (D): The PLL-g-PEG chemical structure (d1) (194) AFM image lithographed area on glass functionalized with PLL-g-PEG (d2), and their profile (d3) are presented.

2.5.3. Ex situ and in-situ nanopore functionalization

Figure 2.17 (C) represents the solid-state nanopore with two windows size of 20 nm and 10×10 μm and 250×250 μm dimensions. Two polymethyl methacrylate (PMMA) compartments make up the reusable flowcell frame. Piranha solution (1:3 mixture of hydrogen peroxide to sulfuric acid) was used to clean the nanopore chips. Then, they were washed with DI water and dried at 60°C for 5 min. Two gaskets were used to sandwich the nanopore in order to prevent liquid from leaking, between two PMMA compartments. The whole system was fixed and compacted with a white ring as shown in Figure 2.17 (B). The gaskets were completely attached to the nanopore chip and were well insulated from one another. As a result, there was no current leakage between the two compartments since no buffer solution leakage was determined. Silver wires were used to fabricate the pseudo-reference electrode by soaking them in the NaOCl : water solution in a ratio of 1 : 1 for 2 min for chlorination and then dried at room temperature. Following each experiment, nanopore chips were immersed for five minutes in water, ethanol, and chloroform, alternatively. Unused chips were stored in chloroform. 0.01 mg/mL of PLL-g-PEG solution was prepared by diluting in 1×PBS and kept at 5 °C. The stimulus voltage of 800 mV was applied to functionalize the nanopore during 37 μL injection of PLL-g-PEG solution to the chamber filled with 1×PBS placed in tip side of the nanopore at pH 7.4 which presented in Figure 2.17 (c2). A chamber oriented on a base and tip side of the nanopore contained pure 1×PBS and PLL-g-PEG solution, respectively. During transferring the PLL-g-PEG chains through the nanopore, in situ functionalization was performed by electrostatically interaction between SiN nanopore sidewall and PLL-g-PEG. Recombinant Human Serpin A1/ α -1-Antitrypsin proteins were reconstituted at 100 $\mu\text{g}/\text{mL}$ in sterile 50 mM Tris, 10 mM CaCl_2 , and 150 mM NaCl at pH 7.5. After diluting to 1 $\mu\text{g}/\text{mL}$, the protein solution was maintained at -20°C for further use.

In summary, by functionalizing 20 nm pore via in-situ, the nanopore coating thickness, which was estimated by AFM on glass coverslip functionalized by PLL-g-PEG, is closely correlated with the ion-current through it. The coating kinetics were monitored in real time using in situ measurements. We introduce an in situ monitorable technique to coat the nanopore in a reproducible manner as small as 20 nm, and the results demonstrate quick reaction kinetics, surface modification, and non-sticky protein translocation behaviour.

2.5.4. Nanopore lifetime characterization

To characterize and study the effect of storage process on nanopore, the PLL-g-PEG functionalization, PLL-g-PEG functionalized and non-functionalized 20nm SiN nanopore were immersed in 0.1×PBS for 6 hours, followed by washing 5 times with DI water and drying by nitrogen gas before and after I-V measurements to remove any PBS or other contaminants and dried by nitrogen gas between each storage process. In order to prevent buffer evaporation during storage, the entire chamber was sealed with parafilm. The storage process was repeated for 10 times (each storage time 6 hours) in 0.1×PBS.

2.6. Measurement device, software and analysis methods

Several aspects must be considered when using the RPS technique for single particle analysis. These include non-tacky translocation of the particle through the micro or nanopore, discrimination of the translocation peak from noise, and counting of translocation spikes as an indicator of the estimated concentration of the target. Therefore, the selection of an appropriate current measurement device with low current noise and the choice of an appropriate bandwidth are also crucial for the study of translocation with true amplitude. Increasing the sampling rate can amplify the noise, which may result in the translocation peak being indistinguishable from the background noise. Conversely, decreasing the sampling rate may result in particles that translocate too quickly not being recorded or the true value of the blockade current for each particle translocation not being observed.

In both projects including polyimide micropore and SiN nanopore, all experiments were done by using Elements nanopore reader (EDR) device and its software which can be downloaded from https://elements-ic.com/edr_setup to record and analyse the ionic current signals. By applying 400 mV through chlorinated wires to the polyimide micropore Cis chamber, the ionic current was measured and recorded by EDR. Elements SRL's nanopore reader collected the data using a 50 kHz and a 5 kHz low-pass filter by using EDR software. SiN nanopore experiments were conducted by the Elements SRL nanopore reader using 100 kHz EDR sampling rate. Ground and working electrodes were connected to the bottom and top sides of the chamber, respectively. For running the nanopore experiment, the electrodes of our nanopore flowcell with 1.6 x 1.6 x 0.8 cm dimension were connected to EDR terminals through a printed circuit board (PCB) as shown in Figure 2.17 (B).

2.6.1. Elements SRL's nanopore reader

Nanopore currents are usually measured with a two-electrode system. As it is capable of applying voltage and measuring very low currents, such as picoamps and nanoamps, the noise level of the current measurement device affects the quality of the signals. The Elements Nanopore Reader (eNPR) device from Elements SRL (Italy) was used during my doctoral projects because it is able to apply voltages in the range ± 2 V and measure current from 1 pA to 200 nA, as well as having a low current noise level, soft signal analysis program, and its small in size.

Here is the eNPR characteristics:

- Open input (RMS) noise (Voltage range ± 2000 mV) : 0.08 pA rms @ 1 kHz; 0.42 pA rms @ 10 kHz; 3.7 pA rms @ 100 kHz
- Current ranges: ± 200 pA (Gain 2.25 G Ω), ± 2 nA (Gain 225 M Ω), ± 20 nA (Gain 22.5 M Ω), ± 200 nA (Gain 2.25 M Ω)
- Voltage hold ranges: ± 2000 mV
- Parametric voltage protocols Max sampling rate: 200 ksps Selectable x4
- Oversampling (max final sampling rate 800 ksps)
- Available bandwidth between 62.5 Hz to 100 kHz
- Auto electrodes voltage offset fine compensation.
- Continuous Capacitance and Resistance estimation
- USB powered
- Size & Weight: 101 x 44 x 18 mm, 140 g

Devices can directly connect to eNPR using USB cables to create a complete, stand-alone system. USB cables can power devices in addition to being used for data transmission. The lid that covers the nanopore flowcell serves as a miniature Faraday cage that is built into the device. By connecting them to the GND plug with the GND cable, more external Faraday cages can be added.

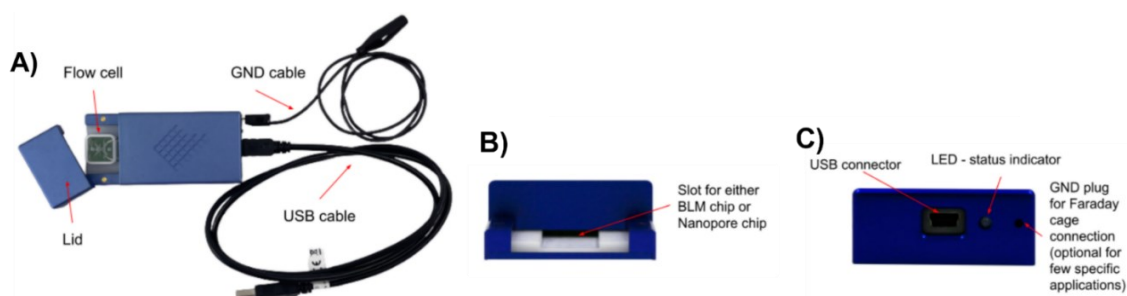


Fig. 2.18. Element SRL's nanopore device (A), eNPR front-side connections (B), eNPR back-side connections (C).

2.6.2. Analysis method

Elements Data Analyzer (EDA) software offers two different ways to evaluate a translocation event: current step detection and current event detection. By setting the amplitudes and diversion steps, the EDA programme provides uphill and downhill steps with positive and negative signs vs. step's time durations in the current step detection technique. For signals comprising events with lengthy dwell durations, this technique was applied. The capabilities of this method are demonstrated in Figure 2.19a by the graphical representation of the detected stages (green line) and their associated parameters. The software uses the current step detection method to construct a histogram of the amplitude and duration of the current steps, and a report is made based on the number of different current values to determine the average current step duration and the average current step amplitude. By assuming two thresholds for the ion current amplitude, the current event detection approach, as illustrated in figure 2.19b, may identify short events. When the current passes the first threshold, current events begin, and when the current passes the second threshold, current events terminate. Any dwell time occurrences that fell outside of a specific range were disregarded.

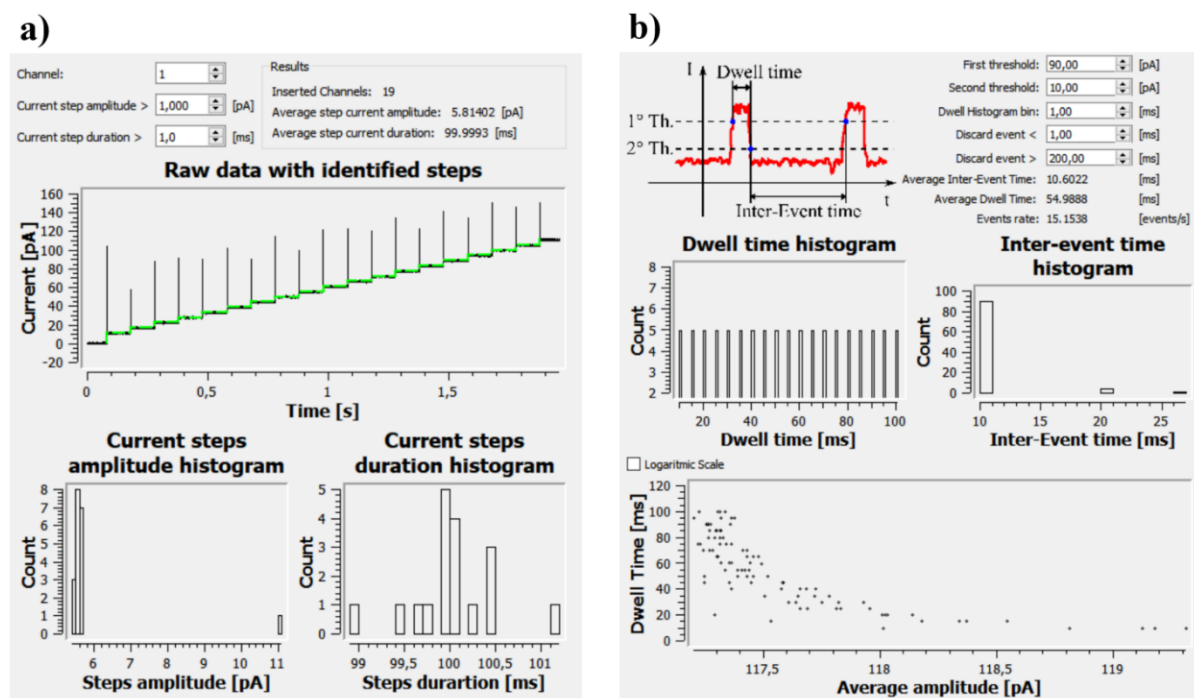


Fig. 2.19. the graphical illustration of the identified steps (green line) and their related parameters (A), and short events detected by current event detection (B).

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3. Result and discussion

3.1. Mass production of polyimide micropore-based flowcell

A translocation event signal is generated when nanosized particles or biomolecules pass through the nanopore. Following the remarkable development in particle size detection with nanopore, considerable effort has been invested towards improving the sensing capability of solid-state nanopores with the aim of achieving high standard level of repeatability and accuracy equivalent to biological pores, since they are robust and stable under stress conditions as well as their ability to be integrated into embedded electronics. Resistive Pulse Sensing (RPS) is a powerful technique used to detect and analyse the particle size in liquids. By using RPS for particle detection, upon applying voltage between two chambers, a funnel-shaped electric field is generated (1). As an example, negatively charged polystyrene (PS) nanoparticles are forced to translocate through the chamber by applying the negative voltage at physiological pH buffer solutions (2,3). The translocation time of nanoparticles can also be used to determine their electrokinetic acceleration, which can be estimated by measuring the voltage and length of the nanopores. Generally, particles with higher surface charges translocate more rapidly, so the slope of the translocation time versus voltage is called the particle's zeta potential (4–7).

The fabrication of solid-state nanopores often involves TEM or vacuum-drilling the membrane, although both methods are difficult and expensive (8). During these laborious, protracted processes, it requires extensive knowledge and specialised equipment to detect single molecules. It has become increasingly popular to use novel materials, particularly polymeric membranes like polyethylene terephthalate (PET) or polyimide film (PI), to streamline the production process by using etching to reduce the need for semiconductor processing cleanroom nanofabrication (9–11). Due to its superior mechanical and electrical properties, thermal stability, chemical resistance, high resistance, and breakdown voltage, PI performs better than PET (12,13). PI shows a promising potential for lowering device capacitance-induced current noise because of its insulating qualities. We proposed a novel,

sensitive, portable, cost-effective and fast production method of micropore flowcell to detect nanoparticles in liquids in order to get over these limitations. It can be utilised in large-scale production because it is easy and affordable. We achieved the first one-step drilling of a micropore in a PI substrate by exposing a 290nm wavelength UV laser with 290 fs pulse and 1.5 J/cm² power. In sections 3.1.1 and 3.1.2, the fabricated micropore was analysed and discussed. Also, its conductance and application in detecting nano-sized particles in fluids was investigated and explained in sections 3.1.3 and 3.1.4.

3.1.1. PI micropores characterization

Drilling the PI film with a femtosecond UV laser produces micro conical shape pores that are highly accurate in size and shape. Due to the resistance of the pore being placed at the tip of the micropore, the asymmetric pore's effective length is shorter. Also, conical micropores exhibit a greater current level at the same voltage than cylindrical ones due to their smaller resistance (14). The signal produced by the ion current can provide valuable data about structural characteristics, the translocation events, and the chemical characteristics of the sensed molecules. A reduction in signal amplitude occurs when large molecules translocate through the micropore. Current signals should remain stable regardless of whether the molecule is dissolved in a buffer. Polymeric micropores also exhibit a fluctuating current behaviour. Although, when a constant potential is biased to PET conical pores, the current fluctuates to 100% of the signal (15–18). Conversely, the electrical currents in PI nanopores are extremely stable over a wide range of electrolytes and pH values. The purpose of this section is to describe a single conical shape micropore in a PI substrate, which is fabricated by femtosecond laser drilling. In fact, the long-term translocation seems to be caused by the large aspect ratio, as it effectively provides a higher temporal resolution. We fabricated and investigated a series of conical shape PI micropores. A material used for the substrate and laser operating parameters can affect drill hole smoothness and accuracy. Figure 3.1a shows a drilled micropore that its tip and base opening holes are 1 μm and 9.5 μm , respectively, and the cross-section of following steps are illustrated in Figure 3.1b, which taken by Focused Ion Beam (FIB). Figures 3.1c, d, and e represent the three final single micropore images. Despite this, there is a difference around 1 to 2 μm in diameters of the narrow part of the micropores' tips. Furthermore, the thicknesses of the PI were ranged between 13 and 14 μm . It was found that some micropores were contaminated with melted PI residue that remained inside the

hole following laser drilling as shown in Figure 3.1d. Due to melted PI, the micropore was closed completely or partially, preventing particles from translocating.

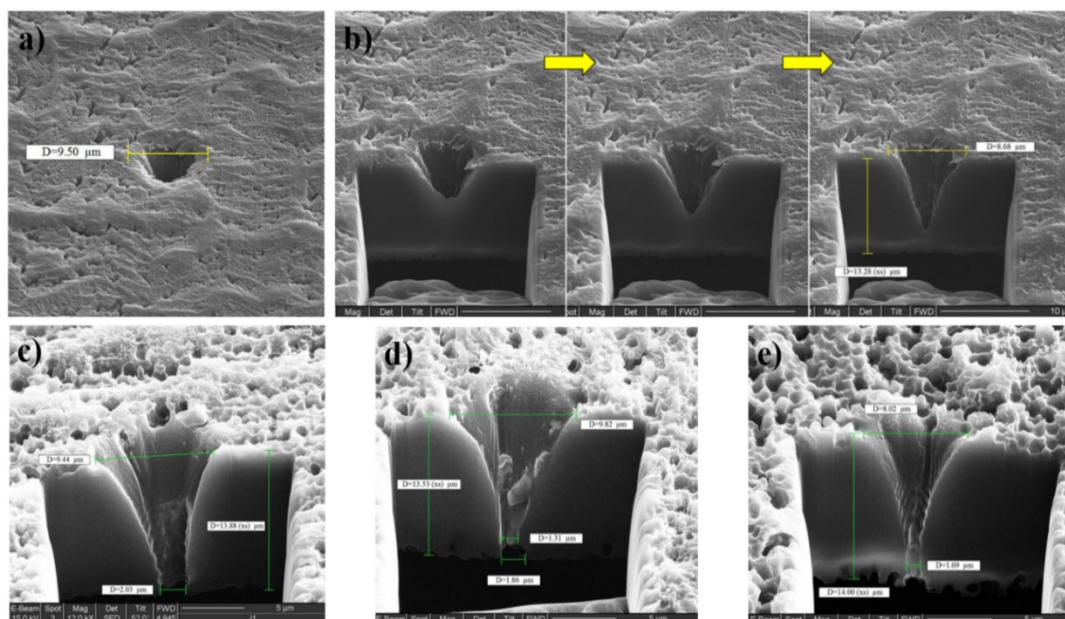


Fig. 3.1. (a) Images of femtosecond laser-drilled micropores in PI films. (b) The cross-section of the hole recorded by FIB (following steps) (c, d, e) cross-sections of different individual micropores recorded by FIB.

3.1.2. Silver electrode chlorination characterization

Silver wires were used to fabricate the quasi-reference electrodes by chlorination, after affixing them on PI membrane. As illustrate in Figure 3.2, upon contacting the PI membrane with NaOCl to chlorinate the Ag wires, conductance, and hydrophilicity of the PI membrane were altered as well. By optimising the chlorination of the mounted silver electrodes on the PI membrane, in addition to the chlorination of the silver wires, the size of the micropores did not change and the wettability of the micropores increased by 50% after 2.5 minutes immersion in NaOCl.

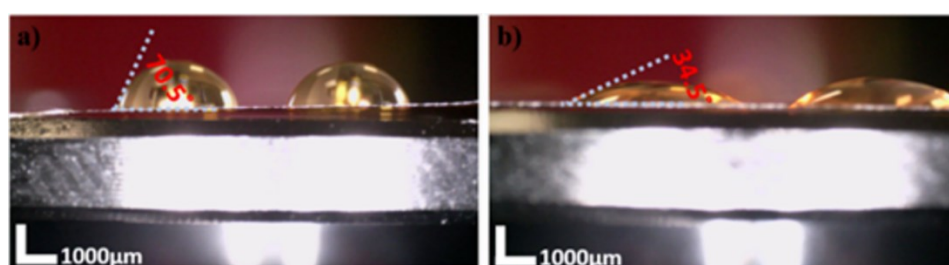


Fig. 3.2. Measuring the contact angle of bare PI membranes (a) and NaOCl treated for 2.5 minutes (b). The scale bar marks 1000 μm .

Moreover, the I-V curve (at pH 7.4 and 1×PBS) was affected as well after electrode chlorination in 50% NaOCl for 2.5 and 5 min, which is represented in Figure 3.3a. Furthermore, it is obvious from Figure 3.3a, that micropore diameters increased with longer chlorination time.

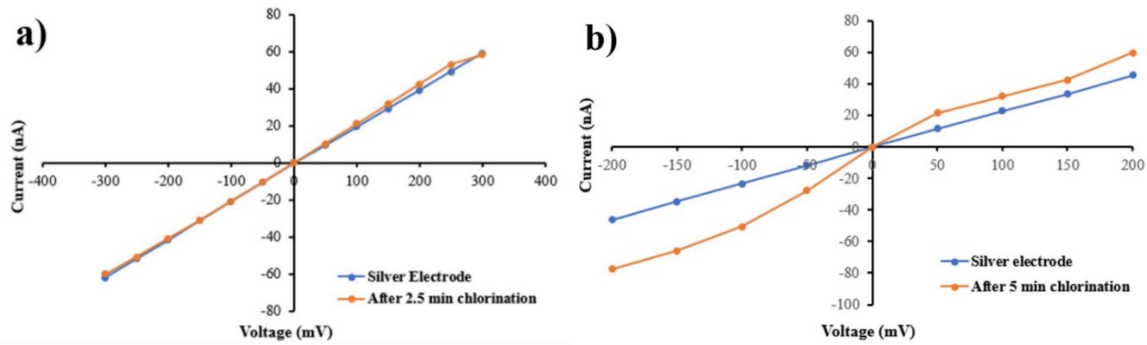


Fig. 3.3. I-V curves for PI micropore after 2.5min (a) and 5min (b) chlorination by NaOCl in 1×PBS and buffered at pH 7.4.

3.1.3. Micropore conductance characterization

The different conductance of the micropore depends on its diameter and shape. An illustration of the conductance distribution of 25 micropores drilled by a femtosecond laser measured in 1×PBS is represented in Figure 3.4. There were micropores with diameters ranging from 1000 to 2200 nm and openings from 4 to 7 μm . The misplaced PI substrates at the laser focal point led to an impact of the laser distance and defocusing distance on the geometrical characteristics of the micropores. So, about 1 μS of conductance corresponds to pores measuring about 2 μm in size and 710 nS corresponds to pores measuring about 1 μm in size. According to figure 3.4, micropores with conductance from 0 to 0.1 nS, which account for about a quarter of the total lasered nanopore chips, were either not perforated at all, or the hole was very small because of contaminations and polyimide deformation.

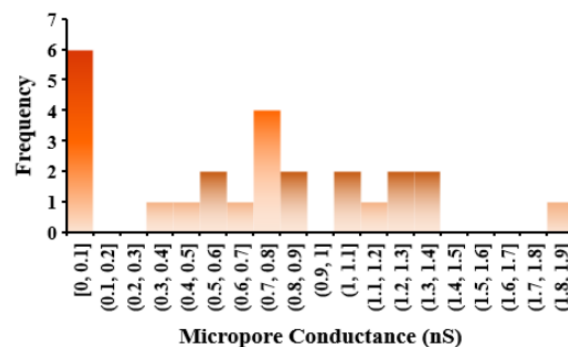


Fig. 3.4. Conductance distribution of 25 micropores drilled by a femtosecond laser.

3.1.4. Particle translocation

In the absence of particles, I_0 is the baseline current of the micropore and ΔI represents the change in current caused by passing nanoparticles as shown in equation 3.1. Shape factor (Υ) of sphere particle has a Υ of 1.5. The volume of a particle affects the translocation of particles the normalized value of the resistive pulse can present as $\Delta I/I_0$.

$$\frac{\Delta I}{I_0} = \frac{4\Upsilon\Lambda}{\pi d_p^2 (I_p + \frac{\pi}{4} d_p)} \quad (3.1)$$

The diameter of the micropore should be large enough to minimize interaction between the nanoparticles and the nanopore sidewalls and clogging, and small enough to allow sufficient space for detection of the smallest targets and particles of interest. PI film with thickness of 12.2 μm , the d_p as 1 μm , and I_{eff} around 12 μm were considered. As a result, the $0.0007 \times I_0$ and $0.0295 \times I_0$ current blockade values were calculated for translocation of 100 and 300 nm PS particle, respectively. Here, in microwell containing 1 $\times$ PBS, an electric field with power of 400 mV was applied across the micropore, at pH 7.4, which is generating a 100 nA baseline current. Then, for a detection purpose, 2×10^9 NPs/mL PS was injected to the cis chamber of flowcell (tip side of the micropore). Figure 3.5 related to the several translocation of 300 nm PS nanoparticles at ± 400 mV stimulus voltage, which can show by the switching the voltage to negative the particles were translocated back to Cis the chambers. As shown in figure 3.5 each single spike is related to the passage of one PS nanoparticle with the current blockade amplitude levels as low as 1% of the baseline (~ 1 nA). The scatter plot in figure 3.5b shows the amplitude of PS nanoparticles translocation versus dwell time. It is clear that the PS translocation from the trans (base side of micropore) to the Cis chamber (tip side of micropore) takes about six times longer than its translocation from the Cis to the trans chamber. Also, the amplitude of translocation peaks from the micropore tip side is twice that of translocation peaks from the base side.

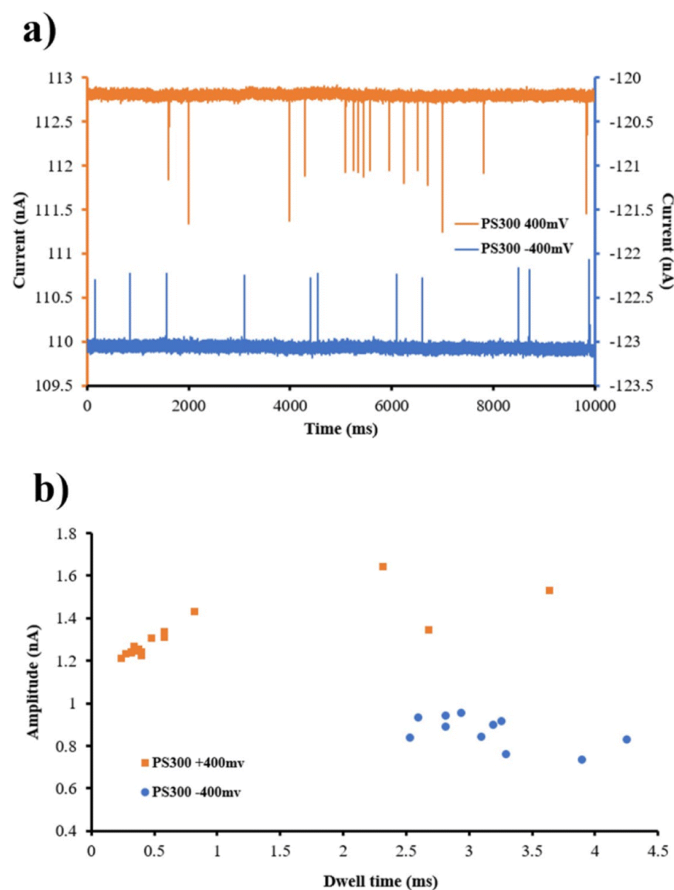


Fig. 3.5. 300nm PS nanoparticle translocations through a PI micropore (a) and Dwell time vs. conductance blockade scatter plot for 300nm PS nanoparticles (b) in +400 mV and -400 mV stimulus voltages.

PI pores are capable of producing stable current signals sampleable at 50 kHz and low pass filtered of 5 kHz. By measuring the amplitude and time of the translocation event at a high sampling rate, we were able to compare the translocation event of different PS sizes. Figure 3.6a shows a continuous 10-second recording of the translocations of PS nanoparticles with different sizes (100, 300, and 350 nm) at a stimulus voltage of 400 mV, and the scatter plot is shown in Figure 3.6b. The number of transactions has increased at the same voltage by increasing the size of the PS particle, but the size of the PS nanoparticle did not significantly affect dwell translocation events. Compared to 100 nm dimensions, 350 nm diameter PS nanoparticles had a shorter dwell time. However, PS nanoparticles whose size increased from 100 to 300 nm experienced an increase in dwell time. For particles with diameter 100 and 300 nm, the current blockade is about 0.1 nA and 2.2 nA, respectively. There is a good match between these values and the estimated current blockade ones (0.07 and 3 nA ($I_0=110$ nA)) for PS particles with 100 and 300 nm sizes, respectively. Several factors that lead to a small

difference between the estimated ΔI value and experimental one are a) shape of the micropore which is conical on this work, b) non uniform thickness and roughness of PI membrane, c) particle aggregation or its stickiness to the sidewall of nanopore, and d) PS particle size distribution.

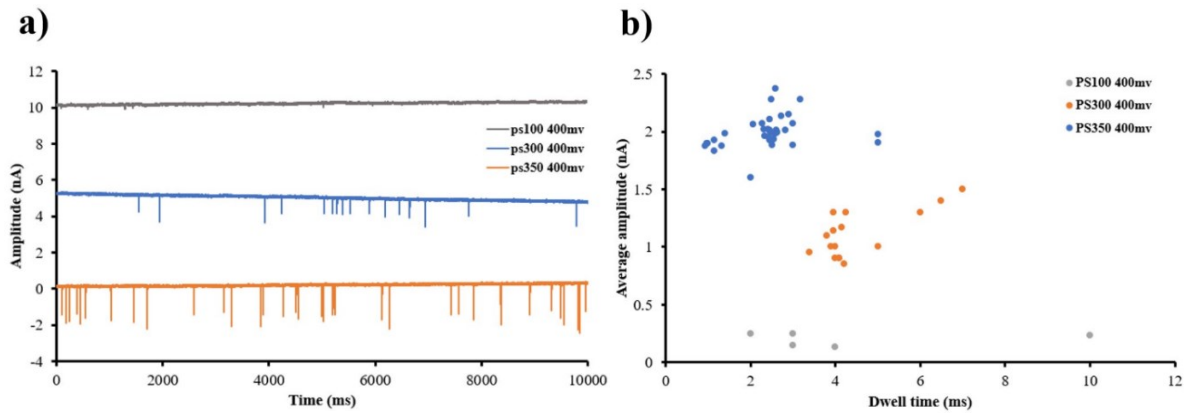


Fig. 3.6. Various PS nanoparticle sizes translocations through a PI hole (a). Dwell time vs. conductance blockade scatter plot of 100, 300, and 350 nm PS nanoparticles (b).

As shown in Figure 3.7, the micropores are viewed from a top-view before and after 100 nm PS translocation. According to Figure 3.7b, it is apparent that agglomerated particles are adhered to the micropore sidewalls as a result of sedimentation and drying.

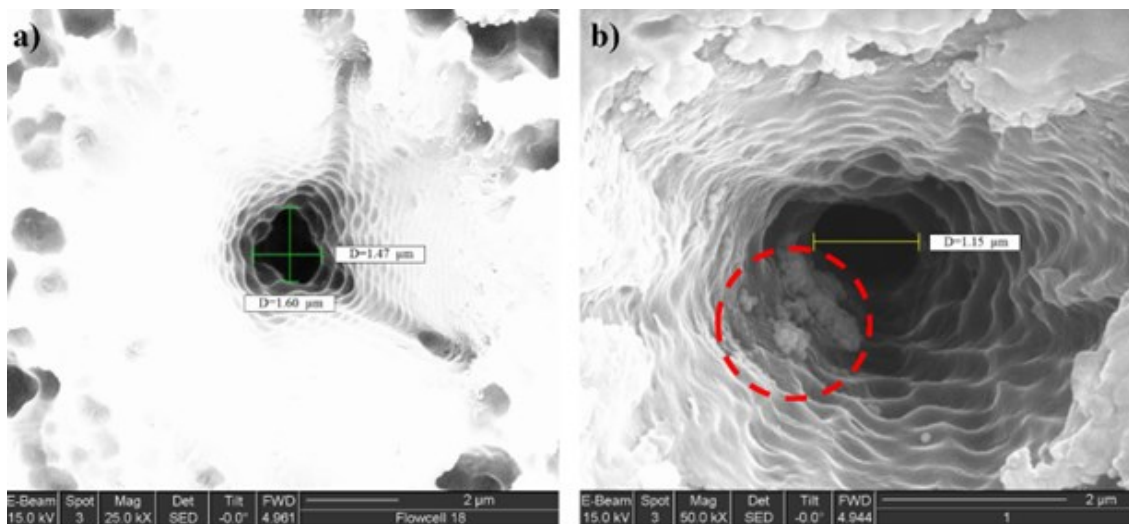


Fig. 3.7. Focused Ion Beam images of the sidewall of micropore (a) before translocation and (b) after 100 nm PS particle translocations. The 100 nm PS particles stuck to the micropore sidewall after translocation are highlighted in a red circle.

In this project we fabricated of a novel laser drilled micropore flow cell and we proposed the resistive pulse sensor (RPS) based method to detect nano-sized particles in liquids. The diameter of 25 micropores drilled into the high breakdown voltage and low-cost polyimide

membrane were analyzed by using a femtosecond laser. Pore size was estimated by calculating micropore conductance by comparing baseline current to applied voltage, indicating that most pores were successfully drilled. In addition, a micropore with a diameter of 1 μm and 2 μm had a conductance of 710 nS and 1 μS , respectively. Various conical micropore sizes with a micropore tip of one micro meter to 2.2 μm and a micropore base of 4 to 7 μm in 1 $\times$ PBS were examined. It was found that 24% of the micropores were not perforated by laser drilling. The exhibited portable and low-cost micropore flow cells were able to detect and discriminate polystyrene nanoparticles of 100, 300, and 350 nm in PBS buffer, recording the corresponding current blockages of 0.1, 1, and 2 nA, respectively. By using aptamer as detection probe, we believe our platform could detect viruses up to 150 nm in size by functionalizing nanoparticles with complementary receptors. After binding the target to our functionalized nanoparticles, the size and volume of the whole particle/aptamer/target could be increase. This may indicates the presence of the target in the liquid sample by RPS analysis. The micropore flowcell was designed and optimized for detecting nano-sized particles in the range of 100 nm to 350 nm in fluids. Considering micropores are unable to detect smaller biomolecules, such as proteins, PLL-g- PEG functionalized SiN nanopores were used for protein detection in the next chapter.

3.2. In-situ PLL-g-PEG functionalized nanopore and its application in protein detection

By applying the voltage across the nanopore, the ionic current can be monitored. An alteration in ionic current level during passage of the biomolecule through the nanopore, can provide the valuable information about the shape, size, and charge of transferred molecule (19,20). One of the most interesting applications of RPS technology is the characterization of biomolecules, particularly the diagnosis of disease by measuring protein content. Currently, massive attempts have been done on protein detection by solid state nanopore because of its integration feasibility into electronic systems and outstanding durability in challenging conditions like electric field, temperature, and pH (21). Meanwhile, prior studies bothered from some issues such as nanopore reversible clogging caused by biomolecules adhering to the nanopore sidewalls (22–25). In order to overcome this issue, solid-state nanopore functionalization by metals (26) and oxides (27,28) deposition is a promising approach (29–31) to eliminate the biomolecule stickiness to the sidewall of the nanopore. As explained in chapter 2, there are several methods and chemicals that can be used to functionalize the nanopore side wall. In section 3.2.1 and 3.2.2 the results from our functionalized PLL-g-PEG nanopore via in-situ technique are investigated. Also, in-situ poly(L-lysine)-g-poly-(ethylene glycol) (PLL-g-PEG) functionalized nanopore application in α 1-antitrypsin (AAT) protein translocation is discussed as well. Finally, protein properties such as size and volume are calculated from the translocation event's data and their results are compared with the previous studies and theoretical information. In addition, Atomic Force Microscope (AFM) was used to study the functionalized PLL-g-PEG flat glass coverslips uniformity and thickness.

3.2.1. Functionalized nanopore morphology characterization

To indicate the thickness of PLL-g-PEG layer, the AFM imaging and lithography were used. The PLL-g-PEG was scraped away from 1 μm^2 glass coverslip by increasing the voltage and time which was applied to the AFM tip until a saturated depth was reached. The thickness of PLL-g-PEG when it was attached on the flat surface was measured around 3.5 nm which is illustrated in Figure 3.8.

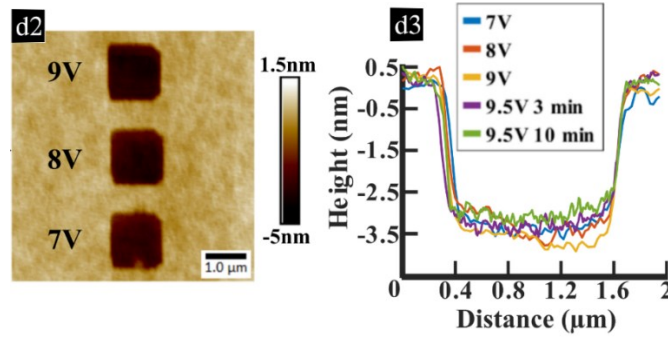


Fig. 3.8. AFM image lithographed area on glass functionalized with PLL-g-PEG (d2), and their profile (d3) are presented.

The PLL-g-PEG has a thickness of 3.5 nm on flat surface, which implies a reduction of 7 nm in nanopore diameter. By injecting PLL-g-PEG solution into the nanopore chamber, we expect that applied voltage leads to flow the PLL-g-PEG chains through the nanopore which causes more uniformly and thinner PLL-g-PEG functionalization than ex-situ method.

3.2.2. Conductance characterization

After using mixture of NaOCl solution and deionized water in a ratio of 1 : 1 to chlorinate the reference electrode for 2 min, the conductance, PLL-g-PEG functionalization, and protein translocation were examined by nanopore wetting step. We used piranha solution to clean the surface and mixture of H₂O₂ and IPA solution in a ratio of 1 : 1 for 15 minutes to improve the wettability of the inner wall of the nanopores.

Here we were able to estimate the size of the nanopore by analyzing the conductivity of the nanopore in different buffer concentrations. To calculate the conductance of the nanopore in the cylindrical model, the following equation can be used (32,33).

$$G = s \left(\frac{4L}{\pi d^2} + \frac{1}{d} \right)^{-1} \quad (3.2)$$

$$d = \frac{G}{2s} \left(1 + \sqrt{1 + \frac{16sL}{\pi G}} \right) \quad (3.3)$$

Where S and L represent the conductivity of the solution and the effective length of the pore or thickness of the membrane, respectively. The conductance of the 20nm cylindrical nanopore with 30 nm membrane thickness in 1×PBS was measured 9.8 nS. So, by applying the calculated conductance in equation 3.3, the estimated nanopore diameter would be 18.7 nm, which is close to the actual diameter.

According to the data depicted in Figure 3.9A, at low concentrations of PBS, the I-V curve displays non-linear characteristics. With different polarities at the same voltage, Ionic Current Rectification (ICR) can be seen in different level of current. For different application of nanopore, theoretical understanding of this nonlinear processes is essential (34). In summary, on the tip of the nanopore, positively charged ions form, followed by a group of counter-charged ions: this is known as the electric double layer (EDL). Therefore, the EDL will not overlap, when Debye length is small (λ_D). As a result, ion migration is not affected. Consequently, the ions are driven at the nanopore's core by the applied potential. In contrast, the ionic current decrease due to increasing the (λ_D), and consequently, resistive forces at the tip of nanopore. Moreover, better ionic permeation coefficient and developed sensitivity are the benefits of short nanopore in compared to the long ones. Generally, the ratio of ICR refers to the forward versus backward ionic currents under equivalent voltage bias conditions. Recent research indicates that the most ICR can be observed in nanopore with more than 500 nm length, but it is rarely possible to occur in nanopore with ≤ 200 nm length (33). For example, the ICR was monitored in 30 nm- long pyramidal silicon nanopore with a ratio less than 2, however it could not be observed in pyramidal silicon nanopore with 55 nm long even at different concentration of KCl (1-1000 mM). Furthermore, the ICR ratio about 2 was seen in 200 nm long conical nanopore (35). The λ_D for the 0.125×PBS and 1×PBS Phosphate-buffered saline (PBS) concentrations are 2.2 nm and 0.7 nm, respectively (36,37). By decreasing the PBS salt concentration the λ_D increased and the I-V curves with non-asymmetric shape were obtained (38). As shown in Figure 3.9A, at 0.125×PBS concentration, the ICR ratio of 1.25 was clearly obtained. Here, the low value of ICR ratio is caused by the short nanopore (30 nm) with large 20 nm diameter. The conductance of the nanopore in 1×PBS, 0.5×PBS, 0.25×PBS, 0.125×PBS were demonstrated in Figure 3.9B theoretically and experimentally, by considering the 1×PBS solution conductivity as 1.6 S/m. Thus, equations 3.2 and 3.3 accurately describe nanopore conductance trend (dash line in figure 3.9B). According to figure 3.9C, increasing stimulus voltage from 200 to 600 mV increased PSD noise of non-functionalized 20 nm SiN nanopores, especially at frequencies less than 10 kHz. A PSD noise vs frequency curve is shown in figure 3.9D for 600 and -600 mV. The PSD noise level and the RMS noise for 600 and -600 mV (0.14 and 0.5 nA, respectively) were affected by the polarity of the stimulus voltage on the nanopore base side. Possibly, these differences can be attributed to the nanopore's conical shape and structure.

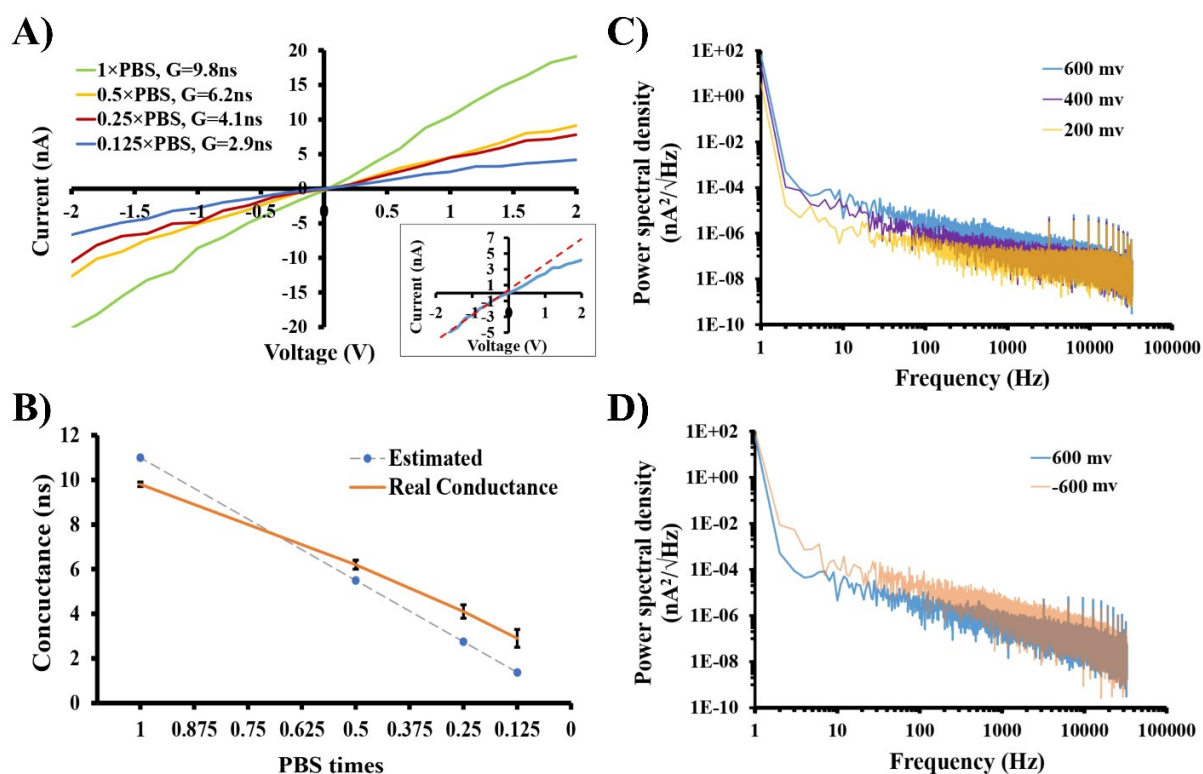


Fig. 3.9. I-V curves of 20 nm SiN nanopore in 0.125×PBS, 0.25×PBS, 0.5×PBS, and 1×PBS at physiological pH (A), The inset displays the ion current rectification in 0.125×PBS. Comparison of estimated (dashed line) and measured (orange line) nanopore conductance at different PBS buffer concentrations (B). PBS time of 1 indicate the PBS without dilutions, and 0.125 means eight time dilutions. PSD noise level of 20nm pore at 200, 400, 600 mV in 1×PBS at pH 7.4 (C). PSD noise level for 600 and -600 mV stimulus voltage applied to the nanopore base side (D).

3.2.3. Real-time nanopore functionalization

Our PLL-g-PEG functionalization was used to prevent the proteins from adhering to nanopore sidewalls. By real-time in-situ method, PLL-g-PEG was functionalized on tip side of the 20 nm SiN nanopore under well-controlled conditions, which is demonstrated in Figure 3.10A. This technique consists of following steps:

1. Injection of the PLL-g-PEG solution by opening the Faraday cage (O).
2. 3 times injection of the PLL-g-PEG solution in order to remove the prior buffer following by closing the Faraday cage (C).
3. Diffusion of PLL-g-PEG chains.
4. Functionalization of the nanopore sidewalls.

Herein, the surface of the negatively charged SiN nanopore adsorbed the positively charge (39,40) polyelectrolyte backbone (PLL) chains by electrostatic interactions (41–44). According to Figure 3.10A, after the first injection of the PLL-g-PEG, about 15% drop in the current level

was observed, which could be due to the different conductivity of the solution in cis and trans chambers. After 50-70s, when the ion current experienced a 31.8% reduction in its level (3.5 nA), the ionic current became stable following completion of PLL-g-PEG functionalization. To study the conductance of our 20 nm solid-state SiN nanopore chip in 1×PBS before and after PLL-g-PEG functionalization via in-situ and ex-situ approaches, the I-V curves were recorded (Figure 3.10B). Ionic currents through nanopores are rectified by the surface charge on the sidewalls of the pores (45). A number of methods have been reported to induce ICR, including concentration gradients (46) and viscosity in nanopores (47,48), shape asymmetry, such as conical nanopores (49), and surface geometry, including asymmetric charge densities (50). Salt concentrations and buffer viscosities were exactly the same in both chambers in our experiments. The ion current rectification ratio of 1.7 is a result of an alteration in the size, shape, and charge of the nanopores. Due to the asymmetric functionalization of PLL-g-PEG layers, in addition to a change in nanopore size, an asymmetric charge and nanopore shape was produced and led to the ICR phenomenon. Additionally, more than 80.7% reduction in the nanopore conductance is obvious after ex-situ coating, which is due to the several layers of PLL-g-PEG functionalization with a thickness of ~11 nm on nanopore sidewalls as shows Figure 3.10B. There was no repeatability or controllability in this method. By considering the AFM data related to the 3.5 nm thickness of PLL-g-PEG layer, a maximum conductance reduction of 55.1% (from 9.8 nS to 4.4 nS) is expected in 18.7 nm nanopore. On the other hand, the slope of the experimental fitting line, showed a 37.7% decrease in nanopore's conductance ($\Delta G=3.7$ nS) due to the 2.3 nm functionalized PLL-G-PEG on sidewalls of nanopore. Therefore, the diameter of 18.7 nm SiN nanopores after functionalization with PLL-g-PEG is estimated to be 14.1 nm.

To overcome adhesion of proteins to the sidewalls of nanopores, we have developed an in situ approach to modify the inner sidewall with PLL-g- PEG that could potentially solve this problem. In addition to reducing protein attachment to the inner sidewall of the nanopore, our in situ approach using PLL-g- PEG could also increase the lifetime and storage capacity of the nanopore.

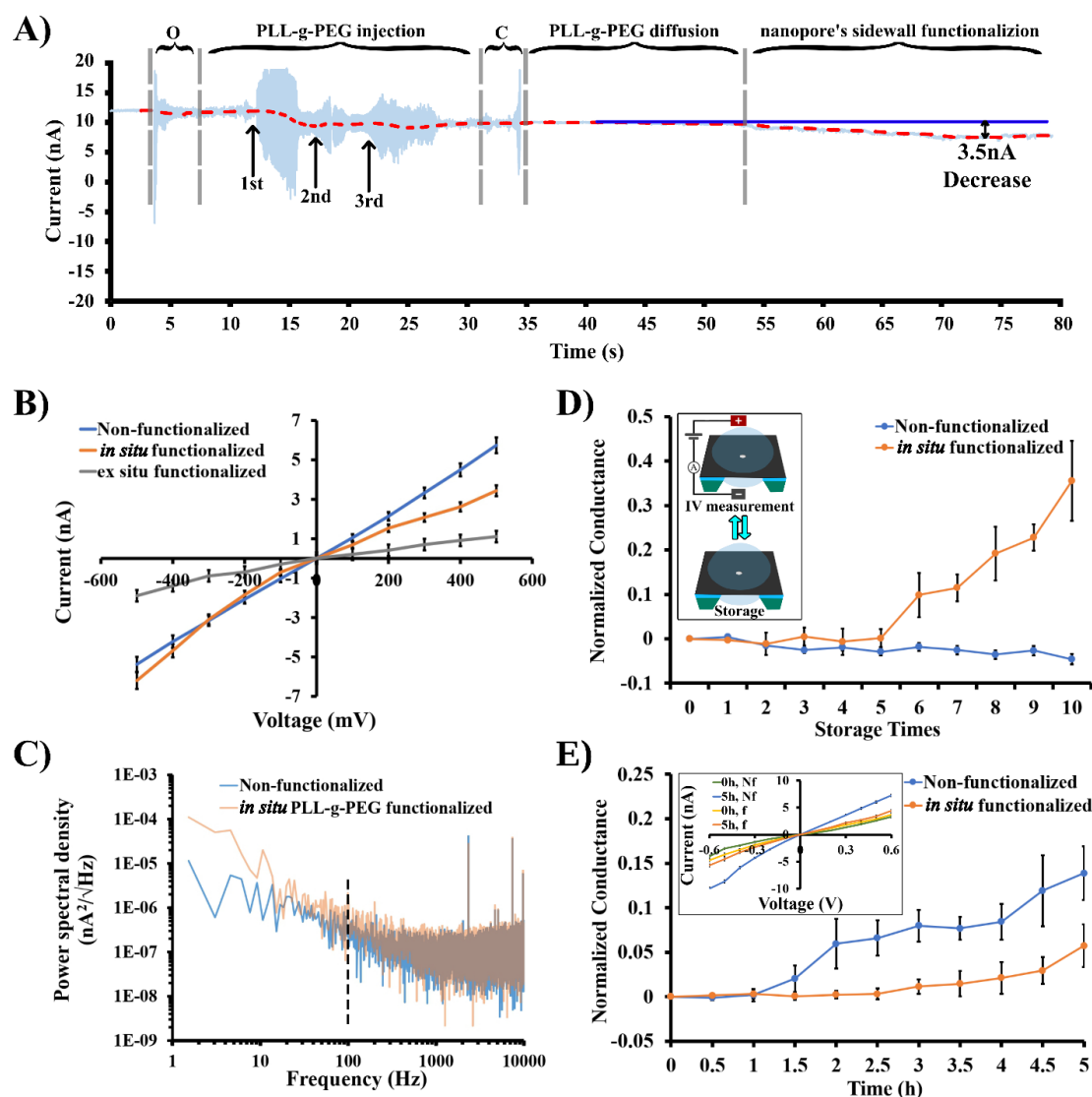


Fig. 3.10. Real-time ionic current trace during PLL-g-PEG functionalizing 20 nm SiN nanopore at 800 mV (A): open Faraday cage (o), 3 times PLL-g-PEG injection, close cage (c), PLL-g-PEG diffusion, and nanopore's sidewall functionalization. (B) The I-V curves of in-situ, ex-situ, and non-functionalized nanopore. (C) PSD noise of non-functionalized (Blue) and PLL-g-PEG functionalized (Orange), dashed lines show the 100Hz line. All measurements were done in 1×PBS at physiological pH. PLL-g-PEG functionalized and non-functionalized nanopore normalized conductance in 1×PBS after storage in 0.1×PBS for 6 hours (over 10 times nanopore chip storage) (D). Continuous measurement of normalized conductance for functionalized and non-functionalized nanopore in 1×PBS at pH 7.4 (E), Inset shows the IV curve for both functionalized and non-functionalized before and after 5 hours measurement.

An illustration of normalized conductance over ten times of storage in 0.1×PBS is shown in Figure 3.10D for both non-functionalized and in situ PLL-g-PEG functionalized ~20 nm SiN nanopores. Figure 3.10D inset, illustrates the conductance measurements of both PLL-g-PEG functionalized and non-functionalized ~20 nm SiN nanopore before and after the storage process. Base on the results, the non-functionalized nanopore conductivity was stable over

storage time, with a slight decrease, as predicted by previous studies because of the water-based buffer in contact with our SiN nanopores (51). In the case of functionalized nanopore, the PLL-g-PEG layers remained stable for 5 times storage, but a portion of the PLL-g-PEG layers have started to disassemble from SiN sidewalls after six times of storage and washing processes, creating an increase in nanopore conductance. A ten-storage period eventually resulted in functionalized nanopore conductivity reaching 8.27 nS, close to bare nanopores (9.8 nS). Over 5 hours of continuous experiment at pH 7.4 under 600 mV, Figure 3E shows the normalized conductance of a ~ 20 nm non-functionalized and PLL-g-PEG functionalized nanopore. From the results, after five hours, the non-functionalized nanopore's conductance increased from 9.8 to 11.16 nS due to the changes in the nanopore edge structure and chemistry (52). In contrast, the PLL-g-PEG layer on the nanopore sidewall was stable and prevented the SiN nanopore structure from changing or expanding under high stimulus voltage for ~ 4 hours, which increased the lifetime of nanopores.

The PLL-g-PEG thickness in functionalized nanopore is about one nanometre thinner than flat glass surface PLL-g-PEG functionalized as shown in Figure 3.8 by AFM. Moreover, the thickness of functionalized PLL-g-PEG layer via in-situ method was thinner than the ex-situ functionalized one, which caused the conductance change from 37.7% to 80.7% by in-situ and ex-situ methods, respectively (Figure 3.10B). In addition, due to the charge of the PLL-g-PEG chains, the tip wall charge of the nanopore was slightly positive after functionalization (53–55), which led to I-V curve be parabolic. Overall, ionic conductivity has a greater impact on measured currents than bulk ionic concentrations. So, ICR occurs as a result of ionic selectivity, when tip of the nanopore has a positive charge, which leads to limitation in cations flow and decrease the resistance of pore (56,57). The other phenomenon we investigated was current noise. As demonstrated in Figure 3.10C, a comparison between the current noise power spectrum density (PSD) of bare and PLL-g-PEG functionalized nanopores was done. Based on the results, in low frequencies (≤ 100 Hz) the power spectral noise was increased in functionalized PLL-g-PEG. Based on the previous studies, this is due to an increase in the polymeric subunit motion on the nanopore sidewall, which caused low-frequency noise and presence of nanometre-sized bubbles (nanobubbles) within the nanopores (22,58,59).

3.2.4. Protein translocation

In order to investigate the unsticking behaviour of PLL-g-PEG functionalized nanopore under the same experimental conditions and environment, the control experiments were carried out by injecting 25 nM AAT protein into Cis chambers while applying 600 mV stimulation voltage by using a 16.5 nm non-functionalized nanopores at physiological pH. Rarely, after adding proteins, the nanopore becomes irreversibly blocked, and only a small number of proteins were translocated by adhering to the sidewalls, as illustrated in Figure 3.11A. A 16 nm nanopore was functionalized with PLL-g-PEG, and its estimated size after functionalization was approximately 9.5 nm. As shown in Figure 3.11B, we observed approximately 15 events/s of the protein translocation through functionalized nanopore, which is 15 times greater than that through non-functionalized nanopore. According to the scatter plot in Figure 3.11C, AAT protein translocations through PLL-g-PEG functionalized nanopores (with a mean dwell time of around 0.4 ms) occur approximately 105 times faster than those through non-functionalized nanopores. Furthermore, we noticed a 25% increase in translocation event amplitude (from 0.24 to 0.3 nA), as a result of the PLL-g-PEG functionalization, which led to decrease in nanopore diameter. Thus, the amplitude of the translocation event increased for the same protein translocation. This effect is roughly equivalent to the volume change in nanopores before and after PLL-g-PEG functionalization. The high number of translocations without pore clogging and/or sticking behaviour suggests that PLL-g-PEG functionalized nanopores are the best choice for AAT protein translocation. According to this work, the single PLL-g-PEG layer efficiently blocks the protein from engaging in ongoing interactions with sidewall nanopores.

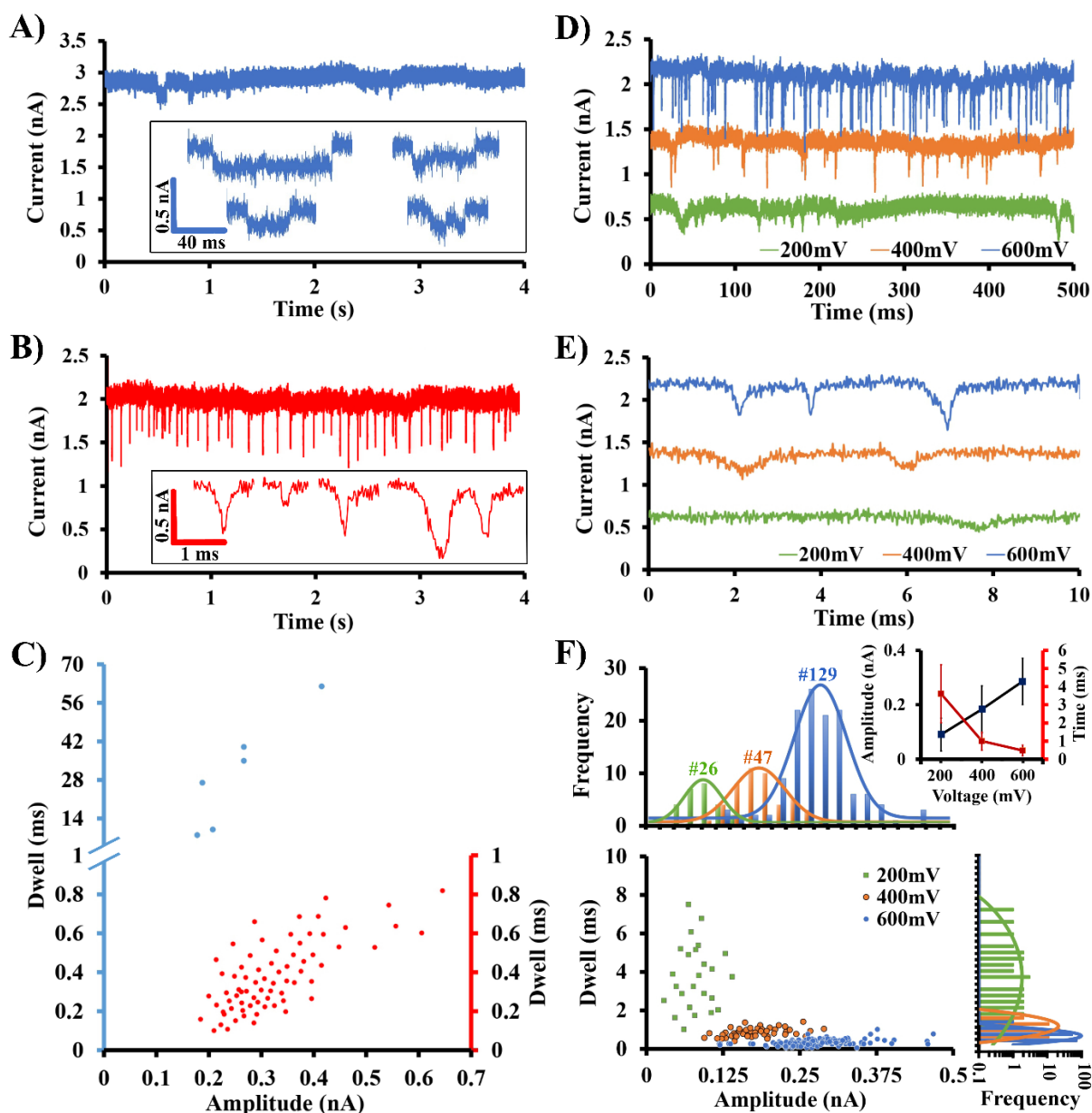


Fig. 3.11. 25nM AAT protein translocation through 20nm non-functionalized (A) and PLL-g-PEG functionalized (B) SiN nanopores (30 nm thick) and their Scatter plots of dwell time vs. blockade amplitude (C), //: represent the vertical (Dwell) axis scale change in the scatter plot of protein translocation through un-functionalized nanopore. Comparison of 500 nM AAT protein translocation events under different stimulus voltages (200, 400, and 600mV) during 500 ms (D) and 10 ms measurements (E). 500 nM AAT translocation events scatter plot of current blockage vs translocation time in different voltages (F), the inset displays the events dwell time and amplitude changes with stimulus voltage based on the fitted Gaussian distribution curves with variance σ^2 , and the # represent the total number of translocations events in each stimulus voltages. All measurements were done by using 1×PBS at physiological pH.

The current traces for the translocation of 500 nM AAT protein through a ~9.5 nm functionalized nanopore at different voltages (200, 400, and 600 mV) while utilising 1×PBS are shown in Figures 3.11 (D and E), at physiological pH. The scatter plots in figure 3.11F display

the dwell time and amplitude distributions of 500 nM AAT protein translocation under different voltages. The scatter plot on Figure 3.11F according to a Gaussian distribution fitted curve on histograms data and the inset graphs demonstrate the reduction in dwell time from 3.6 ± 1.6 to 1 ± 0.5 and 0.473 ± 0.3 ms as the voltage increased from 200 mV to 400 and finally 600 mV, respectively. On the other hand, raising the voltage caused the current blockades amplitude to rise from 0.09 ± 0.06 to 0.18 ± 0.09 and 0.28 ± 0.08 for stimulus voltages of 200, 400, and 600 mV, respectively. As shown in Figure 3.11F, raising the voltage causes an increase in the frequency of AAT protein translocation events; the number of events at 200, 400, and 600 mV is 26, 47, and 129, respectively. These observations are all consistent with earlier studies (32,33,60–62).

3.2.5. Protein Volume estimation

One of the most important globulin proteins in the serum is recombinant Human Serpin A1/ α -1-Antitrypsin Protein with specific features like globular shape, molecular weight around 46 KDa, and 1.02 gr/cm^3 density at 300 K (63,64). AAT protein volume can be calculated by using the mass to density ratio ($V = M/d$). As a result, volume of 74.9 nm^3 and 97.7 nm^3 can be achieved if 46 KDa or 60 KDa (SDS-PAGE molecular weight) are used as molecular weight, respectively. In an AFM study of α 1-antitrypsin by Maria Gaczynska et al., they found that antitrypsin molecule has a length of 4–5 nm and width of 7–8 nm based on its crystal structure model (65). In other study done by J.K. Armstrong, the hydrodynamic radius of α -1 Antitrypsin was demonstrated as 3.81 nm (66), resulting in a hydrodynamic volume of 231 nm^3 , under ellipsoid shape assumptions. AAT molecular volume can be estimated using PLL-g-PEG functionalized nanopores. So, the conductance blockade (ΔG) can be estimated by the following equations, where Λ is protein volume, d is nanopore diameter, h_{eff} is effective thickness (67,68):

$$\Delta G = \frac{\sigma \gamma \Lambda}{(h_{eff} + 0.8d)^2} S(d, a) \quad (3.4)$$

The 1×PBS buffer at 25°C has an electrolyte solution conductivity (σ) of 1.6 (S/m). Based on DeBlois and Bean (69), the correction factor S can be negligible, when the hydrodynamic diameter of the AAT protein ($d_m \sim 7.62 \text{ nm}$) is smaller than the PLL-g-PEG functionalized nanopore diameter, and length of the fully folded AAT is well within the effective length of

the nanopore ($\langle h_{\text{eff}} \rangle$) (70). On the other hand, orientation of the AAT protein can effect on the shape factor (γ). Here, for spherical molecule the γ is 1.5 (71) in a normal distribution of ΔI values (72). Figure 3.11F shows a scatter plot representing the conductance blockage vs event duration of AAT protein translocations at various voltages. With a background current level of 2.2 nA and an average current blockage of 0.26 nA under a 600 mV stimulus voltage, the average ΔG for a single AAT protein is around 0.43 nS. Then, the volume of a single AAT molecule is estimated to be 253 nm³, or the hydrodynamic radius is estimated to be 3.92 nm, at pH 7.4, which is close to the AAT hydrodynamic radius reported by J.K. Armstrong (231 nm³) (66). The thickness of the hydration shell, i.e. the water molecules/layer surrounding the protein, and counter ions, which bind closely to the proteins rather than water molecules, are hypothesised as the causes of the discrepancy between measured and crystal structure volumes (72,73).

First, a nanopore flowcell was designed and fabricated which is reusable, transparent, portable. Then, the conductivity and ion current rectification (ICR) behaviour of SiN nanopores were investigated in different concentrations of PBS, and the noise of the SiN nanopores were studied at different voltages. The non-tacky SiN nanopore by in situ functionalization of PLL-g- PEG layers proposed to study multicharged globular proteins. The lifetime and storage time of SiN nanopores improved and the amplitude of particle translocation signals increased by using PLL-g- PEG nanopores. Finally, with the designed flow cell and PLL-g-PEG functionalized SiN nanopores, two different protein concentrations without sticking to the nanopore side wall were translocated and the approximate size of the protein was estimated. The functionalized SiN nanopores will enable us to provide highly sensitive detection of small changes in protein concentration and in the diagnosis of early-stage diseases.

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4. Conclusion

There are numerous applications for particle size and protein volume detection along with number of particle detection in fluids. Solid-state micro and nanopores can be employed to fulfil a task, but several challenges need to be tackled.

Solid-state micropores are primarily constrained by slow production time, expensive techniques, cumbersome sample preparation steps. To overcome these problems, polyimide (PI) was selected as the substrate due to its chemical and physical durability, and we used femtosecond laser to drill precisely constructed 1 μm pore in PI film and poly(methyl methacrylate) (PMMA) container. Polystyrene (PS) particles with different sizes of 100, 300, 350 nm were derived and passed through the micropore by applying voltage, which caused and alteration in the magnitude of the current blockage related to particle sizes (0.1, 1, and 2 nA, respectively). Finally, 100, 300, and 350 nm PS particles in fluid can be distinguished, which is promising approach for distinguish between unmodified PS and virus-modified PS, virus-specific sensors, and coronavirus detection. However, there are still a number of issues that need to be resolved, such as where the pore is located inside the membrane and how that affects the amplitude of the observed current blockage.

For smaller particles like protein investigations, normally we used SiO_2 or SiN nanopore chips, but still there is a lot of obstacles. As the first issue, lack of small, portable, transparent, leak-free led us to design our reusable nanopore flowcell (2.6×2.6×0.8 cm) with nearly 13 μL volume transparent chambers. Second, the wettability of the SiN nanopore must be improved since it is challenging to fill the pore with aqueous solutions, in this regards we proposed piranha cleaning procedure followed by wetting with Isopropyl alcohol (IPA)-water mixture to improve the hydrophilicity of the nanopore. As a result of increase in the stimulus voltage, the PSD noise level was elevated, however, the polarity of stimulus voltage affected the current noise level (base positive voltage led to 4 times less noise). In situ functionalization of PLL-g-PEG nanopores increased nanopore lifetime by threefold (up to four hours), compared to non-functionalized SiN nanopores. Functionalized PLL-g-PEG layers can be stored in 1×PBS

at pH 7.4, for 5 times (each for 6 hours). Third, the biggest problem which all researches faced, is sticking of proteins to side wall of the nanopore and lack of cheap and monitorable functionalizing method. To translocate the non-sticky single α 1-antitrypsin (AAT) protein through solid-state nanopores, the real-time and monitorable in situ method was proposed to functionalize the 20 nm SiN nanopore sidewalls based on Poly(L-lysine)-g-poly(ethylene glycol) (PLL-g-PEG) polymer chains self-assembly to nanopore sidewall (2.5 nm thick). Our monitorable in situ physical adsorption (self-assembly) could provide a reproducible and homogeneous 2.5 nm thick PLL-g-PEG layer inside the nanopore. We showed that the ion current rectification ratio was achieved from around 1 to 1.25 in a 20 nm SiN nanopore with a 30 nm thickness by lowering the buffer salt concentration from 1×PBS to 0.125×PBS. Additionally, compared to the non-functionalized nanopore, the amplitude of AAT protein translocation events was increased by 25% (from 0.24 to 0.3 nA), along with 105 times reduction in dwell time (from 31 to 0.59 ms) and a noticeably higher number of translocation events (enhancement from 1 to 15 events/s), after nanopore PLL-g-PEG functionalizing. In detail, the molecular volume of 253 nm³ for the AAT protein was measured, and it was shown that the PLL-g-PEG functionalized nanopore can identify single protein molecules. Our in situ PLL-g-PEG functionalizing method can be used for other proteins.

A micropore sensor can be the cheapest and most simple way to detect viruses, bacteria, and any other biomolecule larger than 100 nm in diameter, and it can be used by anyone in their home. Alternatively, these micropores are also capable of detecting contamination or sedimentation in environmental samples, as well as aggregation of nanometer particles. Also, micropore has the potential to use in pandemic situation, like Coronavirus, to detect with high sensitivity and specificity. The rapid advancement of science and technology and the increasing demand of people, it is expected that future methods for protein detection will focus on portability, intellectualization, rapidity, and integration of diagnosis and treatment with solid state nanopore technology, in order to improve clinical practice and testing at home and bedside. Using solid state nanopores, recognition of multiple amino acids is possible in a way that allows discrimination of single amino acid substitutions, providing a greater understanding of individual proteins and their variations for future disease detection and prevention.