Video Article

Monitoring Protein Adsorption with Solid-state Nanopores

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Abstract

Solid-state nanopores have been used to perform measurements at the single-molecule level to examine the local structure and flexibility of nucleic acids [1-6], the unfolding of proteins [7], and binding affinity of different ligands [8]. By coupling these nanopores to the resistive-pulse technique [9-12], such measurements can be done under a wide variety of conditions and without the need for labeling [3]. In the resistive-pulse technique, an ionic salt solution is introduced on both sides of the nanopore. Therefore, ions are driven from one side of the chamber to the other by an applied transmembrane potential, resulting in a steady current. The partitioning of an analyte into the nanopore causes a well-defined deflection in this current, which can be analyzed to extract single-molecule information. Using this technique, the adsorption of single proteins to the nanopore walls can be monitored under a wide range of conditions [13]. Protein adsorption is growing in importance, because as microfluidic devices shrink in size, the interaction of these systems with single proteins becomes a concern. This protocol describes a rapid assay for protein binding to nitride films, which can readily be extended to other thin films amenable to nanopore drilling, or to functionalized nitride surfaces. A variety of proteins may be explored under a wide range of solutions and denaturing conditions. Additionally, this protocol may be used to explore more basic problems using nanopore spectroscopy.

Video Link

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Protocol

1. Manufacture of solid-state nanopores in silicon nitride membranes

1. Bring the FEI Tecnai F20 S/TEM to an acceleration voltage of 200 kV. If using a different S/TEM, the acceleration voltage should be greater than or equal to 200 kV [9]
2. Load a 20 nm thick SPI silicon nitride window grid into the TEM sample holder and clean with Oxygen plasma for 30 seconds to remove any contaminants from the holder.
3. Load the sample into the S/TEM and allow for the vacuum to pump down. Once the S/TEM has pumped down to vacuum, find the nitride window in bright-field TEM mode by looking for a bright square on the Ronchigram. Make sure the TEM is aligned properly, then align and focus the sample.
4. Switch the S/TEM into STEM mode. Use the HAADF (or equivalent) detector to image the sample and make sure it is properly aligned.
5. Set the Monochromator to a low value. A lower value of the Monochromator allows for a higher current of electrons available for drilling. If the S/TEM does not have a Monochromator, ignore this step.
6. Select an appropriate spot size for drilling the nanopore. This corresponds to the diameter of the electron probe. A 3 nm probe works well for drilling 5 nm diameter pores. A larger probe (5 nm) will drill more quickly and create a larger pore. A smaller probe (1 nm) will take longer to drill and create a smaller pore.
7. Adjust the STEM alignments, if necessary.
8. Focus the nitride membrane and bring it to a magnification of x1.3M. Fine focusing should be done by monitoring the Ronchigram.
9. If there is any movement of the sample, then wait for the sample to stabilize. This can take up to an hour. Blank the electron beam while waiting so as not to damage the nitride.
10. Place the electron probe on the sample and begin drilling.
11. Periodically check the sample by scanning with the HAADF detector, both to make sure the nanopore is being drilled (it will look like a dark spot) and to check that there is no movement of the sample. If the sample drifts slightly, adjust the position of the electron probe to be over the nanopore.
12. Watch the Ronchigram to identify when the pore has formed. When in focus, the nitride film has a shimmering appearance. This will disappear when the nanopore forms. Putting the Ronchigram slightly out of focus allows one to see a Fresnel fringe associated with the nanopore.
13. Take an image of the nanopore with the HAADF.
14. Do an intensity profile of the nanopore image. The diameter of the nanopore can be estimated by the darkest region of the profile.

15. Enlargement of the nanopore may be performed by moving the electron probe along the edges of the pore.
16. Once the nanopore is of the desired diameter, put the S/TEM into TEM mode.
17. Bring the Monochromator to its standard setting and image the nanopore using bright-field TEM to confirm the size **(Fig. 1)**.

2. Wetting of the solid-state nanopore

1. De-gas de-ionized water containing 1 M KCl, 10 mM potassium phosphate, pH 7.4. This can be done by putting the solutions under vacuum and placing them in a bath sonicator for 20 minutes.
2. Place the nanopore containing silicon nitride chip into a 10 ml Pyrex beaker. Take care not to break the nitride window as it is very delicate. Place the beaker on a hotplate in a fume hood and set to 100°C.
3. Clean the nanopore chip with piranha solution using great care. First add 3 ml sulfuric acid to the container using a glass pipette. Next, carefully add 1 ml hydrogen peroxide to the sulfuric acid to make piranha solution[14]. Please take all precautions.
4. Allow the nanopore chip to soak in piranha solution for 10 minutes.
5. Remove the piranha solution from the beaker using a glass pipette. Place it into a proper storage receptacle.
6. Fill the beaker with the de-gassed, de-ionized water using a clean glass pipette. Empty beaker of water and repeat at least 5 times.
7. Remove the nanopore chip with clean tweezers and dry it by light suction
8. Immediately, seal the nanopore chip into the chamber **(Fig. 2a, 2b)**. The chip may be secured in the chamber by O-rings or silicone sealant.
9. Fill the chamber with KCl solution
10. Connect the Ag/AgCl electrodes to the chamber **(Fig. 2b)**. Electrodes may be made by soaking silver wire in bleach overnight.
11. Apply a transmembrane potential across the electrodes and monitor the current response using an Axon 200B patch-clamp amplifier in the voltage-clamp mode.
12. Construct an I/V (current-voltage) curve from the measurements. The I/V curve should be highly linear when using 1 M KCl **(Fig. 3)**. The conductance of the nanopore should correspond to its diameter. If the nanopore does not display a linear I/V curve, the conductance is too low, or the open current of the nanopore is not stable **(Fig. 4a)**, it means that the nanopore is not properly wetted and piranha washing should be repeated.

3. Monitoring protein adsorption

1. It is critically important to perform control experiments with a single-nanopore membrane or parallel array of nanopores (see below) to monitor the current with the buffer conditions lacking the protein sample. We recommend the following two steps: (i) the use of a high purity-level buffer (purity > 99.9%; chromatography-grade level), and (ii) the use of a buffer whose interaction with the solid-state nanopore does not produce single-channel events. In addition, we recommend obtaining a high-purity protein sample using standard protein chemistry procedures. The purity of the protein sample should be checked by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel analysis and high-resolution mass spectrometry [15].
2. Add a purified sample of the protein to be studied into the grounded bath of the chamber. Allow time for the sample to diffuse throughout the bath. Typical concentrations of protein that can be measured are in the tens of nM to μM range [7,15-21].
3. Apply a transmembrane potential voltage bias with polarity opposite to that of the total charge of the protein being studied. For example, BSA has an effective net negative charge at pH 7.4, and therefore a positive voltage bias should be applied. The applied voltage creates potential gradient within the nanopore interior and proteins of opposite charges will be driven by opposite forces. Only voltage polarities that drive the proteins into the nanopore will create measurable signals.
4. An applied potential of magnitude of 200 mV should be large enough to observe most protein analytes. Events will appear as transient current deflections from the baseline **(Fig. 4b)**. If no signal is observed, increase the concentration of the protein in the chamber. Higher voltages improve the signal to noise ratio. Nitride nanopores can withstand several volts.
5. Protein adsorptions will appear as long-lived changes in the baseline current of the nanopore **(Fig. 4c)**.

4. Possibilities for functionalization of the nanopores

There exist several methods for applying functional groups to silicon nitride [22,23]. Most nitride films have a thin oxide layer on the outside and exposure to ozone can create an additional oxide layer, if necessary. Different organosilanes can be used and self-assembled onto such layers. Of particular interest is aminosilane, which self-assembles and can be modified further (i.e., carboxylic acid and aldehydes), allowing for the inspection of a range of organic surfaces. After a nanopore has been washed with piranha and secured in the chamber, amine can be added directly to the chamber bath with a supporting electrolyte of 0.5 M TBACl (tetrabutylammonium) and anhydrous MeOH (methanol) used as a solvent. Monolayer formation can be monitored by the application of a 400 mV voltage bias and the measurement of the current drop [23].

5. Determination of the apparent first-order reaction rate constant and the Langmuir adsorption constant using parallel nanopore arrays

5.1 The apparent first-order reaction rate constants for absorption and desorption

We emphasize that the positive aspect of this methodology is its ability to observe individual adsorptions at the single-molecule level. The single-molecule measurements of protein adsorption onto an inorganic surface can be scaled up by employing parallel arrays of solid-state nanopores. The parallel array of nanopores is necessary because of the need to assay many pores to get reliable statistics. To this end, the use of a nanopore array would allow the monitoring of individual adsorptions as well as the measurement of multiple events for further analysis. An array of nanopores in silicon nitride may be formed by the above protocol simply by drilling multiple holes in the sample. Each nanopore should be of similar size. Arrays of nanopores of 6x6 or 7x7 should be adequate. Upon addition of protein analyte in the chamber, the current will decay in an exponential manner with a following expression:

$I_t = I_\infty - (I_\infty - I_0)exp(-k't)$

Here, I_t, denotes the current at an experimental time t. I_0 is the original current passing through the nanopore array. I_∞ indicates the current at saturation level (i.e., infinity). k' is the apparent first-order reaction rate constant, which can be determined from the fit of the experimental curve. A greater k' can be interpreted as a faster adsorption rate. The ratio I_∞/I_0, also called the normalized saturation current, is a dimensionless number between 0 and 1. This parameter is a measure of the occupancy by the adsorbed protein analytes. Therefore, each distinct experimental condition should be associated with two specific output parameters I_∞/I_0 and k'.

It should be noted that the apparent first-order adsorption rate constants and normalized currents are impacted by the effective time spent by proteins within the nanopores. This time is dependent on the concentration of protein analytes in bulk aqueous phase [24]. Therefore, additional correction of these numbers needs to be implemented based upon the effective time spent by the proteins within the nanopore interior. We suggest measuring the frequency of fast (translocation) events and multiplying it by the average dwell time of such events. This will give the average time of proteins spent within the nanopore interior per unit time.

The rate constant of desorption can be determined using a parallel array of nanopores as well. As soon as the current level reaches saturation (I_∞), the voltage should be reversed, so no more proteins are trapped into the nanopores. Desorption of individual proteins will be accompanied by the alteration of the recorded current towards greater values. The rate constant will be then extracted from the rise in the current level.

5.2 The Langmuir adsorption constant

The absorption of protein analytes onto the inorganic surfaces of the nanopores is dependent on the protein concentration in aqueous phase. This phenomenon has already been observed at the single-molecule level [13]. If θ represents the normalized saturation current (I_∞ / I_0), then a typical Langmuir isotherm equation is given by the following expression:

$$\theta = \frac{\alpha C}{1 + \alpha C}$$

where C is the protein concentration in the aqueous phase. α is the Langmuir adsorption constant. This constant increases with an increase in the binding energy of adsorption and with a decrease in temperature. The collection of the θ data points will employ measurements with parallel arrays carried out at various protein concentrations in aqueous phase. Data should be analyzed in combination with other techniques to verify the magnitude of the fitted Langmuir adsorption constant. In addition, full-atomistic molecular dynamics simulations [25,26] might be also used to help the interpretations of the obtained experimental data.

6. Representative Results

Typical results for solid-state nanopores will be as follows. The open pore current should be highly stable, as seen in **Fig. 4a**. The I/V characteristics of the nanopore should be highly linear in 1 M KCl, 10 potassium phosphate, pH 7.4, as seen in **Fig. 2**. The slope of a linear fit to the I/V curve will provide the unitary conductance of the nanopore. The conductance has a direct relationship to the nanopore diameter

and should match the equation: $G \approx \frac{\pi d^2}{4l}\sigma$, where G is the conductance of the nanopore, d is its diameter, l is its length, and σ is the

conductivity of the solution in the chamber [14]. This value should match to within 30%. If it is too small, your pore is likely not wetted. With addition of protein, rapid events should ensue, as seen in **Fig. 4b**. Protein adsorption is a long-lived current drop as displayed in **Fig. 4c**. Some proteins are highly labile and undergo structural transformation within the pore interior. In this case, the long-lived voltage drop will be accompanied by rapid fluctuations.
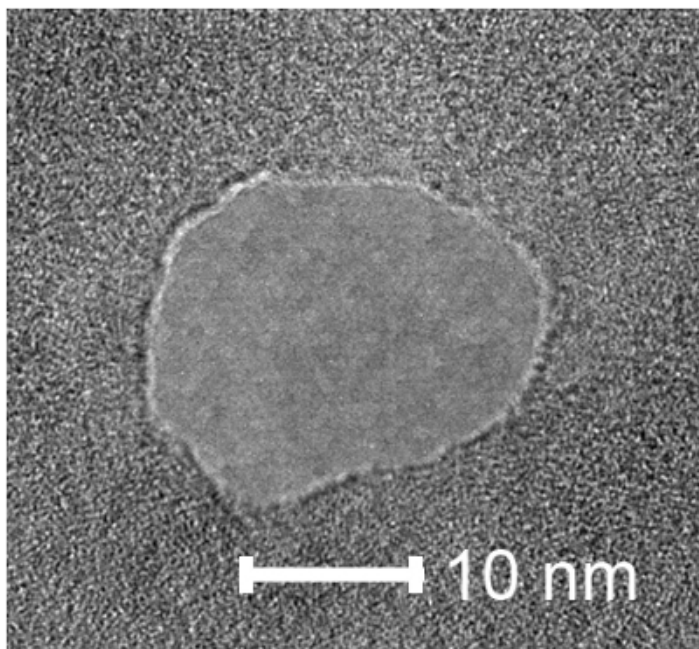
Figure 1. Solid-state nanopore fabricated using a Tecnai F20 S/TEM. The pore was drilled in STEM mode to a diameter of 20 nm. The image was taken in bright-field TEM mode. Nitride was 30 nm thick.
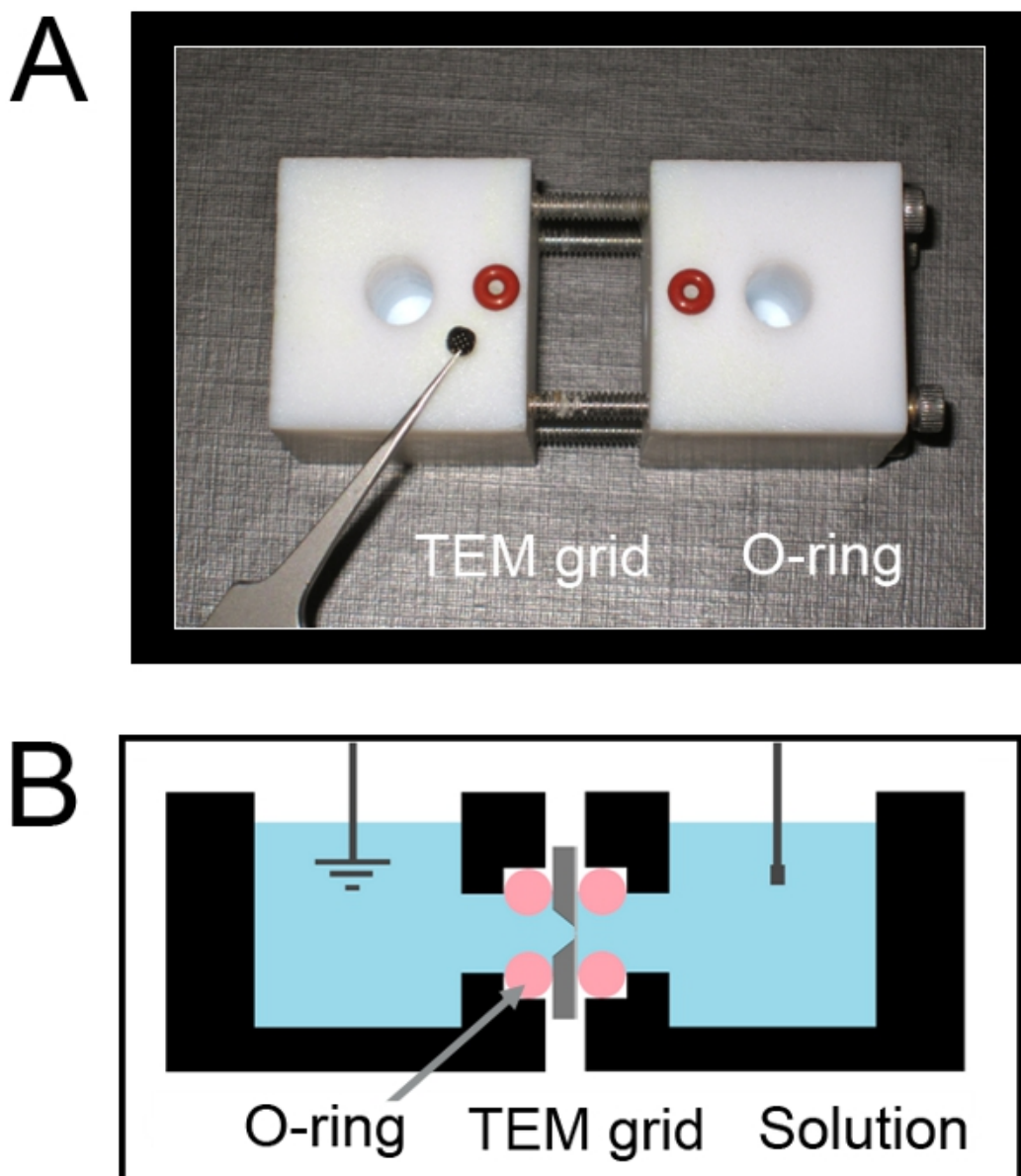
Figure 2. Chamber used for housing a single solid-state nanopore in resistive-pulse measurements. A) Chamber and a silicon chip with free-standing nitride window (TEM grid). The nanopore is drilled into the nitride before loading. Red O-rings are used to form a good seal about the chip, which separates two baths of ionic solution. B) Schematic of the chamber showing placement of solution baths and electrodes with respect to the nanopore.
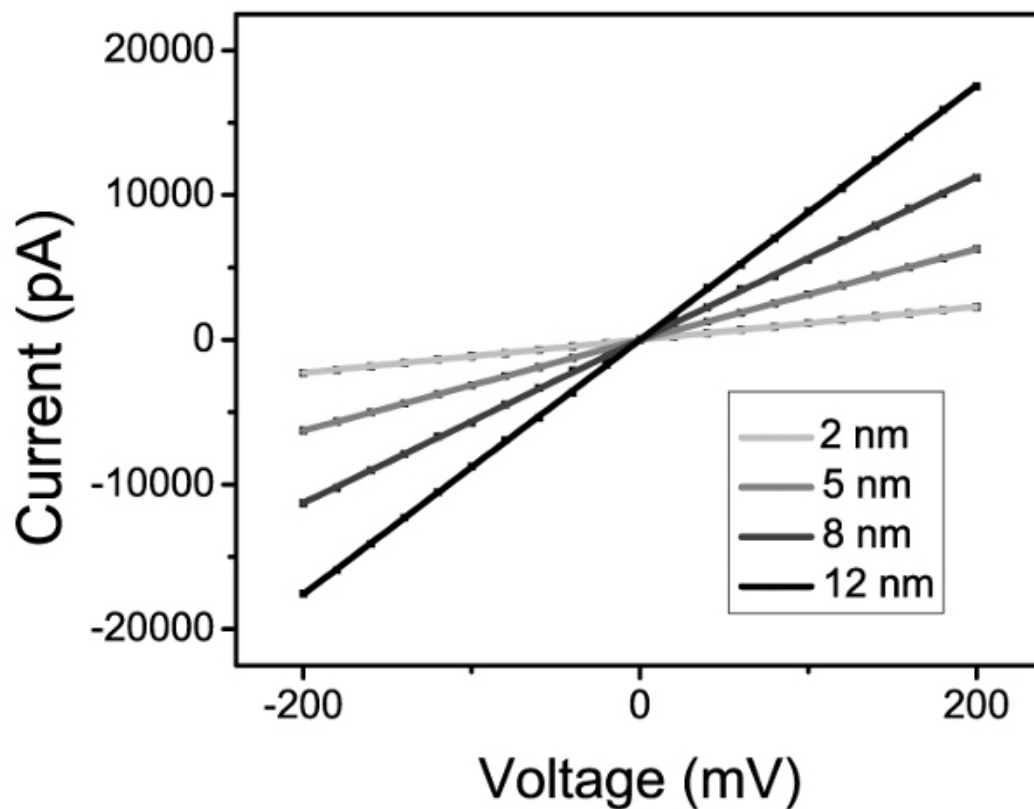
Figure 3. Typical I/V traces for solid-state nanopores of different diameters. Single-channel electrical traces were taken in 1 M KCl, 20 mM Tris, pH 8.5. Nanopores were drilled in 30 nm thick silicon nitride. Note nitride thickness will affect the unitary conductance of the nanopore.
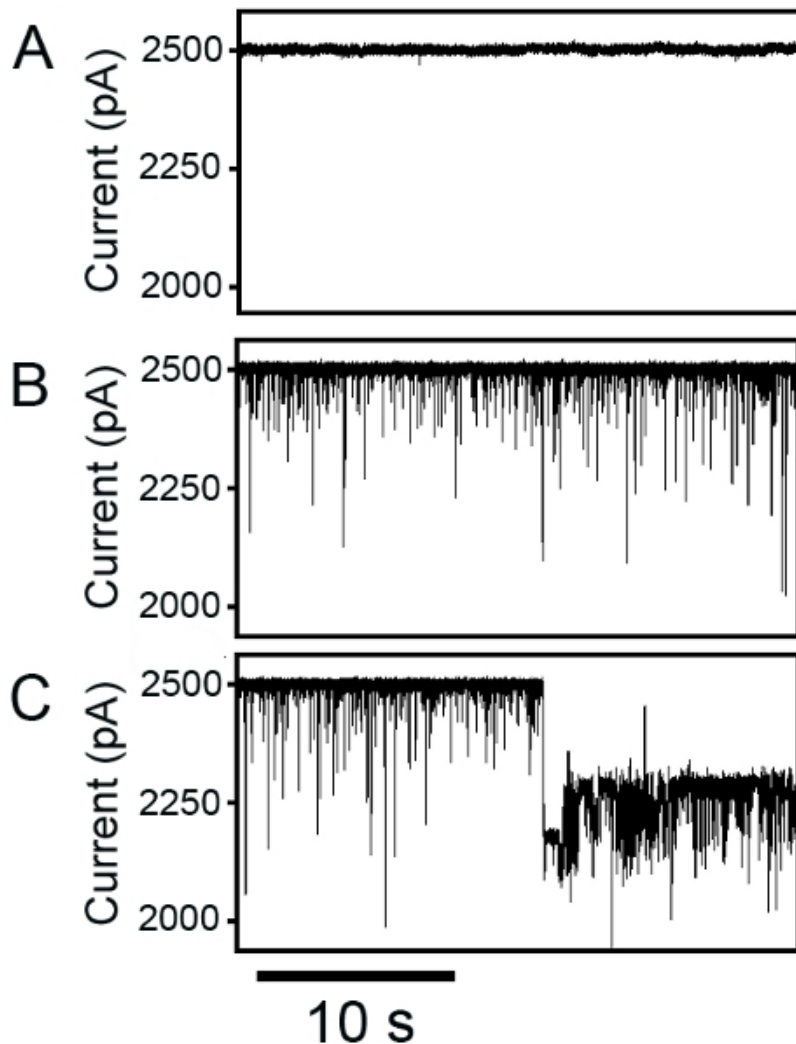
Figure 4. Measured single-channel current traces and the detection of protein adsorption. A) A single-channel electrical trace showing the open current of a 10 nm diameter nanopore. B) Current deflections represent partitioning of bovine serum albumin (BSA) into the interior of the nanopore. C) Adsorption of BSA onto the nitride surface. All traces are from the same nanopore in 1 M KCl, 10 mM potassium phosphate, pH 7.4. The applied transmembrane potential was +40 mV and BSA was added to the grounded chamber bath at a concentration of 120 nM.

Discussion

Spontaneous adsorption of proteins onto solid-state surfaces [27-29] is fundamentally important in a number of areas, such as biochip applications and design of a new class of functional hybrid biomaterials. Previous studies have shown that proteins adsorbed to solid-state surfaces do not show lateral mobility or significant desorption rates, and therefore protein adsorption is generally considered an irreversible and nonspecific process [30-32]. Protein adsorption onto solid state surfaces is thought to be due to several factors, including electrostatic and hydrophobic forces among the side chains of the proteins and the reactive groups at the solid-liquid interface [13].

The process of protein adsorption has been explored by number of approaches, such as quartz crystal microbalance [28,29], electron microscopy,[33,34] ellipsometry,[35] florescent labeling,[36] and planar polarization interferometry (PPI).[37] In contrast, this single-nanopore and nanopore array methodology relies on the detectable single-channel current changes produced by the interactions between single-protein analytes and the nanopore interior.

The most critical step in achieving positive results is the proper wetting of the nanopore interior. Should the pore characteristics not meet the specifications discussed in the results section, several troubleshooting methods may be used. Transient current blockades that occur without any proteins in solution may indicate that the chamber is contaminated. Teflon chambers may be washed with piranha. PDMS chambers should be made fresh from clean molds for each experiment. Nanopores with smaller than expected conductance or non-linear I/V characteristics may indicate that wetting is unsuccessful. A brief application of high voltage (˜5V) may improve the electrical characteristics of the nanopore. Otherwise, the nanopore should be washed again with the piranha solution. Even in the best circumstances, some nanopores fail to wet properly. The ability to wet a nanopore depends on its dimensions. Both thinner nitride nanopores and larger diameter nanopores are easier to wet than thicker nitride nanopores or smaller diameter nanopores. For 20 nm thick nanopores with a radius of 5 nm the success rate is approximately 70%

after first washing. Care should be taken in selecting a nanopore size for your analyte, since nanopores must be large enough to accommodate the protein.

The protocol discussed here only applies to adsorption to a silicon nitride surface. Other surfaces, such as silicon oxide [9] or alumina [23] may also be drilled using this technique, however the number of thin films amenable to this technique is limited to those that can be manufactured so that they are free-standing, thin and free of holes. To overcome this issue, chemical coating of the nanopore may be used [23,38-40]. Another trade off of the single nanopore technique is that it can only look at one molecule at a time. To overcome this limitation, we offered an alternative of using parallel arrays of solid-state nanopores [41]. Current measurements with nanopore arrays provide an opportunity to obtain macroscopic kinetic parameters, such as the apparent first-order reaction rate constants of absorption and desorption. In addition, this approach would allow the determination of the Langmuir adsorption constant. One immediate limitation of this technique is its inability to collect information on flat and wide surfaces. The measurements will only probe protein adsorption within confined spaces of the interior of the fabricated solid-state nanopores. In many instances, it is quite challenging to obtain precise information on the geometry and the inner surface of the nanopore. In the past, protein adsorption was extensively examined by quartz crystal microbalance [28,29]. The protein adsorption onto the crystal surface is accompanied by a dampening of the imposed oscillatory movement of the crystal microbalance, producing a decrease in the resonant frequency. An alteration of the total coupled mass due to protein adsorption induces a frequency shift of the quartz crystal microbalance. The total adsorbed protein mass is linearly related to the frequency shift. This is the underlying principle of this technique, which indirectly probes protein adsorption on a well-defined flat surface. In contrast, the use of nanopore arrays employs the resistive-pulse technique that is closely similar to the Coulter counter approach [42]. Again, the nanopore technique can be scaled down to a limited number of nanopores in a membrane, so individual adsorptions can be watched in real time. In contrast to quartz crystal microbalance, the nanopore measurements cannot asses the adsorption process on a large-scale and flat surface, in which additional events can occur (i.e, lateral diffusion of adsorbed proteins), thus producing alterations in the Langmuir adsorption isotherms. Of course, it is desirable that these approaches devoted to protein adsorption should be used in combination with each other, since they probe only some aspects of the adsorption process. For example, Brewer and colleagues employed a combination of quartz crystal microbalance and ζ -potential techniques to show that BSA binding onto gold nanoparticles and gold surfaces occurs via an electrostatic mechanism.

Disclosures

We have nothing to disclose.

Acknowledgements

References

1. Li, J., *et al.* Ion-beam sculpting at nanometre length scales. *Nature.* **412**, 166-169 (2001).
2. Li, J., Gershow, M., Stein, D., Brandin, E., & Golovchenko, J.A. DNA molecules and configurations in a solid-state nanopore microscope. *Nat. Mater.* **2**, 611-615 (2003).
3. Dekker, C. Solid-state nanopores. *Nature. Nanotechnology.* **2**, 209-215 (2007).
4. Branton, D., *et al.* The potential and challenges of nanopore sequencing. *Nat. Biotechnol.* **26**, 1146-1153 (2008).
5. Wanunu, M., Morrison, W., Rabin, Y., Grosberg, A.Y. & Meller, A. Electrostatic focusing of unlabelled DNA into nanoscale pores using a salt gradient. *Nat. Nanotechnol.* **5**, 160-165 (2010).
6. Peng, H. & Ling, X.S. Reverse DNA translocation through a solid-state nanopore by magnetic tweezers. *Nanotechnology.* **20**, 185101 (2009).
7. Talaga, D.S. & Li, J. Single-molecule protein unfolding in solid state nanopores. *J. Am. Chem. Soc.* **131**, 9287-9297 (2009).
8. Zhao, Q. *et al.* Detecting SNPs using a synthetic nanopore. *Nano. Lett.* **7**, 1680-1685 (2007).
9. Storm, A.J., Chen, J.H., Ling, X.S., Zandbergen, H.W., & Dekker, C. Fabrication of solid-state nanopores with single-nanometre precision. *Nat. Mater.* **2**, 537-540 (2003).
10. Sexton, L.T., *et al.* Resistive-pulse studies of proteins and protein/antibody complexes using a conical nanotube sensor. *J. Am. Chem. Soc.* **129**, 13144-13152 (2007).
11. Martin, C.R. & Siwy, Z.S. Chemistry. Learning nature's way: biosensing with synthetic nanopores. *Science.* **317**, 331-332 (2007).
12. Sexton, L.T., *et al.* An adsorption-based model for pulse duration in resistive-pulse protein sensing. *J. Am. Chem. Soc.* **132**, 6755-6763 (2010).
13. Niedzwiecki, D.J., Grazul, J., & Movileanu, L. Single-molecule observation of protein adsoption onto an inorganic surface. *J. Am. Chem. Soc.* **132**, 10816-10822 (2010).
14. Wanunu, M. & Meller, A. *Single-molecule techniques: a laboratory manual*. Selvin, P.R. & Ha, T. (eds.), Cold Spring Harbor Laboratory Press, New York, 395-420 (2008).
15. Han, A., *et al.* Label-free detection of single protein molecules and protein-protein interactions using synthetic nanopores. *Anal. Chem.* **80**, 4651-4658 (2008).
16. Han, A., *et al.* Sensing protein molecules using nanofabricated pores. *Appl. Phys. Lett.* **88**, (2006).
17. Fologea, D., Ledden, B., McNabb, D.S., & Li, J. Electrical characterization of protein molecules by a solid-state nanopore. *Appl. Phys. Lett.* **91**, nihpa38991 (2007).

18. Firnkes, M., Pedone, D., Knezevic, J., Doblinger, M., & Rant, U. Electrically Facilitated Translocations of Proteins through Silicon Nitride Nanopores: Conjoint and Competitive Action of Diffusion, Electrophoresis, and Electroosmosis. *Nano. Lett.* (2010).
19. Pedone, D., Firnkes, M., & Rant, U. Data analysis of translocation events in nanopore experiments. *Anal. Chem.* **81**, 9689-9694 (2009).
20. Oukhaled, A., *et al.* Dynamics of Completely Unfolded and Native Proteins through Solid-State Nanopores as a Function of Electric Driving Force. *ACS. Nano.* (2011).
21. Yusko, E.C., *et al.* Controlling protein translocation through nanopores with bio-inspired fluid walls. *Nat. Nanotechnol.* **6**, 253-260 (2011).
22. Arafat, A., Schroen, K., de Smet, L.C., Sudholter, E.J., & Zuilhof, H. Tailor-made functionalization of silicon nitride surfaces. *J. Am. Chem. Soc.* **126**, 8600-8601 (2004).
23. Wanunu, M. & Meller, A. Chemically modified solid-state nanopores. *Nano. Lett.* **7**, 1580-1585 (2007).
24. Movileanu, L., Cheley, S., & Bayley, H. Partitioning of individual flexible polymers into a nanoscopic protein pore. *Biophys. J.* **85**, 897-910 (2003).
25. Aksimentiev, A. Deciphering ionic current signatures of DNA transport through a nanopore. *Nanoscale.* **2**, 468-483 (2010).
26. Timp, W., *et al.* Nanopore Sequencing: Electrical Measurements of the Code of Life. *IEEE Trans. Nanotechnol.* **9**, 281-294 (2010).
27. Roach, P., Farrar, D., & Perry, C.C. Surface tailoring for controlled protein adsorption: effect of topography at the nanometer scale and chemistry. *J. Am. Chem. Soc.* **128**, 3939-3945 (2006).
28. Roach, P., Farrar, D., & Perry, C.C. Interpretation of protein adsorption: surface-induced conformational changes. *J. Am. Chem. Soc.* **127**, 8168-8173 (2005).
29. Brewer, S.H., Glomm, W.R., Johnson, M.C., Knag, M.K., & Franzen, S. Probing BSA binding to citrate-coated gold nanoparticles and surfaces. *Langmuir.* **21**, 9303-9307 (2005).
30. Schon, P., Gorlich, M., Coenen, M.J., Heus, H.A., & Speller, S. Nonspecific protein adsorption at the single molecule level studied by atomic force microscopy. *Langmuir.* **23**, 9921-9923 (2007).
31. Rabe, M., Verdes, D., Zimmermann, J., & Seeger, S. Surface organization and cooperativity during nonspecific protein adsorption events. *J. Phys. Chem. B.* **112**, 13971-13980 (2008).
32. Rabe, M., Verdes, D., Rankl, M., Artus, G.R., & Seeger, S.A comprehensive study of concepts and phenomena of the nonspecific adsorption of beta-lactoglobulin. *Chemphyschem.* **8**, 862-872 (2007).
33. Micic, M., Chen, A., Leblanc, R.M. & Moy, V.T. Scanning Electron Microscopy Studies of Protein-Functionalized Atomic Force Microscopy Cantilever Tips. *Scanning.* **21**, 394-397 (1999).
34. Grant, A.W., Hu, Q.H., & Kasemo, B. Transmission electron microscopy 'windows' for nanofabricated structures. *Nanotechnology.* **15**, 1175-1181 (2004).
35. Giannoulis, C.S. & Desai, T.A. Characterization of proteins and fibroblasts on thin inorganic films. *J. Mater. Sci: Mater. Med.* **13**, 75-80 (2002).
36. Gustavsson, J., *et al.* Surface modifications of silicon nitride for cellular biosensors applications. *J. Mater. Sci: Mater. Med.* **19**, 1839-1850 (2008).
37. Shirshov, Y.M., *et al.* Analysis of the response of planar polarization interferometer to molecular layer formation: fibrinogen adsorption on silicon nitride surface. *Biosens. Bioelectron.* **16**, 381-390 (2001).
38. Chen, P., *et al.* Atomic layer deposition to fine-tune the surface properties and diamters of fabricated nanopores. *Nano. Lett.* **4**, 1333-1337 (2004).
39. Siwy, Z., *et al.* Protein biosensors based on biofunctionalized conical gold nanotubes. *J. Am. Chem. Soc.* **127**, 5000-5001 (2005).
40. Ding, S., Gao, C. & Gu, L.Q. Capturing Single Molecules of Immunoglobulin and Ricin with an Aptamer-Encoded Glass Nanopore. *Anal. Chem.* (2009).
41. Kim, M.-J., Wanunu, M., Bell, C.D., & Meller, A. Rapid Fabrication of Uniform Size Nanopores and Nanopore Arrays for Parallel DNA Analysis. *Adv. Mater.* **18**, 3149-3153 (2006).
42. Bezrukov, S.M. Ion channels as molecular Coulter counters to probe metabolite transport. *J. Membr. Biol.* **174**, 1-13 (2000).