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Immunoassays Using Artificial Nanopores

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1. Introduction

1.1 Scope

Artificial nanopores can be loosely defined as materials possessing one or more nanometer-size pores (1-100 nm in diameter). This recent class of nanostructures is generating great interest in the scientific community as a platform for biomolecular analysis. The first fabrication of an artificial nanopore with true nanometer control dates to 2001 (Li et al., 2001) but the application of nanopores for biological studies started 10 years earlier. Following a rapid review of the highlights of 20 years of nanopore science, we explore the advancement of nanofabrication techniques that allowed the creation of individual nanopores, nanopore arrays, and nanoporous materials. Although most of the methods currently used to fabricate nanopores require expensive equipment and highly-skilled technicians, we focus here upon those technologies that allow the fabrication of nanopores at the bench and discuss signal transduction mechanisms that allow nanopores to be used as biosensors. In particular, we review the creative application of nanopipettes, artificial nanopores that can be easily fabricated from inexpensive glass capillaries, as a biosensing platform and discuss their potential for immunosensing.

1.2 History of nanopores

The concept of employing a nanopore for biological studies was initiated in the early 1990's by David Deamer (UC Santa Cruz) in collaboration with Daniel Branton (Harvard University), and independently by George Church and Richard Baldarelli (Harvard Medical School). Their hypothesis was that a DNA molecule threaded through a nanopore would perturb the ionic current in a sequence-specific fashion. This concept, however, required a very small pore of molecular dimensions to thread a long strand of DNA.

The first experimental evidence of whether a nanopore could detect nucleic acids was reported by Kasianowicz and coworkers in 1996 (Kasianowicz et al., 1996). The authors threaded single stranded DNA and RNA molecules through an α-haemolysin pore by means of an external applied voltage. Isolated from *Staphylococcus aureus*, α-haemolysin is a 33 kDa membrane pore protein whose channel remains open at neutral pH and high ionic strength and whose pore diameter is ~2 nm (Song et al., 1996). The passage of each molecule was detected as a transient decrease of ionic current whose duration was proportional to the polymer length. From the very first paper published using nanopore technology, the

authors envisioned that such a device would allow direct, ultra-fast DNA sequencing. The initial optimism was boosted by the discovery that one can distinguish adenine from cytosine within an RNA molecule with a heterosequence $A_{30}C_{70}$ (Akeson et al., 1999).

The initial excitement diminished when scientists realized that the signal-to-noise ratio was insufficient to ever reach single-base resolution in translocation experiments under typical conditions. The main obstacle is that translocation through α-haemolysin occurs at such a high rate that statistical fluctuations mask subtle differences among the four different bases. The fact that well-defined chemical and structural changes can be introduced to α-haemolysin by genetic engineering still offers the promise that a sequencing device can be built out of a biological nanopore. However, biological nanopores hold some inherent limitations. In particular, they need to be embedded in a supporting lipid bilayer whose stability is closely dependent on pH, temperature, electrolyte concentration, and mechanical stress. Even as research on these protein-based pores was progressing, improved nanofabrication techniques have made it possible to artificially sculpt nanopores into inorganic surfaces with nanometer precision. The possibility of precisely tuning nanopore size and geometry has made possible an expansion of nanopore-sensing technologies (Fig. 1). Applications include the detection of small molecules, peptides, enzymes, proteins and protein complexes (Siwy&Howorka, 2010).
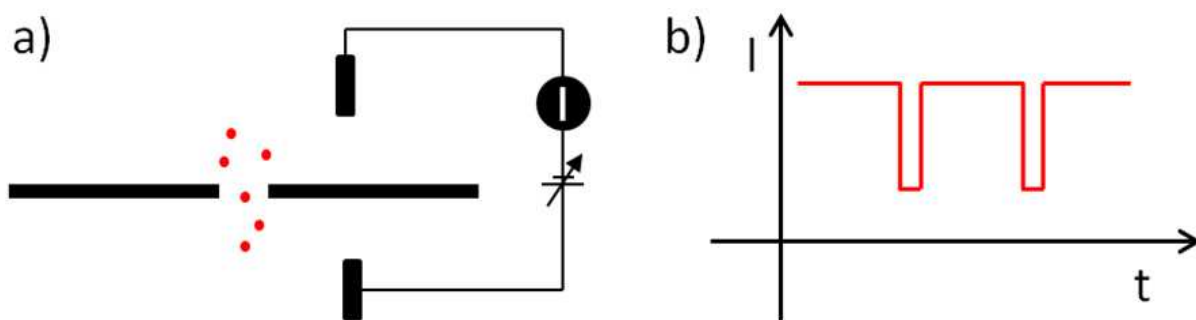

Fig. 1. Scheme illustrating the principle of nanopore measurements. a) A transmembrane potential causes a constant ion flow which is b) temporary perturbed by analytes passing through the nanopore.

Until now, DNA sequencing has been the application of choice for nanopores. The potential of a sequencing device that doesn't require (labelled) nucleotides, polymerases or ligases, requires a minimal sample preparation and allows long reads (>10,000 nt) still inspires several research groups (Branton et al., 2008). Nanopore research is, however, by no means limited to DNA sequencing, and the goal of this chapter is to highlight the recent advances in immunosensing using artificial nanopores, a platform that has the potential for myriad applications.

2. Fabrication of artificial nanopores

Naturally occurring and synthetic zeolites are perhaps the archetypical nanoporous materials, but their sub-nm pores make them more suitable for gas storage rather than for biosensing (Morris&Wheatley, 2008). In this section, we review the fabrication of nanopores and nanoporous materials with cavities of ~2 nm and above. We will first discuss the fabrication of individual nanopores and nanopore arrays using nanolithography, while we

dedicate separate sub-sections to bench-top fabrication methods and fabrication of nanoporous materials.

2.1 Ion and electron-beam sculpting

In 2001, Li and coworkers (Li, et al., 2001) reported the first fabrication of a nanopore with true nanometer control using a method called ion beam sculpting. The authors developed a focused ion beam (FIB) machine that uses ions to mill a tiny hole in a silicon nitride membrane. Their system was equipped with ion detectors as a milling feedback-control. Interestingly, they observed that ion rate and temperature affected pore dimensions, causing shrinkage and enlargement, fine-tunable with nanometer precision. Cees Dekker's group (Storm et al., 2003) developed a different approach based on electron beam lithography followed by etching. As with Li's method, pore size could be modified, in this case by exposure of the nanopore to a high-intensity wide field illumination (Fig. 2D). Holes with a diameter greater than the membrane thickness grew in size, whereas smaller holes shrank. Several other groups reported the use of a transmission electron microscope (TEM) to drill nanopores in thin membranes thus making the laborious preparation for electron-beam lithography unnecessary (Zandbergen et al., 2005) (Kim et al., 2006) (Krapf et al., 2005). Traditionally, insulating oxides were used as the material of choice for nanopore fabrication due to their robustness, chemical stability, and wide availability. More recently, scientists started fabricating nanopores into conductive or semi-conductive materials.

Graphene is a two-dimensional layer of carbon atoms packed into a honeycomb lattice with a thickness of only one atomic layer (~0.3 nm). Despite its minimal thickness, graphene is robust as a free-standing membrane. In addition, graphene is an excellent electrical conductor (Geim&Novoselov, 2007). Independently, three groups showed the fabrication of nanopores in graphene (Garaj et al., 2010, Merchant et al., 2010, Schneider et al., 2010) by electron beam milling (Fig. 2E). Preliminary results showed that current blockades due to DNA translocation through graphene nanopores are larger than what has been observed for silicon nanopores of the same diameter. On the other hand, the authors showed that bare graphene devices exhibited large ion current noise and suffered from low yields.

Another very popular technique to fabricate artificial nanopores is the track-etching method (Fig. 2B,C). This technique is based on the irradiation of a polymer foil with energetic heavy ions followed by preferential chemical etching of the particle tracks (Siwy et al., 2003). Cylindrical or conical pores with diameters from a few nanometers to the micrometer range can be obtained. Individual nanopores can be fabricated by masking the polymer foil with a metal during the ion irradiation. Track-etched nanopores can be coated with gold to create individual conical gold nanotubes or nanotube arrays (Martin et al., 2001).

2.2 Bench top fabrication

All nanopore fabrication methods described above require expensive equipment, such as TEM or FIB, or access to heavy ion accelerators. Nanopipettes are a class of artificial nanopores that can be easily fabricated at the bench starting from inexpensive glass capillaries (Fig. 2A). Quartz is the material of choice to fabricate nanopipettes. It possesses many advantages compared to other glasses in term of optical transparency, electrical noise, and mechanical properties. The most widely adopted fabrication method for nanopipettes is
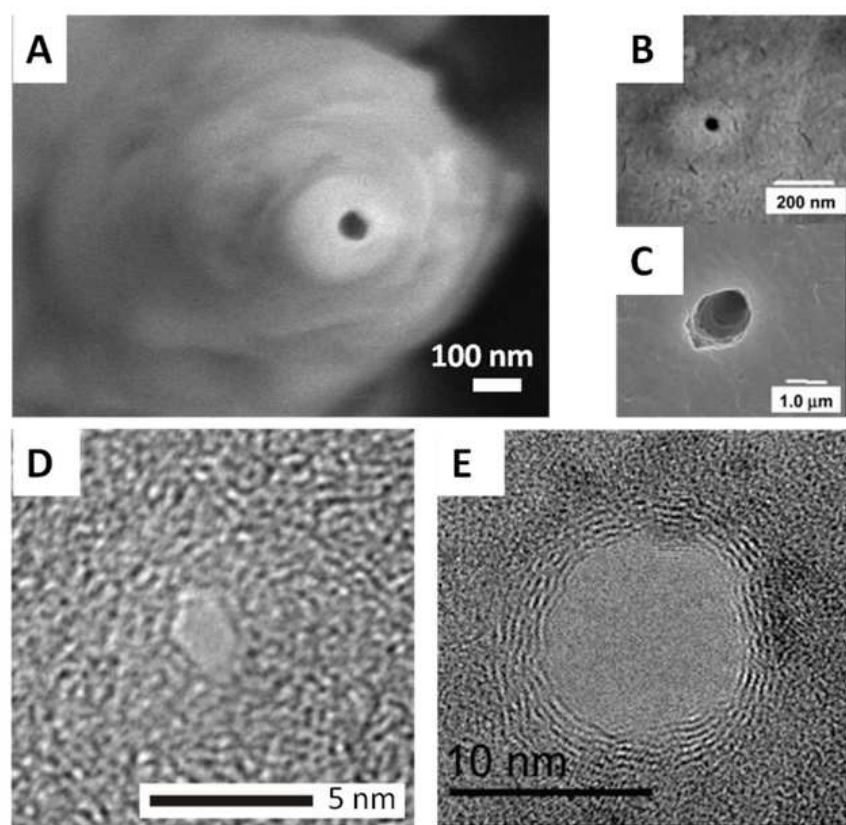
Fig. 2. Electron microscopy of nanopores A) laser-pulled nanopipette, adapted with permission from (Umehara et al., 2006). Copyright 2006 American Chemical Society. Track-etched nanopore in polycarbonate showing respectively B) surface of the membrane exposed to the etch solution and C) surface exposed to the stop solution showing tip opening, adapted with permission from (Harrell et al., 2006). Copyright 2006 American Chemical Society. D) Nanopore in a SiO_2 membrane. Adapted with permission from (Storm, et al., 2003) and E) graphene nanopore. Adapted with permission from (Merchant, et al., 2010). Copyright 2010 American Chemical Society.

laser pulling of a glass capillary. A laser beam is focused on the glass tubing until it reaches its softening point. A hard pull is then applied with a preset delay to generate two identical nanopipettes. The major advantage of the laser pulling method is the simplicity of the approach; there is no need for expensive equipment, clean rooms, or specialized technicians.

Fabrication of nanopipettes with nanometer precision remains technically challenging and alternative methods have been proposed to increase the reproducibility of pore dimensions. Ding and colleagues (Ding et al., 2009) reported an etching protocol to enhance the reproducibility of the nanopore size. First the capillary was pulled into a micropipette and then the very tip was sealed by a heat treatment. A nanopore was opened by external etching and monitored through electrochemical measurements, in order to attain the desired pore size.

Significant effort has been invested in fabricating nanopipettes from materials other than glass. Kim and colleagues (Kim et al., 2005) fabricated carbon nanopipettes with large aspect ratios (length/diameter) based on the glass pulling technique. First, they created an aluminosilicate nanopipette with a conventional laser puller, after which they catalytically deposited carbon layers onto the exterior and interior of the nanopipette. The exterior carbon layer and the glass

layer are subsequently removed by chemical etching, exposing the interior carbon nanopipette tip structure. Freedman and coworkers (Freedman et al., 2007) employed magnetic techniques to affix a magnetized carbon nanotube (mCNT) to the tip of a conventional glass nanopipette. The resulting mCNT-tipped nanopipettes were sufficiently robust that they could be used to penetrate cell membranes and to allow fluidic transport.

Zhang and coworkers (Zhang et al., 2004) developed the "nanopore electrode" which consists of atomically sharp platinum wire sealed into a glass capillary. By fine polishing, they exposed a platinum disk of nanometer dimension, and a subsequent etching step produced a truncated cone shaped nanopore embedded in glass, where the bottom of the pore is defined by the platinum disk.

2.3 Nanoporous materials

Numerous methods have been developed to fabricate nanoporous films and materials. We will limit the description to materials already employed for immunosensing whose applications will be described in the Section 3: "Nanoporous structures as loading materials for immunoassays".

Anodized aluminum oxide (AAO) has been one of the substrates of choice for the fabrication of nanoporous materials. AAO membranes are fabricated by anodic oxidation of an aluminum substrate in acidic solutions. The resulting membrane consists of densely packed hexagonal pores of 10–200 nm in diameter, whose size depends on the oxidation conditions (Stroeve&Ileri, 2011). Architectures consisting of long-range ordered nanochannel arrays can be fabricated out of AAO on the millimeter scale (Masuda et al., 1997).

Nanoporous films can be prepared by template-assisted synthesis as well. The self-assembly of polystyrene nanospheres and 5 nm SiO_2 nanoparticles on a glass slide followed by a calcination process produces an ordered array of nanopores embedded in a SiO_2 matrix (Yang et al., 2008). The application of more volatile template materials was used to create both carbon (Lee et al., 2001) and silica (Schmidt-Winkel et al., 1998) mesocellular foams. Moreover, nanoporous structures can be fabricated out of metallic materials. Ding and coworkers (Ding et al., 2004) demonstrated the synthesis of nanoporous gold film by selective dissolution (dealloying) of silver from a silver/gold alloy. Upon silver dissolution, gold atoms re-organize into an interconnected network of nanopores whose size can be tuned in the nanometer range via simple room-temperature post-processing.

Lin and coworkers (Lin et al., 2010) developed a very original method based on biogenic silica to create a nanoporous film on top of electrode materials. Diatoms are eukaryotic, unicellular photosynthetic algae that are ubiquitous in nearly every aquatic habitat. Diatoms produce diverse three-dimensional, regular silica structures (a.k.a. "frustules") with pores from nanometer to micrometer dimensions.

3. Nanoporous structures as loading materials for immunoassays

As discussed in the previous section, nanoporous materials are fundamentally different than solid state, "single-track" nanopores due to fabrication methods, size and geometry of the nanopores. A distinguishing feature is that nanoporous materials have a very large surface area, a property used in many immunoassay applications. Herein, we will review the application of nanoporous materials for immunosensing.

Piao and co-workers constructed a highly stable immunoassay using signal-generating enzyme (as ELISA label) immobilized in mesocellular carbon foam (Piao et al., 2009). Mesoporous carbon possesses large pores (D ~ 31 nm), interconnected by smaller windows (D ~ 21 nm) that are surrounded by small pores (D ~ 5.6 nm). Acid treatment can easily introduce carboxylic moieties at the surface of mesoporous carbon, thus enabling immobilization of horseradish peroxidase (HRP) by cross-linking with glutaraldehyde. HRP can readily enter the large pores while small pores can act as a substrate transporting channel, facilitating the enzymatic reaction. Furthermore, the immobilization of enzyme into nanoporous materials leads to high enzymatic activity and stability, and protection from proteolysis while enhancing mass transport (Wang&Caruso, 2004). The same group demonstrated that this approach give similar results when mesoporous silica was used (Piao et al., 2009).

Li et al (Li et al., 2011) used ultra-thin nanoporous gold leaf to enhance the electrochemiluminescent detection of carcinoembryonic antigen (CEA). Nanoporous gold leaves are large-area, highly conductive and ultra-thin nanoporous gold membranes with uniform pore size distribution centered around 20–30 nm. The authors showed that the nanoporous film reduces electron injection barrier to quantum dots thus enhancing electrochemiluminescence. Shulga and coworkers synthesized a nanoporous gold (NPG) film by selective dissolution (dealloying) of silver from silver/gold alloy. Upon silver dissolution, gold atoms re-organize into an interconnected network of nanopores. Antibodies were immobilized within the nanoporous gold allowing the spectrophotometric detection of prostate specific antigen (PSA) (Shulga et al., 2008). Wei and coworkers employed the same material for the detection of PSA but used a label-free electrochemical transduction (Wei et al., 2011). Li and coworkers constructed a composite material from a nanoporous gold film and graphene sheets. The synergy between these two materials yielded to a highly conductive composite that could detect human serum chorionic gonadotropin (hCG) within the range 0.5–40.00 ng/ml with a detection limit of 0.034 ng/mL (Li et al., 2011). Ding et al. developed an electrochemical immunoassay using nanoporous gold (NPG) electrodes and enhanced the sensitivity of the method by using gold nanoparticles conjugated with horseradish peroxidase (HRP) labeled secondary antibody. They demonstrated the detection of hepatitis B surface antigen (HBsAg) with a dose response in the range of 0.01–1.0 ng/mL with a detection limit of 2.3 pg/mL (Ding et al., 2010).

Yang and coworkers constructed a highly efficient chemiluminescent immunoassay based on a biofunctionalized three-dimensional ordered nanoporous SiO_2 film (Yang, et al., 2008). Lin and coworkers employed biogenic nanoporous silica (diatoms) to create a high density of nanowells where capture antibodies were immobilized. The performance of the biogenic silica membrane biosensor was tested in comparison with nanoporous alumina and plain metallic thin film biosensor. The authors showed an impressive linear range spanning 6 orders of magnitude, from 1 pg/mL up to 1 µg/mL. Significant enhancement in the sensitivity and response time was attributed to enhanced diffusion of fluids within the diatoms nanochannels (Lin, et al., 2010).

4. Methods of detection using nanopores

Because nanopores comprise a wide variety of materials, there are many possible mechanisms to measure a signal for a nanopore-based assay. The most common techniques

involve measuring electrolyte flux, or ionic current, through a pore that bridges two chambers. These methods are discussed in detail below (Section 4.1), as they often involve properties that only arise with nanopores. Other methods, employing functionalized nanopore sensors, are discussed in Section 4.2.

4.1 Resistive-pulse sensing

The technique known as resistive-pulse sensing derives from the well-known Coulter counter, in which particles such as individual cells pass through an aperture. If the aperture bridges two electrolyte-filled chambers containing polarized electrodes, then a particle causes a "pulse" of electrical resistance as it passes through the opening. By definition, then, this is a single-channel technique. Performing this technique with nanopores provides the exciting potential for the particles to include many types of biomolecules, including proteins and nucleic acids (Siwy&Howorka, 2010).

For the purposes of immunoassays, the resistive-pulse technique offers a way to analyze biomolecules without labelling. The shape and duration of pulses can discriminate biomolecules, reveal conformational states of proteins, and show antibodies that are bound to antigens. As described by Charles Martin's group (Sexton et al., 2010), the size of proteins as well as their affinity with the pore walls contribute to their electrical translocation signature (Fig. 3). Techniques for analyzing proteins with artificial and biological pores have been recently reviewed (Movileanu, 2009). The wide range of sizes covered by proteins, antibodies, and even viruses makes artificial pores an important tool for analyzing biomolecules.
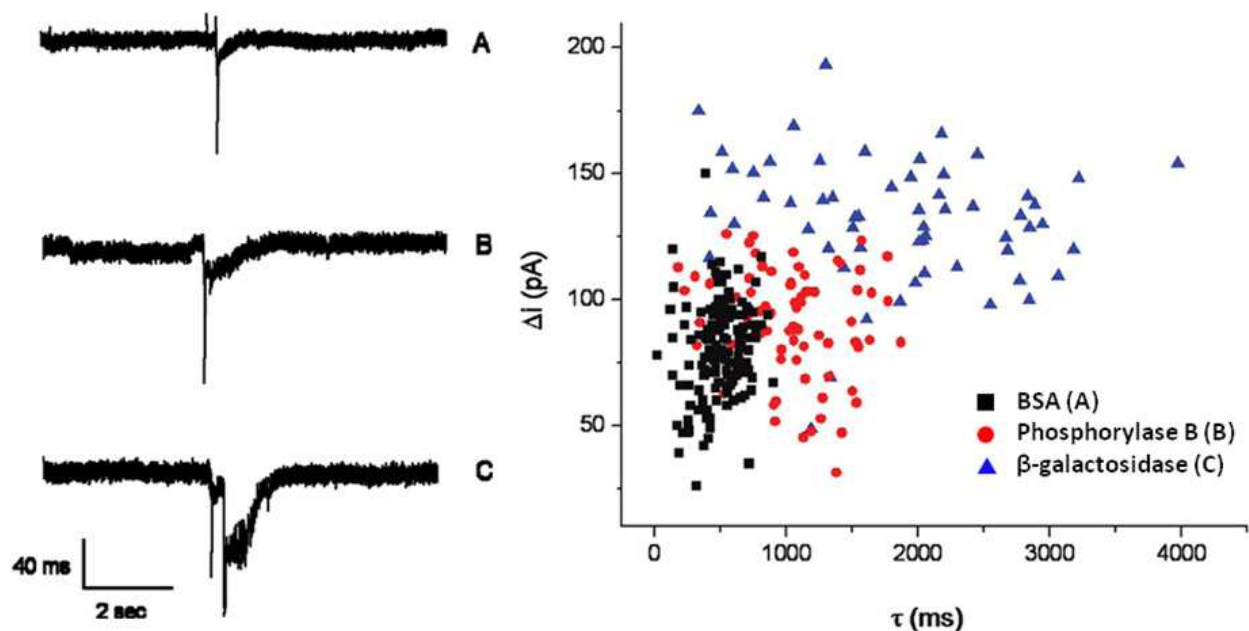

Fig. 3. Left: Ion current pulses arising from translocation of three proteins from tip to base of a conical nanopore with tip diameter of 17 nm. The proteins are bovine serum albumin (A), phosphorylase B (B), and beta-galactosidase (C). Right: scatter plot of the pulse amplitude (ΔI) and pulse duration (τ) for the three proteins. Reprinted with permission from (Sexton, et al., 2010). Copyright 2010 American Chemical Society.

Several recent examples in the literature demonstrate the potential of resistive-pulse sensing to develop powerful immunoassays. In the context of translocation through a nanopore, "immunoprecipitation" can simply mean a change in pore blockade properties compared to individual antigens or individual antibodies. Also, because particles such as viruses can be on the order of 500 nm, we will include micron-scale pores in our consideration of resistive-pulse assays. Using 600 nm pores in glass, Uram, et al. were able to determine antibody binding to a virus based on translocation (Uram et al., 2006). The virions, with diameter of 200 nm, increased in size roughly 60% on antibody binding. A few years earlier, pores of comparable size (1 micron diameter in PDMS) were used to detect binding of antibodies to biotin-derivatized colloids (Saleh&Sohn, 2003).

Moving to pores less than 100 nm in diameter brings the technique of resistive pulse sensing into the realm of detecting native proteins and antibodies. These applications require no labelling of the protein, which can sometimes change binding properties. For example, the Martin group has shown that the electrical signature of a protein can be distinguished from that of the protein bound to an antibody fragment (Sexton et al., 2007). In that report, track-etched nanopores were coated with gold to make a conical nanotube of tip opening 9 to 27 nm in diameter. Electrophoretic translocation of biomolecules from tip-to-base of the nanopores gave characteristic pulse durations that were significantly longer for the immunocomplex than for protein or antibody fragment alone. Silicon nitride pores have been used in several studies of protein translocation. Using 28 nm pores in a membrane 20 nm thick, protein-protein interactions were observed in real-time (Han et al., 2008). More recently, 20 nm pores prepared using the focused ion beam (FIB) technique in silicon nitride membranes were used to discriminate native from unfolded proteins (Oukhaled et al., 2011). Another dimension was recently added to the resistive pulse nanopore techniques, in which artificial nanopores were coated with a "fluid wall" within which biotin was attached (Yusko et al., 2011). The translocation of streptavidin was markedly influenced by the interaction with the antigen. The promise of these new techniques is that they may one day replace immunoassays that require labelling, such as ELISA, and offer greater sensitivity and discriminatory power.

4.2 Ion current modulation methods

The resistive pulse technique measures brief translocation events recorded as "pulses" of resistance (or current). However, ion current can be modulated in other ways in order to give an analytical signal. For example, if a receptor is immobilized to the nanopore surface, then the presence of the target will cause a change in ion current as long as the target is bound. There is a growing arsenal of techniques for chemically modifying the surface of nanopores (Nguyen et al., 2011, Sexton, et al., 2007, Wanunu&Meller, 2007). There is nothing about this particular sensing mechanism that requires nanoscale pores. But as with resistive pulse sensing, bringing the opening down to the scale of biomolecules provides increased sensitivity. Furthermore, there are some properties of ion current in nanopores that are not present in larger structures, and these can be exploited as a signalling mechanism.

The sensitivity of receptor-appended nanopores as analytical instruments depends not only on the binding affinity of the receptor for the analyte, but also on the ability for the binding to result in a modulation of ion current. Predictably, binding many large particles such as antibodies will increase resistance to ion flux through a pore. This was demonstrated with

biotin-modified conical nanopores which showed a dramatic decrease in ion current in the presence of streptavidin at concentrations as low as 1 pM (Ali et al., 2008). Quartz nanopipettes functionalized with biotin likewise responded to streptavidin, and were also responsive to the 18 kD protein VEGF after immobilization of antibodies to the pore surface (Umehara et al., 2009) .

Another important consideration is the property of ion current rectification in conical and other types of asymmetric nanopores. This is a true nanoscale effect, and results from the interaction of ions with the electrical double layer formed at the surface of charged nanopores (Wei et al., 1997). The result is a "nanofluidic diode" (Vlassiouk et al., 2009) in which the ion current shows nonlinear dependence on applied voltage. This property is highly sensitive to surface charge, and thus such conical nanopores lend themselves to detection of charged analytes such as polyelectrolytes (Actis et al., 2011, Fu et al., 2009, Umehara, et al., 2006). To date, polyelectrolytes have been the primary targets for assays using this mechanism. Furthermore, it should be possible to target any analyte in which the binding results in a modulation of surface charge.

The use of receptor-appended nanopores for ion current modulation, rather than Coulter counting methods, broadens the technique to include all manner of nanoporous materials and is not limited to single-channel technique (Gyurcsányi, 2008). Despite the availability of many types of functionalized nanoporous materials, there are few examples of these used as ion current-based biosensors (Wang&Smirnov, 2009). Two recent reports illustrate how nanoporous materials can be used for label-free immunosensing. Using a suspension of nanoparticles functionalized with streptavidin, biotin could be detected at a concentration as low as 1 nM (Lei et al., 2010). This method is particularly versatile because there are many established techniques to functionalize nanoparticles. It is also noteworthy that in this case the analyte is very small compared to the receptor. In another example of modulating ion current with a small molecule analyte, glucose was detected using a receptor protein obtained from E. *coli* (Tripathi et al., 2006) where the histidine-tagged receptor was immobilized to a hybrid material consisting of nanoporous polycarbonate onto which gold was deposited using electroless plating. While the measurement of ion current through such membranes is relatively straightforward, reports in the literature of such sensors are scarce. This could be due to the difficulty in functionalizing nanomaterials, or perhaps to insufficient sensitivity in modulations of ion current.

A more common electrochemical technique for biosensing with nanoporous materials is to perform voltammetry using a redox indicator which can be detected as it diffuses through the nanoporous material. The nanoporous membrane can be affixed directly to the working electrode. A recent example is the measurement of biomarkers in whole blood using a nanoporous membrane derivatized with antibodies (de la Escosura-Muñiz&Merkoçi, 2011). To amplify the signal, a secondary antibody was tagged with gold nanoparticles, which then catalyzed the deposition of silver to further block the pores. While this added some complexity to the assay, the device was able to perform using whole, unfiltered blood. Simpler schemes measuring diffusion of a redox active species have also been successful with a variety of nanoporous materials (Lin et al., 2009, Nguyen et al., 2009, Wei, et al., 2011). Singh and co-workers also showed the application of electrochemical impedance spectroscopy to detect peanut protein Ara h1 with an antibody-functionalized nanopore (Singh et al., 2010).

5.1 Label-free immunoassays with functionalized nanopipettes

Our group pioneered the application of nanopipettes as a platform for label-free biosensing (Umehara, et al., 2009). The principles that govern transport properties through nanopipette are similar to conical nanopores in general. The ion current is rectified due to the surface charge and the conical shape of the pore (Wei, et al., 1997). When receptors are attached, the nanopipette can become an electrical biosensor.

We named this technology STING, as Signal Transduction by Ion Nano-Gating. The tip of a nanopipette was functionalized with protein A/G, a commercially available chimeric protein that captures the Fc region of an IgG molecule. This strategy allows the control over the orientation of antigen-binding sites which are always exposed to the analyte. As a proof of concept, two proteins associated with human colorectal cancer were detected, interleukin-10 (IL-10) and vascular endothelial growth factor (VEGF). Exogenously added antigens instantly reduced the ionic flow through the nanopipette, an effect that was not detected in the control nanopipette functionalized with nonspecific antiferritin IgG. Unfortunately, a lack of reproducibility of pore dimensions and lot-to-lot variations in the surface functionalization did not allow for a more quantitative analysis of the observed current reduction. Theoretically, the limit of detection of the nanopipette platform is determined by the number of molecules that generate a detectable signal at the 50-nm tip. In practice, however, consumption of target molecules on surfaces of no sensitivity, such as the outer nanopipette sidewalls, could prevent devices from achieving the theoretical detection limit. Once the protocols were improved to limit the nanopipette functionalization to its inner walls, we were able to demonstrate the analytical applications of nanopipettes for the detection of mycotoxins (Actis et al., 2010)(Fig. 4).
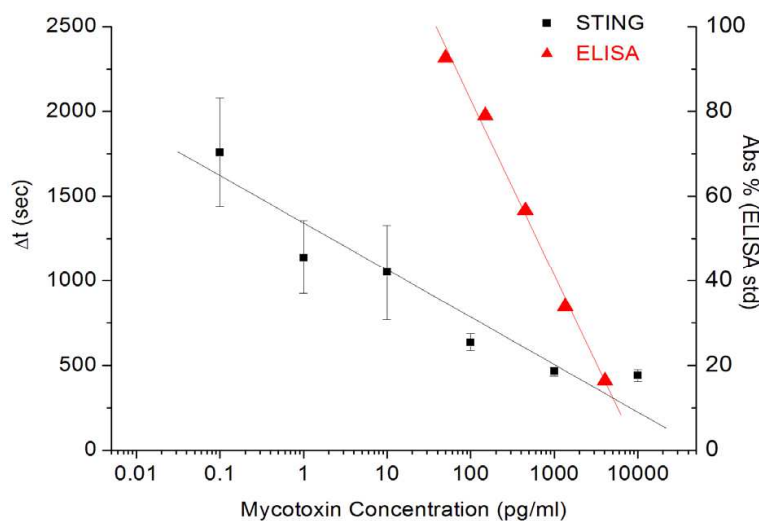

Fig. 4. Comparison between standard curves from ELISA kit (red triangles) and the STING sensor (black squares) for mycotoxin detection. Results show a dynamic range for the STING platform of at least 2 orders of magnitude with a detection limit 3 orders of magnitude lower than ELISA. Adapted with permission from (Actis, et al., 2010).

Mycotoxins are small, non polar molecules produced by fungi and are extremely toxic to mammals even in low concentrations. Using a nanopipette functionalized with antibodies specific to a mycotoxin, we showed a concentration dependent response. Indeed, the

nanopipette platform achieved a dynamic range of 5 orders of magnitude with a detection limit 3 orders of magnitude lower than that of a commercially available ELISA kit for mycotoxin.

Despite these successes, antibody-based detection schemes suffer from several drawbacks including: production, cost, limited target analytes, and limited shelf life. To address these imitations, the use of aptamers instead of antibodies as specific receptors was explored. Aptamers are single-stranded oligonucleotides, designed through an in vitro selection process called SELEX (Systematic Evolution of Ligands by Exponential Enrichment) (Ellington&Szostak, 1990, Tuerk&Gold, 1990). Aptamers have similar affinity and selectivity for targets as antibodies, but they can be chemically synthesized, stored in ambient conditions, and easily regenerated. In addition, they can be engineered to undergo a large-scale conformational response to specific molecules, largely affecting the ion transport through the nanopipette.

As a proof-of-concept, a thrombin-binding aptamer was immobilized on the surface of a nanopipette using standard carbodiimide coupling. The interaction of thrombin with its specific aptamer tethered on the nanopipette caused a partial occlusion of the nanopipette pore thus decreasing the ion flow. The decreased ion current can be directly correlated with thrombin concentration in both pure buffer solution and diluted serum. A major advantage of aptamers over antibodies is their ability to reverse the analyte binding. We showed that nanopipette sensors can be reused up to 5 times with minimal degradation of the sensor performance (Actis et al., 2011). In the long term, nanopipette technology promises to open new avenues for biomedical research, and promises breakthroughs in understanding of diseases at the single cell level.

5.2 Potential of nanopipettes for single-cell immunoassay

Nanotechnology-based tools having high sensitivity and low invasiveness hold great promises as new biomedical devices for single cell manipulation and intracellular analysis. We are currently developing a single-cell manipulation platform based on nanopipettes. As a nanopipette approaches the surface of a cell the ionic current through the pore will decrease due to "current squeezing", a well known effect, exploited to great benefit in scanning ion conductance microscopy (Hansma et al., 1989). By monitoring the ionic current the precise position of the nanopipette can be determined and controlled within ~200 nm of the cell membrane. After initial positioning above the cell membrane the nanopipette can either be scanned over the cell membrane to render a topographical image (Klenerman & Korchev, 2006) or inserted into the cell. The comparatively small size of the nanopipette combined with controlled penetration conditions maximizes cell viability.

By employing functionalized nanopipettes, detection of the interaction of intracellular, soluble antigens with antibodies immobilized on the nanopipette pore should be possible.

6. Other detection methods

To round out the discussion of nanopores and nanoporous materials in immunoassays, we will mention some non-electrochemical techniques. As with redox indicators, the diffusion of an optical indicator can be measured through a nanoporous membrane (Jágerszki et al., 2007). Such sensing schemes can potentially take advantage of distinctive recognition/transport

phenomena that occur in nanostructures. Many of the other properties of nanoporous materials, such as high surface area, can also be exploited for sensing schemes. A recent example is the use of porous alumina functionalized with a capture protein (Alvarez et al., 2009). The binding of an antibody can be detected by optical interferometry. In theory, this method can be used with many types of nanomaterials and is not restricted to nanoporous structures.

7. Conclusions

Artificial nanopores represent a fundamental technological breakthrough. As with many new technologies, nanopores required interdisciplinary collaboration. Fittingly, then, the potential applications of this new technology are equally broad, ranging from sequencing to diagnostics, cell biology to biophysics. Going forward, the integration of nanopores with microfluidic or optofluidic platforms may generate self-contained biodetectors with single molecule sensitivity (Holmes et al., 2010). The fabrication of nanopores into materials of atomic thickness, such as graphene can dramatically improve signal-to-noise ratio in biosensing experiments. Graphene nanopores may lead to sensing devices where the electric potential is controlled locally at the nanopore and transverse current can be measured across the pore aperture. Although artificial nanopore technology is at its infancy, we believe that it holds a tremendous potential for single-molecule sensing as well as single-cell analysis and manipulation.

8. Acknowledgements

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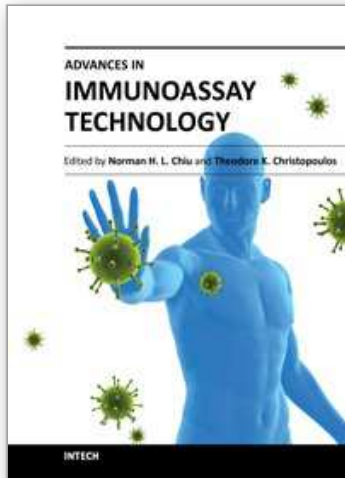
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