Challenging Nanopores with Analyte Scope and Environment

Buddini I. Karawdeniya[2], Y.M. Nuwan D.Y. Bandara[2], Jonathan W. Nichols[3], Robert B. Chevalier[1], James T. Hagan[1], and Jason R. Dwyer[1].

[1]*Department of Chemistry, University of Rhode Island, Richard E. Beaupre Center for Chemical & Forensic Sciences, 140 Flagg Rd., Kingston, RI, 02881, USA*

Present addresses:

[2]*Department of Mechanical Engineering, Lyle School of Engineering, Southern Methodist University, Dallas, TX, 75275, USA.*

[3]*Nova Biomedical, 200 Prospect Street, Waltham, MA 02453-3465 USA.*

jason_dwyer@uri.edu, 401-874-4648

ORCID

Jason R. Dwyer: 0000-0002-2938-2888

Y.M. Nuwan D.Y. Bandara: 0000-0003-1921-8467

Buddini I. Karawdeniya: 0000-0002-2733-5973

Jonathan W. Nichols: 0000-0003-4489-7828

Acknowledgements. This material is based upon work supported by the National Science Foundation under 1808344 and 1150085.

Nanopores are nanofluidic channels formed through thin membranes that can deliver standout single-molecule and single-particle sensing capabilities. Analytical targets include small molecules and nanoparticles, and the DNA, protein, and glycan biopolymers underpinning genomics, proteomics, and glycomics. Detection—notably even in the simplest implementation, resistive-pulse sensing—does not inherently require sample labelling and thus offers the potential for general sensing utility combined with the prospective benefits of reduced sample processing requirements. A key pursuit for biopolymer sensing is the characterization of monomer sequence.

This review article will provide an overview of the use of nanopores for general chemical sensing and –omics-related applications, writ-large. The broad analyte scope provides fertile ground for a discussion of principles governing nanopore sensing and considerations useful for guiding nanopore development. For nanopores to be effective in the face of broad analyte scope, stringent requirements on analytical performance must be met within the particular analyte class without sacrificing the operational flexibility necessary to be responsive across classes presenting very different physical and chemical challenges. These sample-driven challenges provide a unifying framework for discussing aspects of nanopore fabrication, properties, and integration; sensing paradigms, performance, and prospects; fundamental electrokinetic and interfacial phenomena; and practical challenges facing the use and further development of nanopore devices.

Keywords. Nanopore; single-molecule sensing; nanofluidics; point-of-care; silicon nitride; resistive pulse; wearable sensors; quality assurance

1. INTRODUCTION

1.1 Background

Nanopores are nanofluidic channels ≤100 nm in length and ≤100 nm in width that can serve as a powerful enabling technology for single-molecule science and for applications in chemical analysis (Figures 1, 2). The story of nanopores for biomedical diagnostics is one that is usually dominated by visions of DNA sequencing at low cost, with high accuracy and long reads, and with minimal time and sample processing.[1, 2] The ability to achieve this, and more, at the single-molecule level rightly captivates the imagination, and the rich blend of fundamental theoretical and experimental studies alongside impressive experimental milestones and applications provides a firm foundation for ongoing work in chemical analysis with a more general scope.[1-25] To wit: the accomplishments and prospects of nanopore sensing transcend the DNA sequencing and genomics milieu. Early nanopore development work showed the feasibility and promise of using the technique for small molecule sensing, an important touchstone for the consideration of nanopores as a general tool for sensing, including for metabolomics. There have been valuable experiments using nanopores to detect proteins and to characterize aspects of their structure, properties, and function, providing insight into the complex nanoscale world of proteins, and underpinning the prospects of using nanopores for proteomics. There have been other studies bringing into focus the promise of using nanopores for characterizing glycans and for exploring phenomena with glycans in key roles. One can thus conceive of nanopores as a powerful potential tool for glycomics, answering a need for new tools for this tremendous and tremendously important undertaking.[26]

This review article is intended to provide a chemistry-centered perspective on nanopore sensing, and thus has been constructed from a selection of the literature to support that view, rather than an exhaustive review that can be found in citations such as those listed above. Nevertheless,

this focus allows a fairly extensive coverage of key topics within the literature of nanopore sensing, and moreover provides vital insights that will be important for the ongoing development and future prospects of nanopore science in a diversity of application areas (Figure 2). In particular, we wanted to provide a perspective on the interactions between nanopore, matrix, and sample, from the most fundamental challenge—ensuring that analytes interact with the nanopore in a suitable way, without such unwanted interactions such as "sticking"—to a diversity of profound influences on experimental design and sensing performance that arise from choice of nanopore fabrication material (including surface coating), sample matrix (chosen, for example, by application rather than by design), and analyte class (and thus physicochemical properties among other factors). In addition, we wanted to highlight how design and operational choices different from those made within the prevalent nanopore framework of lab-based DNA sequencing might yield tremendous benefits in application areas such as wearable sensors and glycomics.

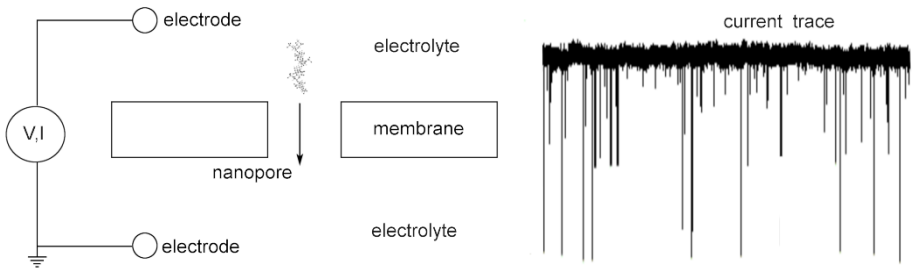


Figure 1. General configuration of a nanopore single-molecule experiment by resistive pulse sensing. At left, a ≤100 nm wide, ≤100 nm long channel through an insulating membrane provides the only ionic conduction path from one reservoir to the other. Electrodes immersed in each reservoir are used to establish a potential difference across the membrane that drives electrolyte ions through the pore, generating an open-pore current. Passage of an analyte through the nanopore by mechanisms such as electrophoresis, electroosmosis, and diffusion, can generate transient changes in the current such as those at right, that are determined by parameters such as nanopore size and surface charge, electrolyte composition, and analyte size and physicochemical properties.

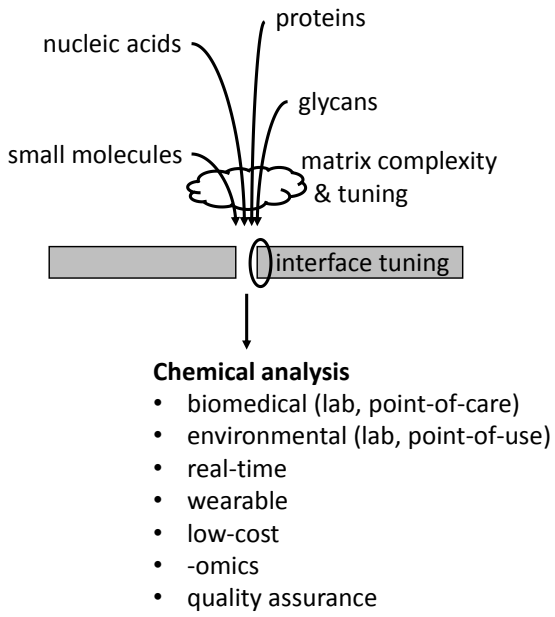


Figure 2. General perspective of the review article. Nanopores have the potential to perform general chemical sensing for a diversity of applications in a range of settings when suitable design and operating criteria are established and implemented.

1.2 Pores: Open For Business

At the core of all nanopore efforts is a nanopore, a nanoscale channel generally—and by design—not much larger in size than the analytes of interest. A not infrequent query in response to this geometric state of affairs is "Don't those things clog?", to which one might fairly reply "Not as often as one might fear, but more frequently than one might hope." Relevant factors affecting clogging severity include electrostatics and entropy, and it should be noted that the entropic penalty for nanoscale confinement is a significant player in preventing nanopore clogging.[4] Efforts to develop nanopores as a *general* tool for chemical sensing—wherein molecules and particles (analytes and sample matrix) possessing a wide range of properties will pass near or through a channel with high surface-area-to-volume-ratio—must contend with the landscape of interactions

that may emerge in this confined sensing volume. The influence of these interactions can be predominantly thermodynamic, but kinetic effects—such as those arising from changes to the analyte transport mechanism—can complicate the observed behavior. Changes of fabrication material can affect the achievable size and shape of nanopores, and also introduce different native nanopore surface chemistry and post-fabrication modification options. The development of surface chemical modifications to decorate the nanopore interior to dictate nanopore-analyte interactions—ranging from suppression of unwanted interactions to enhancing desirable ones—remains a forefront research question. Since such interactions can manifest through the interplay of several complex mechanisms for a given nanopore, it is important to be generally cognizant of how the choice of a particular nanopore size, shape, and composition can have profound consequences for nanopore sensing.

1.3 Nanopore Type

Nanopores can be conveniently (and broadly) circumscribed as nanofluidic channels $\leq$100 nm in length and $\leq$100 nm in width. Circularly-symmetric nanopores are prevalent (at the least as the centerpiece of analytical treatments), and in a common implementation, both ends of the nanopore have largely unrestricted access to bulk solution. Alternative configurations exist, including those with the nanopores present as constrictions within larger nano- or microfluidic channels that don't reach the bulk limit. For those nanopores formed as channels through supporting films, there are four broad classes (Table 1):

1.3.1 Naturally-occurring pores, especially the commonly-used proteinaceous types, provide a fairly narrow selection of discrete pore sizes, with limited size tunability.[27-30] These protein pores, however, offer incredibly rich surface

chemistries with molecular biology transformations available to support efforts to augment the rich chemical complexity of the wild-type pores.

1.3.2 **Chemically synthesized pores** offer tremendous chemical and size tunability limited only by the capabilities of the synthetic chemist's toolbox,[31-33] but have seen much less adoption within the nanopore sensing community. One reason for this less frequent use may be the specialized skills required for chemical synthesis. In this regard, self-assembling DNA origami pores[31, 34-38] offer programmable nanopore formation supported by a robust commercial DNA synthesis industry, but with a far more limited set of base fabrication materials than by organic synthesis.

1.3.3 **Polymer-supported pores** are local constrictions in larger channels through ~10–100 μm-thick polymer membranes.[4, 39] They emerged early-on as a contemporary of the protein nanopores, and see ongoing use, although on a more limited scale than the fourth class, their thin-film cousins. There are several likely reasons for this more limited use, including: the conventional use of large-scale facilities in fabrication (creative fabrication alternatives exist using elastomerics,[40] for example, although the elastomeric support may lead to unwanted behavior); the undesirable sample flow characteristics associated with an often tortuous fluid channel bracketing the nanopore constriction; and the perception of greater ease of integration of nanopores in micro- and nanofabrication-compatible materials into more complex device formats. The polymer class of nanopore, however, has several compelling features that warrant increasing development effort and frequency of use. First, they provide a useful combination of continuous size-tunability with chemical tunability. The latter is

possible by combining a straightforward and common commercial process, electroless metallization, with the well-established process of thiol monolayer self-assembly, optimized for the polymer nanopore surface.[4, 5, 39, 41] Commercial sources can easily provide a range of monolayer terminal groups that can change the nanopore-solution interfacial properties and interactions between the sample and nanopore, itself. In contrast to the chemically and spatially heterogeneous—yet highly reproducible—surface chemistry inside protein nanopores such as α-hemolysin,[28] the use of self-assembled monolayers would generally be expected to result in coatings with either a single surface functional group at the solution interface, or with a heterogeneous mixture of terminal groups if more than one thiol were used. Second, the general methodology of electroless metallization offers more than just the ability to metal-coat a surface.[42-46] In combination with one of a variety of patterning methods, it is possible to exert spatial control over the electroless metallization so that wires and more sophisticated electronic circuit elements can be directly fabricated onto the same polymer base supporting the nanopore (the ionic circuit element).[43, 45] There is thus potential for close integration of electronic circuitry with the nanopore, and this is particularly compelling when considering the third reason: polymer-supported nanopores are supported within flexible substrates that could be readily integrated into ***wearable sensor devices***. It is intriguing to imagine the use of such wearable nanopore sensors for sweat analysis, given that nanopore sensors are almost always immersed in electrolyte as a basis for their use.

1.3.4 Thin-film solid-state pores are the most recent addition to the suite of nanopore tools.[16, 19, 47-50] They offer many of the benefits of the earlier polymer-supported nanopores, with two key benefits: the length of the nanopore is no greater than the ~10-100 nm thickness of the thin films, thus minimizing fluid resistance; and thin film materials such as LPCVD silicon-rich silicon nitride (SiN_x) is a conventional micro- and nanofabrication material,[50] so that there is a ready infrastructure to enable the fabrication of sophisticated nanopore-based devices with additional functional elements, so long as the augmenting materials and methods are compatible with nanopore operating conditions. For example, there are ongoing efforts to perform transverse electron tunneling currents on analytes as they pass through the nanopore orthogonal to tunneling electrodes.[51] It is also conceivable that the recent demonstration that SiN_x thin films can be made to stand truly free of their conventional rigid silicon support frames may be useful in transferring these nanopores over to the realm of wearable sensors as outlined above for the polymer-supported pores.[52]

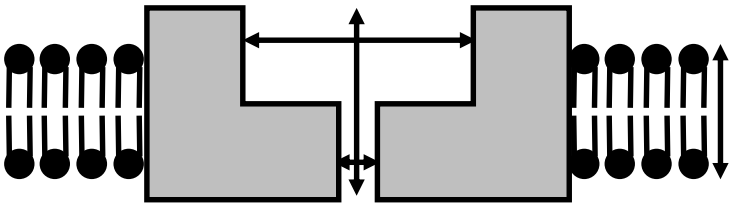
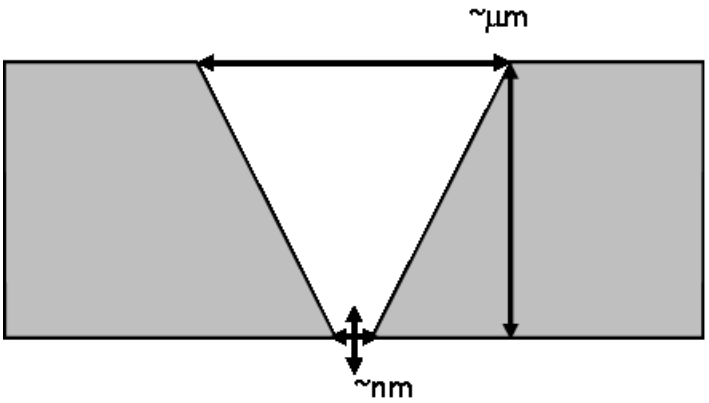
Nanopore class	Schematic
Naturally-occurring pores	
Chemically synthesized pores	
Polymer-supported pores	
Thin-film solid-state pores	

Table 1. Overview of nanopore classes. All four classes share the common element of a nanoscale constriction (the nanopore), but differ in material composition, form factor, and relationship to the supporting or surrounding membrane: inserted through a fragile lipid bilayer (top two examples) or similar thin film; formed at one edge of a much thicker film (polymer example); or having length identical (thin-film example) or similar to the thickness of the supporting membrane.

Beyond the particular focus and target application, the considerable effort and advances in the domain of thin-film solid-state pores can be constructively placed into several different

categories, and there is extensive overlap with similar investigations using the other pore platforms:

a) Optimizing nanopore form, interface, and function
b) Investigating mechanisms and determinants of analyte transport into and through the nanopore; and
c) Exploring and benchmarking analyte scope and information content of nanopore sensing methods;

2 OPTIMIZING NANOPORE FORM, INTERFACE, AND FUNCTION

2.1 Nanopore Formation

While SiN_x is a conventional material with well-established micro- and nanofabrication and modification workflows,[16, 50, 53] the coveted ~1-10 nm length scale for many solid-state nanopores represents a considerable fabrication challenge. The use of transmission electron microscopes to fabricate nanopores on this length scale represented a significant technical advance for the field,[47] but remaining technical and instrumentation burdens meant that nanopore fabrication largely remained the preserve of a rarefied group of nanopore practitioners—and was certainly practically out of reach of commercial-scale applications. Other fabrication tools, including (wet) scanning electron microscopes, helium ion microscopes, and large-scale accelerator facilities offered fabrication alternatives, but not appreciably greater ease or scaling.[8, 9, 11, 16, 19, 20, 47, 54, 55] Only recently, controlled dielectric breakdown (CDB) was discovered and developed to offer a much simpler and much less expensive way to reliably make even the smallest nanopores.[56-58] Requiring little more than a ~20 V source to generate $\lesssim$1 V/nm fields across ~10 nm-thick membranes submerged in buffered electrolyte, the pores could be formed, characterized, and then used with little change to the instrumentation and experimental

configuration required.[56-59] The CDB technique allows for the substitution of 2D materials such as graphene for SiN_x,[57, 60] thereby allowing for tuning of the native pore surface chemistry and the properties—such as transverse conductance—of the film supporting the nanopores.

2.2 Nanopore Surface Chemistry

There is considerable interest in the nanopore community in methods to control and tune nanopore surface chemistry. The motivations can be as simple as passivation (e.g. coating to minimize clogging), to creating functional nanopores by installing chemically responsive agents (including the special case of chemically selective recognition agents) on the nanopore surface. In spite of the considerable need to tune nanopore surface chemistry, many existing methods can be complex, expensive, or unreliable.[16, 50, 61-64] The report of silane-based chemical modification of SiN_x nanopores remains a signal moment for the nanopore field, but significant barriers to preparing reproducible chemical coatings on this platform have meant that the approach has gained little widespread traction in the field.[16, 19, 50, 62, 65-68] Instead, practitioners resort to techniques such as gold-coating followed by the formation of thiol monolayers, or perform no modifications at all.[19, 42, 61] These gold-coating approaches quite naturally harken back to the earlier work in polymer nanopores,[4] provide a means to size-tune the nanopore through the dimensions and disposition of the metal layer, and can be used to alter nanopore performance by, e.g. leveraging the conductivity of the metal layer to press it into service as an additional electrode. The uncertain electrical potential of electrically floating metal films raises some concerns. Nanostructured coinage metal surfaces such as those often generated by electroless plating can add significant function to nanopore interiors, such as providing a catalytic moiety inside a highly constrained volume, by providing a reflective element to optically isolate each side of the nanopore membrane, or by providing the electric field enhancement necessary for optical interrogations by

surface-enhanced Raman spectroscopy (SERS). Thus, suitable metallization of nanopores or their environs might help to create multifunctional sensing platforms with complementary signal readouts. In spite of this, it remains vital to develop new methods independent of metallization to covalently functionalize the highly constrained inner surfaces of nanopores.

2.3 Using Nanopore Conductance to Characterize Nanopore Surface Chemistry

In the nano*particle* field, surface chemistry is an incredibly important question to address, but is also one that is incredibly challenging to answer definitively, even with the functionalized surface exposed to investigation using conventional surface analysis tools. A nano*pore* can be imagined to be a kind of *inverse* nanoparticle, so that the difficulties of characterizing its surface chemistry by conventional instrumental methods are amplified by the difficulty of accessing the internal surface of interest. That internal surface, however, is what the supporting electrolyte must come in contact with in order to carry out most nanopore experiments. Straightforward measurements of the nanopore conductance afford the opportunity to directly assess both the nanopore size and its surface chemistry. Electrodes in each reservoir can be used to establish a potential difference across the nanopore that drives ions through the nanopore and generates the open-pore current flow dictated by the conductance (G). The presence of a nanopore surface charge structures the electrolyte-nanopore interface, with a cationic surface drawing anionic counterions, and vice versa. The resulting Debye layer thickness is determined by the ionic strength of the electrolyte, and adds a *surface* conductance term to the usual bulk nanopore conductance [59, 69-76]

$$G_{\text{access-free}} = G_{\text{bulk}} + G_{\text{surface}} = K \cdot A(r, L) + \mu|\sigma| \cdot B(r, L) \quad (1)$$

There is an additional access resistance term important when the length of the nanopore, L, is comparable to its diameter, $2r$; we set $G \sim G_{\text{access-free}}$ in the subsequent treatment to more clearly

show the enhancement of the nanopore conductance by the presence of a surface charge, but offer the full expression here for a cylindrical nanopore of radius r_0 and length L:

$$G = K\left(\frac{1}{\frac{\pi r_0^2}{L} + \frac{\mu|\sigma|}{K}\cdot\frac{2\pi r_0}{L}} + \frac{2}{\alpha\cdot 2r_0 + \beta\cdot\frac{\mu|\sigma|}{K}}\right)^{-1} \quad (2)$$

where α and β are model-dependent parameters.[76] We note that while the access resistance is typically approximated using only its geometric component, it has been shown experimentally to be affected by a surface chemistry term as shown in Equation (2).[76] The bulk conductance is determined by the number of ions that can pass through the nanopore volume, and is readily calculated as the product of the bulk solution conductivity, K, and a nanopore volume integral $A = \left(\int \frac{dz}{\pi(r(z))^2}\right)^{-1}$, where r is the radius at a particular position, z, along the pore longitudinal axis. The surface conductance is given by product of the nanopore surface charge density (σ)—the charge that governs the attraction of counterions to the surface—with the counterion mobility μ and a nanopore surface area integral, $B = \left(\int \frac{dz}{2\pi r(z)}\right)^{-1}$. The key to using so simple an experimental measurement as the nanopore conductance to profile the surface coating is to recognize that the nanopore surface charge density is dependent upon the pK_a of any ionizable groups on the nanopore surface, the total surface density, Γ, of that group, and the solution pH

$$\sigma\left(\psi_D(\mathrm{pH}-\mathrm{pK_a},\Gamma)\right) = \frac{2\epsilon\epsilon_0\kappa}{\beta e}\sinh\left(\frac{\beta e\,\psi_D(\mathrm{pH}-\mathrm{pK_a},\Gamma)}{2}\right). \quad (3)$$

The calculation of σ is complicated by the dependence of the diffuse-layer potential, ψ_D, on σ, itself.[74] The constants $\epsilon\epsilon_0$, β, and e are the solution permittivity, the inverse of thermal energy, and the electron charge. The Debye screening length, κ^{-1}, depends on the number density of electrolyte ions, n ($\kappa^2 = \beta e^2 n/\epsilon\epsilon_0$), and sets a length scale for the influence of the nanopore

surface into its constrained, nanoconfined "bulk". In brief, the magnitude of the surface conductance term is determined by the native chemical properties of the nanopore surface termination (pK_a, Γ), and can be tuned by solution properties including the pH and ion concentration (n). The change in nanopore conductance with solution pH is perhaps most informative in this sense:

$$\frac{dG}{d\mathrm{pH}} \propto \frac{d|\sigma|}{\mathrm{pH}} \tag{4}$$

where the *chemical* origins of the change in surface charge originate in pH-dependent protonation and deprotonation of surface terminal groups involved in acid-base equilibria such as (for SiN_x nanopores):[50, 77]

$$\text{Si-R-OH } (\sigma = 0) \rightleftharpoons \text{Si-R-O}^- \ (|\sigma| > 0) + H^+; \ \frac{dG}{d\mathrm{pH}} > 0 \tag{5}$$

$$\text{Si-R-NH}_2 \ (\sigma = 0) + \mathrm{H}^+ \rightleftharpoons \text{Si-R-NH}_3^+ (|\sigma| > 0); \ \frac{dG}{d\mathrm{pH}} < 0 \tag{6}$$

For SiN_x, the presence of both such equilibria implies the presence of an isoelectric point at which there is a minimum in the conductance versus pH curve where the net $\frac{dG}{d\mathrm{pH}} = 0$. For SiN_x nanpores, this isoelectric point is ~4.3.[50, 78, 79] For surfaces terminated in moieties that do not undergo acid-base equilibria, $\frac{dG}{d\mathrm{pH}} = 0$ across all pH values (absent other mechanisms to change the nanopore conductance).

Changes in nanopore surface chemistry can thus be assayed through simple measurements: a change in G_{bulk} reveals the thickness of surface coatings (ideally equal to the molecular length for an upright monolayer), and a change in G_{surface}—in particular a change in surface pK_a from a measurement of G versus pH—reveals a change in surface termination. Methods to extract such

key nanopore specifiers as size and surface chemistry have been extensively detailed in the literature.[56, 59, 69, 72, 77, 80]. It should also be clear from this section that nanopore physicochemical properties such as charge can be tuned through chemical modification and changes of solution properties, thus providing different ways to control possible interactions between a nanopore and a passing analyte.

2.4 Analyte-Induced Nanopore Conductance Changes

Passage of a target molecule, nanoparticle, or complex through the nanopore perturbs the open-pore ionic current and provides molecular-level information. That information naturally depends on the target's dimensions and physicochemical properties and the ionic solution composition, but it is also profoundly affected by the size, shape, and surface chemistry of the nanopore. In the case of a (cylinder-like) double-stranded DNA polymer that fills the entire length of a cylindrical nanopore as it transits through, a simple geometric treatment considering only the displacement of bulk ions by the polymer gives a straightforward expression for the macromolecule-induced conductance change (most commonly a blockage) [81]

$$\chi_B \equiv \frac{(\langle G \rangle - \langle G_b \rangle)}{\langle G \rangle} \cong \left(\frac{r_{DNA}}{r_0}\right)^2 \tag{7}$$

where $\langle G \rangle$ and $\langle G_b \rangle$ are the time-averaged conductances through an unobstructed and DNA-containing nanopore, respectively, and r_{DNA} and r_0 are the cross-sectional radii of the molecule and nanopore. When the translocating object, such as a nanoparticle, is smaller than the nanopore extent, the conductance change can be expressed [75, 82]

$$\chi_B = \frac{D}{L}\left(\frac{\arcsin(d/D)}{\sqrt{1-\left(\frac{d}{D}\right)^2}} - \frac{d}{D}\right) \tag{8}$$

where d is the particle diameter, D is the nanopore diameter, L is the length of the nanopore, and similar, albeit more complex, expressions can be derived for analytes with nonspherical shapes.

Careful control experiments in conjunction with rigorous theoretical work and simulations provide detailed insight into the panoply of complex phenomena—including surface charges on analyte and nanopore surfaces—giving rise to conductance perturbations in nanopore sensing.[83, 84] In a more comprehensive framework invoking nanopore surface charge and access resistance, the nanopore conductance change arising from the passage of λ-DNA (cross-sectional radius $r_{\lambda-\mathrm{DNA}}$, effective linear charge density $q_{\lambda\text{-DNA}}$) through the nanopore is given by [84, 85]

$$\Delta G_{\lambda-DNA} = G - K\left(\frac{1}{\frac{\pi r_{\text{with DNA}}^2}{L} + \frac{\mu|\sigma|}{K}\cdot\frac{2\pi r_0}{L} + \frac{\mu}{K}\cdot\frac{q_{\lambda\text{-DNA}}}{L}} + \frac{2}{\alpha\cdot 2r_{\text{with DNA}} + \beta\frac{\mu|\sigma|}{K}}\right)^{-1} \quad (9)$$

where $r_{\text{with DNA}} = \sqrt{r_0^2 - r_{\lambda\text{-DNA}}^2}$. Equations (7) and (8), in convenient closed-form, appropriately underscore the importance of nanopore dimension and provide molecular-level profiling. This geometric basis of the conductance change has been used to infer biopolymer conformation: for example, a folded-over polymer presents a larger effective cross-section than a linear one.[86] When measured with a nanopore of sufficient size, the conformational flexibility of λ-DNA allows it to translocate linearly or folded, with corresponding $\Delta G = \mathrm{n}\Delta G_{\lambda-DNA}$ (n=1, 2, etc.).[85, 87] Analyte length is related to the duration of the current perturbation during translocation, with translocation time also determined by parameters such as analyte charge density, solution viscosity, applied electric field, and interactions between analyte and nanopore surface that can be moderated by controlling the surface chemistry of the nanopore and/or the analyte. The dependence of current change on single-stranded DNA base sequence, for example, underpins

efforts to sequence single strands of DNA using nanopores. In a dramatic demonstration of the dependence of nanopore "blockage" magnitude on analyte properties and sensing conditions, DNA passing through a conventional SiN_x nanopore at pH 7.5, will decrease the transient conductance at high salt concentrations, cause no change at 370 mM KCl, and actually enhance the transient analyte-induced conductance at solution concentrations below that level.[84]

3 ANALYTE TRANSPORT

3.1 Electrophoresis

The dominance of DNA as a target for nanopore sensing has meant that the bulk of the foundation of the field's understanding of nanopore sensing rests on studies focused on this anionic polymer. The forefront featured mechanism for analyte translocation through nanopores is thus electrophoresis,[8, 77, 88] in which the electric field applied across the nanopore interacts with the analyte charge—dictated by the sequence-independent phosphate backbone charge—to drive the biopolymer through the nanopore with an electrophoretic migration rate given by v

$$v = \mu_{\mathrm{ep}} E = \frac{\chi\left(\left\{\mathrm{q_i^{analyte}}\left(\mathrm{pH,pK_a^{i,analyte}}\right)\right\}, t\right)}{f} E \tag{10}$$

where μ_{ep} is the electrophoretic mobility in response to an applied electric field E. The friction coefficient, f, depends on the analyte size. To account for a polymer with variable monomer units having the potential to carry different charges, the notation $\left\{\mathrm{q_i^{analyte}}\left(\mathrm{pH,pK_a^{i,analyte}}\right)\right\}$—replacing the more conventional single value of the analyte charge, q^{analyte}—is used to denote that the segment of analyte subjected to the cross-pore electric field at a particular time can contain several charged moieties, each with its own pK_a that dictates its charge in a particular solution pH. The (unspecified weighting) function $\chi\left(\left\{q_i^{\mathrm{analyte}}\right\}, t\right)$ is used to reflect that while at any moment in time, *t*, a particular set of charges will be physically entrained inside the nanopore, the electric field extends beyond

the physical limits of the nanopore. The length of the nanopore and the extent of the electric field outside the physical nanopore confines would average the effective charge "within" the pore at any moment across multiple monomers. The important part to emphasize, however, is that the electrophoretic mobility depends on the applied electric field and the *analyte* physicochemical properties.

Protein and glycan polymers will show no DNA-like uniformity of charge in general, and thus the segments of each analyte entrained in the nanopore will present different charges. Depending on the size of the biopolymer analyte relative to the nanopore, steric hindrance may forcibly linearize a protein, may induce partial unfolding, or may allow passage of the analyte with its native conformation undisturbed. By size-tuning the nanopore one thus has the ability to probe an analyte's net charge, or to map out its charge distribution (perhaps in concert with its conformational stability). Without careful experimental design and execution, however, one would be forced to *contend* with such a heterogeneous presentation of charge from the same analyte. More broadly, though, it is not enough to consider only the "native" monomer charge. Neutral molecules that can sorb ions from solution can be given an effective electrophoretic mobility by virtue of this charge. This mechanism has been strongly indicated in the case of polyethyleneglycol (PEG), and has in fact allowed exquisitely resolved measurements of polymer length and led to the coining of the term "nanopore single molecule mass spectrometry".[89] Moreover, in the framework of purely electrophoresis-determined motion, a reversal of analyte charge polarity—from solution pH changes in (amphoteric) analyte protonation state, for example—is significant as the direction of driven motion would change.

3.2 Electroosmosis

The importance of nanopore surface chemistry reappears in an additional electrokinetic mechanism for translocating analytes through a nanopore: electroosmosis.[8, 65, 77, 79, 88, 90-92] This mechanism operates with charged and neutral species and thus considerably opens up the potential analyte scope for nanopore sensing. When electroosmosis occurs and the analyte is also charged, the two mechanisms act together,

$$\begin{aligned} v &= \left(\mu_{\mathrm{ep}} + \mu_{\mathrm{eo}}\right)E \\ &= \left(\mu_{\mathrm{ep}}\left(\left\{\mathrm{q_i^{analyte}}\left(\mathrm{pH,pK_a^{i,analyte}}\right)\right\}\right)\right. \\ &\quad \left. + \mu_{\mathrm{eo}}\left(\left\{q_i^{\mathrm{nanopore}}\left(\mathrm{pH,pK_a^{i,nanopore}}\right)\right\}\right)\right)E \end{aligned} \tag{11}$$

but their effect need not be in the same direction (the sign of each mobility is given by the relevant charge polarity) because the direction for electrophoresis is dictated by the *analyte* charge polarity while the direction for electroosmosis is dictated by the *nanopore* surface charge polarity. The relative influence of electrophoresis and electroosmosis for the nanopore-based sensing of a particular analyte can be tuned by adjustment of the solution pH (because it determines the degree of ionization for all acid-base equilibria present), or by changing the surface chemistry of the nanopore (thereby changing the $\mathrm{pK_a^{i,nanopore}}$). Changing the nanopore surface chemistry is more flexible than changing the solution pH because it does not have the potential to change the electrophoretic mobility, but it is more technically challenging. With sufficient device engineering, the effective surface charge—and thus the relative contribution of electroosmosis—can be electronically controlled, as has been illustrated, for example, in the DNA sequencing domain.[9]

3.3 Diffusion

A key point to consider is that these deterministic electrokinetic motions occur on a background of random motion due to diffusion (Figure 3).[1] In the biopolymer sequencing application space, diffusion can lead to back-stepping, and thus the "read" of the same monomer

(or series of monomers) more than once to give the appearance of a repeat sequence when only one is present. This challenge of potential back-stepping should be viewed in concert with the challenges of sequencing biopolymers with repeats: if sequencing measurements sufficiently dominated by deterministic analyte motion cannot be implemented, then measurements such as resequencing the same strand must be performed to gain sufficiently robust statistics to deal with such stochastic effects as backstepping. More broadly, given the potential for electrophoresis and electroosmosis to be of equivalent magnitude but opposite direction, one must also be careful to consider analyte translocation by diffusion, alone.[77]

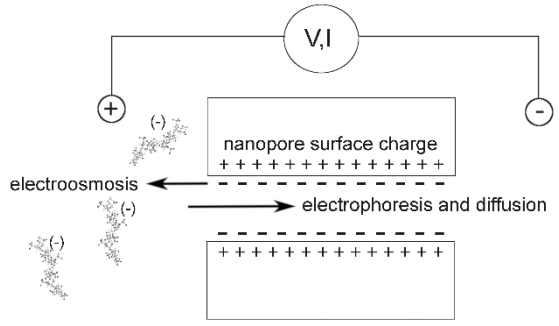


Figure 3. Schematic illustrating the interplay between electrophoresis, electroosmosis, and diffusion in analyte transport through a nanopore. The net direction for diffusion would follow the concentration gradient from left to right, but does not preclude instantaneous motion in the reverse direction. For the anionic analyte and nanopore with fixed positive surface charge shown, the directions for electrophoretic and electroosmotic motion would be opposed, with the relative magnitudes determined by a number of factors. Inversion of the nanopore surface charge would align the electroosmotic and electrophoretic directions for the anionic analyte.

Nanopore surface chemistry thus has two principal effects on analyte transport through the nanopore: it can affect it by direct interaction with the analyte (via electrostatic or chemical

interactions such as hydrogen bonding), or it can affect it through a medium-dependent interaction (e.g. electroosmosis). Clearly the ability to control nanopore surface chemistry could pay dividends in a multitude of ways, and thus efforts to wed organic synthesis to nanopore science should take on a high priority.

4 SENSING VISTAS

4.1 Environment and Sample Complexity

Sensitivity, selectivity, accuracy, and precision are familiar targets for analytical method and instrument development and application. The chemical laboratory with its advanced instrumentation, well-conditioned environment, and highly trained and regulated personnel offers considerable support for achieving good analytical results. Nevertheless, chemical analysis even in that setting remains an impressively challenging undertaking. Point-of-care and point-of-use sensors must operate in significantly more challenging environments and under more difficult conditions. Medical diagnostics targeted for the developing world, or remote or harsh environments, must frequently contend with an insufficiently resourced health care infrastructure. To give metrics to aim for, the World Health Organization (WHO) has expanded on this basic set of familiar targets by setting forth so-called ASSURED characteristics for diagnostics tests.[93] They should be Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Delivered to those who need it. One might well imagine that nanopore sensing—requiring conceptually little more than a <1 V power supply, a sensitive current amplifier, some salt water, and a nanoscale hole—might serve as a promising technology platform. On a more specific technical basis, nanopore sensing is direct in that it does not inherently require chemical labelling of an analyte (such as nonfluorescent molecules requiring pretreatment steps to be labelled with fluorophores to enable single-molecule fluorescence detection) and thus does not demand that

particular technical proficiency of the operator, nor infrastructure for chemical labelling steps, including waste disposal. There is also considerable potential for deployment in challenging environments for a sensor technology that can operates only electronically, as seen in the deployment on the International Space Station, and in West Africa during the 2014-15 Ebola outbreak of small-scale dongles that can plug directly into laptop computers.[94, 95] It is compelling to consider that nanopores might also serve a powerful role if deployed as real-time marine sensors: they work naturally in a salt water environment, and since blood, saliva, synovial fluid and the like can be described in a general sense as complex water-based samples, they bear a formal similarity to typical marine waters. The chemical manufacturing plant is an additional intriguing possibility for a chemical sensor with no moving parts (thus requiring neither "alignment" nor realignment). Given the pharmaceutical industry's need for high purity, and its use of formulation components ranging from small molecules to polymers to nanoparticles, the single-molecule sensitivity and size-scaling (from single to hundreds of nanometers) of nanopore sensing offers a potentially compelling complement to existing process monitoring and quality assurance methods.

4.1.1 Fingerprinting

While the use of nanopores for DNA sequencing has put a spotlight on the potential of nanopores for chemical *identification* and *analysis*, the power of chemical analytical *fingerprinting* should not be overlooked. This strategy is particularly powerful for quality assurance applications:[79] when detecting impurities at the end of, e.g. a pharmaceutical manufacturing run, or when analyzing in-market clinical therapeutics or street drugs, one need not necessarily identify the components, but rather be satisfied with detecting a departure—any departure—of the characteristic signal characteristics from those of a known standard. While one might ask how

much variability in signal characteristic "fingerprint" might emerge from passing analytes through a hole, the platform and technique offers numerous parameters to tune to match a single nanopore to particular conditions, or to design an array for array-based sensing:[96] nanopore size, shape, and surface chemistry; solution conditions such as ionic strength, electrolyte composition, and pH; and experimental parameters such as voltage polarity and magnitude. For an amphoteric nanopore, there is a strong and useful interplay between solution pH and voltage polarity in tuning analyte signal characteristics by changing the interplay between electrophoresis and electroosmosis.[77, 79]

4.1.2 Chemical Selectivity by Nanopore Force Spectroscopy

One particularly compelling method to enhance the selectivity of nanopore sensing is to use molecular recognition agents in conjunction with a technique known as nanopore force spectroscopy (NFS).[13, 97-100] In NFS, an applied electrical force draws a folded molecule or complexed pair against a nanopore that is too small to permit passage of the whole (Figure 4). Under continued force, the intramolecular interactions stabilizing the conformation or the intermolecular attractions stabilizing the complex are ruptured and the molecular structure is unfolded or the complex is dissociated.[97-99, 101-104] The time required for these processes gives direct information about the interaction energetics.[105] In particular, the dissociation timescale for a molecular recognition agent bound to its target is expected to be significantly different than if nonspecifically interacted with a matrix element. Thus, only a timer and molecular recognition agent must be added to the usual complement of nanopore needs: relatively straightforward electronics, salt water, and nanoscale hole. For the rupture of noncovalent interactions, thermal energy contributes appreciably to the dissociation: individual dissociation timescales are stochastically distributed and it is necessary to measure ≥100 individual

dissociations to construct a curve of the survival probability of the association as a function of time.[97-99] This curve can then be fit to yield a single characteristic timescale that corresponds to the dissociation timescale.

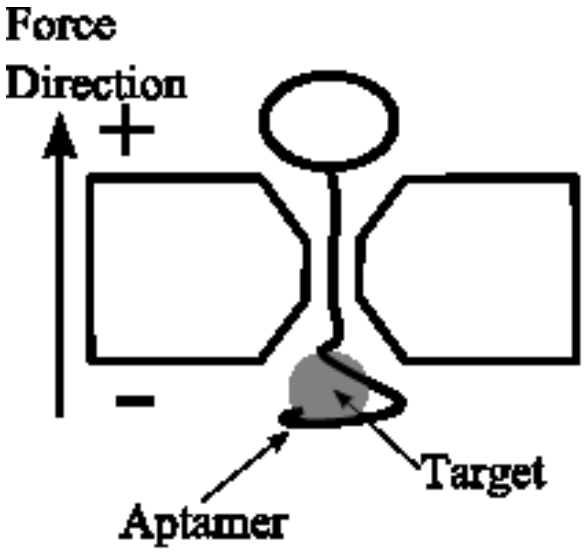


Figure 4. Schematic of a nanopore force spectroscopy experiment using an aptamer. In this implementation, electrophoretic insertion of an aptamer through a nanopore allows it to bind to a target present in the opposite well. The bead (open circle) bound to the aptamer prevents passage of the aptamer through the nanopore. The target species stabilizes a conformation of the aptamer that makes passage of the aptamer back through the pore, in the direction of applied force, impossible without dissociation of the complex.

4.2 Small Molecules

The protein nanopore α-hemolysin and track-etched polymer nanopores have both been used to sense individual small molecules, an application contrasted with the more familiar use of nanopore platforms for sensing macromolecules and nanoparticles. Polymer pores haven been used to detect porphyrin molecules using single pores,[4, 106] and quinine, methyl viologen, and $Ru(bpy)_3^{2+}$ in a multi-pore assay format.[12] Engineered α-hemolysin nanopores have been used to detect metal

ions,[107] a wide range of small molecules from neurotransmitters such as dopamine,[108] and threats such as trinitrotoluene (TNT),[109] and to distinguish the subtlest of chemical differences such as differentiating enantiomers including amino acids and therapeutics such as thalidomide.[110, 111] Given the prevalence of small molecules in medicine, as therapeutics and as the targets of assays, and as the products of biochemical processes, nanopore scientists should not overlook the capability that nanopores have to sense small molecules. In the –omics context, the demonstrated ability to sense chemical entities smaller than the canonical biological macromolecule, DNA,[13, 14] is incredibly exciting and should motivate the expansion of this domain of nanopore science.

4.3 Genomics and DNA-Based Sensing

4.3.1 DNA Sequencing

Genomics has been the beneficiary of the most concentrated effort in nanopore single-molecule sensing, focused primarily on sequencing DNA (and RNA[3]).[1, 13, 112] The efforts can be largely distilled to efforts to answer the following question: how to take the measured current perturbations as a DNA oligo- or polymer passes through a nanopore, and extract the sequence of DNA bases from those perturbations. Advances in nanopore DNA sequencing arose through methodical control experiments, broadly summarized here: early experiments used homopolymer samples of varying lengths so that the current perturbations of unique bases in sequence could be measured (without needing to slow translocation speed to detect single bases); work progressed to DNA block copolymer samples in which an uninterrupted sequence of one base was followed by the next (again allowing the differences in signal between bases to be resolved without the necessity of slowing the translocation speed). Naturally these initial benchmark experiments then progressed to measuring signals from DNA strands with more varied

base sequences. Key issues that have been addressed by measurement, discovery, and technological advance, have been how to control the DNA strand speed to allow for electronic readout with sufficient signal-to-noise ratios (strategies include using enzymes to control translocation, laser optical tweezers, and more prosaic changes such as solution viscosity and supporting electrolyte identity—tetramethylammonium, for example, slows DNA translocation[113]); how to improve limits of detection (high salt concentration gradients from one side of the nanopore membrane to the other can increase molecular detection frequency[114]); how to prevent analyte sticking to the nanopore; and even how to reliably fabricate nanopores (and from what material; SiN_x is a standout choice). Chemical tagging and conversion schemes have also been proposed and developed to label particular sequences of DNA—to provide sequence recognition and increase signal magnitude—and to chemically transform short sequences of DNA into moieties that are easier to detect than a small number of bases. Sequence tagging is particularly interesting: tags can provide selectivity through molecular-recognition-like processes, can provide steric bulk to enhance the magnitude of the current perturbation, and can provide size, charge, and/or solution drag to slow translocations to within the signal bandwidth of the electronics. In recognition of the difficulty of traditional resistive-pulse measurements to provide DNA base sequence, others have pursued alternative and complementary schemes including augmenting nanopore measurements with optical readout, and employing transverse electrodes for tunneling-based measurements of DNA bases as oligomers pass through the nanopore.[1, 9, 16, 115] The first step in these schemes is to optimize analyte translocation through a nanopore formed in materials that can be used as a fabrication platform. In this sense, silicon nitride is an ideal choice for optimizing nanopores: it is a ubiquitous nanofabrication material and can support the integration of more sophisticated components required for alternate readout schemes.[1, 49, 50]

4.3.2 DNA-Based Chemical Selectivity

While DNA sequencing has been the most prominent driving force for the development of nanopore sensing, there are significant benefits of this work for a more general chemical sensing program. For example, chemical sensing schemes to label analytes using DNA-based tags that can be differentiated by nanopore sensing have been proposed and developed.[116] Molecular recognition agents used in conjunction with implementations of NFS hold substantial promise to widen the analyte scope of nanopore biosensing beyond genomics (Figure 4). The use of molecular recognition agents rooted in the firmament of nanopore sensing of nucleic acids can provide a reassuringly solid basis for work to challenge nanopore chemical selectivity with a broader and more varied set of target species. Aptamers are nucleic acid oligomers that are incredibly versatile molecular recognition agents, and are indeed frequently referred to as artificial antibodies. While biomolecular recognition is far more familiar in the form of antibody-antigen interactions, chemically synthesized aptamers have greater reproducibility. Aptamers rival antibodies in target selectivity and affinity, and they can distinguish between molecules that differ only in small degree, such as the presence of a methyl or hydroxyl group,[117-122] or that differ only in conformation, as in the case of misfolded prion proteins responsible for afflictions such as Creutzfeldt-Jakob disease.[123] Aptamers can fold into a conformation that is energetically stabilized by complexation with its designed target: adenine, for example, stabilizes the conformation of the pbuE adenine riboswitch aptamer of *Bacillus subtilis* by 6 kcal/mol.[124] Both the fundamental biophysics of aptamer-ligand interactions[125] and the bioanalytical application of aptamer recognition[121, 122] are promising areas for exploration using NFS. While aptamers do occur naturally, they can be custom designed through a highly optimized process of *in vitro* evolution (SELEX) to be able to target chosen ions, small molecules, and macromolecules such as

proteins and other nucleic acids.[117, 126, 127] The use of aptamers in conjunction with NFS will thus contribute substantially to expanding the analyte scope of nanopore sensing while leveraging the extensive body of knowledge regarding the use of nucleic acids and nanopores.[1, 98, 99, 128, 129] For example, NFS for genotyping through hybridization assays has been shown to readily yield single nucleotide selectivity.[98, 99] A number of experiments have demonstrated the potential for using aptamers to enhance nanopore selectivity.[128, 130-135] By using NFS with aptamers which can be designed to target a tremendous breadth of targets—from small ions to cells—one can imagine sufficient development leading to a single, general biosensing platform capable of achieving a wide range of different biosensing aims.

4.4 Proteomics

Whereas nanopore-based resistive-pulse sequencing of DNA requires a series of nanopore current blockages to be associated with a unique sequence of the *four* naturally occurring DNA bases (and their methylated variants), protein sequencing involves establishing the correct sequence of amino acid monomers drawn from the *twenty* naturally occurring amino acids. There is thus a dramatic leap in complexity in progressing from genomic applications of nanopore sequencing to proteomic applications. Proteins hold out still greater complexity to a prospective analysis tool: the variable charge density allowed by the 20 different amino acid pK_a values, the ability to tune that charge density (and polarity) through solution pH in a way that the charged DNA phosphate backbone doesn't allow, and the prevalence of protein conformation and higher order structures (playing a clear biological functional role). The literature covering nanopore-based protein sensing has included studies of protein and peptide translocation through pores (including interactions with the pore); and protein folding and unfolding in the context of nanopore

measurements (including using NFS to assess protein conformational energetics and kinetics).[6-10, 13, 14, 16, 17, 19, 136-150]

Rather than exhaustively cataloguing the studies, we choose to focus on selected results that foreshadow the challenges faced in extending nanopores to biopolymers beyond those of genomics and proteomics. We feature two reports showcasing nanopore protein analysis that underscore particular complexities of nanopore sensing in the face of analyte diversity. In work reported in 2011, Yusko and co-workers[63] revealed a method to form fluid coatings on SiN_x nanopore surfaces. While impressive as fundamental work in a "bio-inspired" vein, it also helped to prevent pore clogging when studying the translocation of amyloid-beta oligomers and fibrils implicated in Alzheimer's disease. The authors noted that these peptides tend to aggregate and clogged nanopores, and that "despite several attempts, we were unable to detect translocation events from samples of [amyloid-beta] peptides using uncoated pores".[63] Given the chemical and structural diversity of the targets of proteomics (and beyond), it is entirely reasonable to expect that there will be a strong need to develop approaches to control or harness a wide range of possible analyte-pore interactions. The chemical and structural complexity of proteins is also seen in work examining the electrokinetics of protein transport through (surface-charged) silicon nitride nanopores. Depending on nanopore and analyte charge distributions, electroosmosis could overwhelm electrophoresis as the effect determining the direction of analyte motion.[77] Colloquially, this observation has been described as '"anomalous" translocation behavior'—certainly true in comparison to the DNA case: the analytes can travel the "wrong way" with respect to the typical, or assumed, electrophoretic direction.[77] Changing the solution pH changed the distribution and density of charge on both the nanopore and analyte surfaces by changing the degree of dissociation of the species involved in acid-base equilibria in each. While tuning the pH

of the electrolyte solution bathing surface functionalized nanopores has been shown to affect DNA translocation speed and detection frequency, this *reversal of polarity* for detection awaited work with proteins.[65, 77]

4.5 Glycomics Rising

4.5.1 Answering the Call

The National Academies of Sciences Consensus Report Transforming Glycoscience: A Roadmap for the Future[26] made a clear and strong call for "a suite of tools…to detect, describe, and purify glycans from natural sources, and characterize their chemical composition and structure. The development of transformative tools for detection, … separation, and [structure determination] of carbohydrate structures and complex mixtures should be a high priority for [NIH, NSF,… and the FDA]." "Glycans play roles in almost every biological process and are involved in every major disease,"[26] are a source of energy, and provide therapeutic function (e.g. the anticoagulant heparin).[151-162] Polysaccharide complexity challenges conventional chemical analysis:[26, 163] ~120 naturally occurring monomers with variability in e.g. sequence, linkages, and polymer branching.[163] In 2008 contamination of heparin by structurally similar oversulfated chondroitin sulfate (OSCS) resulted in adverse clinical consequences in the U.S., including ~100 deaths.[164-169] While new analysis tools have been broadly called for,[26, 163, 170] single-molecule-sensitive methods that can cope with sample heterogeneity and low abundance—without amplification methods as for DNA[171]–are especially important. Nanopore science thus has the potential to make a contribution to glycomics that could be beyond even its contribution to genomics and proteomics where the existing analysis technology leaves less of a gulf between aims and achievability.

4.5.2 Prior Work

A number of elegant and informative glycan characterizations have been carried out using protein and abiotic nanopores.[79, 172-182] In 2002, Kullman and co-workers[175] studied the translocation of maltodextrins through maltoporin, a naturally occurring membrane protein showing faster uptake of maltooligosaccharides than other oligosaccharides. This study with a single pore was contrasted by the authors with earlier studies using multiple pores, and allowed them to conclude that the measured pore blockages were the result of oligosaccharide translocation and not merely pore binding.[175] Oukhaled and co-workers used anionic dextran sulfate to experimentally study the effect of electrostatic screening on the transport of charged polymers through α-hemolysin nanopores, and found that the kinetics of transport slowed down by nearly two orders of magnitude when the Debye screening length was on par with the nanopore size. In these experiments, electrophoresis was the sole deterministic driving force for biopolymer translocation considered.[181] In 2011 Bacri and co-workers[172] used α-hemoslysin protein pores to study neutral maltose and dextran oligosaccharides which differ by having 1→4 and 1→6 glycosidic bonds, respectively. The more rigid 1→4 glycosidic bond was postulated to explain the more dramatic decrease in event frequency with increased molecular weight for maltose versus dextran oligosaccharides. Dwell time changes with oligosaccharide molecular weight were detected and in addition to providing information on polymerization degree, provided insight on the process of nanoscale confinement in the pore forcing conformational changes (linearization) of the larger oligosaccharides. In 2012, the substrate scope was further expanded. Anionic hyaluronic acid and its enzymatic digestion products were detected by translocation through α-hemoslysin protein pores, with differences in dwell time providing sizing resolution.[173] Similar studies exploring enzyme digestion kinetics were carried out using aerolysin nanopores.[174]

Glycan branching has the potential to increase glycan size beyond the size of the most readily available protein nanopores, so that size-tunable nanopores may hold the greatest relevance as a (general) tool for glycomics. In the arena of glycan analysis, there is an uptick in the adoption of polymer nanopores. In 2011, Ali and co-workers[183] anchored the protein concanavalin A—a protein that can interact with mannosyl and glucosyl residues present in polysaccharides and glycoproteins—inside a polymer nanopore. When the functionalized pore was exposed to glucose, the greater affinity of the protein for the free glucose versus the mannose residues binding it to the surface resulted in liberation of the protein from the surface. This competitive binding assay was transduced as a change in the conductance of the nanopore, owing to the change in the physical pore dimension with the loss of the protein. A much simpler glucose-sensitive polymer nanopore based on current rectification was created by surface termination with a boronic acid, where the response to the sugar could be pH-gated.[180] Reversible binding between the boronic acid and diols of saccharides and glycoproteins in solution produced changes in current rectification for both analyte classes, and changes in the overall conductance for the larger glycoprotein.[178] Prior work has also been done using other pore materials. Nanopore current rectification was achieved, without the need for covalent surface coupling, by immersing a quartz nanopipette nanopore in a sugar-binding polymer that could swell or collapse in response to that target.[179] Nanopore current rectification was used to detect glucose in a quantitative manner through the use of glass nanopores surface-functionalized with phenylboronic acid.[176] Here, the combination of an asymmetric nanopore geometry decorated with a chemically selective recognition element (the boronic acid), was essential for the analytical achievement. Sensitivity to three different stimuli—pH, temperature, and sugars—was engineered into a single glass nanopore device, increasing the complexity of the analytical capabilities.[177]

As in the earlier use of nanopore for DNA sequencing, thin-film nanopores have emerged only very recently for glycomics. The Hall group has recently used He-ion drilled SiN_x nanopores to provide molecular weight discrimination of hyaluronic acid polymers, with synthetic samples ranging in molecular weight from 54 kDa to 2.4 MDa, and natural samples acquired from equine synovial fluid.[182] The latter studies, performed on as little as 10 ng of hyaluronic acid in ~2 h, were performed as a test of this nanopore method for assaying samples acquired from an equine model of osteoarthritis. This work, with fairly small ~6.5-8.6 nm-diameter pores, allowed observation of different conformations of hyaluronic acid, notably linearized and folded-over. In exploratory and proof-of-principle work from our group using SiN_x nanopores prepared by controlled dielectric breakdown,[79] we were interested in challenging SiN_x nanopores with analyte diversity as a first step towards the utility of this basic platform for sequencing applications. We were also interested in testing the fingerprint assay paradigm for the quality assurance of therapeutics. One of our analytes, heparin, is an interesting and important choice addressing both interests. It is a prevalent clinically used anticoagulant that holds the distinction of being the most highly charge-dense biological macromolecule known.[184] As noted above, an undetected contaminant in the clinical supply of this important pharmaceutical caused deaths in 2008.[164-169] We were able to measure linear calibration curves for heparin samples and, through a simple statistical analysis of the nanopore fingerprints of the therapeutic and toxic impurity, detect the impurity when mixed into certified heparin samples. We are excited by the tantalizing application horizons that this result might portend in the pharmaceutical industry, in particular with increasing interest in using glycoengineering to improve protein drugs, and with the importance of glycans as constituents of small molecule drugs.[185, 186] In the case of the demonstration of impurity

sensing in heparin by fingerprint analysis, we have a demonstrated case of the pharmaceutical industry quality assurance prospects proposed above.

4.5.3 Prospects

In the grander context of sensing vistas and how different molecule classes can present opportunities and challenges, the charge density of heparin makes it an extreme case. Certainly, electrophoresis would be expected to be the dominant mechanism for analyte translocation, and the observed direction of translocation was consistent with electrophoretic motion.[79] The high charge density presented no insurmountable obstacles to heparin detection through SiN_x nanopores, in spite of often challenging SiN_x surface chemistry that might have led to pore clogging.[49, 50, 63, 79] The other oligo- and polysaccharides we chose as part of our effort to expand glycan scope for evaluating the prospect of using SiN_x nanopores for glycomics were far from heparin's extremes of composition and behavior. Their lower charge density could have presented challenges for SiN_x nanopore sensing. In particular, potential interactions with the complex surface charge distribution of the SiN_x,[49, 50, 63, 79] might be exacerbated by the potential for slower translocation through the pore because the lower analyte charge density lowers the electrophoretic driving force, and depending on solution pH, might be further diminished by an electroosmotic driving force in the opposite direction. Put in stark terms, unwanted interactions between analyte and nanopore surface might have led to irreversible or long-term clogging. While changes of pH on either side of the SiN_x isoelectric point did, indeed, require a reversal of applied voltage polarity to detect analytes—supporting an increased role for electroosmosis—and affected the frequency of analyte detection (undoubtedly due to an effectively lower net electrokinetic force drawing analyte into and across the pore), none of the analytes explored irreversibly clogged the nanopores.[79] The successful translocation of hyaluronic acid through He-ion milled SiN_x pores,

likely with different surface chemistry than those fabricated by controlled dielectric breakdown, was similarly not prevented by pore clogging.[182] Here, the repeated successful translocation, detection, and characterization of a diverse set of glycans through SiN_x nanopores with a variety of sizes is an exciting precedent for considering the longer-term prospects of nanopores in glycomics. Successful detection without considerable effort at optimizing conditions established for DNA sequencing means that nanopore glycomics might successfully borrow from the core approaches of nanopore genomics. The success of chemical tuning, such as changing electrolyte ionic strength and pH, and using enzymes to selectively and controllably alter the analytes, opens up additional means to use nanopores to profile a diversity of glycans. This should all be considered in the context of the straightforward compatibility of SiN_x thin films with nanofabrication work flows, thereby encouraging the consideration of adding complementary analyte control and readout elements to the core nanopore platform.

5 OUTLOOK

Nanopores have come to occupy a prominent role in genomics, through the demonstrated ability of devices using protein nanopores to provide DNA sequencing information, and through the prospect of solid-state pores to allow the development of still more sophisticated nanopore devices that promise greater sensing capabilities for genomics. This development arc is underway for protein characterizations and is beginning for glycan profiling, and impressive accomplishments in small molecule detection and analysis should not be overlooked. The performance horizons for proteomics, glycomics, metabolomics, wearable sensors, environmental monitoring, and quality assurance applications remain bright and tantalizing. There is ample precedent in the literature to generate considerable excitement for the possibility that nanopore scientists can dramatically expand analyte scope, increase nanopore sensing capabilities through fabrication and method

development, and put powerful single-molecule sensing capabilities in the hands of non-experts in challenging environments to assay complex samples.

REFERENCES

1. Branton D, Deamer DW, Marziali A, Bayley H, Benner SA, Butler T et al. The potential and challenges of nanopore sequencing. Nat Biotechnol. 2008;26(10):1146-53. doi:10.1038/nbt.1495.
2. Wanunu M. Nanopores: A journey towards DNA sequencing. Physics of Life Reviews. 2012;9(2):125-58. doi:http://dx.doi.org/10.1016/j.plrev.2012.05.010.
3. Egatz-Gomez A, Wang C, Klacsmann F, Pan Z, Marczak S, Wang Y et al. Future microfluidic and nanofluidic modular platforms for nucleic acid liquid biopsy in precision medicine. Biomicrofluidics. 2016;10(3):032902. doi:10.1063/1.4948525.
4. Sexton LT, Horne LP, Martin CR. Developing synthetic conical nanopores for biosensing applications. Molecular BioSystems. 2007;3(10):667-85.
5. Choi Y, Baker LA, Hillebrenner H, Martin CR. Biosensing with conically shaped nanopores and nanotubes. PCCP. 2006;8(43):4976-88.
6. Oukhaled A, Bacri L, Pastoriza-Gallego M, Betton J-M, Pelta J. Sensing Proteins through Nanopores: Fundamental to Applications. ACS Chem Biol. 2012;7(12):1935-49. doi:10.1021/cb300449t.
7. Shi W, Friedman AK, Baker LA. Nanopore Sensing. Anal Chem. 2017;89(1):157-88. doi:10.1021/acs.analchem.6b04260.
8. Haywood DG, Saha-Shah A, Baker LA, Jacobson SC. Fundamental Studies of Nanofluidics: Nanopores, Nanochannels, and Nanopipets. Anal Chem. 2015;87(1):172-87. doi:10.1021/ac504180h.
9. Taniguchi M. Selective Multidetection Using Nanopores. Anal Chem. 2015;87(1):188-99. doi:10.1021/ac504186m.
10. Mathwig K, Albrecht T, Goluch ED, Rassaei L. Challenges of Biomolecular Detection at the Nanoscale: Nanopores and Microelectrodes. Anal Chem. 2015;87(11):5470-5. doi:10.1021/acs.analchem.5b01167.
11. Kocer A, Tauk L, Déjardin P. Nanopore sensors: From hybrid to abiotic systems. Biosens Bioelectron. 2012;38(1):1-10.
12. Bayley H, Martin CR. Resistive-Pulse Sensing-From Microbes to Molecules. Chem Rev. 2000;100(7):2575-94. doi:10.1021/cr980099g.
13. Reiner JE, Balijepalli A, Robertson JWF, Campbell J, Suehle J, Kasianowicz JJ. Disease Detection and Management via Single Nanopore-Based Sensors. Chem Rev. 2012;112(12):6431-51. doi:10.1021/cr300381m.
14. Howorka S, Siwy Z. Nanopore analytics: sensing of single molecules. Chem Soc Rev. 2009;38(8):2360-84.
15. Hou X, Guo W, Jiang L. Biomimetic smart nanopores and nanochannels. Chem Soc Rev. 2011;40(5):2385-401. doi:10.1039/C0CS00053A.

16. Miles BN, Ivanov AP, Wilson KA, Dogan F, Japrung D, Edel JB. Single Molecule Sensing with Solid-State Nanopores: Novel Materials, Methods, and Applications. Chem Soc Rev. 2013;42(1):15-28. doi:10.1039/c2cs35286a.
17. Albrecht T, Edel JB, Winterhalter M. New developments in nanopore research—from fundamentals to applications. J Phys: Condens Matter. 2010;22(45):450301.
18. Majd S, Yusko EC, Billeh YN, Macrae MX, Yang J, Mayer M. Applications of biological pores in nanomedicine, sensing, and nanoelectronics. Curr Opin Biotechnol. 2010;21(4):439-76.
19. Kudr J, Skalickova S, Nejdl L, Moulick A, Ruttkay–Nedecky B, Adam V et al. Fabrication of solid-state nanopores and its perspectives. Electrophoresis. 2015;36(19):2367-79. doi:10.1002/elps.201400612.
20. Healy K, Schiedt B, Morrison AP. Solid-state nanopore technologies for nanopore-based DNA analysis. Nanomedicine. 2007;2(6):875-97. doi:10.2217/17435889.2.6.875.
21. Healy K. Nanopore-based single-molecule DNA analysis. Nanomedicine. 2007;2(4):459-81. doi:10.2217/17435889.2.4.459.
22. Haque F, Li J, Wu H-C, Liang X-J, Guo P. Solid-state and biological nanopore for real-time sensing of single chemical and sequencing of DNA. Nano Today. 2013;8(1):56-74. doi:http://dx.doi.org/10.1016/j.nantod.2012.12.008.
23. Lee K, Park K-B, Kim H-J, Yu J-S, Chae H, Kim H-M et al. Recent Progress in Solid-State Nanopores. Adv Mater. 2018;30(42):1704680. doi:doi:10.1002/adma.201704680.
24. Fu K, Bohn PW. Nanopore Electrochemistry: A Nexus for Molecular Control of Electron Transfer Reactions. ACS Central Science. 2018;4(1):20-9. doi:10.1021/acscentsci.7b00576.
25. Piruska A, Gong M, Sweedler JV, Bohn PW. Nanofluidics in chemical analysis. Chem Soc Rev. 2010;39(3):1060-72. doi:10.1039/b900409m.
26. National Research Council. Transforming Glycoscience: A Roadmap for the Future. Washington, DC: The National Academies Press; 2012.
27. Soskine M, Biesemans A, De Maeyer M, Maglia G. Tuning the Size and Properties of ClyA Nanopores Assisted by Directed Evolution. J Am Chem Soc. 2013;135(36):13456-63. doi:10.1021/ja4053398.
28. Song L, Hobaugh MR, Shustak C, Cheley S, Bayley H, Gouaux JE. Structure of Staphylococcal α-Hemolysin, a Heptameric Transmembrane Pore. Science. 1996;274(5294):1859-65. doi:10.1126/science.274.5294.1859.
29. Derrington IM, Butler TZ, Collins MD, Manrao E, Pavlenok M, Niederweis M et al. Nanopore DNA sequencing with MspA. P Natl Acad Sci USA. 2010;107(37):16060-5. doi:10.1073/pnas.1001831107.
30. Ma L, Cockroft SL. Biological Nanopores for Single-Molecule Biophysics. ChemBioChem. 2010;11(1):25-34.
31. Sakai N, Matile S. Synthetic Ion Channels. Langmuir. 2013;29(29):9031-40. doi:10.1021/la400716c.
32. Sakai N, Mareda J, Matile S. Artificial beta-Barrels. Acc Chem Res. 2008;41(10):1354-65. doi:10.1021/ar700229r.
33. Litvinchuk S, Tanaka H, Miyatake T, Pasini D, Tanaka T, Bollot G et al. Synthetic pores with reactive signal amplifiers as artificial tongues. Nat Mater. 2007;6(8):576-80. doi:http://www.nature.com/nmat/journal/v6/n8/suppinfo/nmat1933_S1.html.
34. Hernández-Ainsa S, Misiunas K, Thacker VV, Hemmig EA, Keyser UF. Voltage-Dependent Properties of DNA Origami Nanopores. Nano Lett. 2014;14(3):1270-4. doi:10.1021/nl404183t.

35. Bell NAW, Thacker VV, Hernandez-Ainsa S, Fuentes-Perez ME, Moreno-Herrero F, Liedl T et al. Multiplexed ionic current sensing with glass nanopores. Lab on a Chip. 2013;13(10):1859-62. doi:10.1039/c3lc50069a.
36. Hernández-Ainsa S, Bell NAW, Thacker VV, Göpfrich K, Misiunas K, Fuentes-Perez ME et al. DNA Origami Nanopores for Controlling DNA Translocation. ACS Nano. 2013;7(7):6024-30. doi:10.1021/nn401759r.
37. Plesa C, Ananth AN, Linko V, Gülcher C, Katan AJ, Dietz H et al. Ionic Permeability and Mechanical Properties of DNA Origami Nanoplates on Solid-State Nanopores. ACS Nano. 2013;8(1):35-43. doi:10.1021/nn405045x.
38. Langecker M, Arnaut V, Martin TG, List J, Renner S, Mayer M et al. Synthetic Lipid Membrane Channels Formed by Designed DNA Nanostructures. Science. 2012;338(6109):932-6. doi:10.1126/science.1225624.
39. Wharton JE, Jin P, Sexton LT, Horne LP, Sherrill SA, Mino WK et al. A Method for Reproducibly Preparing Synthetic Nanopores for Resistive-Pulse Biosensors. Small. 2007;3(8):1424-30. doi:10.1002/smll.200700106.
40. Vogel R, Willmott G, Kozak D, Roberts GS, Anderson W, Groenewegen L et al. Quantitative Sizing of Nano/Microparticles with a Tunable Elastomeric Pore Sensor. Anal Chem. 2011;83(9):3499-506. doi:10.1021/ac200195n.
41. Menon VP, Martin CR. Fabrication and Evaluation of Nanoelectrode Ensembles. Anal Chem. 1995;67(13):1920-8. doi:10.1021/ac00109a003.
42. Whelan JC, Karawdeniya BI, Bandara YMNDY, Velleco BD, Masterson CM, Dwyer JR. Electroless Plating of Thin Gold Films Directly onto Silicon Nitride Thin Films and into Micropores. ACS Appl Mater Interfaces. 2014;6(14):10952-7. doi:10.1021/am501971n.
43. Bandara YMNDY, Karawdeniya BI, Whelan JC, Ginsberg LDS, Dwyer JR. Solution-Based Photo-Patterned Gold Film Formation on Silicon Nitride. ACS Appl Mater Interfaces. 2016;8(51):34964-9. doi:10.1021/acsami.6b12720.
44. Møller P, Nielsen LP. Advanced Surface Technology. Denmark: Møller & Nielsen APS; 2013.
45. Zabetakis D, Dressick WJ. Selective Electroless Metallization of Patterned Polymeric Films for Lithography Applications. ACS Appl Mater Interfaces. 2009;1(1):4-25. doi:10.1021/am800121d.
46. Karawdeniya BI, Bandara YMNDY, Whelan JC, Dwyer JR. General Strategy To Make an On-Demand Library of Structurally and Functionally Diverse SERS Substrates. ACS Applied Nano Materials. 2018;1(2):960-8. doi:10.1021/acsanm.7b00385.
47. Storm AJ, Chen JH, Ling XS, Zandbergen HW, Dekker C. Fabrication of solid-state nanopores with single-nanometre precision. Nature Materials. 2003;2(8):537-40. doi:http://www.nature.com/nmat/journal/v2/n8/suppinfo/nmat941_S1.html.
48. Li J, Stein D, McMullan C, Branton D, Aziz MJ, Golovchenko JA. Ion-beam sculpting at nanometre length scales. Nature. 2001;412(6843):166-9.
49. Dwyer JR, Harb M. Through a Window, Brightly: A Review of Selected Nanofabricated Thin-Film Platforms for Spectroscopy, Imaging, and Detection. Appl Spectrosc. 2017;71(9):2051-75. doi:10.1177/0003702817715496.
50. Dwyer JR, Bandara YMNDY, Whelan JC, Karawdeniya BI, Nichols JW. Silicon Nitride Thin Films for Nanofluidic Device Fabrication. In: Edel J, Ivanov A, Kim M, editors. Nanofluidics, 2nd Edition. 2 ed. RSC Nanoscience & Nanotechnology, vol 41: Royal Society for Chemistry; 2016.

51. Di Ventra M, Taniguchi M. Decoding DNA, RNA and peptides with quantum tunnelling. Nat Nano. 2016;11(2):117-26. doi:10.1038/nnano.2015.320.
52. Joshua JM, Robert NC, Kelly BM, Jon-Paul SD, Christopher CS, Joshua DW et al. Lift-off of large-scale ultrathin nanomembranes. Journal of Micromechanics and Microengineering. 2015;25(1):015011.
53. Williams KR, Muller RS. Etch rates for micromachining processing. J Microelectromech Syst. 1996;5(4):256-69. doi:10.1109/84.546406.
54. Vlassiouk I, Apel PY, Dmitriev SN, Healy K, Siwy ZS. Versatile ultrathin nanoporous silicon nitride membranes. P Natl Acad Sci USA. 2009;106(50):21039-44. doi:10.1073/pnas.0911450106.
55. Duan C, Wang W, Xie Q. Review article: Fabrication of nanofluidic devices. Biomicrofluidics. 2013;7(2):026501-41.
56. Bandara YMNDY, Karawdeniya BI, Dwyer JR. Real-Time Profiling of Solid-State Nanopores During Solution-Phase Nanofabrication. ACS Appl Mater Interfaces. 2016;8(44):30583-9. doi:10.1021/acsami.6b10045.
57. Kwok H, Briggs K, Tabard-Cossa V. Nanopore Fabrication by Controlled Dielectric Breakdown. PLoS ONE. 2014;9(3):e92880. doi:10.1371/journal.pone.0092880.
58. Briggs K, Kwok H, Tabard-Cossa V. Automated Fabrication of 2-nm Solid-State Nanopores for Nucleic Acid Analysis. Small. 2014;10(10):2077-86. doi:10.1002/smll.201303602.
59. Bandara YMNDY, Nichols JW, Iroshika Karawdeniya B, Dwyer JR. Conductance-based profiling of nanopores: Accommodating fabrication irregularities. Electrophoresis. 2018;39(4):626-34. doi:10.1002/elps.201700299.
60. Kuan AT, Lu B, Xie P, Szalay T, Golovchenko JA. Electrical pulse fabrication of graphene nanopores in electrolyte solution. Appl Phys Lett. 2015;106(20):203109. doi:doi:http://dx.doi.org/10.1063/1.4921620.
61. Wei R, Pedone D, Zürner A, Döblinger M, Rant U. Fabrication of Metallized Nanopores in Silicon Nitride Membranes for Single-Molecule Sensing. Small. 2010;6(13):1406-14. doi:10.1002/smll.201000253.
62. Wanunu M, Meller A. Chemically modified solid-state nanopores. Nano Lett. 2007;7(6):1580-5. doi:10.1021/nl070462b.
63. Yusko EC, Johnson JM, Majd S, Prangkio P, Rollings RC, Li J et al. Controlling protein translocation through nanopores with bio-inspired fluid walls. Nat Nanotechnol. 2011;6:253-60. doi:10.1038/nnano.2011.12.
64. Lepoitevin M, Ma T, Bechelany M, Janot J-M, Balme S. Functionalization of single solid state nanopores to mimic biological ion channels: A review. Adv Colloid Interface Sci. 2017;250:195-213. doi:https://doi.org/10.1016/j.cis.2017.09.001.
65. Anderson BN, Muthukumar M, Meller A. pH Tuning of DNA Translocation Time through Organically Functionalized Nanopores. ACS Nano. 2012;7(2):1408-14. doi:10.1021/nn3051677.
66. Valentina M, Paola F, Giuseppe F, Luca R, Ugo V. Size and functional tuning of solid state nanopores by chemical functionalization. Nanotechnology. 2012;23(43):435301.
67. Mussi V, Fanzio P, Repetto L, Firpo G, Scaruffi P, Stigliani S et al. DNA-functionalized solid state nanopore for biosensing. Nanotechnology. 2010;21(14):145102. doi:10.1088/0957-4484/21/14/145102.
68. Mussi V, Fanzio P, Repetto L, Firpo G, Scaruffi P, Stigliani S et al. Electrical characterization of DNA-functionalized solid state nanopores for bio-sensing. J Phys: Condens Matter. 2010;22(45):454104.

69. Liebes Y, Drozdov M, Avital YY, Kauffmann Y, Rapaport H, Kaplan WD et al. Reconstructing solid state nanopore shape from electrical measurements. Appl Phys Lett. 2010;97(22):223105.
70. Ayub M, Ivanov A, Instuli E, Cecchini M, Chansin G, McGilvery C et al. Nanopore/electrode structures for single-molecule biosensing. Electrochim Acta. 2010;55(27):8237-43.
71. Stein D, Kruithof M, Dekker C. Surface-Charge-Governed Ion Transport in Nanofluidic Channels. Phys Rev Lett. 2004;93(3):035901.
72. Frament CM, Dwyer JR. Conductance-Based Determination of Solid-State Nanopore Size and Shape: An Exploration of Performance Limits. J Phys Chem C. 2012;116(44):23315-21. doi:10.1021/jp305381j.
73. Kowalczyk SW, Grosberg AY, Rabin Y, Dekker C. Modeling the conductance and DNA blockade of solid-state nanopores. Nanotechnology. 2011;22(31):315101.
74. Behrens SH, Grier DG. The charge of glass and silica surfaces. J Chem Phys. 2001;115(14):6716-21.
75. DeBlois RW, Bean CP. Counting and Sizing of Submicron Particles by the Resistive Pulse Technique. Rev Sci Instrum. 1970;41(7):909-16.
76. Lee C, Joly L, Siria A, Biance A-L, Fulcrand R, Bocquet L. Large Apparent Electric Size of Solid-State Nanopores Due to Spatially Extended Surface Conduction. Nano Lett. 2012;12(8):4037-44. doi:10.1021/nl301412b.
77. Firnkes M, Pedone D, Knezevic J, Döblinger M, Rant U. Electrically Facilitated Translocations of Proteins through Silicon Nitride Nanopores: Conjoint and Competitive Action of Diffusion, Electrophoresis, and Electroosmosis. Nano Lett. 2010;6(8):895-909. doi:10.1021/nl100861c.
78. Hoogerheide DP, Garaj S, Golovchenko JA. Probing Surface Charge Fluctuations with Solid-State Nanopores. Phys Rev Lett. 2009;102(25):256804.
79. Karawdeniya BI, Bandara YMNDY, Nichols JW, Chevalier RB, Dwyer JR. Surveying silicon nitride nanopores for glycomics and heparin quality assurance. Nat Commun. 2018;9(1):3278. doi:10.1038/s41467-018-05751-y.
80. Frament CM, Bandara N, Dwyer JR. Nanopore Surface Coating Delivers Nanopore Size and Shape through Conductance-Based Sizing. ACS Appl Mater Interfaces. 2013;5(19):9330-7. doi:10.1021/am4026455.
81. Wanunu M, Sutin J, McNally B, Chow A, Meller A. DNA Translocation Governed by Interactions with Solid-State Nanopores. Biophys J. 2008;95(10):4716-25. doi:10.1529/biophysj.108.140475.
82. Carbonaro A, Sohn LL. A resistive-pulse sensor chip for multianalyte immunoassays. Lab on a Chip. 2005;5(10). doi:10.1039/b504827c.
83. Aksimentiev A. Deciphering ionic current signatures of DNA transport through a nanopore. Nanoscale. 2010;2(4):468-83. doi:10.1039/b9nr00275h.
84. Smeets RMM, Keyser UF, Krapf D, Wu M-Y, Dekker NH, Dekker C. Salt Dependence of Ion Transport and DNA Translocation through Solid-State Nanopores. Nano Lett. 2006;6(1):89-95. doi:10.1021/nl052107w.
85. Beamish E, Kwok H, Tabard-Cossa V, Godin M. Precise control of the size and noise of solid-state nanopores using high electric fields. Nanotechnology. 2012;23(40):405301.

86. Tabard-Cossa V, Trivedi D, Wiggin M, Jetha NN, Marziali A. Noise analysis and reduction in solid-state nanopores. Nanotechnology. 2007;18(30). doi:http://dx.doi.org/10.1088/0957-4484/18/30/305505.
87. Li J, Gershow M, Stein D, Brandin E, Golovchenko JA. DNA molecules and configurations in a solid-state nanopore microscope. Nature Materials. 2003;2:611. doi:10.1038/nmat965.
88. Schoch RB, Han J, Renaud P. Transport phenomena in nanofluidics. Reviews of Modern Physics. 2008;80(3):839-83.
89. Reiner JE, Kasianowicz JJ, Nablo BJ, Robertson JWF. Theory for polymer analysis using nanopore-based single-molecule mass spectrometry. P Natl Acad Sci USA. 2010;107(27):12080-5. doi:10.1073/pnas.1002194107.
90. Weerakoon-Ratnayake KM, O'Neil CE, Uba FI, Soper SA. Thermoplastic nanofluidic devices for biomedical applications. Lab on a Chip. 2017;17(3):362-81. doi:10.1039/C6LC01173J.
91. Uba FI, Pullagurla SR, Sirasunthorn N, Wu J, Park S, Chantiwas R et al. Surface charge, electroosmotic flow and DNA extension in chemically modified thermoplastic nanoslits and nanochannels. Analyst. 2015;140(1):113-26. doi:10.1039/C4AN01439A.
92. Haywood DG, Harms ZD, Jacobson SC. Electroosmotic Flow in Nanofluidic Channels. Anal Chem. 2014;86(22):11174-80. doi:10.1021/ac502596m.
93. Mabey D, Peeling RW, Ustianowski A, Perkins MD. Diagnostics for the developing world. Nature Reviews Microbiology. 2004;2:231. doi:10.1038/nrmicro841.
94. Quick J, Loman NJ, Duraffour S, Simpson JT, Severi E, Cowley L et al. Real-time, portable genome sequencing for Ebola surveillance. Nature. 2016;530:228. doi:10.1038/nature16996.
95. Castro-Wallace SL, Chiu CY, John KK, Stahl SE, Rubins KH, McIntyre ABR et al. Nanopore DNA Sequencing and Genome Assembly on the International Space Station. Sci Rep-UK. 2017;7(1):18022. doi:10.1038/s41598-017-18364-0.
96. Peveler WJ, Yazdani M, Rotello VM. Selectivity and Specificity: Pros and Cons in Sensing. ACS Sensors. 2016;1(11):1282-5. doi:10.1021/acssensors.6b00564.
97. Tabard-Cossa V, Wiggin M, Trivedi D, Jetha NN, Dwyer JR, Marziali A. Single-Molecule Bonds Characterized by Solid-State Nanopore Force Spectroscopy. ACS Nano. 2009;3(10):3009-14. doi:10.1021/nn900713a.
98. Tropini C, Marziali A. Multi-nanopore force Spectroscopy for DNA analysis. Biophys J. 2007;92(5):1632-7. doi:10.1529/biophysj.106.094060.
99. Nakane J, Wiggin M, Marziali A. A nanosensor for transmembrane capture and identification of single nucleic acid molecules. Biophys J. 2004;87(1):615-21. doi:10.1529/biophysj.104.040212.
100. Mathe J, Visram H, Viasnoff V, Rabin Y, Meller A. Nanopore unzipping of individual DNA hairpin molecules. Biophys J. 2004;87(5):3205-12. doi:10.1529/biophysj.104.047274.
101. Renner S, Geltinger S, Simmel FC. Nanopore Translocation and Force Spectroscopy Experiments in Microemulsion Droplets. Small. 2010;6(2):190-4. doi:10.1002/smll.200901435.
102. Hornblower B, Coombs A, Whitaker RD, Kolomeisky A, Picone SJ, Meller A et al. Single-molecule analysis of DNA-protein complexes using nanopores. Nature Methods. 2007;4(4):315-7. doi:10.1038/nmeth1021.
103. Dudko OK, Mathe J, Szabo A, Meller A, Hummer G. Extracting kinetics from single-molecule force spectroscopy: Nanopore unzipping of DNA hairpins. Biophys J. 2007;92(12):4188-95. doi:10.1529/biophysj.106.102855.

104. McNally B, Wanunu M, Meller A. Electromechanical Unzipping of Individual DNA Molecules Using Synthetic Sub-2 nm Pores. Nano Lett. 2008;8(10):3418-22. doi:10.1021/nl802218f.
105. Evans E. Looking inside molecular bonds at biological interfaces with dynamic force spectroscopy. Biophys Chem. 1999;82(2-3):83-97.
106. Heins EA, Siwy ZS, Baker LA, Martin CR. Detecting Single Porphyrin Molecules in a Conically Shaped Synthetic Nanopore. Nano Lett. 2005;5(9):1824-9. doi:10.1021/nl050925i.
107. Braha O, Gu L-Q, Zhou L, Lu X, Cheley S, Bayley H. Simultaneous stochastic sensing of divalent metal ions. Nat Biotechnol. 2000;18:1005. doi:10.1038/79275.
108. Boersma AJ, Brain KL, Bayley H. Real-Time Stochastic Detection of Multiple Neurotransmitters with a Protein Nanopore. ACS Nano. 2012;6(6):5304-8. doi:10.1021/nn301125y.
109. Guan X, Gu L-Q, Cheley S, Braha O, Bayley H. Stochastic Sensing of TNT with a Genetically Engineered Pore. ChemBioChem. 2005;6(10):1875-81. doi:10.1002/cbic.200500064.
110. Boersma AJ, Bayley H. Continuous Stochastic Detection of Amino Acid Enantiomers with a Protein Nanopore. Angew Chem Int Ed. 2012;51(38):9606-9. doi:10.1002/anie.201205687.
111. Kang X-f, Cheley S, Guan X, Bayley H. Stochastic Detection of Enantiomers. J Am Chem Soc. 2006;128(33):10684-5. doi:10.1021/ja063485l.
112. Niedringhaus TP, Milanova D, Kerby MB, Snyder MP, Barron AE. Landscape of Next-Generation Sequencing Technologies. Anal Chem. 2011;83(12):4327-41. doi:10.1021/ac2010857.
113. Wang Y, Yao F, Kang X-f. Tetramethylammonium-Filled Protein Nanopore for Single-Molecule Analysis. Anal Chem. 2015;87(19):9991-7. doi:10.1021/acs.analchem.5b02611.
114. Wanunu M, Morrison W, Rabin Y, Grosberg AY, Meller A. Electrostatic focusing of unlabelled DNA into nanoscale pores using a salt gradient. Nat Nano. 2010;5(2):160-5. doi:http://www.nature.com/nnano/journal/v5/n2/suppinfo/nnano.2009.379_S1.html.
115. Pitchford WH, Kim H-J, Ivanov AP, Kim H-M, Yu J-S, Leatherbarrow RJ et al. Synchronized Optical and Electronic Detection of Biomolecules Using a Low Noise Nanopore Platform. ACS Nano. 2015;9(2):1740-8. doi:10.1021/nn506572r.
116. Morin TJ, McKenna WL, Shropshire TD, Wride DA, Deschamps JD, Liu X et al. A handheld platform for target protein detection and quantification using disposable nanopore strips. Sci Rep-UK. 2018;8(1):14834. doi:10.1038/s41598-018-33086-7.
117. Jayasena SD. Aptamers: An Emerging Class of Molecules That Rival Antibodies in Diagnostics. Clin Chem. 1999;45(9):1628-50.
118. O'Sullivan C. Aptasensors - the future of biosensing? Anal Bioanal Chem. 2002;372(1):44-8. doi:10.1007/s00216-001-1189-3.
119. Luzi E, Minunni M, Tombelli S, Mascini M. New trends in affinity sensing: aptamers for ligand binding. TrAC, Trends Anal Chem. 2003;22(11):810-8.
120. Bunka DHJ, Stockley PG. Aptamers come of age – at last. Nat Rev Micro. 2006;4(8):588-96.
121. Tennico YH, Hutanu D, Koesdjojo MT, Bartel CM, Remcho VT. On-Chip Aptamer-Based Sandwich Assay for Thrombin Detection Employing Magnetic Beads and Quantum Dots. Anal Chem. 2010;82(13):5591-7. doi:10.1021/ac101269u.

122. Lubin AA, Plaxco KW. Folding-Based Electrochemical Biosensors: The Case for Responsive Nucleic Acid Architectures. Acc Chem Res. 2010;43(4):496-505. doi:10.1021/ar900165x.
123. Takemura K, Wang P, Vorberg I, Surewicz W, Priola SA, Kanthasamy A et al. DNA Aptamers That Bind to PrPC and Not PrPSc Show Sequence and Structure Specificity. Exp Biol Med. 2006;231(2):204-14.
124. Greenleaf WJ, Frieda KL, Foster DAN, Woodside MT, Block SM. Direct Observation of Hierarchical Folding in Single Riboswitch Aptamers. Science. 2008;319(5863):630-3. doi:10.1126/science.1151298.
125. Lin L, Wang H, Liu Y, Yan H, Lindsay S. Recognition Imaging with a DNA Aptamer. Biophys J. 2006;90(11):4236-8.
126. Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Nature. 1992;355(6360):564-6.
127. Tasset DM, Kubik MF, Steiner W. Oligonucleotide inhibitors of human thrombin that bind distinct epitopes. J Mol Biol. 1997;272(5):688-98.
128. Shim JW, Gu L-Q. Encapsulating a Single G-Quadruplex Aptamer in a Protein Nanocavity. J Phys Chem B. 2008;112(28):8354-60. doi:10.1021/jp0775911.
129. Wiggin M, Tropini C, Tabard-Cossa V, Jetha NN, Marziali A. Nonexponential Kinetics of DNA Escape from alpha-Hemolysin Nanopores. Biophys J. 2008;95(11):5317-23. doi:10.1529/biophysj.108.137760.
130. Ding S, Gao C, Gu L-Q. Capturing Single Molecules of Immunoglobulin and Ricin with an Aptamer-Encoded Glass Nanopore. Anal Chem. 2009;81(16):6649-55. doi:10.1021/ac9006705.
131. Abelow AE, Schepelina O, White RJ, Vallee-Belisle A, Plaxco KW, Zharov I. Biomimetic glass nanopores employing aptamer gates responsive to a small molecule. Chem Commun. 2010;46(42):7984-6.
132. Kawano R, Osaki T, Sasaki H, Takinoue M, Yoshizawa S, Takeuchi S. Rapid Detection of a Cocaine-Binding Aptamer Using Biological Nanopores on a Chip. J Am Chem Soc. 2011;133(22):8474-7. doi:10.1021/ja2026085.
133. Ying Y-L, Wang H-Y, Sutherland TC, Long Y-T. Monitoring of an ATP-Binding Aptamer and its Conformational Changes Using an α-Hemolysin Nanopore. Small. 2011;7(1):87-94. doi:10.1002/smll.201001428.
134. Kong J, Zhu J, Chen K, Keyser UF. Specific Biosensing Using DNA Aptamers and Nanopores. Adv Funct Mater. 2018;0(0):1807555. doi:doi:10.1002/adfm.201807555.
135. Winters-Hilt S. The α-Hemolysin nanopore transduction detector – single-molecule binding studies and immunological screening of antibodies and aptamers. BMC Bioinformatics. 2007;8(7):S9. doi:10.1186/1471-2105-8-s7-s9.
136. Harms ZD, Haywood DG, Kneller AR, Jacobson SC. Conductivity-based detection techniques in nanofluidic devices. Analyst. 2015;140(14):4779-91. doi:10.1039/C5AN00075K.
137. Freedman KJ, Haq SR, Edel JB, Jemth P, Kim MJ. Single molecule unfolding and stretching of protein domains inside a solid-state nanopore by electric field. Sci Rep. 2013;3. doi:10.1038/srep01638.
138. Pastoriza-Gallego M, Breton M-F, Discala F, Auvray L, Betton J-M, Pelta J. Evidence of Unfolded Protein Translocation through a Protein Nanopore. ACS Nano. 2014;8(11):11350-60. doi:10.1021/nn5042398.

139. Payet L, Martinho M, Pastoriza-Gallego M, Betton J-M, Auvray L, Pelta J et al. Thermal Unfolding of Proteins Probed at the Single Molecule Level Using Nanopores. Anal Chem. 2012;84(9):4071-6. doi:10.1021/ac300129e.
140. Merstorf C, Cressiot B, Pastoriza-Gallego M, Oukhaled A, Betton J-M, Auvray L et al. Wild Type, Mutant Protein Unfolding and Phase Transition Detected by Single-Nanopore Recording. ACS Chem Biol. 2012;7(4):652-8. doi:10.1021/cb2004737.
141. Cressiot B, Oukhaled A, Patriarche G, Pastoriza-Gallego M, Betton J-M, Auvray L et al. Protein Transport through a Narrow Solid-State Nanopore at High Voltage: Experiments and Theory. ACS Nano. 2012;6(7):6236-43. doi:10.1021/nn301672g.
142. Singh PR, Bárcena-Uribarri I, Modi N, Kleinekathöfer U, Benz R, Winterhalter M et al. Pulling Peptides across Nanochannels: Resolving Peptide Binding and Translocation through the Hetero-oligomeric Channel from Nocardia farcinica. ACS Nano. 2012;6(12):10699-707. doi:10.1021/nn303900y.
143. Jetha NN, Semenchenko V, Wishart DS, Cashman NR, Marziali A. Nanopore Analysis of Wild-Type and Mutant Prion Protein (PrP^C): Single Molecule Discrimination and PrP^C Kinetics. PLoS ONE. 2013;8(2):e54982.
144. Radu IS, Dhruti T, Andre M, Jeremy SL. Evidence that small proteins translocate through silicon nitride pores in a folded conformation. J Phys: Condens Matter. 2010;22(45):454133.
145. Yusko EC, Bruhn BR, Eggenberger OM, Houghtaling J, Rollings RC, Walsh NC et al. Real-time shape approximation and fingerprinting of single proteins using a nanopore. Nat Nanotechnol. 2016;12:360. doi:10.1038/nnano.2016.267

https://www.nature.com/articles/nnano.2016.267#supplementary-information.
146. Sexton LT, Horne LP, Sherrill SA, Bishop GW, Baker LA, Martin CR. Resistive-Pulse Studies of Proteins and Protein/Antibody Complexes Using a Conical Nanotube Sensor. J Am Chem Soc. 2007;129(43):13144-52. doi:10.1021/ja0739943.
147. Movileanu L, Schmittschmitt JP, Martin Scholtz J, Bayley H. Interactions of Peptides with a Protein Pore. Biophys J. 2005;89(2):1030-45.
148. Siwy Z, Trofin L, Kohli P, Baker LA, Trautmann C, Martin CR. Protein Biosensors Based on Biofunctionalized Conical Gold Nanotubes. J Am Chem Soc. 2005;127(14):5000-1. doi:10.1021/ja043910f.
149. Sha J, Si W, Xu B, Zhang S, Li K, Lin K et al. Identification of Spherical and Nonspherical Proteins by a Solid-State Nanopore. Anal Chem. 2018;90(23):13826-31. doi:10.1021/acs.analchem.8b04136.
150. Restrepo-Pérez L, Joo C, Dekker C. Paving the way to single-molecule protein sequencing. Nat Nanotechnol. 2018;13(9):786-96. doi:10.1038/s41565-018-0236-6.
151. Dong X, Zhou S, Mechref Y. LC-MS/MS analysis of permethylated free oligosaccharides and N-glycans derived from human, bovine, and goat milk samples. Electrophoresis. 2016;37(11):1532-48. doi:10.1002/elps.201500561.
152. Imberty A, Pérez S. Structure, Conformation, and Dynamics of Bioactive Oligosaccharides: Theoretical Approaches and Experimental Validations. Chem Rev. 2000;100(12):4567-88. doi:10.1021/cr990343j.
153. DeMarco ML, Woods RJ. Structural glycobiology: A game of snakes and ladders. Glycobiology. 2008;18(6):426-40. doi:10.1093/glycob/cwn026.
154. Dove A. The bittersweet promise of glycobiology. Nat Biotechnol. 2001;19:913-7. doi:doi:10.1038/nbt1001-913.

155. Ernst B, Magnani JL. From carbohydrate leads to glycomimetic drugs. Nat Rev Drug Discov. 2009;8(8):661-77.
156. Helenius A, Aebi, Markus. Intracellular Functions of N-Linked Glycans. Science. 2001;291(5512):2364-9. doi:10.1126/science.291.5512.2364.
157. Rudd PM, Elliott T, Cresswell P, Wilson IA, Dwek RA. Glycosylation and the Immune System. Science. 2001;291(5512):2370-6. doi:10.1126/science.291.5512.2370.
158. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. Nat Rev Cancer. 2015;15(9):540-55. doi:10.1038/nrc3982.
159. Seeberger PH. Chemical glycobiology: why now? Nat Chem Biol. 2009;5(6):368-72.
160. Lichtenstein RG, Rabinovich GA. Glycobiology of cell death: when glycans and lectins govern cell fate. Cell Death Differ. 2013;20(8):976-86. doi:10.1038/cdd.2013.50.
161. Dalziel M, Crispin M, Scanlan CN, Zitzmann N, Dwek RA. Emerging Principles for the Therapeutic Exploitation of Glycosylation. Science. 2014;343(6166). doi:10.1126/science.1235681.
162. Essentials of Glycobiology. 2 ed. Cold Spring Harbor Laboratory Press; 2009.
163. Czjzek M. Biochemistry: A wine-induced breakdown. Nature. 2017;544(7648):45-6. doi:10.1038/nature21901.
164. Lester J, Chandler T, Gemene KL. Reversible Electrochemical Sensor for Detection of High-Charge Density Polyanion Contaminants in Heparin. Anal Chem. 2015;87(22):11537-43. doi:10.1021/acs.analchem.5b03347.
165. Kim D-H, Park YJ, Jung KH, Lee K-H. Ratiometric Detection of Nanomolar Concentrations of Heparin in Serum and Plasma Samples Using a Fluorescent Chemosensor Based on Peptides. Anal Chem. 2014;86(13):6580-6. doi:10.1021/ac501089m.
166. Liu H, Zhang Z, Linhardt RJ. Lessons learned from the contamination of heparin. Nat Prod Rep. 2009;26(3):313-21. doi:10.1039/B819896A.
167. Korir A, Larive C. Advances in the separation, sensitive detection, and characterization of heparin and heparan sulfate. Anal Bioanal Chem. 2009;393(1):155-69. doi:10.1007/s00216-008-2412-2.
168. Kishimoto TK, Viswanathan K, Ganguly T, Elankumaran S, Smith S, Pelzer K et al. Contaminated Heparin Associated with Adverse Clinical Events and Activation of the Contact System. New Engl J Med. 2008;358(23):2457-67. doi:10.1056/NEJMoa0803200.
169. Guerrini M, Beccati D, Shriver Z, Naggi A, Viswanathan K, Bisio A et al. Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events. Nat Biotech. 2008;26(6):669-75. doi:10.1038/nbt1407.
170. Kailemia MJ, Ruhaak LR, Lebrilla CB, Amster IJ. Oligosaccharide Analysis by Mass Spectrometry: A Review of Recent Developments. Anal Chem. 2014;86(1):196-212. doi:10.1021/ac403969n.
171. Kasianowicz JJ, Brandin E, Branton D, Deamer DW. Characterization of individual polynucleotide molecules using a membrane channel. P Natl Acad Sci USA. 1996;93(24):13770-3.
172. Bacri L, Oukhaled A, Hémon E, Bassafoula FB, Auvray L, Daniel R. Discrimination of neutral oligosaccharides through a nanopore. Biochem Biophys Res Commun. 2011;412(4):561-4. doi:http://dx.doi.org/10.1016/j.bbrc.2011.07.121.
173. Fennouri A, Przybylski C, Pastoriza-Gallego M, Bacri L, Auvray L, Daniel R. Single Molecule Detection of Glycosaminoglycan Hyaluronic Acid Oligosaccharides and

Depolymerization Enzyme Activity Using a Protein Nanopore. ACS Nano. 2012;6(11):9672-8. doi:10.1021/nn3031047.
174. Fennouri A, Daniel R, Pastoriza-Gallego M, Auvray L, Pelta J, Bacri L. Kinetics of Enzymatic Degradation of High Molecular Weight Polysaccharides through a Nanopore: Experiments and Data-Modeling. Anal Chem. 2013;85(18):8488-92. doi:10.1021/ac4020929.
175. Kullman L, Winterhalter M, Bezrukov SM. Transport of Maltodextrins through Maltoporin: A Single-Channel Study. Biophys J. 2002;82(2):803-12. doi:http://dx.doi.org/10.1016/S0006-3495(02)75442-8.
176. Zhao S, Zheng Y-B, Cai S-L, Weng Y-H, Cao S-H, Yang J-L et al. Sugar-stimulated robust nanodevice: 4-Carboxyphenylboronic acid modified single glass conical nanopores. Electrochem Commun. 2013;36:71-4. doi:http://dx.doi.org/10.1016/j.elecom.2013.09.009.
177. Zheng Y-B, Zhao S, Cao S-H, Cai S-L, Cai X-H, Li Y-Q. A temperature, pH and sugar triple-stimuli-responsive nanofluidic diode. Nanoscale. 2017;9(1):433-9. doi:10.1039/C6NR07339E.
178. Nguyen QH, Ali M, Neumann R, Ensinger W. Saccharide/glycoprotein recognition inside synthetic ion channels modified with boronic acid. Sensor Actuat B-Chem. 2012;162(1):216-22. doi:http://dx.doi.org/10.1016/j.snb.2011.12.070.
179. Vilozny B, Wollenberg AL, Actis P, Hwang D, Singaram B, Pourmand N. Carbohydrate-actuated nanofluidic diode: switchable current rectification in a nanopipette. Nanoscale. 2013;5(19):9214-21. doi:10.1039/C3NR02105J.
180. Sun Z, Han C, Wen L, Tian D, Li H, Jiang L. pH gated glucose responsive biomimetic single nanochannels. Chem Commun. 2012;48(27):3282-4. doi:10.1039/C2CC17277A.
181. Oukhaled G, Bacri L, Mathé J, Pelta J, Auvray L. Effect of screening on the transport of polyelectrolytes through nanopores. EPL (Europhysics Letters). 2008;82(4):48003.
182. Rivas F, Zahid OK, Reesink HL, Peal BT, Nixon AJ, DeAngelis PL et al. Label-free analysis of physiological hyaluronan size distribution with a solid-state nanopore sensor. Nat Commun. 2018;9(1):1037. doi:10.1038/s41467-018-03439-x.
183. Ali M, Ramirez P, Tahir MN, Mafe S, Siwy Z, Neumann R et al. Biomolecular conjugation inside synthetic polymer nanopores via glycoprotein-lectin interactions. Nanoscale. 2011;3(4):1894-903. doi:10.1039/C1NR00003A.
184. Linhardt RJ. 2003 Claude S. Hudson Award Address in Carbohydrate Chemistry. Heparin: Structure and Activity. J Med Chem. 2003;46(13):2551-64. doi:10.1021/jm030176m.
185. Solá RJ, Griebenow K. Glycosylation of Therapeutic Proteins. BioDrugs. 2010;24(1):9-21. doi:10.2165/11530550-000000000-00000.
186. Seeberger PH, Cummings RD. Glycans in Biotechnology. In: A V, RD C, JD E, al. e, editors. Essentials of Glycobiology [Internet]. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2017.