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Nanometer-scale pores: Potential applications for analyte detection and DNA characterization

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Abstract. Several classes of transmembrane protein ion channels function in vivo as sensitive and selective detection elements for analytes. Recent studies on single channels reconstituted into planar lipid bilayer membranes suggest that nanometer-scale pores can be used to detect, quantitate and characterize a wide range of analytes that includes small ions and single stranded DNA. We briefly review here these studies and identify leaps in technology that, if realized, might lead to innovations for the early detection of cancer.

1. Introduction

Proteins that form nanoscale pores in cell membranes perform many cellular functions, including the propagation of the action potential in nerve [1,2], protein translocation [3,4] and cell-cell coupling [5]. One type of pore-forming proteins, receptor channels in nerve that bind neurotransmitters, convert a chemical signal (i.e. the neurotransmitter secreted by presynaptic neurons) into an electrical one (i.e., a change in the postsynaptic transmembrane potential). Receptor channels accomplish this by changing their ionic conducting state depending on the number of neurotransmitters that are bound to them.

There are several mechanisms by which analytes could alter channel conductance. These include: physically occluding the pore lumen, altering the pore geometry; competing for the site to which the permeant species occupy before they are translocated or altering the local electrostatic potential and thus the concentration of permeant ions near the pore. If channels or artificially produced nanopores are to eventually be used in real-world sensing applications, an understanding of the mechanism(s) for analyte-induced conductance changes would certainly be advantageous.

Well over a million ions per second flow through a fully open single channel. In some cases, this flux can be leveraged (or "gated") by a few analytes that bind to critical regions on the channel. In a sense, analyte-gated channels are Biology's equivalent of the solid-state transistor. It follows that the amplification created by this leverage provides the basis for using nanopores as detectors for analytes.

I briefly review here recent studies on the ability of nanoscale pores to function as sensing elements for a wide range of analytes including individual molecules of single-stranded DNA.

2. Lessons learned from ion channels gated by small ions

In addition to receptor channels, there are other proteinaceous pores that are gated by small ions or other effectors. For example, it was shown that the reversible binding of protons to amino acid side chains on a particular Ca^{2+} channel [6,7].

Suppose that the reversible binding of protons to the critical side chains causes the channel to switch

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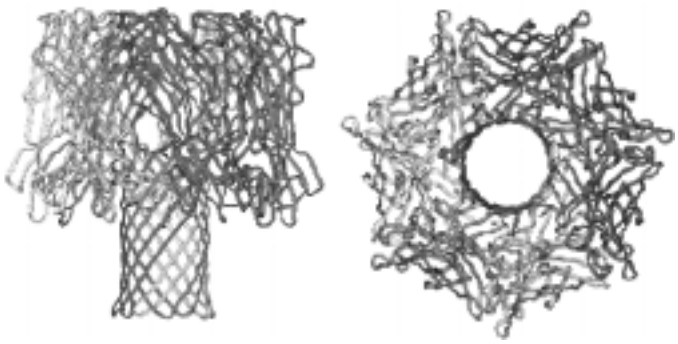

Fig. 1. Ribbon diagram views of the α-hemolysin ion channel crystal structure [11]. (Left) The channel is $\sim$ 10 nm long from top to bottom. The anti-parallel β-Sheets in the bottom half of the structure spans a bilayer membrane. (Right) The top view of the channel illustrates the 7-fold symmetry of the molecule. Although not shown explicitly in this representation, the smallest constriction inside the pore is $\sim$ 1.5 nm in diameter [11].

between two different conductance states, C_1 and C_2 and that this reaction can be described by the equation

$$\boldsymbol{H}^+ + \boldsymbol{C}_1 \underset{k_{\rm off}}{\overset{k_{\rm on}}{\rightleftharpoons}} \boldsymbol{C}_2. \qquad (1)$$

The forward and reverse rate constants for the reaction, $k_{\rm on}$ and $k_{\rm off}$, are defined by $K = k_{\rm on}/k_{\rm off}$, where $1/k_{\rm off}$ is the mean time that a particular amino acid side chain is bound with a proton, K is the reaction equilibrium constant, and the pK is defined as $-\log_{10}$K.

If the side chains are acidic, then for $pH \ll pK$or $pH \gg pK$, the proton binding site is virtually always occupied or unoccupied, respectively. At $pH = pK$, the channel spends, on average, half the time in each of the two conducting states. If the individual current fluctuations between states can be resolved, the proton concentration and reaction rate constants are easily determined from the relative time the channel spends in each conducting state.

During the past decade, it was posited that single ion channels might prove useful as components of sensors for specific analytes [8,9]. The principle of analyte detection and identification is simple. The binding of analyte to a site inside the pore or near the pore mouth causes fluctuations in the single channel current. These conductance fluctuations could be caused by changes in the electrostatic potential inside or near the pore, by changes in the conformation of the channel, or by occlusion of the pore.

More recent studies on proton binding to the channel formed by the bacterial exotoxin *Staphylococcus aureus* α-hemolysin (αHL) demonstrated that the reaction rate constants and the H^+ concentration can be determined from the frequency content of the H^+-induced current fluctuations [8,9]. The αHL channel is

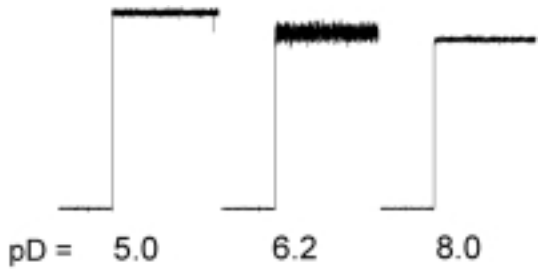

Fig. 2. The α-hemolysin ion channel as a model sensor for small ions. The single channel conductance is pH dependent [8,9] and analyses of the mean conductance and the frequency content of the current fluctuations provided an estimate of the pK and kinetic rate constants for the binding of H^+ or D^+ ions to the channel. The pH (or pD in D_2O solutions) is determined directly from the mean current and/or the statistical variance of the current noise. Adapted from [9].

formed from seven identical monomers [10,11], as is illustrated in Fig. 1. Thus, even if the reversible protonation of only one of the side chains per monomer caused the current fluctuations, resolving individual conductance state changes is made more difficult because of the channel's seven-fold symmetry. However, it was shown that "spectral" or "noise" analysis of the fluctuations could be used to measure the reaction parameters (i.e., the pK, $k_{\rm on}$ and $k_{\rm off}$), the pH, and the number of relevant ionizable residues (Fig. 2).

Based on these results, we concluded that nanoscale pores could be used to measure chemical reaction rates, which are characteristic for particular molecular species, and the concentration of analytes in solution [8]. We subsequently used noise analysis [12–14] to distinguish between D^+ and H^+ induced conductance fluctuations in the αHL channel [9].

To broaden the range of analytes that could be detected using an ion channel, genetic engineering was

then used to affix novel analyte binding sites for heavy metal divalent cations to the αHL channel [15–18]. Shortly thereafter, the detection of molecular species from analyte-induced single channel current fluctuations became known as "stochastic sensing". It should be noted that Menestrina and his colleagues demonstrated that higher concentrations of divalent and trivalent cations caused the wild-type αHL channel to slowly gate from a fully open to a less conducting state [19].

Krasilnikov, his colleagues and others demonstrated that the radius of the αHL channel could be estimated from the ability of size-selected neutral polymers, e.g., poly(ethylene glycol) or PEG, to partition into the pore [20–22]. Higher bandwidth recordings and spectral analysis of PEG-induced current fluctuations permitted Bezrukov and ourselves to show that the polymer most likely binds to the αHL channel walls [23–25]. This effect markedly increases the mean residence time of PEG in the pore over that predicted using either a one dimensional diffusion equation or any other simple relationship for polymer mobility in the bulk aqueous phase. Thus, the transit of even a single polymer through the pore can be detected using conventional electrophysiological techniques. For example, random-flight polymers of poly(ethylene glycol), i.e. PEG, that are small enough to partition into the αHL pore should diffuse the length of the channel in about 100 nanoseconds as they apprently do in the alamethicin channel [25]. In contrast, PEGs that enter the αHL channel spend some 100 μs in the pore [23].

Others took advantage of this property of the αHL channel and demonstrated that cyclodextrins with 7-fold symmetry also partition into the pore [26]. These cyclic macromolecules bind to virtually the same region inside the pore as PEG seems to [27]. Because the interior cavity of cyclodextrins can bind some small analytes with specificity, when the cyclodextrin was bound to the pore (lifetimes on the order of 1 to 10 ms), the single channel conductance fluctuations were characteristic of the analyte types and concentration. This approach to using single channels to detect analytes in solution is referred to as sensing with "molecular adapters".

To date, there are several significant barriers to using single protein ion channels as nanopore-based sensing elements. First, they function in planar lipid bilayer membranes, structures that are fragile and not yet easy to form in real-world applications. Second, the "molecular adapter" approach [26] took advantage of a property of the α-hemolysin channel, i.e. its ability to bind macromolecules in its pore lumen. It may not be easy to design a site that binds such adapters in a robust nanopore. Another potentially complicating factor is that the molecular adapter has to bind simultaneously to two molecules: the analyte of interest and a site or sites inside the nanopore. Lastly, the molecular adapter approach substitutes one difficult problem (i.e., designing analyte binding sites in a nanopore) with a potentially more difficult one (doing the same thing in an even smaller pore-shaped molecule). If these difficulties can be overcome, the frequency content of analyte-induced single channel current fluctuations could prove useful in distinguishing between different analytes [8].

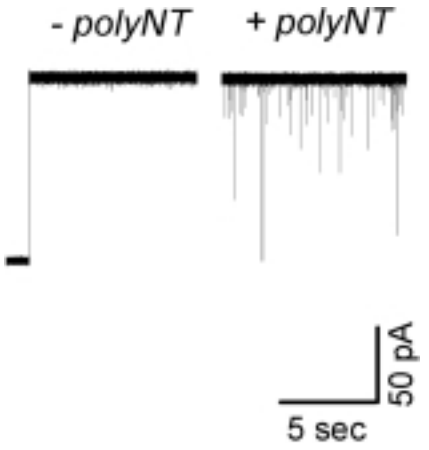

Fig. 3. DNA transport in the α hemolysin channel. (Left) In the absence of polynucleotides, the single channel current is large and quiescent. (Right) The addition of single-stranded DNA to the solution bathing one side of the membrane causes transient current blockades. Most of the blockades correspond to polynucleotides that completely thread through the channel [28].

3. Single nanopore-based DNA characterization

We recently demonstrated that individual molecules of single-stranded DNA and RNA can be detected and characterized as they are driven electrophoretically through single αHL channels [28]. The passage of a polynucleotide through the channel causes a well-defined transient blockade in the single channel current, as is shown in Fig. 3. The polymer-induced blockade lifetime is proportional to the polynucleotide length, which suggested that the polymer threads through the channel as a linear rod [28]. Because the polynucleotides thread through the αHL channel at ~ 1 to 10 μs/base, it was suggested that a single nanopore might prove useful for ultra rapid DNA sequencing [28]. However, in order for that *ansatz* to become

practicable, some formidable challenges lie ahead (see below).

More recent experiments demonstrated that the depth of the current blockade was different for two different homopolymers (i.e. poly[C] and poly[A]) [29]. Moreover, the transient single channel current blockades caused by a di-block copolymer of poly [C] and poly[A] contained sequential blockade patterns that suggested that the poly[C] segment first entered the pore followed by the poly[A] segment. At first glance, this result could be taken as proof-of-concept that a single nanopore can be used to sequence DNA. However, it is likely that the different signals are caused by different structures adopted by the two different homopolymers [30] and not by the interactions of individual bases with a narrow constriction inside the αHL channel.

There are several significant technological hurdles that must be overcome to reduce nanopore based sequencing to practice. First, as noted above, it may not be desirable to use a protein ion channel for this application. Thus, nanopores made from robust materials (e.g., [31]) might prove useful for this task. Second, polynucleotides thread through the αHL channel at about 1 to 10 μs/base. Because the single channel conductance is less than 1 nS in 1 M NaCl [9], roughly hundreds of ions pass by each base when a polynucleotide is driven through the channel. Thus, the ability to distinguish between different bases with only slightly different molecular volumes is confounded by the expected small signal differences.

In a different approach, it was recently demonstrated that single channel current blockades caused by hairpins of slightly different base composition could be distinguished from each other [32]. A related study showed that a polynucleotide complementary to another affixed inside the αHL can occlude the pore [33]. Although these techniques may be limited to the analysis of small snippets of RNA and DNA, they might become powerful tools for detecting single nucleotide polymorphisms if the problems with using protein ion channels can be resolved or if the technique also works with artificially produced single nanopores if they become readily available. Because the observed effects may depend on the particular geometry of the αHL channel, it is not certain whether the latter hurdle will be easily cleared.

4. Single nanopore-based analyte detection

In the previous section, we discussed how single nanopores were used to detect and characterize polynucleotides and how they might be used in future DNA sequencing applications. We describe below how the controlled polymer transport through a single nanopore could be used in analyte detection.

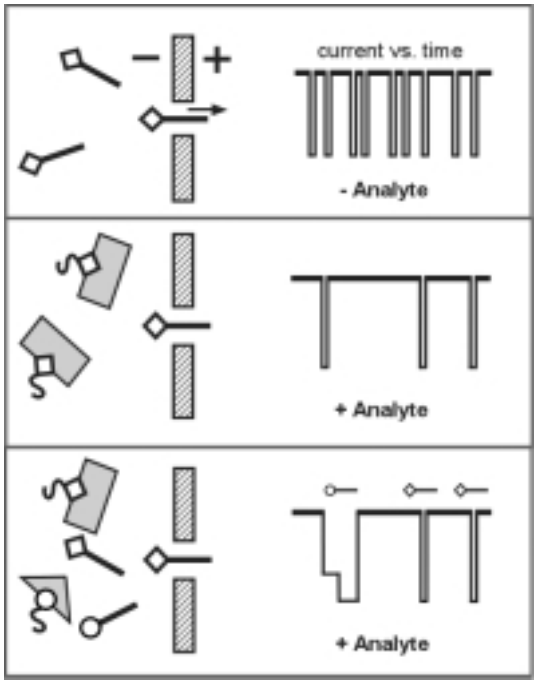

Fig. 4. Schematic of analyte quantitation method using polymers and a single nanopore. (Top) Polymers with covalently attached ligand binding sites are driven through, and transiently occlude, the pore. The time-averaged number of current blockades is proportional to the concentration of free polymer [34]. (Middle) Polymer bound with analyte (rectangles) is less likely to traverse the channel. Therefore, the reduction in the number of blockades caused by the decrease in the free polymer concentration provides a measurement of the analyte concentration. (Bottom) Multiple analytes (represented by the rectangles and triangles), can be detected simultaneously with a single nanopore because different polymers cause unique ionic current blockade patterns [35]. Adapted from [35].

As was noted above, it is possible to add novel analyte binding sites to a protein ion channel. However, because of the potential problems of using single ion channels in real-world sensing applications, we developed a method that could ultimately take advantage of artificially produced nanometer-scale pores. Instead of placing the binding site inside the pore or near one of the pore mouths, it is placed on a macromolecule that can normally thread completely through the nanopore [34]. Figure 4 illustrates schematically this sensor concept and experimental verification of it was demonstrated earlier [35].

Polynucleotides can be electrophoretically driven through a protein ion channel [28]. For low concen-

trations of short polymers, the mean number of current blockades is proportional to the polymer concentration [34,35] (Fig. 4, top). The binding of analyte to a site on the polymer alters the ability of the polymer to transport through the pore. For example, when bound with analyte, relatively short polynucleotides were inhibited from entering and thus occluding interacting with the pore (Fig. 4, middle). As the analyte concentration increases, the time-averaged rate of polymer-induced transient current blockades decreases monotonically [35].

At nanomolar polynucleotide concentrations, the αHL channel is virtually always free of polymer because the rate of polymer entry into the channel is low [34] and the current blockades are relatively short-lived [28,34]. In addition, different polymers cause different (but characteristic) current blockade types. Because different ligand binding sites can be attached to different polymers, the analyte-induced loss of the signals caused by each polymer type can be detected (Fig. 4, bottom) [35]. In this way, multiple analytes can be detected with a single nanopore.

Figure 5 illustrates several other methods for detecting analytes using a polymers and a single nanopore. For example, as is shown in Fig. 5 (top), the binding of analyte to a relatively long polymer may cause the polymer to occlude the pore for a time that is commensurate with $1/k_{\rm off}$ (in this case, $k_{\rm off}$ is the rate constant for the dissociation of analyte and polymer). In this scheme, the analyte concentration is estimated from the mean time that it takes the nanopore to be occluded by the analyte:polymer complex after an electric field is applied (the greater the analyte concentration, the shorter the time interval). It is good to note that the direct measurement of $k_{\rm off}$ could provide some statistical assurance that the molecules bound to the polymer are the analyte of interest, and not an interfering species.

If the sensing polymers would interfere with or contaminate the sample, they could be retained on one side of the nanopore (Fig. 5, bottom). In this variation, a large macromolecule is permanently affixed to one end of the polymer. This allows the polymer to enter the pore but prohibits it from threading all the way through the nanopore. In the absence of analyte, the polymer can be driven into and out of the pore by a time-varying applied potential. However, in the presence of analyte, the free end of the polymer cannot be readily pulled back through the pore. Thus, the analyte concentration can readily be determined [34,36].

Although it is possible to detect and quantitate analytes using polymers and a single protein ion channel, it

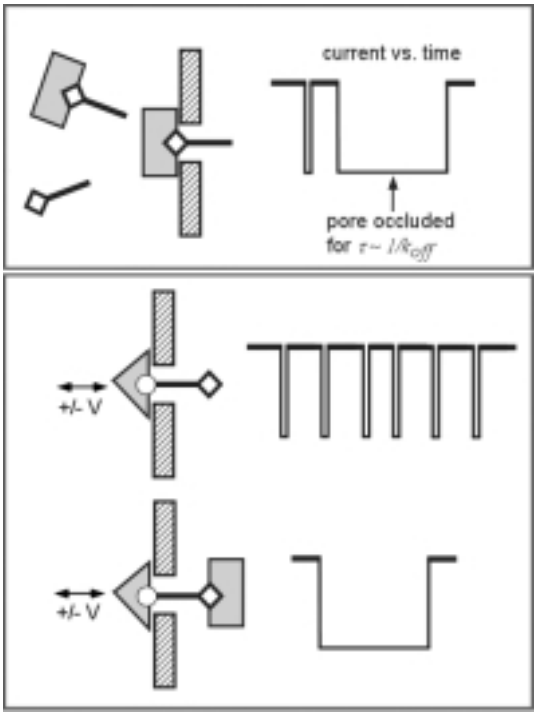

Fig. 5. Two additional analyte quantitation methods using polymers and a single nanopore. (Top) When bound with analyte (rectangle), the polymer, which otherwise threads through the nanopore, blocks the pore for a time that is commensurate with the mean lifetime of the complex. Adapted from [35]. (Bottom) A variation of the above sensor, which retains the sensing polymer, even if side that contains analyte is continuously flushed. A large macromolecule (triangle), which is permanently affixed to the sensing polymer, prevents the polymer from threading through the pore. To test for the presence of analyte (rectangle) on the RHS, the applied potential is transiently reversed (for simplicity, the effect of the transient change in potential on the current is not shown). In the absence of analyte, the polymer can be pulled back out of the pore. However, when analyte bound to the RHS of the polymer, the complex cannot be pulled back out of the pore until the analyte dissociates from binding site on the polymer (adapted from [34]).

remains to be seen if this technology can be transferred to robust single nanopores. One potential complication is that the detailed structure of single nanopores may be difficult to control and the current blockade signals produced by a particular polymer may be substantially different for each nanopore. However, a wide variety of statistical measures (e.g. mean, variance, mean lifetime, autocorrelation, spectral analysis Hidden Markov Models and Viterbi decoding algorithms) [37] might permit the calibration of each nanopore using a set of well-defined polymers. Also, the rational design of polymer-nanopore sensing systems should be aided by recent theoretical studies for the entry of polymers into nanopores [34,38] and the transport of linear polymers

through these nanostructures [38–45]. Lastly, it remains to be seen if the methods illustrated in Figs. 4 and 5 can be used to detect specific sequences of DNA.

5. Conclusions

As we briefly reviewed here, protein ion channels are a good model system for testing novel analyte detection schemes. In fact, Cornell and his colleagues demonstrated an impressive proof-of-concept for a sensor scheme based on impedance measurements of modified gramicidin channels reconstituted into a supported bilayer membrane [46]. To obtain relatively rapid signal averaging, that method uses of many thousands of channels. If technological breakthroughs can be made to enable the use of single nanopores for analyte detection, the distribution of lifetimes of the analyte:binding site complex can be directly measured. This additional information should provide a statistical measure of confidence that the analyte being detected is indeed the one of interest.

The use of glass pipettes for patch clamp analysis provided a major breakthrough for the study of single channel activity [47]. If single nanopores become practicable for sensing applications, it would be interesting if they could also be placed at the end of a pipette tip. It is conceivable that this system might provide a real-time inventory of molecules inside the cell. If this becomes possible, the state of single cells, and how cells respond to chemical and biological effectors, could be directly determined. That might significantly enhance our understanding of the development of certain cancers.

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