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A Smart Nanopore for Bio-detection

L. Q. Gu

Biological Engineering and Dalton Cardiovascular Research Center, University of Missouri, Columbia, Missouri 65211, USA

Abstract

The molecular scale pore structure, called nanopore, can be formed from protein ion channels by genetic engineering or fabricated on solid substrates using fashion nanotechnology. Target molecules in interaction with the functionalized lumen of nanopore, can produce characteristic changes in the pore conductance, which act as fingerprints, allowing us to identify single molecules and simultaneously quantify each target species in the mixture. Nanopore sensors have been created for tremendous biomedical detections, with targets ranging from metal ions, drug compounds and cellular second messengers, to proteins and DNAs. Recently, we have used the nanopore technique to dissect folding and unfolding mechanism of a single G-quadruplex DNA aptamer regulated by a variety of ions; we also created a portable and durable molecular device that integrated a protein pore sensor with a solidified lipid membrane for real-time detection.

The nanopore is a receptive single-molecule detector (1). Single target molecules in interaction with the functionalized pore lumen, will block the pore conductance. The block amplitude, duration and occurrence is specific for each target, therefore serving as the signature or fingerprint, for simultaneous identifying and quantifying targets in the mixture (Figure 1).

The nanopore single-molecule technique has abroad applications, from biosensing (2;3), nucleic acids detection (4–10), and regulation of membrane transportation (11–13), to the study of single-molecule chemistry(14), single-molecule force measurements (15;16), and the construction of biochips(17). In this mini-review, we would like to introduce our recent discoveries with nanopores. First, the α -hemolysin (α HL) protein pore, which has a diameter of 2 nm, has been applied to the detection of folding and unfolding of a single G-quadruplex DNA aptamer regulated by a variety of ions; and second, a portable and durable molecular device has been created that integrated a protein pore sensor with a solidified lipid membrane for real-time detection.

Folding and Unfolding of a Single G-quadruplex Aptamer in a Nanopore

Guanine-rich single-stranded nucleic acids can form four-stranded complexes called G-quadruplex (18–20) in the human genome. G-quadruplexes participate in gene regulation (21), and serve as targets for drugs in cancer treatment (22). Quadruplexes created *in vitro* are building blocks for nano-structures (23) and nanomachines (24;25). Their high affinity for target proteins make them ideal as powerful biosensors (26) and potent pharmaceuticals (27). The thrombin-binding aptamer (TBA, GGTTGGTGTGG TTGG) is a well-known G-quadruplex that efficiently inhibits the thrombin clotting activity (26). TBA is also a

sophisticated biosensor element for protein detection (28). This 15-base single-stranded DNA can fold into a two-tetrad, one-cation quadruplex (Figure 2a) (29), with the formation capability varying with the cation species. Although the formation properties of TBA have been well studied, the cation-dependent kinetics for folding and unfolding of the TBA G-quadruplex is not well understood. Gaining an understanding of the kinetics is important because a properly folded quadruplex is necessary for the molecular recognition involved in many quadruplex functions and is beneficial for designing quadruplex applications.

We first discovered that TBA can produce long current blocks in addition to short spike-like blocks (Figure.2b). The short block should be due to the translocation of a linear DNA through the pore, while we have a number of evidences to prove that the long blocks are caused by the encapsulation of a single G-quadruplex in the nanocavity enclosed by the α HL pore (Figure 2c) (30). Evidences are that, 1) translocation of linear form DNA never produces long blocks; 2) The dimension of the G-quadruplex is 2.1 nm, slightly narrower than the cis opening of α HL (2.6nm) and larger than the β -barrel in middle of the pore (1.4nm), suggesting that the TBA G-quadruplex can enters the nanocavity and be trapped in the nanocavity; 3) Upon binding of TBA, the unoccupied space in the nanocavity forms an ion pathway, as evidenced by the partially-blocked conductance for long events; 4) No long blocks can be observed when the human α -thrombin is presented in the TBA solution, because the formed G-quadruplex/thrombin complex is too large to be trapped in the cavity; 5) When detecting with a tag-TBA (a variant TBA with a tag at the sequence terminal), the linear tag is able to contact with the β -barrel, producing additional level-2 blocks in long events. Overall, current signatures (Figure 2b) allow discriminating a single DNA molecule, either in the G-quadruplex form (Figure 2c) or the linear form (Figure 2e). In particular, we identified that the G-quadruplex trapped in the nanocavity can spontaneously unfold and leave the pore as a linear DNA, producing spike-like short block at the long block terminal (Figure 2d).

Based on these findings, we further developed an analytical approach to determine the equilibrium (K_f , Equation 1), unfolding (k_u , Equation 2) and folding (k_f , Equation 3) rate constants for the G-quadruplex from current signatures.

$$K_f = f_{Ctrl-2} / f_{TBAL} - 1 \quad [1]$$

$$k_u = 1/\tau \quad [2]$$

$$K_f = k_f / k_u \quad [3]$$

f_{Ctrl-2} and f_{TBAL} are the occurrences of short blocks with Ctrl-2 (control) and the linear form (unfolded) TBA; τ is the duration of the G-quadruplex-produced long blocks. Through this approach, we discovered a series of properties for the G-quadruplex formation capability and

regulation of its folding and unfolding reactions by cations. K^+ , Ba^{2+} and NH_4^+ are favorite cations over Cs^+ , Na^+ and Li^+ for forming G-quadruplexes with TBA, while Mg^{2+} and Ca^{2+} did not induce the formation of the G-quadruplex. The cation selectivity in G-quadruplex formation is correlated with the volume of the G-quadruplex, which varies with the cation species. The high formation capability of the K^+ -induced G-quadruplex may be largely due to the slow unfolding reaction. Although the Na^+ - and Li^+ -quadruplexes feature similar equilibrium properties, they undergo radically different pathways. The Na^+ -quadruplex folds and unfolds most rapidly, while the Li^+ -quadruplex performs both reactions at the slowest rates. Through this study, the nanopore is proven to be a useful single-molecule tool for probing molecular processes that enrich our understanding of the ion-regulated properties and processes of oligonucleotides.

These findings may prove useful for molecular recognition and design in the aptamer-target complex. The method used in this study may be expanded for the kinetic study of other quadruplexes and their variants. Potential targets include various biologically-relevant intramolecular quadruplexes, such as the i-motif (quadruplexes formed by cytidine-rich sequences) and chemically-modified quadruplexes with unique functionalities. The contribution of each guanine to the quadruplex's folding capability may be detected by combining our guest-nanocavity approach with site-directed nucleotide substitution. Since the protein-DNA interaction has been probed using a nanopore-based molecular force detector, analog methods could be introduced for detecting target-quadruplex aptamer interactions. This work has already begun: the influence of thrombin on the encapsulation of the TBA G-quadruplex in the nanocavity has been observed. This research may also be helpful in constructing new molecular species with tunable properties for nano-constructions and the manufacture of biosensors.

A Robust Chip Device Integrating Protein Pore Sensor with Solidified Lipid Bilayer

Engineered ion channels have been demonstrated for many potential applications. It is predicted that a future generation of biosensor could be an array of target-specific ion channels where each protein pore acts as a sensor element in a long-lived, highly sealed (>10 G Ω) lipid membrane. This modular device should be portable for both independent storage and free transportation, while in real-time applications, it should act as an independent pluggable component in coupling with other systems. However, the creation of such a robust, versatile device that works with single protein pores is very challenging, and has not been achieved so far. The main limitation comes from the fragile lipid membrane where protein pores are embedded. For example, lipid bilayers have been formed on micrometer-sized apertures fabricated on a thin substrate(31;32), but their lifetimes have not been substantially prolonged. Bilayers tethered on solid surfaces(33;34) suffer from low insulating seals and high resistance of electrolyte reservoir between the bilayer and the substrate, making it problematic in single molecule detection and long-term electrical measurement. Lipid membrane by painted method(35) has also been formed on pre-cast gel slab, and then covered with another gel slab as double support(36). But this membrane may not suit single ion channel measurement due to the reported low resistance. In an

improvement, the UV-triggered hydrogel has been used in replacing agarose gel(37), but this work still lacks convincing evidences for portability and durability required for device design.

We have for the first time created a portable, long-lived, modular biochip that integrates ion channels with a solidified membrane structure for both biomedical detection and membrane protein research. The core of the device is a long-lived lipid membrane that has been sandwiched between two air-insulated agarose layers which gel *in situ*. A single protein pore embedded in the membrane serves as the sensor element. Figure 3 shows the assembling and usage of the modular ion channel chip. 1) The Teflon partition with a 100 μm aperture in the center is clipped with two compartment blocks to form two separate compartments. 2) The 1 M KCl solution containing 1.5% (w/v) ultra-low gelling temperature agarose was melted, then cooled down to room temperature, at which this type of agarose is capable of remaining in liquid form for days. 3) The lipid bilayer membrane is formed by the mono-layer folding process(38), and single protein pore is insertion into the membrane. The single ion channel embedded in the membrane serves as the sensor element. 4) The whole chip is then gently transferred to a 10 $^{\circ}\text{C}$ refrigerator so that the gel solution on both sides is solidified, and the membrane sandwiched between two gel layers is stabilized. 5) Finally the top openings of two compartments were sealed by two block lids for keeping the water content in the gel. The electrodes fixed in the middle of blocks can connect with gel, providing the membrane potential and receiving the pico-ampere current.

When the sample cell cover on the upper compartment wall is removed, the analyte solution can be added directly to the sample cell. Since agarose gel is permeable to analytes of various molecular weights (pore size of 2% agarose gel is 470 nm)(39), the analyte in the solution can transport or diffuse through the gel and reach the protein pore in the membrane for single molecule detection. Since the chip is pluggable, it can also be incorporated into other devices.

The chip device that we have constructed is highly durable. By using the smallest potassium channel Kcv encoded by chlorella viruses(40) as an marker, we have determined that the agarose-sandwiched membrane in the chip can last 65 hours on average, and consistently retain high-sealing property ($>10\text{ G}\Omega$ in 1M KCl) throughout the recording, whereas a qualified agarose-free membrane is only 3–6 hours. This is a dramatically improved property for both biosensing and research applications.

The ion channel chip is also highly portable, another important capability required for practical applications in biosensing and research. It can be repeatedly disconnected and reconnected to any device, such as an electrophysiology recording instrument; the disconnected chip is capable of independent storage, and can be transported from place to place, whereas a membrane without solid support does not have these capabilities. In addition to the safe transport capability, we can also rotate the chip to any angle and flip it over to suit different application platforms. In one example, the chip can be laid horizontally for stochastic sensing. By comparison, a membrane in solution without solid support (agarose-free configuration) could not survive such mechanical impact. It easily breaks

down when the chamber is tilted at such an angle that causes solution to level down below the central aperture on the partition, leaving the bilayer exposed to the air.

The unique portability and durability make the ion channel chip an independent, pluggable, modular biosensor device, useful for real-time sensing applications. For example, Figure 4 demonstrates the detection of the second messenger inositol-1,4,5-triphosphate (IP3). The engineered α HL pore (M113R/T145R) has been made for high specific discrimination of various phosphate compounds (2). Without analyte, this pore installed in the chip exhibits a clear background conductance (Figure 4a). When loading 20 μ l of 500 nM IP3 to the same chip, IP3 produces characteristic long blocks (5.7 ms, Figure 4b), while the presence of 0.3 mM ATP in a separate chip produces distinctly faster blockade (0.28 ms, Figure 4c). As predicted, when 500 nM IP3 is presented in the mixture with 0.3 mM ATP, like it is in a living cell, we can clearly discriminate two binding patterns from a single current recording: the short and long blocks caused by ATP and IP3 respectively (Figure 4d). Because the event occurrence for each component in mixture is corresponding to its concentration(2), its quantity in the mixture could be readily determined by single-molecule stochastic analysis(3).

There are several advantages of the modular chip biosensor that contains a single protein nanopore for stochastic sensing. One is the simultaneous analysis of the identity and quantity of multi-analyte in mixture by a single sensor element. Another advantage is the ability to create digital output with a high Signal/Noise ratio, due to the single molecule binding. As a modular device, the chip may be hybridized with other applicable devices. For example, the chip can be coupled with a micro-fluidic system and perform electrical detection combined with optical technologies. Furthermore, the chip may provide a micro-array in future for high throughput screening, with each array element containing a single stochastic sensor. This speculation is reasonable because micro-patterned hydrogels have been created. In addition, the chip features quick fabrication. The bilayer formation by a mono-layer folding process takes as little as 5 minutes. The single channel incorporation time is no more than 10 minutes. The gelling time of agarose at 10 °C is 15 minutes. Therefore the biosensor fabrication time is less than 1 hour.

The robust, versatile device we created is capable of being programmed because any membrane protein can be used to make the chip for various biomedical detections, such as screening of enzyme and detection of glucose or neural transmitters. For research purposes, the chip also has potential applications: examples are DNA and protein detection in genomics and proteomics, or dynamics of protein-membrane interaction. In addition to agarose, any polymer material, such as smart polymer, that solidifies with pH or salt concentration and is permeable to ions and molecules but does not damage bilayer membrane and protein, could be used as membrane support for the biosensor.

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Figure 1.

Diagram of nanopore single-molecule detection. Different analytes in the mixture can be identified and quantified simultaneously according to the amplitude, duration and occurrence of characteristic blocks to the pore conductance.

Figure 2.

Molecular structure of TBA and scheme of nanopore detection method. **a.** Left, the sequence and structure of TBA G-quadruplex; Right, the two G-tetrad planes in the TBA G-quadruplex (29), with the top tetrad formed by guanines at the position 1, 6, 10 and 15, and the bottom one by guanine 2, 5, 11 and 14. A cation in between is coordinated with eight carbonyls. The average cation-carbonyl distance is 2.86 Å. **b.** Current trace showing signature blocks. **c.** The long-lived block for capturing a single G-quadruplex in the nanocavity enclosed by the α HL pore; **d.** The spike at the long block terminal produced by translocation of the unfolded G-quadruplex in the nanocavity. **e.** Short-lived block formed by translocation of linear form TBA.

Figure 3. Fabrication, prototype and usage of the modular ion channel chip. **a.** Assembly of the chip. **b.** A device prototype. **c** Usage of the chip as an independent device. The analyte can be added from the sample cell on the back of either compartment and delivered to the sensor element through agarose between the sample cell and the membrane.

Figure 4. Current recordings showing the detection of second messenger IP₃ on chips. **a.** control test without analyte. **b.** 500 nM IP₃ was loaded into the sample cell of the same chip. **c.** 0.3 mM ATP was added in a separate control test. **d.** mixture of 500 nM IP₃ and 0.3 mM ATP was loaded in the chip. All traces were captured after the block occurrence reached the maximum.