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Aptamer-Encoded Nanopore for Ultrasensitive Detection of Bioterrorist Agent Ricin at Single-Molecule Resolution

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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. Here we will review our recent discoveries with a lab-in-hand glass nanopore: single-molecule discrimination of chiral enantiomers with a trapped cyclodextrin, and sensing of bioterrorist agent ricin.

THE nanopore, a pore structure made with a dimension at the molecular scale, is an ultrasensitive technique for single-molecule detection. The principle is that, when a target molecule traverses or binds in the lumen nanopore, it characteristically alters the pore conductance. The resulting change in conductance is target specific, therefore serving as a fingerprint for target identification and quantification [B1]. Nanopore technology has demonstrated great potential in biosensing [B1;B2;B3], DNA and peptide detection [B4;B5;B6;B7;B8;B9;B10;B11;B12;B13;B14;B15;B16], control of molecular transportation [B17;B18], and the study of single-molecule chemistry [B19].

One type of nanopores comprises self-assembled protein pores. The protein pore is unique because it allows for structure-directed genetic engineering and chemical modification[B20], but is limited by the fixed pore size and fragility of the lipid bilayer in which the pore is embedded. While the biochip is being developed to improve the membrane stability [B21], sophisticated nanotechnologies such as ion beam and electron beam lithograph have been employed that can puncture a single nanopores in solid substrates such as SiO_2 and polymer film [B10;B22;B23;B24;B25;B26;B13]. However, these methods of pore fabrications require expensive instruments, well-trained operators or special materials.

We recently developed a novel glass nanopore by externally penetrating a nanocavity enclosed in the terminal of a capillary pipette (Fig.1) [B27]. To fabricate this nanopore[B28], we sealed the pipette tip with a melting process so that a wineglass-shaped nanocavity was formed inside the terminal (Fig.1). The tip was exposed to hydrofluoric acid/ammonium fluoride for external etching, and monitored by the ionic current between solutions inside and outside the pipette (Fig.1). A nanopore is formed once the enclosed nanocavity was perforated (Fig.1). The pipette tip was then transferred to an etchant-free solution to determine the pore conductance. The tip can be repeatedly etched until the desired conductance is achieved. Due to the uniform nanocavity profile, the corresponding pore size can be evaluated from the conductance according to the pore size-conductance correlation [B28] (in 1 M NaCl). This nanopore can be fashioned to accommodate almost any molecular complex under investigation, and features several distinguishable benefits: ease of fabrication by virtually any laboratory at low cost, precisely manipulated pore size, from one to several hundred nanometers, an experimentally verified ability to capture single molecules and perform stochastic sensing, reduced electrical noise, bio-friendly surface engineering, and the ability to perform as a probe platform for in situ and high throughput applications.

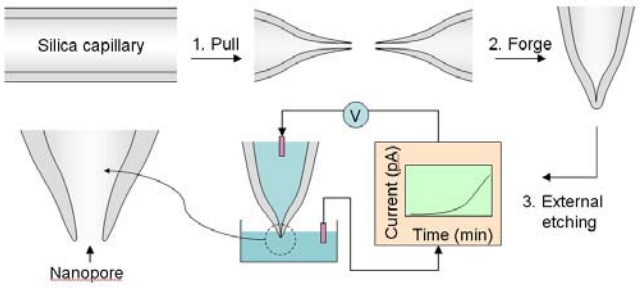

Fig.1. Fabrication of nanopore-terminated probe. Firstly, a micropipette terminal is formed by pulling a silica capillary. Secondly, the terminal is completely sealed by micro-forging to enclose a wineglass-shaped cavity with an assumed atomic-scaled dimension at its narrow end (inlet picture). Thirdly, the closed terminal is bottom-up etched externally and electronically monitored until the perforation of the nano-cavity and formation of a nanopore (inlet diagram). Etching solution is 20mM/120mM HF/NH_4F.

The pore size on the scale of a single molecule have been verified by translocation of molecules of known sizes, including double-stranded DNA (2 nm), gold nanoparticles (10 nm) and ring-shaped cyclodextrin (1.5 nm) [B28]. The small pore size allows entrapment of a single cyclodextrin molecule. Cyclodextrin in the nanopore has proven useful as

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a molecular adapter for chiral enantiomer discrimination (Fig.2a). For example, the duration of blocks with (S)-ibuprofen was 2.0 ms (Fig.2b, top trace), compared with 0.79 ms for the (R)-ibuprofen blocks (Fig.2b, middle trace). When the mixture of (S)- and (R)-ibuprofen is applied, we can not only separate the two molecules from their block durations, but also the amplitude of block current: (S)-ibuprofen (~17 pA) reduced more current than (R)-ibuprofen (11 pA) (Fig.2b, bottom trace).

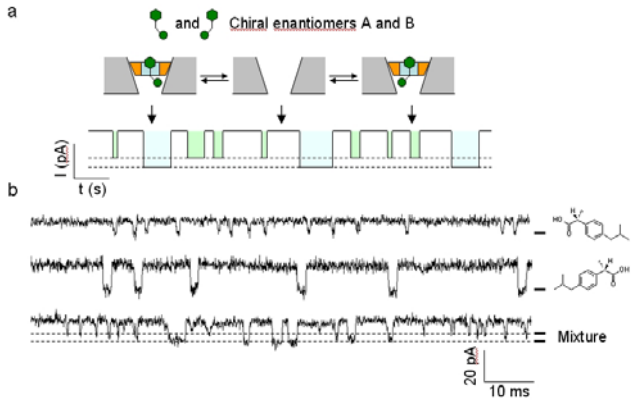

Fig.2. Glass nanopore-terminated probe for single-molecule chiral-discrimination. (a) Glass nanopore fabrication. Left, sealed glass ball (100 μm in radius) enclosing a nanocavity on the pipette terminal; middle, external etching from bottom-up with electrical monitoring; right, perforation on nanocavity terminal and nanopore formation. (b) Chiral-discrimination by cyclodextrin trapped in nanopore. The diagram shows single-molecule discrimination of chiral enantiomers with the cyclodextrin trapped in nanopore. Current traces show the binding of individual enantiomers of ibuprofen to the trapped βCD: 100 μM R-(-)-ibuprofen (top), 100 μM S-(+)-ibuprofen (middle) and a mixture 100 μM R-(-)-ibuprofen and 100 μM S-(+)-ibuprofen (bottom). The current block levels by R- and S-ibuprofen were marked with dash lines.

Indeed solid nanopores offer higher stability, flexible pore sizes, and array platforms; these greatly expand the potential of nanopore applications in biotechnology and the life sciences. However, compared with protein pores that allow for the attachment of a receptor to selectively recognize the specific target, the target selectivity of solid nanopores is low. The binding of single-molecule has not been able to be detected, even though the nanopore has been functionalized with recognizing groups such as antibodies.

We have invented an aptamer-encoded nanopore (AEN) that overcomes these challenges. With AEN, we can study single-molecule binding and release processes in a solid nanopore. The aptamers are synthetic short, structured DNA and RNA [B29;B30], that recognize and bind with high affinity (nano- to pico-molar) to broad species of proteins ligands [B31;B32;B33;B34;B35;B36] and pathogen targets [B37;B38;B39], thus very useful in bioanalysis [B40], diagnostics [B41], therapy [B42], bio-catalysis, and cell modulation [B43]. Aptamers outperform antibodies for single-molecule detection in nanopores because they are much smaller than their targets. When the aptamers are immobilized on a nanopore, the pore current retains small electrical noise; when bound by target, the target signal is pronounced. Additionally, aptamers are more durable than most protein receptors, resisting most denaturing and degrading conditions, including immobilization [B44], yet simpler to synthesize, modify, and immobilize using low cost methods. The affinity and specificity of aptamer-target interactions can also be fine-tuned through rational design or molecular evolution.

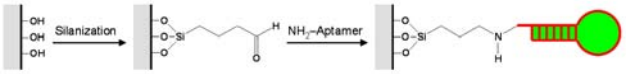

Fig.3. Functionalization of nanopore with aptamer

By using aptamer, we for the first time use label-free method to visualize single molecules of bioterrorist agent ricin that are sequentially captured by the immobilized aptamer in the sensing zone of the nanopore. According to the US Environmental Protection Agency and the Center for Defense Information, ricin is the third most toxic substance known, after plutonium and botulism, with a high potential for use as an agent of biological warfare, a weapon of mass destruction (WMD), or as a terrorist weapon. The anti-ricin A-chain aptamer (A_{ricin}) [B45] was immobilized on the nanopore by two steps, glass surface silanization and attachment of amino-terminated DNA or RNA aptamers to the surface aldehyde (Fig.3). When A_{ricin}-encoded nanopore (56 nS) was merged into a ricin-A chain protein solution (100 nM), we observed a series of stepwise current blocks (-100 mV), as shown in Fig.4a. In the control tests, we did not observe such a block profile when presenting ricin in an unmodified nanopore, nor in a pore modified with the control aptamer that is different from A_{ricin}, suggesting that both ricin and immobilized A_{ricin} are necessary for stepwise blocks, and the possibility of current variation by non-specific adsorption is excluded. The stepwise blocks are also distinguished from those produced by protein molecule translocation, which typically range 10^2 μs to 10^2 ms [B46;B47;B48].

The stepwise blocks are associated with single ricin molecules that sequentially bind to the immobilized aptamers in the nanopore, one molecule per block level. The average unbinding time for these blocks was 10 ± 5.2 mins and mean amplitude of conductance change was 14 ± 13 nS. Further, no discrete current increase was observed for the release of ricin from its aptamer, suggesting a long binding time for A_{ricin}•ricin. This property is in concordance with the high affinity (7.4 nM) for A_{ricin}•ricin reported earlier [B45]. For sensor calibration, we measured the unbinding time before the first ricin block occurred (t_1) in a variety of ricin concentrations (Fig.4b). t_1 decreased from 42 mins to 4.1 mins, as the ricin concentration increased from 3 nM to 3 μM.

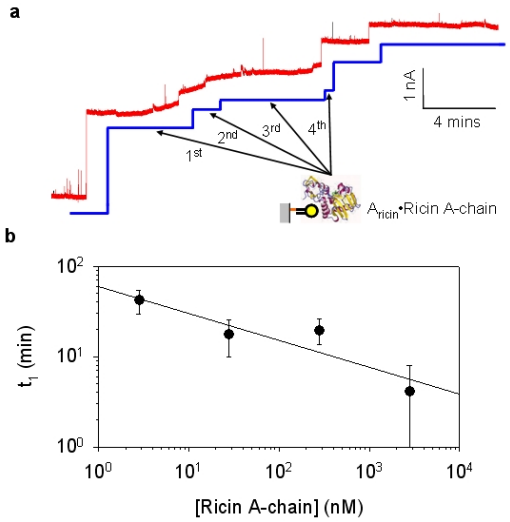

Fig.4. Single-molecule detection of ricin A-chain protein in the external solution using an A_{ricin}-encoded nanopore. (a) Current in a 56 nm A_{Ricin}-encoded nanopore at -100 mV, with 100 nM ricin A-chain protein in the external solution. (b) The unbinding time before the first ricin block occurred (t_1) in various concentrations of ricin A-chain protein.

The integration of sensitive aptamers with our glass nanopore-terminated probe may overcome the main challenges encountered in most nanopore-based single-molecule biosensors. First, this nanopore adopts the receptor attachment mode, rather than the molecular translocation mode, for single-molecule detection. Only molecules that are recognized by the immobilized receptor are able to yield featured block signals, providing high selectivity. Second, by using aptamers as the receptor, our nanopore gains the ability to produce stepwise single-molecule blocks, which has not been reported in other functionalized nanopores. Unlike antibodies, aptamers are much smaller than their targets, making target blockades much more distinguishable. Through stepwise blocks, we were able to "visualize" single protein molecules that were sequentially captured in the aptamer-encoded nanopore. This capability increased the detection sensitivity to ~100 fM. The discrete single-molecule blocks are particularly useful in real-time applications where the background current drifts or fluctuates along over time, because the digital signal of the discrete blocks distinguishes them from the analog background signal. Third, using aptamer-encoded nanopores, we simultaneously detect binding at multiple sites at a single-molecule resolution. In the case of very long target•receptor binding duration, such as target•aptamer, this multi-site detection mode would be much more sensitive than single-site detection. This is because the rate of binding occurrence (s^{-1}) will increase with the number of binding sites. Lastly, the aptamer-encoded nanopore should be programmable, because nanopore can be functionalized with various DNA and RNA aptamers that target high impact substrates.

In conclusion, we have constructed an aptamer-encoded nanopore that allows for selective, sensitive and quantitative protein detection at a single-molecule resolution. The benefits of these capabilities include the possibility of establishing an advanced strategy for multi-target detection with a universal nanopore.

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