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Polycationic probe-guided nanopore single-molecule counter for selective miRNA detection

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Summary

MicroRNAs (miRNAs) are a class of non-coding RNAs that are being explored as a new type of disease biomarkers. The nanopore single-molecule sensor offers a potential non-invasive tool to detect miRNAs for diagnostics and prognosis applications. However, one of challenges that limits the clinical applications is the presence of a large variety of non-target nucleic acids in the biofluid extracts. Upon interacting with nanopore, Non-target nucleic acids produce “contaminative” nanopore signal that interference target miRNA discrimination, thus severely lowering the accuracy in target miRNA detection. We have reported a novel method that utilizes a designed polycationic peptide-PNA probe to specifically guide the target miRNA migration toward the nanopore, whereas any non-target nucleic acids without the probe bound is rejected by the nanopore. Consequently, non-target species are driven away from the nanopore and only the target miRNA can be detected in a low concentration. This method is also able to discriminate miRNAs with single-nucleotide difference by using PNA to capture miRNA. Considering the significance and impact of this substantial advance for the future miRNA detection in biofluid samples, we prepare this detailed protocol, through which the readers can have a view on the experiment procedure, data analysis, and result explanation.

Keywords

nanopore; miRNA (miRNA); single molecule; biosensor; cancer; HIV-1 TAT peptide; PNA; probe; diagnostics; nucleic acids

1. Introduction

The nanopore offers a single-molecule platform for developing the next-generation biosensors and exploring various life science problems.^{1–14} The nanopore technology can be utilized for rapid and low cost gene sequencing, because individual base identity can be read out through the current change during gene fragment translocation in nanopore.^{15–23} The sensitivity of nanopore have also been applied to analyse epigenetic biomarkers, such as DNA methylation²⁴ and gene damage.²⁵ Our recent effort has been developing the nanopore sensor for electric detection of microRNAs (miRNAs).^{26, 27} The motivation is that miRNAs are a class of noncoding RNAs playing important roles in gene expression modulation.^{28–31}

Numerous research results have found that miRNAs are potential cancer biomarkers.^{32–40} Aberrant expression of specific miRNAs in the cells and biofluids are associated with cancer development. Our nanopore-base single-molecule counting approach for circulating miRNA detection would offer a potential non-invasive tool for cancer diagnostics.

However, current nanopore sensors are limited to detect miRNAs directly from patient's samples. One of challenges is the “contaminative signals” generated by non-target nucleic acids in the samples. The RNA extraction from biofluids not only contains the target miRNAs, but also numerous non-target nucleic acids species, such as miRNAs, mRNAs, tRNAs, et al. Due to carrying negative charge, all these nucleic acids species can be driven to interact with the nanopore and change its conductance under the voltage. In addition to linear-form single-stranded and hybridized double-stranded nucleic acids, RNAs can also fold tertiary structures that may clog the nanopore. Overall, the signals produced by non-target nucleic acids can severely interference the accuracy for target miRNA detection. Accurate detection of miRNA in real sample highly demands a method that enables the nanopore to only selectively capture the target miRNA, but reject any non-target nucleic acids from entering the nanopore.

We recently invented a polycationic probe guiding approach that can completely overcome this challenge.⁴¹ Briefly we designed a polycationic probe as a cargo to specifically guide the migration of the target miRNA toward an engineered nanopore, whereas any non-target species without probe binding migrates away from the nanopore driven by the voltage. Consequently, we can only observe signature signals of the target miRNA binding with the the probe, but not any signals of non-target nucleic acids. The probe comprises a short polycationic peptide linked with a peptide nucleic acids (PNA). The PNA is used to specifically capture target miRNA, while the polycationic peptide is a leader that guides the miRNA to be captured by the nanopore. Considering the broad application value and the important nanobiophysics mechanism of this method, we prepared this short protocol to introduce the detailed experiment procedure, including nanopore formation, nanopore current recording, signal analysis, and miRNA quantification, with a discussion on accuracy, sensitivity, specificity, versatility, multiple detection, and broad impact of the approach.

2. Materials

2.1. Reagents

1. Pre-treatment solution: 1:10 hexadecane:pentane. Mix 250 μ L hexadecane with 2.5 mL pentane.
2. Lipid solution: 10 mg/mL. Dissolve 25 mg 1,2-diphytanoyl-sn-glycerophosphocholine (Avanti Polar Lipids) in 2.5 mL pentane.
3. Nucleic acids stock solution: 1 mM. Dissolve synthetic miRNAs including miRNA *let 7a*, *7b* or *7c* and peptide-PNA probes in RNAase-free water to 1 mM respectively.
4. Recording solution: 0.5, 1 or 3M KCl, buffered with 10 mM Tris and titrated to pH 7.2.

5. α -hemolysin protein: 10–100 ng per 50 μ L. Dissolve the K131D protein with ddH₂O.

2.2. Setup and instrument

1. Preparation of the chamber
 - 1) Compartments. Fabricate two Teflon compartments. Each compartment has a volume of 0.2–2 mL depending on specific design.
 - 2) Partition. Prepare a piece of 25- μ m thick Teflon film (Goodfellow) as partition and puncture a 100–150 μ m wide tiny aperture in the center of the film. The lipid bilayer membrane will span over the aperture.
 - 3) Chamber assembling. Chamber is assembled by tightly click the partition with two compartment blocks as shown in Fig. 1a. Seal the partition/compartment interface with vacuum grease. The cells on both sides of the partition are named as *cis* and *trans*. The chamber is placed on the chamber holder, which has been pre-installed with two stirring motors under the bottom of the two cells for stirring. The chamber and the holder are placed in a Faraday box for shielding.
2. A pair of electrodes. Fabricate the Ag/AgCl electrode by merging a 0.5- or 1-mm wide silver wire in bleach overnight. A qualified electrode should have a dark brown surface. Fill 1.5% agarose resolved in 3M KCl in a 50 μ L plastic pipette tip and fix the electrode in the gel.
3. Pico-Ampere current amplifier. Record pico-Ampere ion current through the nanopore using an Axopatch 200B amplifier (Molecular Devices).
4. AD converter. Acquire the nanopore current data from the amplifier and save data in the computer through a Digidata 1440 AD converter (Molecular Devices).
5. Software. Record and analyze the nanopore current using a pClamp software (Molecular Devices).

3. Methods

3.1. α HL mutagenesis and nanopore purification

1. The K131D mutant α HL can be constructed using site-direct mutagenesis⁴². Digest the wild-type α HL plasmid by *Eco*NI or *Hind*III (Promega) respectively into a linear template for PCR. Add 20–30 ng linearized plasmid DNA and 200 pmol each of a non-mutagenic and a mutagenic primer with Expand Long Template PCR Synthesis Kit (Roche Diagnostic GmbH) to 50 μ L PCR mixture. Perform PCR in the following temperature condition: 94°C >5 min; 20 cycles of 94°C 1 min, 50°C 1 min and 72°C 3min; 72°C 6 min. The two long PCR fragments, each from an enzyme digest product, contain two overlap sequences. They are extracted from the agarose gel, mixed, and transformed into XL10-gold super-competent *E.coli* cells, to form recombinant plasmid. The mutant plasmid is finally extracted from *E.coli* cells.

2. Synthesize the K131D α HL protein using Coupled *in vitro* Transcription and Translation kit (IVTT, Promega) by following the company's protocol. A 25 μ l reaction contains 4 μ l DNA template (400 ng/ μ l in stock solution) and complete amino acid mixture (including [35 S]methionine) in IVTT components, and is incubated at 37 °C for 1 h to produce protein monomers. Mix the supernatant of reaction with rabbit blood cell membrane (rabbit blood from Pel-Freez Biologicals) to assemble the protein heptamer. Separate the assembled heptameric protein by electrophoresis on a 7.5% SDS-polyacrylamide gel. Cut the oligomeric protein band and dissolve the gel band in 500 μ l water. Collect the protein using a YM-10 Microcon centrifugal filter (Millipore), divide the protein solution into 10 aliquots, and store at -20 °C.

3.2. Nanopore formation

1. Formation of lipid bilayer. Drop 2–5 μ L of pre-treatment solution by a 10 μ L micropipette over the aperture of Teflon film, and immediately blow to dry. It is helpful to form a stable lipid bilayer. Then put a stirring bar into each cell of the chamber and place the chamber on the holder. Connect the chamber to the amplifier through a pair of Ag/AgCl electrodes. Voltage is applied from the *trans* electrode and *cis* cell is grounded. Form the lipid bilayer according to the monolayer folding process described in reference⁴³: inject 0.1–1 mL recording solution into each cell (0.2–2 mL), then drop 5 μ L of lipid solution by a micropipette onto the surface of the solution. Wait for 2–3 min to evaporate the pentane so as to form a molecular monolayer on the solution surface. Gently inject another 0.1–1 mL recording solution from the bottom of each cell. The liquid surfaces on both sides are raised over the aperture of the partition. Immediately, the lipid monolayers on both sides automatically hybridize and form a bilayer membrane covering the aperture. Use pClamp software to monitor capacitance and current thermal noise during membrane formation. A qualified lipid bilayer for single channel recording should have membrane resistance ~100 G Ω , capacitance ~100–200 pF, and current noise of 1.2–1.8 pA (the value of I_{RMS} on the amplifier panel).
2. Insertion of nanopore in the lipid bilayer. Upon bilayer formation, release 0.5–2 μ l of protein solution to *cis* solution close to the bilayer, followed by gently stirring the recording solution, while watching the current using pClamp software. After a short time (tens of seconds to several minutes), the current suddenly increases sharply from zero to a specific level at a holding voltage, such as ~180 pA at +120 mV for K131D α HL, which indicates the insertion of a single nanopore in the lipid bilayer. Upon insertion, the wide entrance (nanocavity) of the pore faces *cis* solution and the narrow entrance (β -barrel) to *trans* solution (see the nanopore orientation in Fig. 1b).

3.3. Simultaneous enrichment and detection of miRNAs with a polycationic probe

1. Probe•miRNA hybridization. The probe P_{7b} contains a 10-base PNA sequence designed to specifically hybridize with miRNA Let-7b. The PNA was extended

at the N-terminal with an 11 amino acids HIV-1 TAT polycationic peptide,⁴⁴ which includes eight positively charged amino acids (six arginines and two lysines) (Fig. 2a). P_{7b} and Let-7b mixture is added in *trans* solution.

2. Observation of single probe•miRNA molecules. Apply a positive voltage, such as +180mV, from *trans* side to the grounded *cis* side and record the current by pClamp software. This voltage polarity can drive cationic molecules toward pore, while repelling anionic molecules away from the pore.
3. Analysis of the signals. P_{7b} alone in *trans* solution shows a large number of Level-1 blocks (Fig. 2b). The duration of these blocks (τ_{off}) was 4.8ms and their relative conductance I_R/I was 8.2% (I_R and I are currents of the block and the empty pore, Fig. 2b). When the P_{7b} and miRNA Let-7b mixture is added in *trans* solution, a large number of distinct Level-2 blocks appear while Level-1 blocks diminishes. Compared with the Level-1 blocks, the Level-2 blocks were 6-fold longer with duration of 28 ± 4 ms and featured higher relative conductance at 26% (Fig. 2b). Therefore the Level-2 blocks serve as signatures for Let-7b identification. The occurring frequency of the signature signals generated by the P_{7b}•Let-7b complex (f_{sig} , the number of signature blocks per second) is linearly correlated with the Let-7b concentration, i.e. $f_{sig} \approx k_{on}[\text{Let-7b}]$, where k_{on} is the capture rate of the P_{7b}/ Let-7b complex and $[\text{Let-7b}]$ is the target miRNA concentration. Thus, f_{sig} is used to quantify miRNA concentration. To calculate f_{sig} is measuring the interval between adjacent signature blocks using the Clampfit software. τ_{sig} is obtained by fitting the histogram of all interval values to an exponential distribution by function below:

$$f(t) = Ae^{-t/\tau}$$

The inverted τ_{sig} is f_{sig} , i.e. $f_{sig} = 1/\tau_{sig}$.

4. Quantification of miRNAs. Repeat the P_{7b}/ Let-7b experiments described above in a series concentrations of miRNAs, such as 50 pM, 500 pM and 5 nM. Make a calibration curve using the set of f_{sig} at each concentration. The concentration of unknown Let-7b sample can be determined according the calibration curve.

4. Note

1. Polycationic probe. The polycationic probe is the key to guide the selective capture of target miRNA species. As shown in Fig. 2a, the probe is a peptide-PNA conjugate. PNA is used to capture miRNA, and the polycationic peptide is the leader that enables the nanopore to attract the probe•miRNA complex and guide its capture by the nanopore. The peptide leader is not necessary long, but need contain high density of positively charged amino acids to generate driving force near the nanopore entrance. The peptide-PNA co-polymer can be synthesized (commercially available) at once without additional chemical crosslinking, as peptide and PNA have the same backbone. The sequence of the

peptide leader is programmable. Its properties can be tuned by changing the peptide length and the number, position and type of charged amino acids. The peptide can also be functionalized in any position of the sequence by chemical modification (such as crosslinking at cysteine), making it possible to generate unique signatures for multiplex detection.

2. **Accuracy and specificity.** The most unique and useful advantage of this polycationic probe guiding method is that the probe (peptide-PNA) can guide the target miRNA to migrate toward the nanopore and is detected, whereas any non-target nucleic acid species that are not binding with the probe migrate away from the nanopore without generating any “contaminative” or interference signals. As a result of this “noise-free” detection, only target miRNA signal are specifically generated and accurately recognized. We have used this method to test the mixture of target miRNA and various non-target RNA and DNA. It has been found that the capture rate for the target miRNA alone and that in the mixture are the same, suggesting the accuracy of this method. This specificity of this method can be reflected by the capability to discriminate single-base difference between miRNAs with similar sequence, such as the Let-7 miRNA family. For example, the probe P_{7b} has been designed to detect Let-7, and can form fully matched hybrid P_{7b}•Let-7b. When P_{7b} hybridizes with another miRNA Let-7c, which has one-base difference from Let-7b, the hybrid P_{7b}•Let-7c forms a single mismatched base pair. Recorded in 3 M/0.5 M *cis/trans* KCl (pH 7.2) asymmetric solution at +130 mV. The duration of Level-2 signature of fully-hybridized P_{7b}•Let-7b was 2.3 s (Fig. 2c), suggesting that P_{7b}•Let-7b is so stable that it cannot be unzipped prior to releasing from the pore. In contrast, the P_{7b}•Let-7c hybrid containing one mismatch generates a distinct type of two-level block from Level 2 to Level 1 (Fig. 2c). The Level-2 signature was only 19 ms, about 120 times shorter than Level-2 blocks for P_{7b}•Let-7b; and the terminal Level-1 block corresponds to the unzipping of the hybrid followed by translocation of the probe through the pore, suggesting that one mismatch induced in a RNA/PNA duplex can greatly reduce its stability. Overall, different stabilities of RNA/PNA hybrids without and with mismatch can generate distinct signatures that can be utilized to discriminate single-base difference in miRNAs.
3. **Sensitivity.** The K131D mutant pore, which has been engineered with a ring of anionic aspartic acids at the *trans* opening, plays an important role in attracting cationic molecules. It greatly increases the target capture rate k_{on} . The P_{7b}•Let-7b complex was rarely trapped in the wild-type, while its k_{on} was $80 \pm 9 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ in the K131D. Also, it is much higher than the k_{on} for the miRNA in complex with a DNA probe, which was reported as $1.4 \mu\text{M}^{-1} \cdot \text{s}^{-1}$.²⁶ Therefore the combination of the polycationic probe and the engineered nanopore enhance k_{on} over 50-fold. The frequency of P_{7b}/ Let-7b signatures consistently increases with increasing Let-7b concentrations ranging from 50 pM to 5 nM at +180 mV. It can be fitted to a straight line in the log-log scale. Therefore, Let-7b can be detected as low as tens of picomolar. Note that the K131D pore itself may spontaneously generate blocks⁴⁵, but the occurrence is very low ($\sim 6 \times 10^{-3} \text{ s}^{-1}$

above +140 mV), and its current pattern (amplitude) is different from the signature of the probe•miRNA complex. Therefore, the background noise signal from the pore itself can be excluded.

4. **Multiplex detections.** This method has potential to be developed for multiplex detections, in which multiple target miRNAs as a biomarker panel can be simultaneously detected using one nanopore. Specific cancer diagnostics requires accurate detection of a biomarker panel that consists of multiple miRNAs. But currently one nanopore is only used to detect one miRNA. We have developed barcoded DNA probes for simultaneous detection of multiple miRNAs in one nanopore.²⁷ We designed a series of barcode probes to encode different targets. When the probe binds with the target, the barcode group such as polyethylene glycol attached on the probe can specifically modulate the pore's ion flow. The resulting signature serves as a marker for the encoded target, and counting different signatures allows simultaneous analysis of multiple targets in one pore. This approach was verified by using a panel of lung cancer-derived miRNAs as the target. Supported by these result, we can develop barcoded polycationic peptide-PNA probes to simultaneously detect multiple cancer-derived miRNAs in one nanopore. Specifically, the polycationic peptide sequence can contain a cysteine at a designated position, and various chemical groups can be attached to cysteine to generate specific blocking level when the probe is trapped in the nanopore. Each miRNA will be driven by a barcoded carrier-probe that can generate a distinct signature for target recognition. As a result, multiple miRNAs can be quantified by counting their distinct signatures in one pore.
5. **Versatility.** We have developed the polycationic probe guiding method to selective detect miRNA in the complex mixture with non-target species. The biophysical mechanism behind this nano-phenomenon is not the electrophoretic and the electroosmotic effect. The specific driving force comes from a short range (several nanometers) of electrical field formed by a group of negatively charged residues engineered around the *trans* entrance of the K131D pore. This field can only generate an attractive driving force on the polycationic peptide of the probe, but has much less repulsive force acting on the attached miRNA. Therefore the net force on the probe•miRNA complex remains attractive. Our unpublished data supports that this method is also applicable to long nucleic acids by using the probe that has the same polycationic peptide head as that we used for miRNA detection. We expect that that this biophysical mechanism can be utilized to selectively capture any gene fragment, long or short, in complex samples that contain a large number of non-target species.
6. **Significant and broad impact.** (1) The single-molecule counting method described here has potential to become a new player to meet clinical needs for miRNA and other non-coding RNA detections. In addition to high sensitivity and specificity; it is label-free, without amplification or secondary detection; it is fast (<1 hr), inexpensive and simple to operate, and offers a multiplex detection. It may provide higher accuracy than PCR in assaying of the patient samples.²⁶ For example, it could potentially become a new platform to develop a confirmatory

test when the lung nodule is identified by low-dose helical computed tomography (LDCT) in high risk populations for lung cancer screening. In a broad impact, the validated nanopore technology would be applicable in detection of any pathogenic DNA/RNA, single nucleotide polymorphism (SNP), and epigenetics such as DNA methylation, for early diagnosis, prediction of cancer metastasis and monitoring of response to therapy. Notably, the nanopore's SNP discrimination capability can be used not only to distinguish the family members of miRNA, but also to detect a point mutation of the oncogene DNA in blood (circulating nuclear acids) that holds a huge potential in cancer early detection and cancer monitoring. (2) The method we described in this report represents a substantial step toward the nanopore medical applications. Unlike traditional electrophoretic and electroosmotic driving that lack selectivity, the polycationic guiding approach we invented provides a new route to the nanopore selective capture of any nucleic acids target in the mixture for noise-free detection. Therefore for the first time it becomes possible to translate the nanopore sensor into a clinically usable tool. Our long-term vision is a compact, durable and reliable nanopore device for rapid, accurate, low cost and high-throughput assay in a clinical diagnostic setting. If this method can be validated, we would be able to selectively and sensitively detect any type of nucleic acids, DNAs or RNAs, long or short, in complex clinical samples. The outcome will open a new avenue with significance not only in cancer detection and other human disease molecular diagnostics, but more broadly in fields such as plant science and foodborne detection where rapid genetic detection is required.

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Figure 1. Diagrams showing the lipid bilayer/protein pore experimental setup and components for electrical detection of miRNA in the engineered nanopore. **a**, Schematic of experimental setup. **b**, Artificial lipid bilayer covers the aperture of Teflon film. **c**, The structure of α -hemolysin and the engineered site.

Figure 2. Polycationic probe-guided specific detection of miRNA using the K131D protein nanopore. **a**, Diagram showing cartoon of the *trans* opening of K131D with a group of negatively charged residues engineered around the *cis* entrance, and the structures (sequences) of the polycationic probe, miRNA (Let-7b) and the probe•miRNA hybrid. The probe contains a HIV-1 TAT polycationic peptide (cationic Lys and Arg residues are marked in blue) and PNA sequence complementary to Let-7b sequence (marked in green). The sequence of miRNA Let-7b is marked in red; **b**, (Upper) current trace of K131D pore showing that no block was observed in the presence of miRNA (Let-7b). (Middle) current trace of K131D pore showing that the polycationic probe (P_{7b}) alone generated a large number of Level-1 blocks. (Lower) current trace showing a large number of new Level-2 blocks that were generated by the probe•miRNA hybrid (P_{7b} •Let-7b) in the presence of the miRNA (Let-7b) and its probe (P_{7b}). Models of suggested molecular processes are illustrated on the right of

traces. Traces were recorded at +180 mV in 1 M KCl and 10 mM Tris (pH 7.2); **c**, (Upper) Current traces and cartoon model showing characteristic blocks generated by the P_{7b}•Let-7b hybrid that is fully matched. (Lower) Current traces and cartoon model showing characteristic blocks generated by the P_{7b}•Let-7c hybrid that contains a single mismatched base-pair (lower). Both blocks can be discriminated from the Level-1 block duration and the unzipping signature of the Level-2 terminal of the block. Traces were recorded at +130 mV in 3 M/0.5 M (*cis/trans*) asymmetric solutions.

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