MicroRNA Detection at Femtomolar Concentrations with Isothermal Amplification and a Biological Nanopore

Haolin Zhang[a], Moe Hiratani[b], Kentaro Nagaoka[a] and Ryuji Kawano[b, *]

[a] *Laboratory of Veterinary Physiology, Cooperative Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan* [b] *Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology (TUAT), 2-24-16 Naka-cho Koganei-shi, Tokyo 184-8588, Japan.*

* Corresponding author: Ryuji Kawano, Ph.D. E-mail: rjkawano@cc.tuat.ac.jp

Materials and Methods

Chemicals and Regents

We used the following reagents: 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC; Avanti Polar Lipids, Alabaster, AL, USA), *n*-decane (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and potassium chloride (KCl; Nacalai Tesque). Buffered electrolyte solutions [1.0 M KCl, 1×NE buffer 2 (New England Biolabs Japan Inc., Tokyo, Japan), pH 7.9] were prepared from ultrapure water. Wild-type alpha-hemolysin (Sigma-Aldrich, St. Louis, MO, USA and List Biological Laboratories, Campbell, CA, USA) was obtained as the monomer polypeptide, isolated from Staphylococcus aureus in the form of a powder and dissolved at a concentration of 1 mg/mL in ultrapure water. All aqueous solutions were prepared with ultrapure water from a Milli-Q system (Millipore, Billerica, MA, USA). For use, samples were diluted to the designated concentration using a buffered electrolyte solution and stored at 4°C. High performance liquid chromatography (HPLC)-grade DNA oligonucleotides and miRNA were synthesized by FASMAC Co., Ltd. (Kanagawa, Japan) and stored at −20°C and −80°C, respectively. The polymerase Bst is an N-terminal truncation of DNA polymerase I obtained from New England Biolabs Japan Inc. (Japan). Nt.AlwI, a nicking endonuclease that cleaves only one strand of DNA on a double-stranded DNA substrate, was also purchased from the same company. dNTPs were from Takara Bio Inc. (Shiga, Japan). 10× TBE buffer was obtained from Takara Bio Inc. (Japan) and was 1:10 diluted for gel electrophoresis. The power supply and an LED transilluminator were obtained from Bio Craft Co., Ltd. (Tokyo, Japan) and Optocode Corporation (Tokyo, Japan), respectively.

Isothermal amplifications

MiRNA amplification and polyT(20) generation were based on the three-way junction structure with the catalytic enzyme reaction. Before the enzyme reactions, the mixture of each miRNA and DNA was heated to 95°C for 5 min and then rapidly cooled to 0°C on ice. miR-20a (100 nM for gel and 1 fM for nanopore experiments) was added to 10 μL of 1× NE buffer 2 containing primer DNA (100 nM for gel and 1 fM for nanopore experiments), template DNA (100 nM for gel and 1 fM for nanopore experiments), 1.6 units of polymerase Bst, 4.0 units of Nt.AlwI, and 0.2 mM dNTPs. The solutions were incubated at 37°C for 1 hour prior to detection and the enzyme reaction was stopped by heating at 80°C for 20 min. The enzyme reaction without miR-20a was used as the negative control.

Gel electrophoresis

For gel electrophoresis, the product of miRNA-triggered polyT(20) generation were analyzed by 15% denaturing polyacrylamide gel electrophoresis [containing 19/1 acrylamide/bis (w/w)] in 1× TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3) at a constant power of 7.5 W for 45 min at room temperature. The gel was stained with diluted SYBR Green II (Takara Bio Inc., Japan) solution for 10 min, followed by the imaging process under blue LED irradiation and recorded by a digital camera.

Bilayer lipid membrane (BLM) preparation and reconstitution of α-hemolysin (αHL)

BLMs were prepared using a device produced by microfabrication. BLMs can be simultaneously formed in this device by the droplet contact method. In this method, the two lipid monolayers contact each other and form BLMs on a parylene C film that separates two chambers. BLMs were formed as follow: the wells of the device were filled with n-decane (2.7 μL) containing DPhPC (10 mg/mL). The recording solutions (4.7 μL) on each side of the BLMs contained 2 M or 0.2 M KCl and 1× NE buffer 2 (pH 7.9). αHL was reconstituted in BLMs to form a nanopore from the ground side. The reaction solution was also added to the ground side. Within a few minutes of adding the solutions, BLMs were formed, and αHL formed nanopore was incorporated into BLMs. When the BLMs ruptured during this process, they were recreated by tracing with a hydrophobic stick at the interface of the droplets.

Channel current measurements

For the channel current measurements, the channel current was recorded with an Axopatch 200B amplifier (Molecular Devices, USA), filtered with a low-pass Bessel filter at 10 kHz at a sampling rate of 50 kHz. A constant voltage of +120 mV was applied from the recording side, and the ground side was grounded. The recorded data from Axopatch 200B was acquired with

Clampex 9.0 software (Molecular Devices, USA) through a Digidata 1440A analog-to-digital converter (Molecular Devices, USA). The collected data was analyzed using Clampfit 10.6 (Molecular Devices, USA). DNA translocation and blocking were detected when >80% of open αHL channel currents were inhibited. All values represent the mean ± standard error. Nanopore measurements were conducted at 22 ± 2°C.

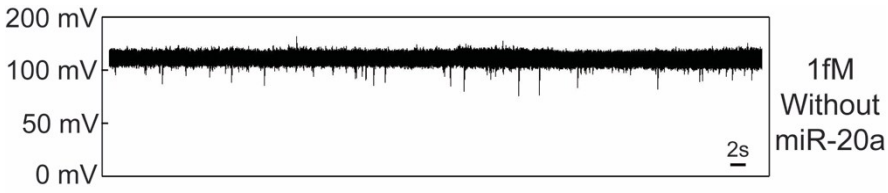

Figure S1. The nanopore detection of DNA amplification (1 fM DNA template and 1 fM primer) without miR-20a.

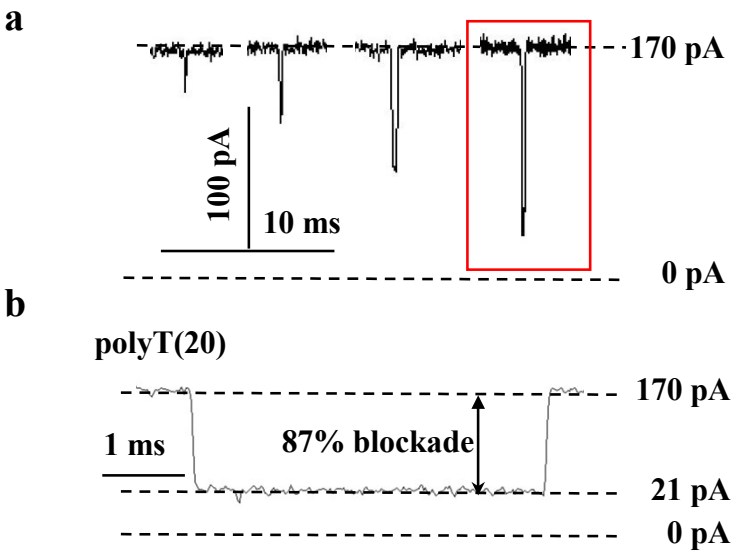

Figure S2. (a) The outline of the current blocking events during the nanopore detection of DNA amplification generated polyT(20). Red box represented the generated polyT(20) passing through nanopore. The other current blocking events represented the transient partial entry of DNA into nanopore. (b) The expanded views of the current blocking events by polyT(20). The average duration of the single event is 3.75 ms and the percentage of blocking current is 87%.

Table S1. The detailed information of translocation frequency for DNA and polyT(20) generated from miR-20a.

Nucleotides	Concentration	Event	Duration (s)	Frequency (1/s)
DNA*	1000 pM	129	570.95	0.23
	100 pM	103	648.84	0.16
	10 pM	27	295.52	0.09
	1 pM	9	242.96	0.04
miR-20a**	1 fM	75	763.45	0.10
	20 fM	14	111.99	0.13
	100 fM	60	309.64	0.19

*The known concentrations of polyT(20) were used for nanopore detection.
**The generated polyT(20) from miR-20a by isothermal amplification were used for nanopore detection.

Table S2. The comparasion of single translocation of polyT(20) and dT(50)

Concentration	Mean Duration (ms)	Blocking current percentage
polyT(20)	3.75	~87%
dT(50)*	~4	~89%

*The data was cited from Bulter et al., Biophysical Journal 2007(93): 3229-3240.