Fabrication of a Deoxyribonuclease Sensor Based on the Electrical Characteristics of DNA Molecules

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ABSTRACT: In this study, we developed a sensing device that can detect deoxyribonuclease (DNase) based on the electrical properties of deoxyribonucleic acid (DNA). We estimated the equivalent circuit between the electrodes with immobilized DNA and investigated whether the characteristics of the electrodes change before and after the DNase reaction. This method detects DNase by simply evaluating the electrical properties of DNA without using a fluorescent reagent. Therefore, inexpensive and highly accurate measurements can be performed with simple operations. However, detection sensitivity must be increased for practical feasibility. Hence, we investigated whether DNA immobilization is restricted by changing the shape of the electrode to a triangle with sharp edges, which may improve the sensitivity of DNase. Additionally, we attempted to detect DNase from an extremely small amount of sample solution using a microchannel. The device was able to quantitatively analyze DNase I activity with a detection limit of $5.5 \times 10^{-5}$ unit/$\mu$L. The results demonstrate the effectiveness of the proposed sensing device for various medical applications.

INTRODUCTION

Deoxyribonucleic acid (DNA) exhibits excellent molecular recognition and self-organizing functions, which make it a viable option for constructing various nanostructures.1,2 For instance, DNA origami is a nanostructure created by weaving long single-stranded DNA (ssDNA) and short ssDNA via self-assembly.3 Additionally, DNA has an electrical conductivity that relies on the base sequence and environmental conditions.4–6 It can also bind to specific biomolecules spontaneously.7,8,9 These characteristics enable DNA to serve as an effective sensing material in the medical field.

Deoxyribonuclease (DNase) is an enzyme that nonspecifically hydrolyzes the phosphodiester bonds of DNA. It exists in the cells and tissues of living organisms; in body fluids, including blood and urine; on the human skin surface; in the atmosphere; and in tap water.10–12 The DNase concentration in blood is being increasingly investigated as a diagnostic marker owing to its active involvement in specific diseases, such as myocardial infarction16,17 and systemic lupus erythematosus.18,19 In other words, the development of DNase measurement sensors can facilitate the early diagnosis of these diseases. Additionally, studies on DNA, such as genetic manipulation experiments, have demonstrated that DNase contamination causes major problems. Therefore, a small sensor for DNase detection can significantly improve the reliability of DNA-based experiments. One of the conventional methods for the detection of DNase is the single radial-enzyme-diffusion method,20 which uses a fluorescent reagent and employs fluorescence resonance energy transfer.21 Although these methods are highly sensitive, they present certain disadvantages, such as a long duration from preparation to detection and a relatively high cost of the fluorogenic oligonucleotide. Therefore, an inexpensive and highly accurate DNase measurement sensor with simpler operations is required. DNase can be electrochemically detected using ferrocenyloligonucleotide (FcODN)-immobilized electrodes. The FcODN is immobilized on the electrode through Au-S linkages.22 DNase I was detected by measuring the oxidation current with a detection limit of $1 \times 10^{-4}$ unit/$\mu$L. A highly effective sensing method was recently developed to detect endonuclease activity using copper nanoparticles as the electrochemical reporters. This sensor had a wide linear range of $10^{-3}$ to $10^{-1}$ unit/mL and a limit of detection of $10^{-5}$ unit/mL.23

In this study, we developed a highly accurate sensing device that can detect DNase in small volumes using the electrical properties of DNA. As DNA molecules typically form a complex random coil in solutions, the molecules must be stretched into a linear shape and immobilized on a substrate for sensing applications.24,25 Therefore, we used an electrostatic orientation to immobilize and stretch the DNA between the two...
microelectrodes. The behavior of DNA can be manipulated using the electric-field strength and applied frequency based on the electrostatic orientation.\textsuperscript{10,26,27} Furthermore, DNA can be stretched and immobilized at arbitrary positions depending on the shape and arrangement of the electrodes. In a previous study, we investigated the electrical properties of lambda phage DNA ($\lambda$DNA) molecules, which were manipulated by electrostatic orientation, based on their current–voltage characteristics and complex impedance plots.\textsuperscript{28} Additionally, we developed a DNase-detecting device using multiple $\lambda$DNA nanowires and performed several experiments using rod-shaped parallel electrodes.\textsuperscript{29} The device comprised a PDMS reservoir with a volume of 400 $\mu$L and two aluminum electrodes. This method enabled a simple and highly reproducible detection of DNase without DNA pretreatment or electrode surface modification. Furthermore, this DNase detection was simpler and faster than that presented by conventional fluorometric detection methods. Furthermore, the conductivity of DNA and the properties of the triangular electrodes were determined through the experimental analysis of DNase detection using the triangular electrodes.\textsuperscript{30} In this study, we developed a simple method based on a microfluidic channel that can be precisely handled with extremely small volumes. The microchannel allows sample small volumes to be filled between electrodes without requiring micropipettes or other tools to control the volume of the sample. Therefore, the dead volume of our device is extremely small, which avoids wastage of the sample and facilitates the conduction of experiments with minimal sample volume. We also estimated the equivalent circuit between the triangular electrodes with multiple $\lambda$DNA molecules using the electrochemical impedance spectroscopy\textsuperscript{31–33} and investigated whether the properties between the electrodes change before and after the DNase treatment. As triangular electrodes facilitate the immobilization of DNA molecules in a specific location, they are expected to exhibit more noticeable impedance changes than those exhibited by parallel electrodes. The experimental results indicate that the detection sensitivity of DNase can be improved, owing to the limited immobilization site of DNA molecules.

## MATERIALS AND METHODS

**Device for Electrostatic Orientation.** Figure 1a depicts a schematic of the device used to detect DNase. This device comprises an aluminum thin-film electrode fabricated on a glass substrate and a microchannel (50 $\mu$m in depth, 500 $\mu$m in width, and 10 mm in length)\textsuperscript{28} composed of polydimethylsiloxane (PDMS, KE-106, Shin-Etsu Chemical Co., Ltd.). Initially, an aluminum film (thickness of 200 nm) was evaporated on a 30 mm square glass substrate using a vacuum evaporation system. Subsequently, a photosist was applied, and ultraviolet exposure and development were performed. The electrode pattern was then formed via wet etching. To limit the locations for immobilizing the DNA molecules, the edges of the electrodes were sharpened. In this experiment, the electrode gap was set to 14 $\mu$m, which was slightly shorter than the length of DNA (16 $\mu$m).

**DNA Immobilization and Electrical Characterization.** The DNA specimens used in this study were the $\lambda$DNA extracted from *Escherichia coli* and procured from Takara Bio Inc. $\lambda$DNA is a double-stranded DNA helix that comprises 48,502 base pairs (length 16 $\mu$m). A DNA solution (1 nmol/L) was introduced into the microchannel with a volume of less than 10 $\mu$L, and an alternating current (AC) voltage was applied between the triangular electrodes to immobilize the $\lambda$DNA molecules. The formation of the AC electric field immobilized the $\lambda$DNA molecules on the sharp edges of the electrode,\textsuperscript{10} and their electrical properties were evaluated (Figure 1b). An impedance analyzer (HIOKI, IM3570) was used for the evaluation. The frequency was varied between 500 Hz and 5 MHz, and the impedance, $Z$, and phase, $\theta$, between the electrodes were measured at different frequencies in steps. The impedance was divided into real and imaginary parts based on these measured values, and a complex impedance plot of the DNA immobilized between the electrodes was generated. Subsequently, the equivalent circuit between the electrodes was estimated using the shape of the obtained complex impedance plot. The impedance of $\lambda$DNA was determined by calculating the appropriate values for each element of the equivalent circuit.

**Optimization of Flow Rate.** Typically, the DNA molecules may peel off from the electrodes when a solution is introduced through a microchannel at an extremely high speed, regardless of the presence or absence of DNase. Therefore, we attempted to optimize the flow rate of the solution using the following procedure.

1. Initially, a $\lambda$DNA solution was introduced into a PDMS microchannel, and an AC voltage of 1 MHz and 20 V<sub>pp</sub> were applied between the two electrodes using a function generator (TEXIO FGX-295). The AC voltage was applied for 15 min to stretch and immobilize the $\lambda$DNA molecules between the electrodes.

2. Ultrapure water was then introduced into the microchannel using an electric syringe pump (Fusion Touch 200, CX07200) to remove the scattered $\lambda$DNA molecules, which were not immobilized from the microchannel. Subsequently, the AC voltage application was terminated.

3. An impedance analyzer (HIOKI, IM3570) was used to measure the impedance, $Z$, between the two electrodes and the phase difference, $\theta$, between the voltage and current at each frequency. The measurements were performed at frequencies ranging from 500 Hz to 5 MHz, with an applied potential of 100 mV.

4. Ultrapure water was introduced again into the microchannel at the same flow rate as in Step (2), and $Z$ and $\theta$ were measured six times for every 10 min in 1 h from the beginning of immobilization.

5. The impedance value of the immobilized $\lambda$DNA molecules was estimated by creating a complex...
impedance plot with the $Z$ and $\theta$ values measured from the initiation of DNA immobilization up to 60 min after the washing with ultrapure water. The frequency of the applied voltage was used as a parameter.

6. The impedance change of DNA was evaluated corresponding to each flow rate, and the decrease rate of conductance was graphically presented to verify the exfoliation of DNA caused by the solution operation.

**DNase Detection.** When DNase was introduced between the electrodes after immobilizing DNA, λDNA molecules were cleaved by an enzymatic reaction (Figure 2). This reaction changes the impedance between the electrodes, and the extent of change depends on the concentration of DNase. In this study, we attempted to detect DNase based on the electrical characterization between the electrodes before and after the enzymatic reaction using the following procedure. The concentration of the DNase solution was adjusted using an appropriate buffer because DNase I requires Ca$^{2+}$ and Mg$^{2+}$ to hydrolyze double-stranded DNA.

1. A λDNA solution was introduced into a PDMS microchannel, and an AC voltage was applied for 30 min to stretch and immobilize the λDNA molecules between triangular electrodes with 12 gaps.
2. The DNA molecules were stretched and immobilized between the triangular electrodes with 12 gaps using an electrostatic orientation.
3. The impedance, $Z$, and phase difference, $\theta$, were measured, and a complex impedance plot was generated. After estimating the equivalent circuit, the resistance component of λDNA molecules before the introduction of DNase ($R_{\text{before}}$) was calculated.
4. A solution of DNase (Recombinant DNase I, Takara Bio Inc.) with a concentration in the range of $10^{-5}$ to $10^{-1}$ unit/$\mu$L was introduced into the microchannel at an optimized flow rate. After the microchannel was filled with the DNase solution, the device was placed in an incubator maintained at 37 °C for 30 min.
5. After removing the device from the incubator, the microchannel was washed with a solution of ultrapure water for 1 h.
6. The $Z$ and $\theta$ values between the electrodes were then measured once again. The resistance value of the equivalent circuit after the DNase treatment ($R_{\text{after}}$) was determined, and the resistance values before and after the DNase treatment were compared.

### RESULTS AND DISCUSSION

**Optimization of Flow Rate.** In a previous study, we evaluated the electrical properties before and after DNA immobilization. We observed that the complex impedance plot before DNA immobilization consisted of a circular arc, while that after DNA immobilization consisted of two semicircles, with a semicircle in the high-frequency range and a circular arc in the low-frequency range. In this experiment, we focused on the diameter of the semicircle that appeared in the high-frequency range (the real part of the impedance, i.e., the resistance component). This was used to determine any changes in the number of DNA immobilized between the electrodes. Figure 3 depicts the impedance change between the electrodes obtained when DNA was stretched and immobilized between the 12 sharp electrodes and ultrapure water was supplied continuously at a flow rate of 1.0 $\mu$L/min. This indicates that the size of the semicircle that appears in the high-frequency range increases as a function of the liquid transfer time. In the real part, the resistance value was 760 kΩ immediately after DNA immobilization, 890 kΩ 30 min after feeding, and 1.7 MΩ after 1 h. The increase in the resistance value from 30 min to 1 h indicates that the immobilized DNA molecules were unable to maintain their state and gradually peeled off between the electrodes at a flow rate of 1.0 $\mu$L/min.

Furthermore, the flow rate was changed from 0.3 to 5.0 $\mu$L/min, resistance component at the start of liquid feeding ($R_0$) changed after 1 min of liquid feeding ($R_1$), and increase ratio was determined to be $R_1/R_0$. Figure 4 depicts the relationship between the solution-sending time and impedance-increase ratio for each flow rate. When the flow rate was 5.0 $\mu$L/min, a large increase was observed in the resistance value of approximately two times 10 min after the onset of pumping, indicating that the DNA molecules were detached. Additionally, the resistance value increased slightly when the liquid feeding operation was performed for 1 h even at a flow rate of 0.5 $\mu$L/min. Conversely, the resistance remained unchanged at a flow rate of 0.3 $\mu$L/min. This analysis indicated that 0.3 $\mu$L/min was the optimal flow rate for operating the proposed device.

![Image](https://doi.org/10.1021/acsomega.2c02069)
The increase ratio was calculated as $R_n/R_0$, using the resistance component before pumping, $R_0$, and the resistance after $n$ minutes of pumping, $R_n$. The impedance increased by a factor of two within 10 min of pumping at flow rates of 5 and 10 μL/min. The impedance increased by a factor of two when the pumping operation was performed for over 60 min even at a flow rate of 1.0 μL/min. The increase ratio remained at one for the flow rate of 0.3 μL/min, even after 60 min of pumping, indicating that the impedance remained unchanged.

Detection of DNase. Following the immobilization of the DNA molecules between 12 sharp electrodes, a DNase solution was introduced at a flow rate of 0.3 μL/min. Typically, when the immobilized DNA molecules were cleaved by the enzymatic reaction, the impedance between the electrodes changes according to the number of cleavages. Therefore, we attempted to quantify the DNase concentration by analyzing the characteristics of the electrodes before and after the enzymatic reaction.

To determine the stretch/immobilization of DNA between the electrodes, we observed the λDNA molecules that were preliminarily labeled with YOYO-1. Figure 5a presents a fluorescence image of λDNA stretched and immobilized between the electrodes in the microchannel. The stretched λDNA was cleaved by the enzymatic reaction after the DNase treatment, and a fluorescent image of the curled molecules was obtained at the tip of the electrode, as shown in Figure 5b. Figure 5c illustrates the complex impedance plot of the immobilized λDNA molecules before and after the DNase treatment. The red circles represent the complex impedance obtained after the DNase treatment with 10−4 unit/μL, which increased resistance value of DNA ($R_{after}$) to 1.6 MΩ. Blue squares represent the complex impedance plot obtained after the DNase treatment with 10−4 unit/μL, which determined to be 400 kΩ after introducing the DNase solution. The resistance value of λDNA ($R_{before}$) was estimated to be 400 kΩ.

Figure 5. Fluorescence image of λDNA stretched and immobilized between two electrodes (a) before and (b) after DNase treatment. The λDNA was labeled with the fluorescence dye, YOYO-1, prior to alignment. (c) Complex impedance plots and (d, e) equivalent circuits of the DNase treatment. Red circles represent the complex impedance plot obtained from the immobilized λDNA molecules before the DNase treatment. The equivalent circuit after λDNA immobilization is assumed to be a series connection of two parallel R-CPE circuits. The resistance value of λDNA ($R_{before}$) was estimated to be 400 kΩ. Blue squares represent the complex impedance plot obtained after the DNase treatment with 10−4 unit/μL, which increased the resistance value of DNA ($R_{after}$) to 1.6 MΩ.

We were able to successfully obtain the increased impedance ratio, which was dependent on the DNase concentration (Figure 6a), when DNase solutions of various concentrations were introduced. Their definite correlations in the range of 10−3 to 10−1 units/μL of DNase concentration demonstrated that the device can be used to quantitatively analyze DNase. The limit of detection for DNase I was expected to be 5.5 × 10−3 unit/μL based on the correlations obtained for DNase I concentrations in the range of 10−5 to 10−4 unit/μL. It was demonstrated that detection in the 10−3 to 10−4 unit/μL range is possible for obtained after the DNase treatment. When the DNase solution was introduced at a concentration of 10−4 unit/μL, the complex impedance changed, and the semicircle increased slightly. Comparing the estimated resistance values of λDNA, the value was determined to be 400 kΩ after λDNA immobilization and changed to approximately 1.6 MΩ after introducing the DNase solution. The dashed and dotted black lines in Figure 5c represent the fitted curves obtained using the R-CPE circuit model and the parameter values listed in Table I. The value increases by four times when expressed as an increase ratio ($R_{after}/R_{before}$). This increase can be attributed to the cleavage of λDNA molecules immobilized between the electrodes by DNase, since the liquid transfer operation does not present a significant effect. In other words, DNase can be detected by measuring the impedance using a microfluidic device.

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Table I. Parameter Values of the R-Constant Phase Element (CPE) Circuit Model that Were Used for Curve-Fitting

| label               | \( R_1 \) (kΩ) | \( X_1 \) (Fm\(^{-1}\)) | \( p_1 \) | \( R_2 \) (MΩ) | \( X_2 \) (Fm\(^{-1}\)) | \( p_2 \) |
|---------------------|----------------|-------------------------|-----------|----------------|-------------------------|-----------|
| before treatment    | 400            | 1.0 \times 10^{-11}     | 0.9       | 70             | 4.0 \times 10^{-9}     | 0.75      |
| after treatment     | 1600           | 3.5 \times 10^{-12}     | 0.98      | 80             | 1.5 \times 10^{-4}     | 0.8       |

\(X\) represents the CPE coefficient, and \(p\) represents the CPE exponent (0 > \(p\) > 1). When \(p = 0\) and \(p = 1\), the corresponding CPEs are equivalent to the ideal resistance and ideal capacitance, respectively.

Figure 6. (a) Relationship between the deoxyribonuclease (DNase) concentration and increased impedance ratio. Red circles represent the relationship between the DNase concentration and increase ratio obtained from the triangular electrodes. The open circle on the ordinate represents the increase ratio in the absence of DNase I. The blue triangle represents the increase ratio obtained from the parallel electrodes. (b) Comparative images of \(λ\)DNA stretched and immobilized between the triangular electrodes and the parallel electrodes. The parallel electrodes could not detect impedance changes at DNase concentrations smaller than the \(10^{-5}\) unit/μL level. When the triangular electrodes were used, an increased ratio by a factor of two times approximately was obtained even at the \(10^{-3}\) unit/μL level. The standard deviation for the increased impedance ratio of the \(λ\)DNA molecules was less than 20%, indicating that the immobilized \(λ\)DNA molecules can be used for DNase detection.

In this study, we fabricated a microdevice for detecting DNase by immobilizing \(λ\)DNA molecules between two triangular electrodes. When the concentration of DNase was \(10^{-4}\) unit/μL, an impedance-increase rate of approximately four times was obtained after the DNase treatment. Furthermore, a definite correlation between the DNase concentration and increased impedance ratio was obtained. This result can be applied to the quantitative analyses of DNase. Moreover, the difference in the increased impedance ratio was investigated based on the shape of the electrode. We observed that the detection sensitivity of DNase improved when the immobilization site of DNA was restricted. In future, the proposed device can be used as a simple DNase sensing kit prior to genetic research experiments or can be mounted on ultrapure water production systems for genetic research.

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