ELECTROCHEMICAL REAL-TIME MONITORING OF ISOTHERMAL NUCLEIC ACID AMPLIFICATION FOR QUANTITATIVE ANALYSIS

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ABSTRACT

Label-free miniaturized electrochemical devices have been developed to monitor the nucleic acid amplification. We successfully monitored released protons during amplification reaction in real-time manner using miniaturized pH sensors and demonstrated quantitative detection of nucleic acid. Because the sensitivity of ion-sensitive field effect transistor (ISFET) (-58.0 mV/pH) and iridium/ iridium oxide (Ir/IrOx) electrode (-57.4 mV/pH) as a pH sensor showed close values to theoretical value (-59.2 mV/pH), the combination between isothermal nucleic acid amplification and the electrochemical detection was compatible with potentially stable measurement. The proposed system would be cost-effective and portable, and potentially implemented in clinical use such as diagnosis of infectious disease and cancer.

KEYWORDS

Electrochemical real-time monitoring, isothermal nucleic acid amplification, ion-sensitive field effect transistor (ISFET), iridium/ iridium oxide (Ir/IrOx) electrode

INTRODUCTION

Detection technologies of nucleic acids are more and more important in infectious disease and cancer diagnosis. Over a decade, much research has indicated that development of nucleic acid detection technique leads to improvement of the quality-of-life. Although the optical detection method by fluorescence labeling such as SYBR Green in polymerase chain reaction (PCR) is generally used for quantitative detection of nucleic acid, there are still several challenges to realize a point-of-care systems because of expensive reagents and apparatus, and also complicated operation. By contrast, label-free methods make systems simpler, and more cost-effective. A lot of nucleic acid assays without labeling for quantitative analysis have been reported, indicating surface plasmon resonance (SPR) [1], quartz crystal microbalance (QCM) [2], and electrochemical methods [3-5].

Recently, the combined electrochemical approach with PCR using the highly integrated device by making full use of semiconductor technology is reported as promising platform for unlabeled pH sensing during amplification reaction [6, 7]. In this approach, extension by DNA polymerase produces protons in a template-dependent manner, high-throughput reading and high parallel analysis are subsequently achieved by high-integrating ISFET sensors. However, PCR requires precise temperature control and rapid thermal cycling for denaturation, annealing, and extension. Since characteristics of semiconductor devices are very sensitive to temperature changes, the performance of the transistor-PCR device would be limited due to the potential instability during the measurement.

In order to overcome these problems, we propose the miniaturized electrochemical sensors in combination with isothermal nucleic acid amplification for real-time nucleic acid quantification without labeling materials aiming at improvement of potential instability and cost reduction of a system. These simple and miniaturized platforms will provide potential possibility for point-of-care testing and home-care system in the future.

EXPERIMENTAL METHODS

Isothermal nucleic acid amplification

The principle of isothermal amplification methods we used in this research are shown in Fig. 1. Rolling circle amplification (RCA) is the isothermal amplification process in which long single stranded (ss)DNA molecules are synthesized on a short circular ssDNA template by using a single DNA primer (Fig. 1a)). RCA is often employed for sensitive detention of DNA and RNA due to the simplicity, robustness, and high signal amplification [8, 9]. One of the advantages over the PCR is that the isothermal amplification progresses at 30^{o}C or even at room temperature by phi29 DNA polymerase. By modifying this linear amplification process, we employed two kinds of exponential amplification reaction for increasing proton generation. One was double-primed RCA (DP-RCA) which we originally modified linear RCA (Fig. 1b)), and the other was primer generation-RCA (PG-RCA) [10] which exponentially amplifies PG-RCA products with a circular DNA probe, phi29 DNA polymerase, and nicking enzyme (Fig. 1c)). In both cases, we confirmed the progress of a reaction through SYBR Green based real-time monitoring and gel electrophoresis.

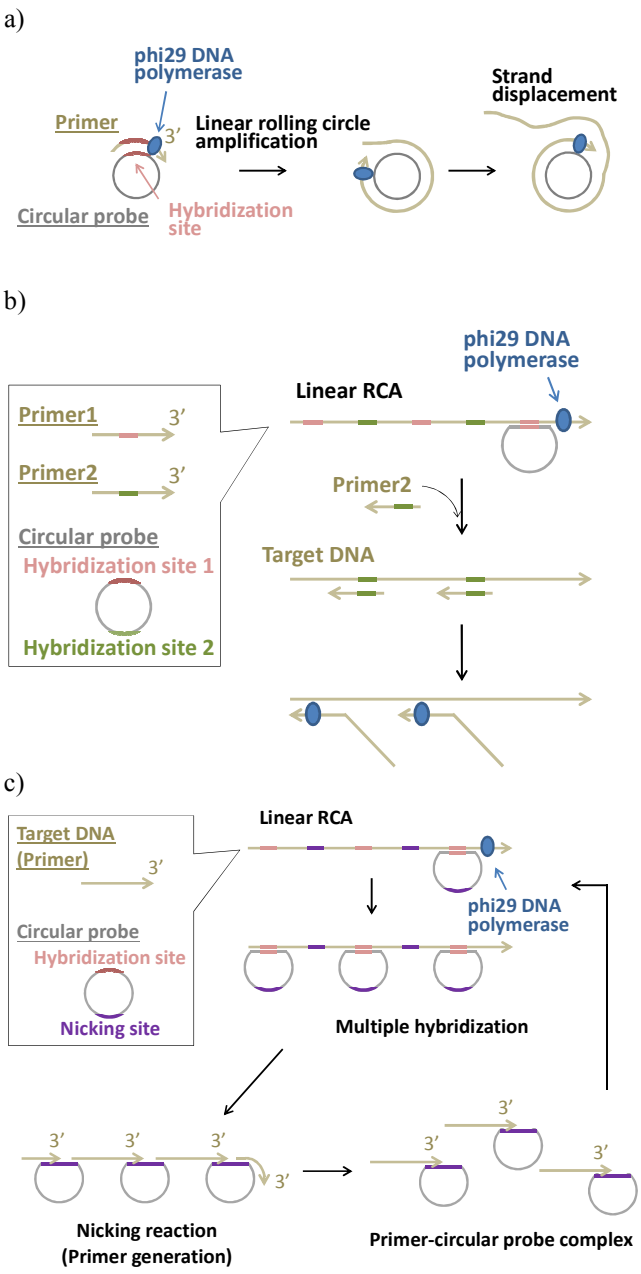


Figure 1: Schematic illustrations of isothermal nucleic and amplification. a) Rolling circle amplification (RCA), which shows linear amplification. b) Double primed RCA (DP-RCA) and c) Primer-generation RCA (PG-RCA) are exponentially-amplified methods.

pH sensing systems

For isothermal amplification experiments, we employed two kinds of pH monitoring system, namely, ISFET and Ir/IrOx electrode. ISFET was commercially available device (ISFETCOM Co. Ltd., Saitama, Japan), and Ir/IrOx was prepared by thermal oxidation method [11]. Briefly, thermal oxidation processing of Ir wire (φ 0.3 mm) was carried out at 800°C for 30 min under air atmosphere 3 times after immersing in 5 M NaOH solution for 2 days. The pH response was calculated from Nernstian equation (1):

$$E = E_0 + (RT/zF) \ln a_{ox}/a_{red} \quad (1)$$

whre E is electrode potential, E_0 is standard potential, R is molar gas constant, T is temperature, z is number of electrons exchanged, F is Faraday's constant, and a is activity. Fabricated pH sensing systems are shown in Fig. 2.

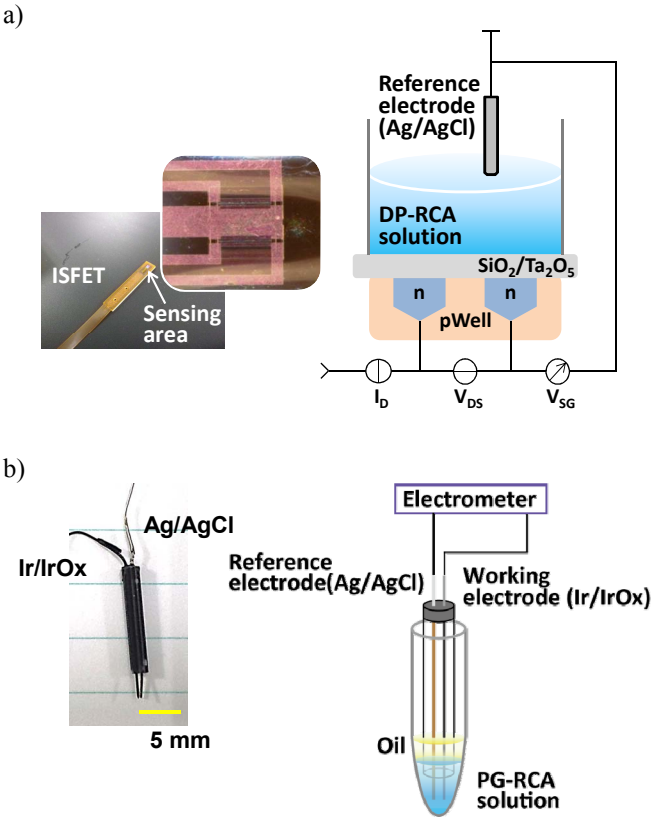


Figure 2: Electrochemically pH monitoring systems of isothermal nucleic acid amplification. a)DP-RCA with ISFET and b) PG-RCA with Ir/IrOx electrode.

RESULTS AND DISCUSSION

pH sensing property of ISFET and Ir/IrOx electrode

To monitor pH change during the amplification reaction, ISFET and Ir/IrOx electrode were used as a pH sensor. The ISFET sensor is an n-channel FET without metal gate and with a 40 nm thick layer of tantalum pentoxide (Ta_2O_5) as an insulator material. On the other hand, Ir/IrOx electrodes have been studied as a typical pH sensing material [9].

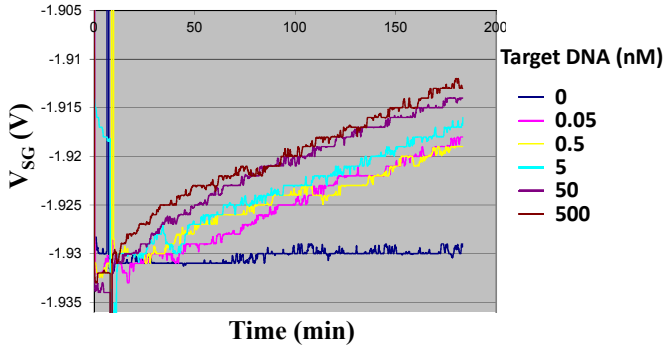
The pH sensitivity of ISFET and Ir/IrOx electrode in different pH buffer solutions were evaluated against Ag/AgCl. In evaluation of pH range from 4 to 8, the potentiometric response of ISFET and the Ir/IrOx electrode were -58.0 and -57.4 mV/pH, respectively. These values are close to the Nernst slope (-59.2 mV/pH at 25°C), indicating the excellent proton sensitivity of Ta_2O_5 and IrO_2. In addition, these pH sensors kept satisfied stability and pH sensitivity during test period.

Electrochemical real-time monitoring of isothermal nucleic acid amplification

We then compared the behavior of the sensor potential by monitoring with/without target DNA in DP-RCA with ISFET (Fig. 3) and PG-RCA with Ir/IrOx electrode (Fig. 4). PCR, which the system operates thermal cycle in Amplification reaction, generally needs Taq DNA polymerase having high-activity at 72 ^{o}C. On the other hand, using strand displacement-type phi 29 DNA polymerase, which works around 30^{o}C, is the one of features in DP - RCA and RG - RCA. It is effective in miniature of a detection device because thermal cycler is unnecessary.

DP-RCA_ISFET assay was performed using Ag/AgCl electrode as reference electrode. As shown in Fig. 3 a), the initial amplification speed in these potential curves increased with increasing concentration of target DNA. This amplification curve showed the similar behavior to real-time fluorescence monitoring. After the electric potential monitoring, the amplification product and progress of the DP-RCA reaction were confirmed by gel electrophoreses (Fig. 3 b)).

a)



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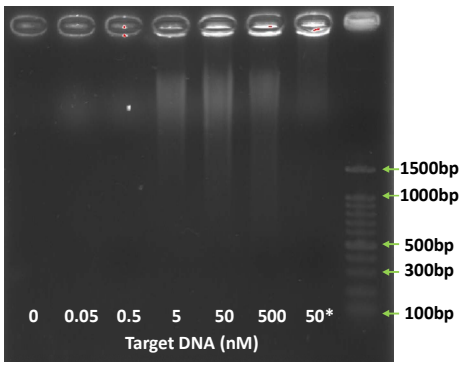
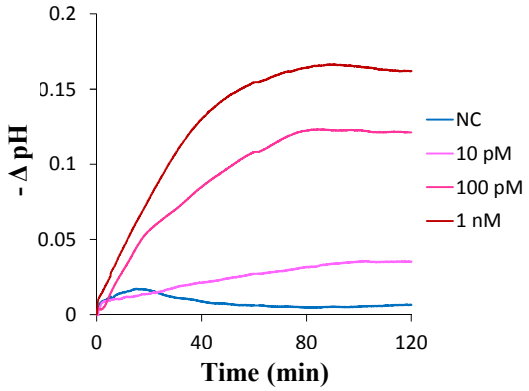


Figure 3: Isothermal nucleic acid amplification monitoring of DP-RCA with ISFET. a) Potential change against time. Concentration of target DNA was varied from 0 to 500 nM. b) Electrophoresis image of amplification products (shows second time analysis.).*

Subsequently, we investigated further in high-speed RCA reaction and miniature of a pH detection device i. e., PG-RCA_Ir/IrOx electrode assay. As in case of the DP-RCA_ISFET assay, the initial amplification curves increased according to the target DNA concentration. The observed potential value was calculated to pH change (Fig. 4 a)), and then the amplification product of PG-RCA was checked by gel electrophoresis (Fig. 4 b)). When a target DNA was not present in the PG-RCA solution, we could get slightly increase of the pH signal. While after several tens of minutes of pH monitoring, the obvious increase was observed in the presence of target DNA. Compared with DP-RCA, amplification reaction of PG-RCA progressed and reached equilibrium quickly. Although the measured detection limit was 10 pM, it seems to be lower than another biosensing system. We could further improve the sensitivity by optimizing of buffer capacity in the future.

Furthermore, this wire-typed pH sensor has some advantages with regard to miniaturization and mechanical strength, and may be a potential material for a portable nucleic acid detection device.

a)



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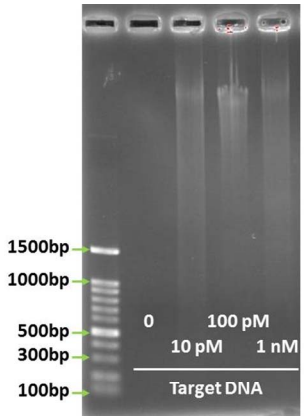

Figure 4: Isothermal nucleic acid amplification monitoring of PG-RCA with Ir/IrOx electrode. a) pH change against time. Concentration of target DNA was varied from 0 to 1 nM. b) Electrophoresis image of amplification products.

From these results, we successfully demonstrated electrochemical real-time monitoring of isothermal nucleic acid amplification by proton detection. It becomes possible to

determine the quantity of unknown target DNA concentration by making a calibration graph beforehand using known target DNA concentration. These devices would be also easily-worked with the shape. We believe that we can take the small instrument out from the laboratory to detect nucleic acids of viruses or microorganisms on site for the purpose of infectious disease testing based on our detection principle. As shown in Fig. 5, by connecting with IT technology, our small and simple device could be useful for medical treatment and clinical diagnosis in the future.

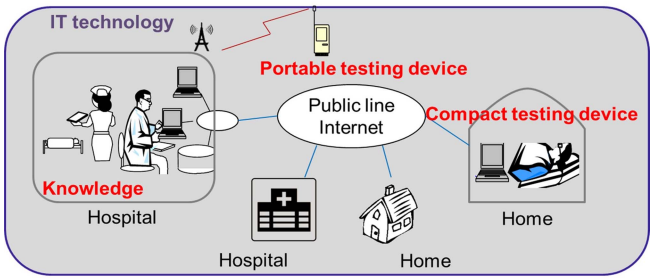

Figure 5: Use of portable testing device for treatment and diagnosis of disease.

CONCLUSION

In conclusion, we developed a micro pH sensing systems for quantitative nucleic acid amplification analysis. DP-RCA and PG-RCA which are isothermal nucleic acid amplification methods achieved stable electrochemical measurement. The ISFET and the Ir/IrOx electrode were employed as the device which monitors pH released during nucleic acid amplification. Our developed systems maintained abundant pH sensitivity during amplification reaction, and the potentiometric technique were performed label-free and real-time detection for target DNA. This feature would help to promote personalized medicine for individual patients in the future.

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