Label-free Nucleic Acid Amplification Detection using Electrochemical Sensors for Liquid Biopsy

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In recent years, isothermal nucleic acid amplification techniques have been developed as alternatives to polymerase chain reaction (PCR) requiring thermal cycles. The integration of isothermal amplification into electrical or electrochemical devices realizes high throughput nucleic acid-based assays and confers high sensitivity. In this research, we tried to develop electrical/electrochemical biosensors detecting nucleic acids aiming at application to future liquid biopsy. For detection of small DNAs or microRNAs (miRNAs), isothermal amplification methods were employed, and the signal changes based on the extension reaction by polymerases were detected using electrical/electrochemical devices, namely, a chronocoulometric sensor and a micro pH sensor. These small sensors based on electrochemical technique might be a promising tool in on-site detection of nucleic acids related to cancer diagnosis.

Keywords: Electrical/electrochemical biosensors, Isothermal amplification, Liquid biopsy, Chronocoulometry, pH sensors

1. Introduction

Nucleic acid amplification is an indispensable technique in many fields of life science from molecular diagnosis in foods to clinical research of tumor or other diseases for precision medicine. Most of the present real-time PCR systems employ fluorescent detection using intercalating reagents (e.g., SYBR Green) or sequence-specific reporter probes to quantify the amplified PCR products.

Liquid biopsies have got attention more than conventional biopsies by clinicians to diagnose and control disease, since it is possible to screen for the presence of disease through minimally-invasive body fluid test. Circulating cells, vesicles, and nucleic acids in patient’s blood (i.e. circulating cancer cells (CTC), circulating free DNAs (cfDNAs), exosomes, and miRNAs) have been actively researched recently as potential diagnostic biomarkers in liquid biopsy targeting cancers. For example, it is known well that the level of cfDNA is higher in diseased than healthy individuals [1,2]. A typical detection method in those researches is optical detecting techniques using fluorescent labeling, because the devices realize highly sensitive detection. On the other hand, electrical/electrochemical biosensing methods without labeling agents are also attracting attention owing to the advantage in miniaturization of instrument’s size, which is an unnecessary optical detection system such as laser excitation systems and fluorescence detectors. The electrochemical detection approach using the highly-integrated sensors based on semiconductor technology is reported as a promising platform for pH sensing [3, 4]. In this approach, extension reaction by DNA polymerase produces protons in a template-dependent manner. Such electrochemical pH detection devices offer advantageous in high-throughput reading and high parallel analysis because of the compatibility with microfabrication technique, and realize a reliable sensing without labeling materials.

In this research, we propose the electrical/electrochemical biosensors in combination with isothermal nucleic acid amplification for quantitative evaluation of nucleic acids aiming at miniaturization of devices [5,6]. The platforms using gold electrodes in
chronocoulometry (CC) and iridium/iridium oxide (Ir/IrOx) electrodes in pH detection were used for small DNAs or miRNA detection.

2. Experimental

2.1. Isothermal amplification

Rolling circle amplification (RCA) employs linear-type amplification method. RCA reaction is characterized by using a strand displacement type enzyme that is 29 bacteriophage DNA polymerase, and the extension reaction proceeds at a constant temperature close to room temperature (approximately 30 °C). A short primer DNA or RNA hybridized with circular probe is extended to a long single strand DNA or RNA with phi29 DNA polymerase. The reaction time requires approximately 4 hours normally. In the RCA-CC assay, microRNAs, DNA probes were purchased from Sangong (Shanghai, China) and listed in Table 1.

Exponential RCA reaction, which is primer-generation RCA (PG-RCA) developed by Murakami et al. [7], were also monitored nucleic acid amplification in real-time and label-free manner with micro pH sensors. PG-RCA achieves multiple primer generation during the reaction by incorporating a nicking site in circular probe, resulting in exponential amplification (Table 2).

2.2. CC detection

As shown in Fig. 1, solid-phase RCA was performed on gold electrode. For CC measurements, we fabricated a patterned thin-film Au electrode. The thiolated ss DNA was immobilized on the surface of the Au electrode. After adding target microRNA, phi29 polymerase and hexaammineruthenium (RuHex), solid-phase rolling circle amplification (RCA) was performed on the surface of the Au electrode at constant temperature of 30 °C. RCA products were detected in situ using CC with RuHex.

The amount of current in CC follows the Cottrell equation. The charge Q, which is a function of time, is expressed as the expanded Cottrell equation (Eq. 1).

\[ Q = 2FnA\left(\frac{Dt}{\pi}\right)^{\frac{3}{2}} + Q_c + Q_{ads} \]  

where \( F \) is the Faraday constant, \( n \) the number of electrons involved in electrode reaction, \( A \) the electrode area (cm\(^2\)), \( C_b \) the bulk concentration (mol cm\(^{-3}\)), \( D \) the diffusion coefficient (cm\(^2\) s\(^{-1}\)), \( t \) the time (s), \( Q_c \) the capacitive charge (coulomb), and \( Q_{ads} \) the charge produced by the adsorbed reactant. From Faraday’s law, \( Q_{ads} \) could be expressed in Eq. 2.

\[ Q_{ads} = nFA\Gamma \]  

where \( \Gamma \) is quantity of adsorbed reactant (mol cm\(^{-2}\)).

Table 1. Oligo nucleic acid sequences employed RCA-CC assay.

<table>
<thead>
<tr>
<th>Oligo DNA / microRNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circular probe</td>
<td>5'-phos-GTGGTTGTCTTTCTCTCAGCTCTAT CCGAATTGATCTCTCTCAGCTCTAT CCGAATTGATCTCTTAACGAGT</td>
</tr>
<tr>
<td>DNA probe1</td>
<td>5'-thiol-AAAAAAAAAAAAAAAAAAAAAGAG CTACAG</td>
</tr>
<tr>
<td>DNA probe2</td>
<td>5'-TGCTTCATCTCAAAAAAAAAAAAAAAGACAACCACACTGTTAGA</td>
</tr>
<tr>
<td>mir-143</td>
<td>5'-UGAGAUGAAGACACUGAUGCUC</td>
</tr>
</tbody>
</table>

Table 2. Oligo DNA sequences for PG-RCA-pH sensing.

<table>
<thead>
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<tbody>
<tr>
<td>Circular probe</td>
<td>5'-phos-GTGGTTGTCTTTCTCTCAGCTCTAT CCGAATTGATCTCTCTCAGCTCTAT CCGAATTGATCTCTTAACGAGT</td>
</tr>
<tr>
<td>Target DNA</td>
<td>5'-CAACCACACTGCTT</td>
</tr>
</tbody>
</table>
2.3. pH detection

Needle type-Iridium/Iridium oxide (Ir/IrOx), which is a typical proton sensitive material, was employed for electrochemical monitoring of PG-RCA. IrOx electrode was fabricated with thermal oxidation method [8,9]. The Ir wire (diameter: 0.3 mm) was oxidized at 800 °C for 30 min under air atmosphere. Subsequently, the oxidized Ir wire was immersed in 5 M NaOH solution for 2 days. The electrical property on proton sensitivity of Ir/IrOx shows Nernstian behavior. According to the Nernstian equation (Eq. 3), the linear dependence of electrode potential on \( \log [H^+] \) can be obtained.

\[
E = E_0 + \left( \frac{2.303RT}{zF} \right) \log a
\]  

where \( E_0 \) is the standard electrode potential, \( R \) the ideal gas constant, \( T \) the temperature in Kelvin, \( z \) the number of moles of electrons, \( F \) the Faraday constant, and \( a \) the ion activity.

3. Results and discussion

3.1. RCA-CC assay

It is known that miRNA143 (mir-143) works as a tumor suppressor for various cancers. We tried to detect mir-143 using the developed RCA-CC biosensor [5]. RuHex could be electrostatically trapped onto the three of phosphate groups of nucleic acids. Therefore, CC is able to give direct signal proportional to the number of phosphate groups. Effect of RCA reaction time to CC signal was evaluated, and the endpoint data were acquired at 10, 30, 60, 120, 240, 480, and 960 min. The signal intensity increased with increasing of reaction time, and then reached to saturation at 240 min. Sensitivity of this RCA-CC assay was examined by using stepwise dilution of target mir-143 ranging from 10 fM to 10 nM. The detection limit of RCA-CC assay was 1 fM. In the detection of mir-143 spiked in human blood samples, the detectable concentration was 1 pM. From these results, we successfully detected miRNA using RCA-CC system. As further experiments, it is necessary to fabricate label-free detection system without RuHex for clinical use.

3.2. PG-RCA-pH sensing

In general, modified RCA reactions are advantageous for electrochemical signal amplification compared with the linear RCA method. Primer-generation RCA (PG-RCA), which was developed by Murakami et al., achieved exponential amplification of target nucleic acids by incorporating nicking site into the circular probe [7]. When the circular probes hybridize to the target DNA, linear RCA reaction is initiated by strand displacement type of phi29 DNA polymerases. Subsequently, multiple primers are produced by nicking enzymes. Exponentially amplification proceeds under the constant temperature around 30 °C. To confirm the progression of PG-RCA reaction, the amplification test was performed using SYBR green method. The reaction was accelerated according to the increase of the concentration of target DNA from 0 to 1000 pM.

In a nucleic acid extension reaction by polymerase, when one dNTP is bound to the template strand, one pyrophosphate and one proton are released as by-products. Needle-type Ir/IrOx electrode was prepared to monitor the pH change in PG-RCA [6]. To confirm pH sensitivity of the prepared micro pH sensor, three-point calibration was carried out with standard buffer solutions at pH 4.0, 7.0 and 9.2. The potential slope against pH change of the pH sensor showed -57.5 mV/pH. This value was close to the ideal Nernstian slope (-59.2 mV/pH at 25 °C), which demonstrated the excellent proton buffering capacity. Figure 2 illustrates pH monitoring system for PG-RCA. The released protons were detected at the surface of the IrOx, and change in the potential was monitored with high resistance electrometer. Electrical detection of PG-RCA was conducted where the micro pH sensor recognizes the proton concentration as an indicator of amplification. The pH values calculated from Fig. 2. Micro pH sensor for label-free nucleic acid amplification detection. The pH shift during PG-RCA was monitored with the needle-type Ir/IrOx electrode.
potential measurement data were shifted to the acid side depending on the concentration of the target. This behavior is the same tendency with that of the conventional method using SYBR Green, and label-free detection method based on pH change found the possibility of quantitative nucleic acid analysis. Furthermore, we have been trying the microRNA assay using the Ir/IrOx chip.

Transistor-based DNA sequencer, which detects pH change during microbeads-based PCR, has been already commercially available [3,4]. To perform the high-performance sequencing on the transistors, beads bearing the templates that are amplified by emulsion PCR are employed. The transistor-based DNA sequencer system has realized highly sensitive detection because of integrated transistors; meanwhile, further increase of signal-to-noise ratio is required. Since the DNA sequencer system is combined with PCR, the thermal cycling process fluctuates electrical properties of the transistors in turn leading to unstable measurement. In contrast, the developed pH sensor in principle achieves stable measurement, because the sensor works on the basis of isothermal amplification strategy. Of note, the developed device is cost-effective due to the absence of semiconductor in the system, and it can miniaturize the size of the entire system. The device based on isothermal nucleic acid amplification would facilitate the success for future clinical use.

4. Conclusion
We developed nucleic acids detecting systems by combining isothermal amplification methods and electric/electrochemical measurement. Our devices are compact, easy to carry and easy to fabricate. Many researches are now addressing a range of challenges in biosensing: miniaturization, parallelization for high-throughput analysis, sensor integration, functionalization, and increasing the S/N ratio to analyze bigdata. Our platforms should be promising input parts of these IoT devices for liquid biopsy and health-check system in precision medicine.

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References