비수식화 DNA를 이용한 유전자 검출 및 새로운 DNA칩의 개발

論 文
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Development of New DNA Chip and Genome Detection Using an Indicator-free Target DNA

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Abstract - This research aims to develop an indicator-free DNA chip using micro-fabrication technology. At first, we fabricated a DNA microarray by lithography technology. Several probe DNAs consisting of thiol group at their 5-end were immobilized on the gold electrodes. Then indicator-free target DNA was hybridized by an electrical force and measured electrochemically in potassium ferricyanide solution. Redox peak of cyclic-voltammogram showed a difference between target DNA and mismatched DNA in an anodic peak current. Therefore, it is able to detect various genes electrochemically after immobilization of various probe DNAs and hybridization of indicator-free DNA on the electrodes simultaneously. It suggested that this DNA chip could recognize the sequence specific genes.

Key Words : Indicator-free DNA Chip, Micro-fabrication Technology, Immobilization, Hybridization

1. Introduction

The development of DNA chips, miniaturized arrays of immobilized single-strand DNA, is motivated by their potential for applications in disease diagnosis and genome sequencing[1]~[4]. In an array-based sensor, DNA probes of varying sequence are immobilized on a surface where the location of each probe is known. DNA diagnostics are performed by exposing the array to a solution containing single-strand DNA of unknown sequence, the target, that can be labeled with a fluorescent or radioactive marker. The suquence of the target is determined by imaging the array and correlating the position of "hot spots" with a probe sequence.

DNA chip microarray technology using the photolithography or stamping methods enables simultaneous analysis of thousands of sequences of DNA for genetic and genomic diagnostics and gene expression monitoring. Affymetrix[5] has developed GeneChip® using the photolithography technique. Brown[6]~[8] has developed DNA microarray using DNA arrayer which is an automated instrument to fabricate DNA chips. Conventional fluorescent detection style DNA chips and microarray scanners are too expensive to use. Only large research institutes or hospital can afford them.

On the other hand, as for the electrochemical measurement method, there are advantages such as the low cost of an analysis device, the simplification of the whole equipment and the analysis time, and the development to the portable DNA chip in comparison with fluorescence measurement method, and the research is carried out[9]. Recently, some electrochemical DNA sensors[10], [11] have been developed using electrochemically active DNA intercalators (metal coordination complexes, antibiotics etc.). Thorp[10] used $Ru(bpy)_3^{2+}$ (bpy=2,2′-bipyridine) as a detection marker for hybridization reaction and detected a single base pair mismatch. Also, it is detecting with an indicator-free method, or redox material is modified to probe DNA or target DNA mainly. There are the problems that should be guanine (G) in a base pair, or these methods introduce intercalator that reacts to DNA specifically, or redox material is modified to probe or target DNA[12], [13].

DNA chips must offer lower cost and greater material efficiencies to gain acceptance over traditional DNA diagnostic methods. Further reduction in the cost of performing DNA diagnostics can be realized by implementing less expensive detection methods.

Therefore, this research aims to develop the multi-channel label-free DNA chip array that has the above advantages and be able to solve the problems. At first, we fabricated a DNA microarray by microfabrication

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technology. It is able to detect various genes electrochemically after immobilization of various probe DNA and hybridization of label-free target DNA on the electrodes simultaneously. Probe DNAs were immobilized on the gold electrodes. Then indicator-free target DNA was hybridized and measured electrochemically. Redox peak of cyclic-voltammogram showed a difference between target DNA and mismatched DNA in an anodic peak current. It suggested that this DNA chip could recognize the sequence specific genes.

2. Experimental

2.1 Materials and Instrumentation

SH-p72 (5'-HS-AGGCTGCTCCCCCCGTGGCC-3'; MW: 6207.3, Tm: 80.5 [℃]) and SH-m72 (5'-HS-AAGCTGCTC CCCCCGTGGCC-3'; MW: 6191.3, Tm: 78.5[℃]) probe DNAs having thiol group at their 5' end and these target DNAs [p72; 3'-TCCGACGAGGGGGGCACCGG-SH-5' and m72; 3'-TTCGACGAGGGGGGCACCGG-5'], which was complementary to the probe, were synthesized and purified. The thiol modified DNA probes were purified to remove a protection group with NAP 10 columns (Pharmacia). The concentrated stock solutions were stored in a 10mM Tris-HCl (pH8.0) - 1mM EDTA solution denoted as a TE buffer at 5[℃]. Micropipette was used to immobilize probe DNAs.

Potassium ferricyanide ($K_3Fe(CN)_6$) and potassium chloride (KCl) were purchased from Wako Pure Chemicals, Ltd. (Tokyo, Japan). Other reagents were commercially available or were laboratory grade. High-purity water (=18.2[MΩ · cm]) was used in all solutions.

The electrochemical measurements were carried out using an electrochemical analyzer manufactured by CH Instruments Inc. Systems, Model 1030 and a computer system with data storage. Voltammetric experiments were carried out in a conventional cell with three electrodes at 25±1[℃] [a reference electrode Ag/AgCl (Bioanalytical Systems), a counter electrode Pt and another electrode mentioned later]. Unless otherwise indicated, voltammetry was carryout at 100[mV/s] in 5[mM] potassium ferricyanide and 100[mM] potassium chloride solution. Cyclic-voltammetry (CV) was measured in the range of -100~+700[mV] at 100[mV/s]. CV took the data of the 5th cycles of potential scans.

2.2 Fabrication of Microelectrode Array

Figure 1 shows fabrication process of micro-electrode array. About 200[nm] gold layer was deposited over a 20[nm] titanium adhesion layer on a glass by RF sputtering. Next, the chip was spin-coated with positive photoresist (S1818) and baked at 110[℃] for 1min in a oven (CLEAN OVEN PUHC-211, ESPEC). UV light (MA-10, MIKASA) was irradiated to a resist film for 25sec through a photo-mask. It was developed to form electrode, lead wires, and their connections by dipping it in developer (MF319) and filtrated water for 60sec. The lead wires were photolithographically covered with photoresist for insulation. Eight individually addressable gold electrodes (diameter : 0.7[mm]) and a Pt counter electrode were arranged on the chip. Each microelectrode was connected to an external potentiostat by insulated gold track.

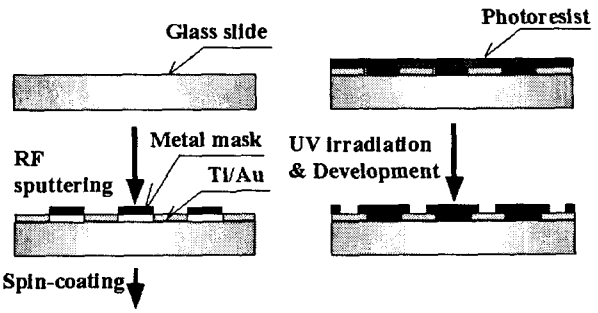


Fig. 1 Fabrication process of microelectrode array for DNA chip.

2.3 Immobilization of Probe DNA

The gold electrodes were reversibly cycled in a 10[mM] H_2SO_4 solution from 0 to 1.7[V] (vs Ag/AgCl) at 100[mV/s] until an ideal redox wave of H_2SO_4 was observed. Then, the electrodes were immersed in the solution of the DNA probe and the mismatched DNAs (5 [μM], 1[$\mu\ell$]) for 2h at 25[℃], and allowed to react utilizing the affinity between gold and sulfur and washed with distilled water to remove probes which were not adsorbed.

2.4 Electrochemical Gene Detection with the DNA Chip Microarray

The electrode modified with double-stranded 20-mer DNA (dsDNA-electrode) was prepared by the following procedure. An electric field was applied to the electrode modified with the single strand DNA probe (ssDNA-electrode) to hybridize the targeted gene (1[μM], 1[$\mu\ell$]), which was complementary to the probe as the target DNA, at 300[mV] for 5sec using a probe station. The specific hybrids (dsDNA) were formed on the ssDNA-electrode. After washing the electrodes, the immobilized DNA probe on the gold electrode was confirmed by voltammetric method using 5[mM] potassium

ferricyanide in 100[mM] potassium chloride solution at 100[mV/s]. The modified electrodes were stored in TE buffer. DNA prevents a redox response of potassium ferricyanide ion after immobilization or hybridization on the Au surface.

3. Results and Discussion

3.1 Fabrication of Microelectrode Array

Figure 2 shows microelectrode chip array including 8 gold electrodes and a Pt counter electrode. Each gold electrode was formed with electrode, lead wire, and their connection. The lead wire was covered with photoresist for insulation. Eight individually addressable gold electrodes and a Pt counter electrode were arranged on the chip. Each microelectrode was connected to an external potentiostat by insulated gold track.

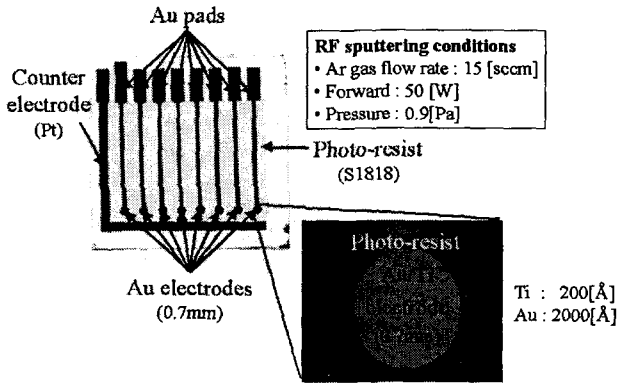
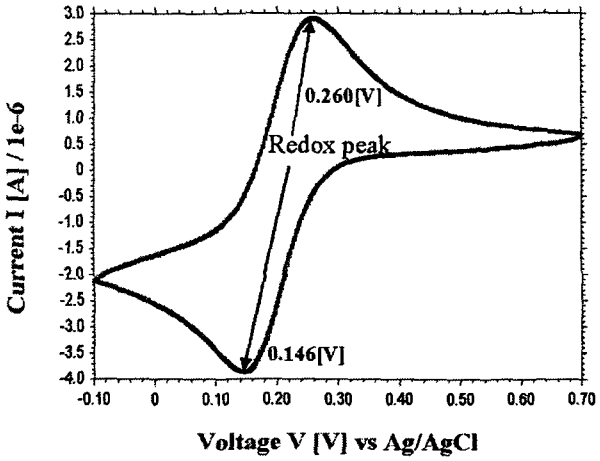


Fig. 2 Photograph of DNA chip microarray with 8 channels and 1 counter electrode.

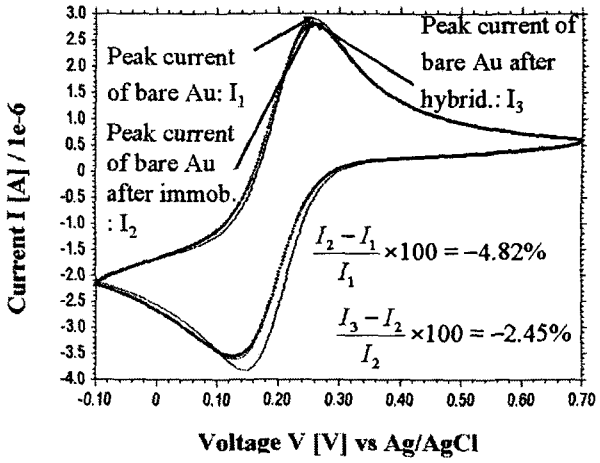
Cyclic-voltammograms of potassium ferricyanide with the bare gold electrodes are shown in Figure 3. In Figure 3 (a), a typical redox peak of potassium ferricyanide observed at 0.260[V] and 0.146[V], respectively. The redox peak currents of eight gold electrodes were almost coincided. This electrode array was used to immobilize and to hybridize DNA as it is. Figure 3 (b) shows a stability of cyclic-voltammogram of the bare gold electrode only before and after immobilization and hybridization of DNA. The redox peak of the bare gold electrode before and after immobilization and hybridization of DNA was decreased about 4.82% and 2.45%, respectively.

3.2 Immobilization of Probe DNA

The DNA probe having the thiol group was immobilized on the gold electrode. Cyclic-voltammograms of potassium ferricyanide with the bare gold electrode and the electrode modified with the DNA probe (ssDNA-modified) are shown in Figure 4. In Figure 4 (a) and (b), the peak currents of potassium ferricyanide decreased about 59.5% and the peak-to-peak separation (Ep) increased when the ssDNA-electrode was used compared with that of the bare electrode (Figure 4 (a)). On the other hand, the gold electrode treated with the DNA probe not having the thiol group made no changes in the current and E_p (data not shown). This suggested that the DNA on the electrode blocked the electrochemical reaction between DNA and the anionic redox couple ions. This result shows that the DNA probe is immobilized on the gold electrode through the thiol group of the 5' end. The ssDNA-electrode was used for the detection of a specific gene.



(a) Typical redox peak of potassium ferricyanide.



(b) Stability of cyclic-voltammogram of the bare gold electrode only without surface modification before and after immobilization and hybridization of DNA.

Fig. 3 Cyclic-voltammograms of potassium ferricyanide with the bare gold electrodes.

The ssDNA (SH-p72) was used for specific gene detection. When biological molecules are exposed to the electric field, molecules with a negative charge move rapidly to an area with a positive charge. Thus, hybridization of DNA having the negative charge through electronics is more accelerated than traditional passive methods. The ssDNA-electrodes were reacted with single stranded p72 by applying the electric field in the hybridization buffer. After hybridization, voltammetric experiments were carried out in 5[mM] potassium ferricyanide in 100[mM] potassium chloride buffer at 100[mV/s]. When the bare gold electrode was reacted with 1[μM] p72, the I_{pa} values were almost the same to that of the bare gold electrodes. In Figure 4 (c), the voltammetric data showed that when the ssDNA-electrode was reacted with 1[μM] p72, the I_{pa} value was decreased, and the change ratio of oxidation peak current [$(I_3/I_2-1)\times 100$, where I_2 is oxidation peak after immobilization and I_3 is oxidation peak after hybridization] is 72.9%. It is considered that the decreased value is derived from potassium ferricyanide ion due to DNA hybridization. Even if The same experiments were repeated 10 or more times the reproducibility was obtained. These results suggest that target DNA, p72, can be detected specifically by using potassium ferricyanide and ssDNA-electrode.

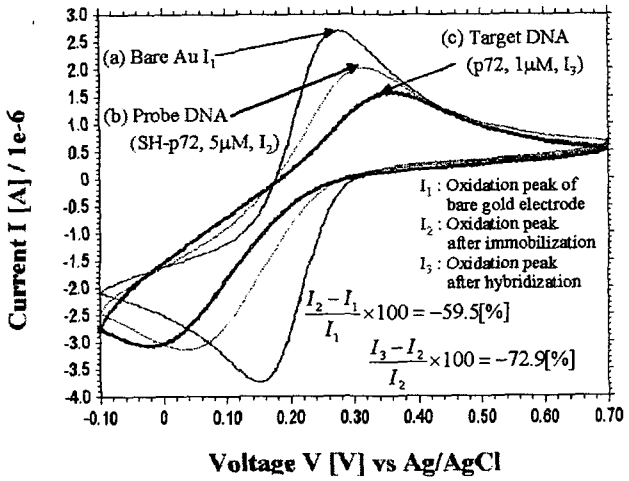


Fig. 4. Cyclic-voltammograms of 5[mM] potassium ferricyanide in 100[mM] potassium chloride at 100[mV/s] using (a) bare gold electrode and (b) probe-modified electrode (SH-p72) and (c) after hybridization with target DNA by the electrical field.

3.3 Electrochemical Gene Detection with the DNA Chip Microarray

In Figure 5 (a) and (b), the peak currents of potassium ferricyanide decreased about 86.4% and the E_p increased when the ssDNA-electrodes (SH-m72) were used in comparison to those of the bare electrodes. The ssDNAs (SH-m72) were used for non specific gene detection. The ssDNA-electrodes were reacted with single stranded p72 with the same method as Figure 4 (c) and measured the voltammetric experiments. In Figure 5 (c), after hybridization, the voltammetric data showed that when the ssDNA-electrode was reacted with 1[μM] p72, the I_{pa} values were almost the same, and the change ratio of oxidation peak current [$(I_3/I_2-1)\times 100$] was 8.9%. However, the change ratio of the peak current was -57.1%, when SH-m72 reacted with m72 of the complementary DNA at the same condition. It could be considered that the ssDNA probes (SH-m72) almost did not hybridize with the targeted gene.

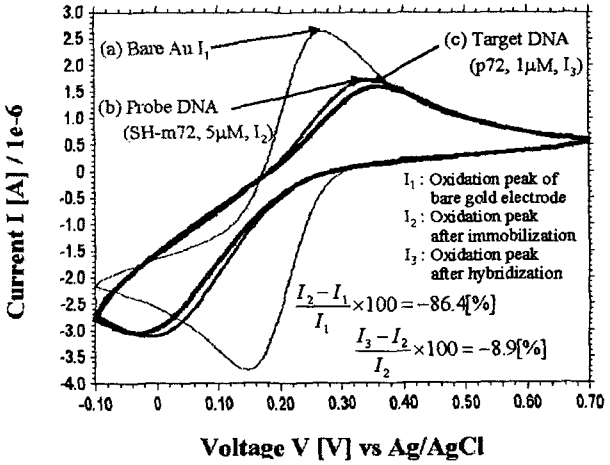


Fig. 5. Cyclic-voltammograms of 5mM potassium ferricyanide in 100mM potassium chloride at 100mV/s using (a) bare gold electrode, (b) after probe-modified electrode (SH-m72) and (c) after hybridization with target DNA.

4. Conclusions

In this study, the integrated microelectrode array was fabricated on slide glass using microfabrication technology. Probe DNAs consisting of thiol group at their 5-end were spotted on the gold microelectrode using micropipette utilizing the affinity between gold and sulfur. Cyclic-voltammogram of 5mM ferricyanide in 100[mM] KCl solution at 100[mV/s] confirmed the immobilization of probe DNA on the gold electrodes.

After hybridization of target DNA using the electric field, when several DNAs were detected electrochemically, there was a difference between target DNA and mismatched DNA in the anodic peak current values. It was derived from ferricyanide ion due to hybridization of

target DNA. These results suggest that target DNA can be detected specifically by using the indicator-free DNA chip array.

In principle, this method requires non-labeling of target DNA. This feature provides simple pretreatment of target DNA. It suggested that multichannel electrochemical DNA microarray is useful in developing a portable device for the clinical gene diagnostic system. Advantages of this method are process simplicity and wide applicability. This method can also be applicable to a new detection technology to develop various biosensors.

감사의 글

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