Articles

Hybridization by an Electrical Force and Electrochemical Genome Detection Using an Indicator-free DNA on a Microelectrode-array DNA Chip

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This research aims to develop DNA chip array without an indicator. We fabricated microelectrode array by photolithography technology. Several DNA probes were immobilized on an electrode. Then, indicator-free target DNA was hybridized by an electrical force and measured electrochemically. Cyclic-voltammograms (CVs) showed a difference between DNA probe and mismatched DNA in an anodic peak. Immobilization of probe DNA and hybridization of target DNA could be confirmed by fluorescent. This indicator-free DNA chip microarray resulted in the sequence-specific detection of the target DNA quantitatively ranging from 10^{-18} M to 10^{-5} M in the buffer solution. This indicator-free DNA chip resulted in a sequence-specific detection of the target DNA.

Key Words : Indicator-free DNA chip, Immobilization, Target DNA, Hybridization, Ferricyanide

Introduction

The development of DNA chips, miniaturized arrays of immobilized single-strand DNA, is motivated by their potential 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[5] or a radioactive marker. The sequence of the target is determined by imaging the array and correlating the position of "hot spots" with a probe sequence.

DNA-based sensors have potential applications that range from genomic sequencing to mutation detection and pathogen identification.[1-4,6] Conventional DNA chip systems employ confocal fluorescence detection.[7-10] Fluorescent detection DNA chips and microarray scanners are too expensive to use. Electrochemical detection is superior to fluorescent detection as regards the cost, portability and convenience. Recently, electrochemical DNA sensors[11-15] have been developed using electrochemically active intercalators. The above mentioned researches have been performed using fluorescent substances or indicators. Thorp[16,17] used Ru $(bpy)_3$2+ (bpy=2,2'-bipyridine) as a detection marker for hybridization reaction and detected a single base pair mismatch.

Steel[4] and Hahn[18] *et al*. have reported electrochemical quantization of DNA using ferricyanide. However, no integrated multichannel electrochemical DNA sensor has been developed. Microfabrication technology has already been established and enables to advance miniaturization and mass production of DNA chips. An integrated multichannel DNA chip can be microarray and this has advantages of low-cost and high through-put.

Therefore, this research aims to develop the multi-channel label-free DNA chip array that has the above advantages to solve the problems. At first, we fabricated a DNA microarray by microfabrication technology. It is able to detect various genes electrochemically after immobilization of various probe DNAs and hybridization of label-free target DNA on the electrodes simultaneously. 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. Redox peak of cyclic-voltammogram showed a difference between target DNA and mismatched DNA in an anodic peak current. This indicator-free DNA chip microarray resulted in the sequence-specific detection of the target DNA quantitatively ranging from 10^{-18} M to 10^{-5} M in the buffer solution. It suggested that this DNA chip could recognize the sequence specific genes.

Experimental Section

Materials and Instrumentation. SH-p72 probe DNA (5'-HS-AGGCTGCTCCCCCCGTGGCC-3'; MW: 6207.3, Tm: 80.5 °C), mismatched DNAs, SH-m72 (5'-HS-AAG-CTGCTCCCCCCGTGGCC-3'; MW: 6191.3, Tm: 78.5 °C) and SH-R72 (5'-HS-AGGCTGCTCCCCGCGTGGCC-3'; MW: 6247.3, Tm: 80.5 °C), having a thiol group[19,20] at their

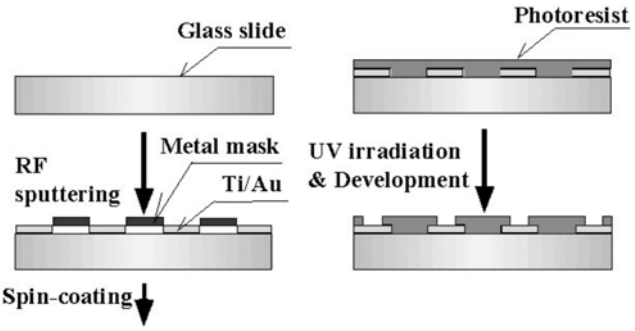

Figure 1. Fabrication process of microelectrode array for DNA chip.

5'-end and these target DNA [p72; 3'-TCCGACGA-GGGG GGCACCGG-SH-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 at 5 °C in 10 mM Tris-HCl (pH 8.0)-1 mM EDTA solutions denoted as TE buffers. 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 the 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 at 25 ± 1 °C in a conventional cell with three electrodes [a reference electrode Ag/AgCl (Bioanalytical Systems), a counter electrode Pt and another electrode mentioned later]. Unless otherwise indicated, voltammetry was carried out 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.

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 °C for 1 min in a oven (CLEAN OVEN PUHC-211, ESPEC). UV light (MA-10, MIKASA) was irradiated to a resist film for 25 sec 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 60 sec. 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.

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 μL) for 2 h at 25 °C, and allowed to react utilizing the affinity between gold and thiol group and washed with distilled water to remove probes which were not adsorbed.

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 aM-50 μM, 1 μL), which was complementary to the probe as the target DNA, at 300 mV for 5 sec using a probe station. After the reaction, the electrodes were sequentially treated with a reverse voltage and washed with the purified water for 1 min at room temperature to remove the non-specifically bound DNAs. 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 buffers. The DNA prevents a redox response of potassium ferricyanide ion after immobilization or hybridization on the Au surface.

Results and Discussion

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.

Confirmation of Immobilization of Probe DNA and Hybridization of Target DNA. Figure 3 shows the results that confirmed immobilization of the probe DNA (SH-p72, 5 μM) and hybridization of the target DNA (p-72, 1 μM). The target DNA was modified with FITC (fluorescence

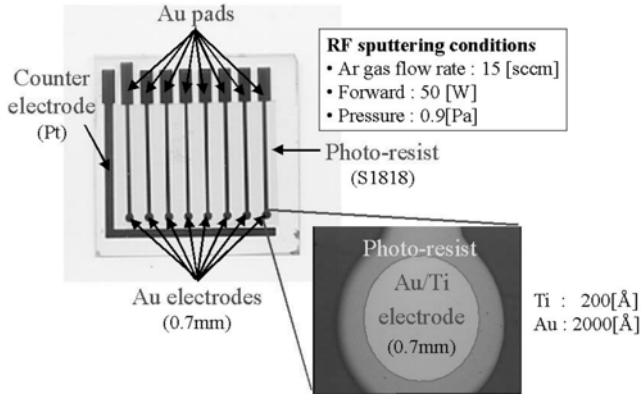

Figure 2. Photograph of the DNA chip microarray with 8 channels and 1 counter electrode.

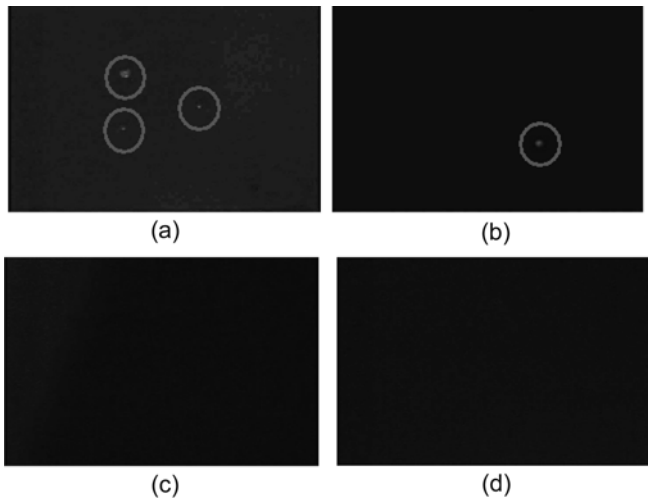

Figure 3. Confirmation of immobilization and hybridization. (a) Immobilization of the probe DNA modified with SH and FITC. (b) Hybridization of the target DNA modified with FITC by an electric force. (c) No SH group was modified to the probe DNA. (d) The mismatched target DNA was hybridized to the probe DNA by an electric force.

isothiocyanate) and detected using a fluorescence microscope.

In Figure 3 (a) and (b), because a fluorescent was confirmed, we could know that probe DNA was immobilized and the target DNA was hybridized to Au electrode.

However, we were able to know that the probe DNA was immobilized with a SH group if a SH group was not modified because the fluorescence was not observed (Figure 3(c)). On the other hand a fluorescent was not observed if probe DNA was hybridized with mismatched target DNA (Figure 3(d)).

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, 5 μM) 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 (E_p) 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 without 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.

The ssDNA (SH-p72, 5 μM) 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-

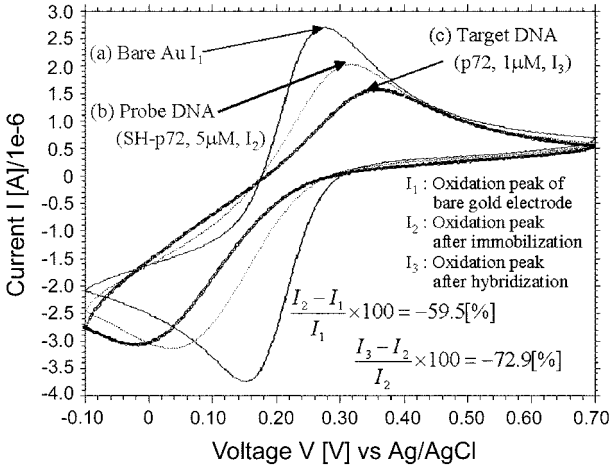

Figure 4. Cyclic-voltammograms of 5 mM potassium ferricyanide in 100 mM potassium chloride at 100 mV/s using (a) bare gold electrode, (b) probe-modified electrode (SH-p72, 5 μM) and (c) after hybridization with target DNA (p-72, 1 μM) by the electrical field.

electrodes were reacted with single stranded p72 (1 μM) 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 with 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] was 72.9%. It is considered that the decreased value is derived from potassium ferricyanide ion due to DNA hybridization. Reproducibility was observed to be kept when the same experiments were repeated 10 or more times.

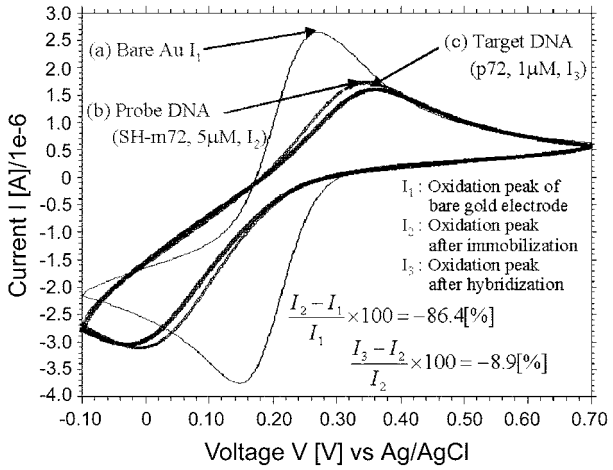

Figure 5. Cyclic-voltammograms of 5 mM potassium ferricyanide in 100 mM potassium chloride at 100 mV/s using (a) bare gold electrode, (b) after probe-modified electrode (SH-m72, 5 mM)) and (c) after hybridization with target DNA (p72, 1 μM).

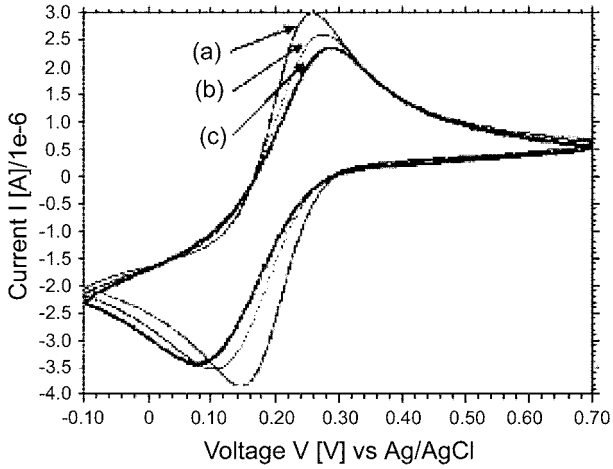

Figure 6. CVs of 5 mM potassium ferricyanide in 100 mM potassium chloride using (a) bare gold electrode (I_1), (b) probe-modified electrode (SH-R72, 5 μM; I_2) and (c) after hybridization with target DNA (p72, 1 μM; I_3), where I_1 is oxidation peak of bare gold electrode, I_2 is oxidation peak after immobilization and I_3 is oxidation peak after hybridization. $(I_2 - I_1) \times 100/I_1$ is −21.1% and $(I_3 - I_2) \times 100/I_2$ is −21.5%.

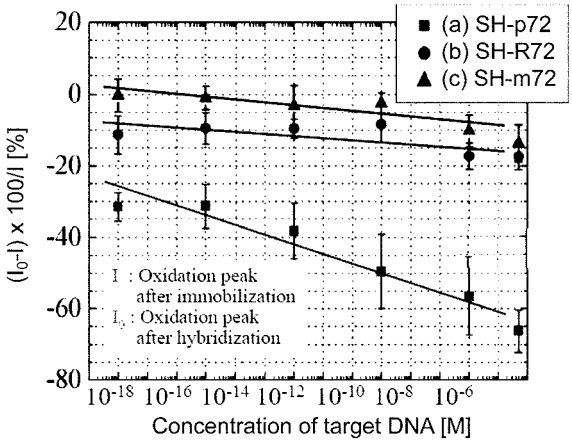

Figure 7. Calibration curves for concentration of target DNA p72 against (a) SH-p72, (b) SH-R72 and (c) SH-m72.

These results suggest that target DNA, p72, can be detected specifically by using potassium ferricyanide and ssDNA-electrode.

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, 5 μM)) 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 (1 μM)) with the same method as in 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.

In Figure 6(a) and (b), the peak currents decreased about 21.1% and the E_p increased when the ssDNA-electrodes (SH-R72, 5 μM)) were used in comparison to those of the bare electrodes. The ssDNAs (SH-R72) were used for non-specific gene detection. The ssDNA-electrodes were reacted with single stranded p72 with the same method as in Figure 4(c) and CV curves were measured. After hybridization, the voltammetric data (Figure 6(c)) showed that when the ssDNA-electrodes were reacted with 1 μM p72, the I_{pa} values were almost the same, and decreased by 21.5%. It could be considered that the ssDNA probes (SH-R72) almost did not hybridize with the targeted gene.

Calibration Characteristics. Figure 7 shows calibrating curves for concentration of the target DNA. In Figure 7(a), the calibrating experiments for the target gene p72 against SH-p72 probe DNA showed that the change ratio of peak current values, $[(I_0/I - 1) \times 100]$ where I is oxidation peak after immobilization and I_0 is oxidation peak after hybridization, were linearly related to the concentration of the target DNA p72 in the hybridization reactions ranging from 10^{-18} to 10^{-5} M. In Figure 7(b) and (c), however, when the target DNA p72 and the single base mismatched DNAs, SH-m72 and SH-R72, were reacted, there were almost no difference in the change ratio of peak current compared to the complementary probe DNA ranging from 10^{-18} to 10^{-5} M. It is considered that the new indicator-free DNA chip microarray can detect the targeted gene quantitatively with high sensitivity.

Conclusions

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

After hybridization of the 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. Immobilization of the probe DNA and hybridization of the target DNA could be confirmed by fluorescent. This indicator-free DNA chip microarray resulted in sequence-specific detection of the target DNA quantitatively ranging from 10^{-18} M to 10^{-5} M in the buffer solution. 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 a clinical gene diagnostic system. Advantages of this method are simplicity in process and wide applicability. This method can also be applicable to a new detection technology to develop various biosensors.

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