Electrochemical Sensor Array Chips
for Multiple Gene Detection

Hiroshi Aoki*, Akiko Kitajima and Hiroaki Tao

National Institute of Advanced Industrial Science and Technology (AIST),
16-1 Onogawa, Tsukuba, Ibaraki 305-8569, Japan

Electrochemical gene sensor array chips were prepared on the basis of photolithographically fabricated nine-channel gold electrode arrays modified with synthesized probe peptide nucleic acids (PNAs). The dimensions and cleanliness of the electrode array were defined by topographical and electrochemical methods. The prepared sensors, whose working principle is based on ion-channel sensing, detected the target DNAs with sequences complementary to those of the probe PNAs distinctively, selectively, and reproducibly. Multichannel responses to the DNAs were measured by cyclic voltammetry and were expressed on a gray scale. While this investigation for label-free multiple gene sensors with a simple detection mechanism is still preliminary, the studied technique helps to distinguish the presence or absence of the target DNAs in sample solutions, and is expected to supersede conventional DNA microarrays for diagnosis of gene expression.

1. Introduction

Gene detection methods based on fluorescence labeling of target nucleic acids are currently widely employed for genetic diagnosis and are able to simultaneously detect thousands of target genes.[1,2] However, fluorescence labeling is time-consuming and costly, owing to the requirement to thoroughly wash away unbound targets. Consequently, gene diagnostic techniques are still too limited to be performed in laboratories for now. Over the past decade, many researchers have devoted much effort to the development of label-free gene detection methods, including optical, microgravimetric, and electrochemical approaches.[3] Among them, electrochemical detection has significant advantages in terms of its simple design, small dimensions, and low-power requirements.[4–8]

We have developed functional gene probes and rapid and simple electrochemical

---

*Corresponding author: e-mail: aoki-h@aist.go.jp

gene sensors, whose working principle is based on ion-channel sensing, that enables the detection of genes without the need to label target genes.[9–12] The negatively charged $[Fe(CN)_6]^{4-}$ ions in the measurement solutions act as an electroactive marker on the electrode surface modified with noncharged peptide nucleic acid (PNA) probe molecules. The hybridization of the gene probe with the target DNA changes the surface charge to become negative. The electrostatic repulsion between the surface and the marker thus inhibits the redox reaction of the marker. The noncharged character of PNA works to enhance the change in the surface charge. The observed decrease in redox current of the marker indicates the presence of the target DNAs in the solutions. Ion-channel gene sensors based on the probe PNAs detected complementary target DNAs with the detection limit at the femtomolar level by optimizing the probe length and surface charge on the sensors, but not for noncomplementary DNAs even with only one-base mismatch (single-nucleotide polymorphisms (SNPs)).[9,10] This sensing technique has the advantage of being based on a simple electrochemistry-based detection mechanism; therefore, with the aim of applying this technique to electrochemical DNA microarrays, we tried to integrate these gene sensors to investigate their capability for multichannel gene detection.

In this study, electrochemical gene sensor array chips were prepared on the basis of photolithographically fabricated gold electrode arrays modified with synthesized probe PNAs. The dimensions and cleanliness of the electrode array were determined by topographical and electrochemical methods. The prepared sensors detected the target DNAs with sequences complementary to those of the probe PNAs distinctively, selectively, and reproducibly. Multichannel responses to the DNAs were measured by cyclic voltammetry and were expressed on a gray scale ranging from white (low) to black (high) for legibility. This study is a preliminary investigation of label-free multiple gene sensors with a simple detection mechanism, aimed at superseding conventional DNA microarrays for analysis of gene expression.

2.   Materials and Methods

2.1   *Reagents*

The gene probes, purchased from Fasmac (Kanagawa, Japan), were designed as conjugates of PNAs and cysteine as the recognition and anchor parts, respectively. Their structures are Cys-O-CTG GCT TTG GTC CGT CT-NH$_2$ (PNA_1), Cys-O-TAC TGT GGT TAT TGC TGT CT-NH$_2$ (PNA_2), Cys-O-ACC TCG TGA CCA TTA CTC-NH$_2$ (PNA_3), Cys-O-GCA ACC TTC CCT ATT ACT CCA C-NH2 (PNA_4), and Cys-O-ATT CTG TCT TTC ACG-NH$_2$ (PNA_5), where Cys and O denote a cysteine group and ethylene glycol unit, respectively (Fig. 1). The target oligonucleotides with the sequences of 5' AGA CAG CAA TAA CCA CAG TA 3' (DNA_2) and 5' GTG GAG TAA TAG GGA AGG TTG C 3' (DNA_4) complementary to PNA_2 and PNA_4, respectively, were purchased from Operon Biotechnologies (Tokyo, Japan). Six-hydroxy-1-hexanethiol (6-HHT) was from Dojindo (Kumamoto, Japan). OMR 83, OMR Developer, and Remover 502A were from Tokyo Ohka Kogyo (Tokyo, Japan). SU-8 3000 and SU-8 Developer were from Kayaku Microchem (Tokyo, Japan). An
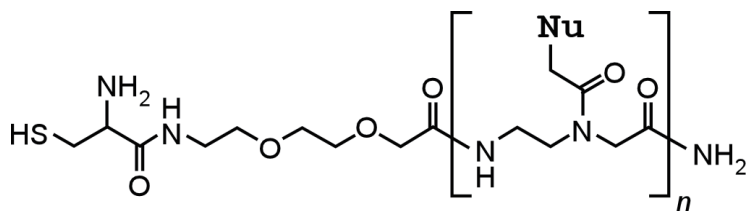
Fig. 1.   Chemical structure of the probe PNAs. Nu represents a single nucleobase (A, T, G, or C) and *n* probe length.

anisotropic conductive film (ACF, ANISOLM) was a gift from Hitachi Chemicals (Tokyo, Japan). All of the chemicals used were of analytical reagent grade. All aqueous solutions were prepared with deionized and charcoal-treated water (specified resistance >18.2 MΩ cm) obtained using a Milli-Q reagent grade water system (Millipore, Bedford, MA).

2.2   *Preparation of sensor array chips*

Electrode array chips (9 channels; array interval, 4–5 mm; diameter, 1.6 mm) were fabricated by photolithography (Fig. 2(a)). A 15-Å-thick chromium adhesion layer and a 2000-Å-thick gold film were deposited on a mechanically polished barium borosilicate glass plate (24×36 mm², #1737, Corning, NY) by a radio-frequency magnetron sputtering method employing CFS-4EP-LL sputter equipment (Shibaura Mechatronics; Kanagawa, Japan). An OMR 83 negative photoresist was spun on the plate for 30 s at 3,000 rpm, exposed to UV irradiation using a Supercure-203S UV light source (San-ei Electronic; Osaka, Japan) for 8 s for patterning with the electrode array, and developed for 5 min in OMR Developer. The pre- and post-bakes were performed for 30 min at 85 and 120°C, respectively. Excess gold and chromium were etched off by immersion in KI/I$_2$/water = 4:1:40 and HClO$_4$/Ce(NH$_4$)$_2$(NO$_3$)$_6$/water = 5:17:100 solutions, respectively. The photoresist was removed by immersion in Remover 502A. SU-8 3000 was spun on the plate for 30 s at 2,000 rpm, exposed for 8 s to cover the plate surface to delineate the electrode regions, and developed for 5 min in SU-8 Developer. After developing, the plates were rinsed with 2-propanol. The pre- and post-bakes were performed for 1 min at 65°C and for 3 min at 95°C, respectively. The electrodes have recessed structures similar to wells owing to the SU-8 cover, which is 10 μm thick as revealed by topographical measurement. For signal output terminals, flexible polyimide cables (9 pins; interval, 0.5 mm) were connected to the edges of the array chip with ACF by thermal compression bonding. The fabricated electrode array chip is shown in Fig. 2(b). Prior to use, the electrode array chips were surface-cleaned by subjecting them to Ar plasma emitted from a PIB-10 plasma ion bombarder (Vacuum Device; Ibaraki, Japan) for 2 min (Ar gas pressure, 10 Pa; plasma discharge power, 50 W). Gene sensor array chips were prepared by modifying the electrode surfaces with probe PNAs and 6-HHT as follows. One-microliter aliquots of 100 μM aqueous probe PNA solutions were dispensed onto the

(a)

Glass plate

Deposition of Cr and Au

Spin-coating photoresist

Exposure

Development

Etching

Removing photoresist

Spin-coating SU-8
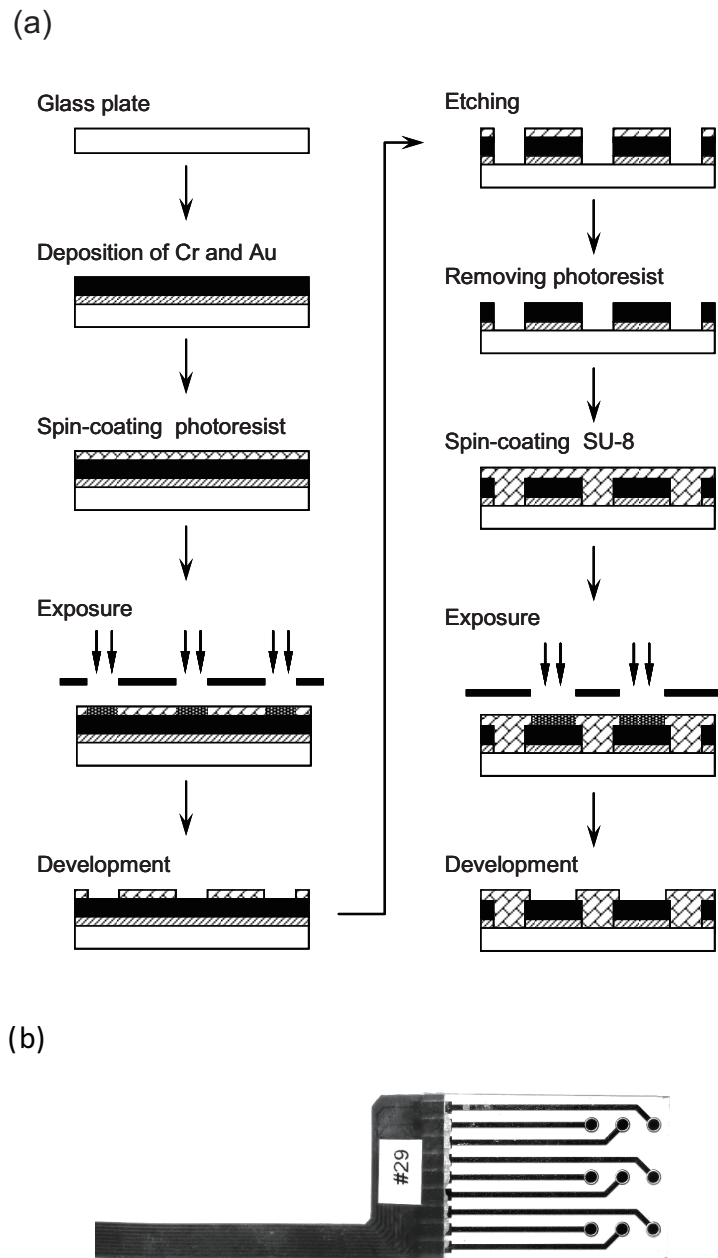
Exposure

Development

(b)

#29

Fig. 2. (a) Schematic overview of the fabrication process of nine-channel electrode array chips. (b) Photograph of a fabricated nine-channel electrode array chip (3×3 array; diameter, 1.6 mm).

corresponding electrode surfaces. After a 30 min immersion, the array chip was rinsed with a 0.1% trifluoroacetic acid aqueous solution and dried by blowing N_2. A 200 µL aliquot of 1 mM 6-HHT aqueous solution was then dropped on the chip to cover all the electrodes. After a 30 min immersion, the array chip was rinsed with water and subjected to electrochemical measurement.

2.3 *Electrochemical measurements*

Electrochemical measurements for the sensor array chip were performed in a 0.1 M $NaClO_4$ + 2.5 mM phosphate buffer solution (pH 7.0, Na^+ salt) containing 1 mM $K_4[Fe(CN)_6]$ as the electroactive marker at 25°C before and after incubation in an aqueous solution containing 100 µM DNA_2 or DNA_4. Cyclic voltammograms were recorded using an ALS-730C electrochemical analyzer (Bioanalytical Systems (BAS); Tokyo, Japan), with the three-electrode configuration consisting of the prepared sensor as a working electrode, an Ag/AgCl reference electrode (an internal solution: 3 M NaCl), and a platinum auxiliary electrode. The prepared sensor array chips were connected to the analyzer with the polyimide flexible cable. The potential was scanned from 0 to +0.5 V and again back to 0 V at a scan rate of 0.1 V s^{-1}. The sensor responses were observed using three or more than three sensors for each probe under the same conditions. The sensor response to the target DNAs was expressed as the ratio of peak current (i_0) to the current decrease ($i_0 - i$) at the peak potential (E_p) in the voltammograms measured before hybridization with target DNAs, similarly to other ion-channel sensors.[10] The normalized current change of $(i_0 - i)/i_0$ was depicted on a gray scale, where smaller or larger responses were expressed in white or black, respectively. Some sensors detected a current increase after incubation. In this case, the normalized current changes are calculated to be negative, rather indicating no responses to the DNAs. The responses less than 0 in such cases were expressed as 0 in a gray scale.

2.4 *Topographical measurements*

Topographical measurements of the dimension of the patterns fabricated on the plates were performed using an Alpha-Step IQ surface profiler (KLA-Tencor; San Jose, CA) and a VK-8500 color laser 3D profile microscope (Keyence; Ohsaka, Japan).

3. **Results and Discussion**

3.1 *Characterization of electrode array chips*

Generally, gold thin films deposited on glass plates have a uniform surface character. With the goal of the preparation of consistent electrode surfaces, a gold thin film was fabricated on an electrode array chip. Prior to the modification of the electrode surfaces on the chip to prepare a sensor array, the surfaces were characterized electrochemically. Cyclic voltammetry of 4.0 mM $[Fe(CN)_6]^{4-}$ marker at a scan rate of 0.1 V s^{-1} in an aqueous solution of 1 M KCl for the thus prepared electrode array chip yielded reversible voltammograms (value of peak potential separation, ΔE_p, was about 60 mV) for all the electrodes. This result shows that the electrode surfaces are free from contaminants

and have a uniform characteristic as a gold thin film on a glass substrate. In reversible systems, the peak current in the observed voltammogram, i_p, at 25°C can be described using the following equation:[4]

$$i_p = (2.69 \times 10^5) n^{3/2} \, AD^{1/2} C v^{1/2} \qquad (1)$$

$$[Fe(CN)_6]^{4-} \rightleftharpoons [Fe(CN)_6]^{3-} + e^-, \qquad (2)$$
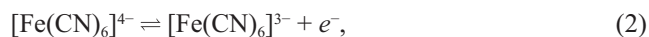
where n is the number of electrons involved in the reaction of the marker in eq. (2), A is the geometric electrode surface area, D is the diffusion coefficient for the marker of 0.632×10^{-5} cm^2 s^{-1},[13] C is the marker concentration of 4.0 mM, and v is the scan rate of 0.1 V s^{-1}. Using eq. (1), the geometric electrode surface area was calculated to be 2.07 ± 0.0270 mm^2 ($n = 8$, for 8 independent electrodes), showing the reproducible fabrication of the electrode. Independently, the topographical measurement yielded the diameter of the electrode to be 1.6 mm. Here, the pattern of the electrode on the mask used for photolithography was a circle with a diameter of 1.6 mm and the area is 2.01 mm^2. It was found that electrodes whose surface areas are nearly equal to that of the pattern on the mask were formed on the fabricated electrode array chip.

3.2 *Sensor responses*

In the previous section, the electrodes were revealed to be cleanly and reproducibly fabricated on the array chip. Gene sensor array chips were prepared by immobilizing the probe PNAs on the electrode array surfaces. The sensor responses were electrochemically measured using the $[Fe(CN)_6]^{4-}$ anion as the marker. The working principle of the sensors employed for the sensor array chips is based on voltammetric ion-channel sensing.[9,10] The hybridization of the gene probe with the target DNA changes the surface charge to become negative. The redox reaction of negatively charged markers is thus inhibited. The sensor response was expressed as the ratio of peak current (i_0) to the current decrease ($i_0 - i$) at the peak potential (E_p) in the voltammograms measured before hybridization with target DNAs, normalized current change. As typical sensor responses, Figs. 3(a) and 3(b) show voltammograms for electrodes modified with the probe PNA_1 or PNA_2 before (dashed lines) and after (solid lines) hybridization with 100 μM target DNA_2, respectively. Before hybridization, the voltammogram was quasi-reversible and the peaks were distinct. After hybridization, in the case of the sensors for PNA_2, the voltammogram became irreversible and current decreased at the potential of Ep, whereas the voltammograms for PNA_1 remained unchanged. These results indicate that DNA_2 selectively hybridized with PNA_2 and that the negative charge of the DNA on the electrode surface decreased the electron transfer rate constant of the $[Fe(CN)_6]^{4-}$ marker owing to electrostatic repulsion between the surface and the marker; that is, the current decrease indicates the presence of the target DNAs. The sensor response curves for the 5 studied probe PNAs for the corresponding complementary oligonucleotides were studied previously.[9,10,14]
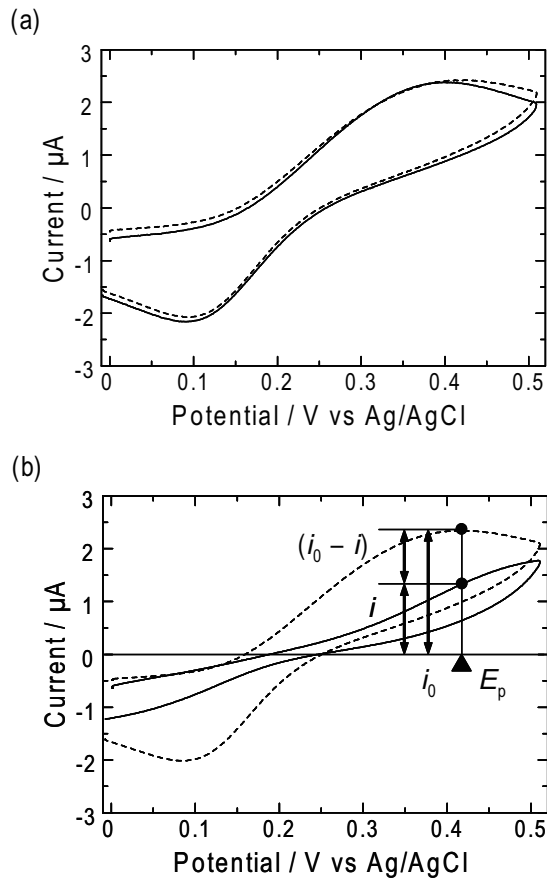
(a)

(b)

Fig. 3.   Cyclic voltammograms for the sensor based on PNA_1 (a) and PNA 2 (b) before (dashed lines) and after (solid lines) hybridization with target DNA 2.

3.3 *Responses of gene sensor arrays*

Figure 4(A) depicts the layout of four gene sensors for PNA_1 and PNA_2 on the electrode array chip (Fig. 4A(a)) and the sensor responses after incubation in an aqueous solution containing 100 µM DNA_2 expressed as the normalized current change of $(i_0 - i)/i_0$ (Fig. 4A(b)) on a gray scale (Fig. 4A(c)), where smaller or larger responses are expressed in white or black, respectively. The electrode at the lower right corner was used as a reference (not shown). The sensors for PNA_2 showed large responses, whereas the sensors for PNA_1 showed quite small responses. The normalized current changes are summarized in Table 1. In Fig. 4A(b), one sensor for PNA_1, noncomplementary to DNA_2, detected the current increase after incubation, causing a negative current change. This apparently extraordinary behavior rather
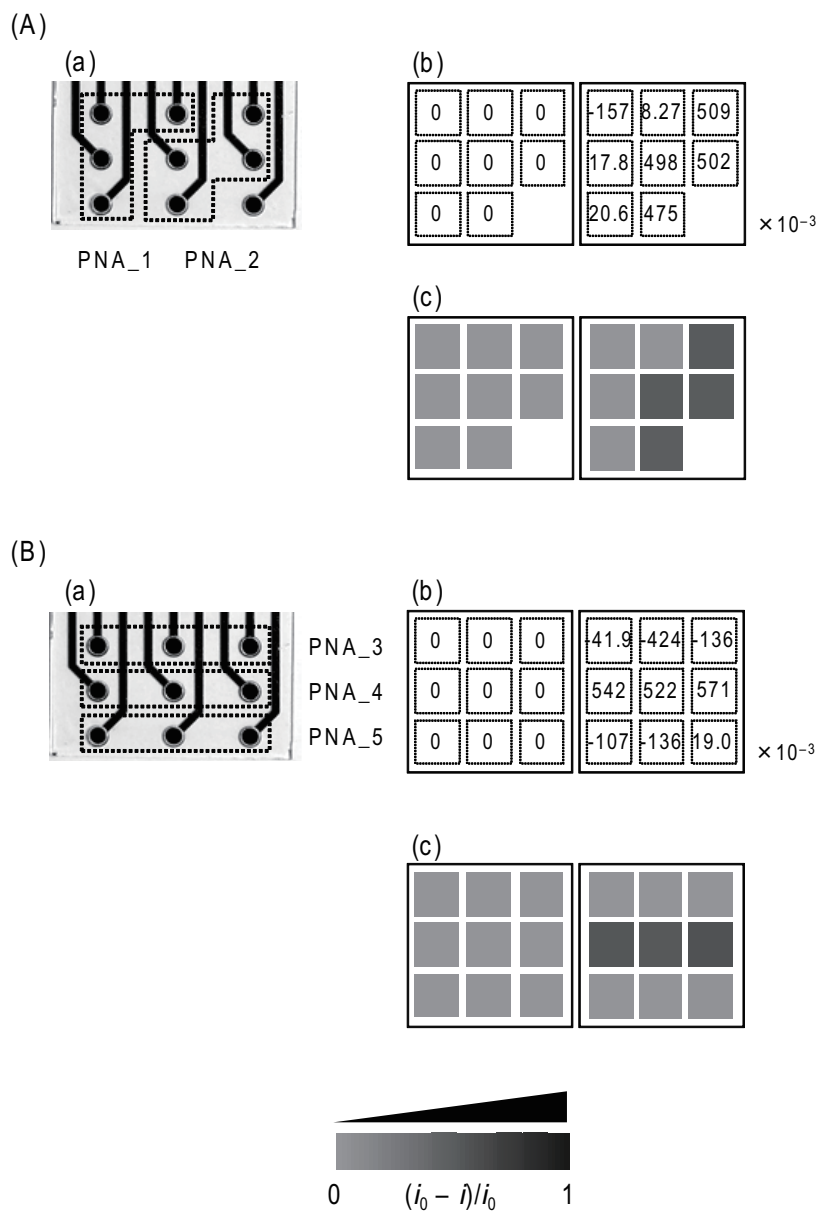
(A)

(a)

PNA_1   PNA_2

(b)

0	0	0
0	0	0
0	0	

-157	8.27	509
17.8	498	502
20.6	475	

× 10⁻³

(c)

(B)

(a)

PNA_3
PNA_4
PNA_5

(b)

0	0	0
0	0	0
0	0	0

-41.9	-424	-136
542	522	571
-107	-136	19.0

× 10⁻³

(c)

0     $(i_0 - i)/i_0$ 1

Fig. 4. Layouts of gene sensors on the electrode arrays (a) and sensor responses after incubation in an aqueous solution containing 100 μM target DNAs for the patterns (A) and (B), expressed as the value of $(i_0-i)/i_0$ (b) on a gray scale (c) ranging from white (low) to black (high). Pattern (A) or (B) contains four or three electrodes modified with probes PNA_1 and PNA_2 or probes PNA_3, PNA_4, and PNA_5, respectively.

Table 1
Normalized current changes of $(i_0-i)/i_0$ before and after incubation in an aqueous solution containing DNA_2 or DNA_4 for sensors based on probe PNA_1 and 2 or PNA_3, 4, and 5, respectively.

PNA_*l*	DNA_*m*	Sample number	$(i_0-i)/i_0$
1	2	4	-0.0275 ± 0.0862
2	2	4	0.496 ± 0.0147
3	4	3	0.545 ± 0.0246
4	4	3	-0.201 ± 0.199
5	4	3	-0.0746 ± 0.0825

confirms that no PNA_1 responds to DNA_2. Selective responses to DNA_2 were thus demonstrated, indicating that droplets of the gene probes were separately dispensed on the corresponding electrodes without mixing with each other, and that, as a result, the probe-modified electrodes discriminated the target DNA in a sequence-specific manner. As the other array layout, three gene sensors for PNA_3, PNA_4, and PNA_5 on an electrode array chip (Fig. 4B(a)) are shown with the sensor responses after incubation in an aqueous solution containing 100 µM DNA_4 (Figs. 4B(b), (c)). Similarly, sequence-specific responses to DNA_4 were demonstrated. The normalized current changes are summarized in Table 1. Similarly to the case for PNA_1, some sensors for PNA_3, noncomplementary to DNA_4, detected the current increase after incubation (Fig. 4B(b)), showing no responses to DNA_4. These results show that the sensor responses are distinct, selective, and reproducible. These gene sensor arrays are expected to be useful in distinguishing the presence or absence of target DNAs in sample solutions. On the other hand, the deviation in fabrication of electrodes in an array might cause a difference in color. Similarly, as in other conventional gene detection techniques based on the difference in color, averaging the responses of several sensors may reduce the number of false results, particularly for samples with SNPs. In this study, the sensor responses were observed using three or more sensors for verification.

4. Conclusions

Electrochemical gene sensor array chips were prepared on the basis of photolithographically fabricated gold electrode arrays modified with the synthesized probe PNAs. The dimensions and cleanliness of the electrode array were determined by topographical and electrochemical methods. The prepared sensors detected the target DNAs with sequences complementary to those of the probe PNAs distinctively, selectively, and reproducibly.

In this study, some sensors detected negative current changes, possibly attributed to the deference between electrodes caused from irregular surfaces of glass substrates. Fabrication of more integrated electrode arrays may reduce surface irregularity.

Future work will extend these results towards application to more integrated microelectrode arrays for evaluating the biological effects of chemicals, a task that

requires several hundred types of gene expression to be investigated simultaneously.[15,16] The authors are planning to fabricate more integrated gene sensor array chips and to analyze multiple gene expressions using the integrated gene chips.

Acknowledgements

References

1   M. J. Heller: Annu. Rev. Biomed. Eng. **4** (2002) 129.
2   R. B. Stoughton: Annu. Rev. Biochem. **74** (2005) 53.
3   A. Sassolas, B. D. Leca-Bouvier and L. J. Blum: Chem. Rev. **108** (2008) 109.
4   A. J. Bard and L. R. Faulkner: Electrochemical Methods: Fundamentals and Applications (John Wiley & Sons, New York, 2001).
5   E. Paleček and M. Fojta: Anal. Chem. **73** (2001) 74A.
6   J. Wang: Anal. Chim. Acta **469** (2002) 63.
7   P. de-los-Santos-Állvarez, M. J. Lobo-Castañón, A. J. Miranda-Ordieres and P. Tuñón-Blanco: Anal. Bioanal. Chem. **378** (2004) 104.
8   F. Lucarelli, G. Marrazza, A. P. F. Turner and M. Mascini: Biosens. Bioelectron. **19** (2004) 515.
9   Y. Umezawa and H. Aoki: Anal. Chem. **76** (2004) 320A.
10 H. Aoki and H. Tao: Analyst **130** (2005) 1478.
11 H. Aoki and H. Tao: Analyst **132** (2007) 784.
12 H. Aoki and H. Tao: Anal. Sci. **24** (2008) 929.
13 D. T. Sawyer, A. Sobkowiak and J. L. Roberts, Jr.: Electrochemistry for Chemists, (John Wiley & Sons, New York, 1995) p. 219.
14 H. Aoki, K. Hasegawa, K. Tohda and Y. Umezawa: Biosens. Bioelectron. **18** (2003) 261.
15 H. K. Hamadeh, P. R. Bushel, S. Jayadev, O. DiSorbo, L. Bennett, L. Li, R. Tennant, R. Stoll, J. C. Barrett, R. S. Paules, K. Blanchard and C. A. Afshari: Toxicol. Sci. **67** (2002) 232.
16 M. McCormick and E. F. Nuwaysir: Handbook of Toxicogenomics, ed. J. Borlak (Wiley-VCH, New York, 2005) p. 83.