

Quantitative Evaluation of DNA Probe Density by Electrochemical Surface Plasmon Resonance Measurement

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Electrochemical surface plasmon resonance (EC-SPR) measurement enables changes in surface refractive index induced by an electrochemical reaction to be detected. Here, we carried out EC-SPR measurement to quantitatively evaluate a DNA sensor surface, which was constructed by immobilizing thiolated DNA probes on a gold film surface by a self-assembled-monolayer technique. This is because the performance of the sensor significantly depends on the probe density. Although the probe density can be evaluated quantitatively by measuring the current (charge) induced by electrochemical reductive desorption, this method removes all DNA probes on the sensor surface, meaning that the sensor cannot be used for further DNA sensing. In contrast, we can evaluate the DNA probe density more quantitatively by SPR measurement after obtaining the relationship between the charge induced by electrochemical desorption and the SPR angle shift by EC-SPR measurement. Both the amount of charge induced by reductive desorption and the SPR angle shift increased with increasing immobilization time from 0 to 24 h, and a strong relationship between the amount of charge and the SPR angle shift was observed. Therefore, the density of immobilized DNA probes can be evaluated without destroying the surface. We used EC-SPR results to evaluate the hybridization efficiency of target DNA by comparing the angle shifts observed by probe immobilization and target hybridization.

1. Introduction

A number of biosensors based on molecular recognition reactions have been developed and widely applied in clinical tests and healthcare devices. Immunoassay and DNA hybridization provide incomparable sensitivity and selectivity due to the strong and selective interaction with target molecules. However, the design of biointerfaces for molecular recognition significantly

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affects the analytical performance characteristics of biosensors, such as the detection limit and linear range.

This is a particularly significant factor for DNA-based biosensors. Although an interface with a sufficient density of immobilized DNA probes can have high sensitivity, an excessive density of DNA probes will hinder DNA hybridization through steric effects and reduce the sensitivity of the sensor.⁽¹⁾ To immobilize DNA probes, a thiol-terminated DNA probe is widely utilized and immobilized on a gold (Au) film to form self-assembled monolayers (SAMs). The probe density of the DNA sensor can be evaluated by measuring the current induced by electrochemical reduction by linear sweep voltammetry (LSV).⁽²⁾

This method enables the density of immobilized probe molecules to be evaluated accurately from the charge in the reductive desorption current of DNA probes. However, the sensor surface cannot be reused after being evaluated by electrochemical methods. This is because the DNA probe molecules on the surface are electrochemically reduced and desorbed from the sensor surface.

On the other hand, the surface plasmon resonance (SPR) method can be used to evaluate the density of immobilized probes without destroying the biointerface. However, it is more difficult to quantitatively evaluate the density of DNA probes using SPR than by electrochemical desorption methods, because SPR measurement detects changes in surface refractive index, which varies not only with the density of DNA probes but also with their orientation, such as whether they are parallel or perpendicular to the Au film surface. Therefore, it will be possible to determine the density of fixed DNA probes by nondestructive and nonlabeled methods if we can obtain the relationship between the SPR signal intensity and the charge in electrochemical desorption for a DNA-probe-immobilized surface.

The electrochemical-SPR (EC-SPR) method, namely, the method of simultaneously measuring the electrochemical response and SPR, has been studied to achieve this purpose. So far, redox reactions, mediator films, and enzymatic reactions on Au film electrodes have been reported.^(3–7) For bioassay, the EC-SPR method has been used to study the formation of electroactive microbial biofilms⁽⁸⁾ and the nonspecific detection of proteins.⁽⁹⁾ Blidar *et al.* measured the SPR angle shift during aptamer immobilization to detect ampicillin, which was improved by using a pulsed potential electrochemical method.⁽¹⁰⁾ Salamifar and Lai reported the application of EC-SPR to the characterization of DNA sensors.⁽¹¹⁾ They monitored both optical and electrochemical signals simultaneously, from sensor fabrication to target interrogation and sensor regeneration.

In this study, we used the EC-SPR method to evaluate DNA probes immobilized on a Au film by applying LSV and SPR measurements at the same time. The relationship between the charge induced by electrochemical desorption and the SPR angle change can be obtained with a single DNA-probe-immobilized Au film. Using DNA probes with a fixed size (number of bases), we were able to evaluate the density of newly immobilized DNA probes accurately without destroying the surface by simply measuring the SPR angle after obtaining the above relationship by EC-SPR measurement.

2. Materials and Methods

2.1 Chemicals and materials

A 15-mer thiol-terminated DNA probe and target DNA were purchased from Gene Design Co., Ltd. Inc. (Osaka, Japan). The sequences of the probe and target are shown below.

DNA probe: HS-(CH₂)₆-AATGAAGTAAGAGGAC

Target DNA: GTCCTCTTACTTCAT

A Tris-EDTA(TE) buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) was also obtained from Nippon Gene Co., Ltd. (Osaka, Japan), and an ultrafiltration spin column was purchased from Apollo Science Co., Ltd. (Tokushima, Japan). A PDMS flow channel, spacer tape, and Au film chip (a Au/Ti film of 4 mm diameter at the center and a Au lead of 1 mm width from the center to the end formed on a 1.6 cm × 1.6 cm square microscope BK7 cover glass) were purchased from NTT Advanced Technology (Kanagawa, Japan).

Magnesium chloride (MgCl₂), (±)-dithiothreitol (DTT), sulfuric acid (H₂SO₄), hydrogen peroxide (H₂O₂), potassium hydroxide (KOH), sodium dihydrogen phosphate (NaH₂PO₄), disodium hydrogen phosphate (Na₂HPO₄·H₂O), and potassium ferricyanide K₃[Fe(CN)₆] were purchased from Wako Pure Chemical Industries (Osaka, Japan). A 0.5 M phosphate buffer (pH 8.0) was prepared from NaH₂PO₄ and Na₂HPO₄·H₂O. Ultrapure water (Milli-Q) was used in all experiments. All chemicals were of analytical grade or better and were used as received.

2.2 DNA probe pretreatment method

The received DNA probe was pretreated as follows. First, DNA was lysed in 250 μL of TE buffer. Next, 0.12 M DTT solution (18.5 mg/mL Milli-Q) and 0.5 M phosphate buffer solution (pH 8.0) were mixed in a ratio of 2:1. The above two solutions were mixed in a ratio of 1:1 and left at room temperature for 6 h. Finally, desalination treatment was performed with an ultrafiltration spin column. For immobilization, the DNA was diluted to 10 μM with 20 mM magnesium chloride (MgCl₂) solution.⁽¹²⁾

2.3 Evaluation of immobilization

The pretreated DNA probe was cast on a Au thin film (Au/Ti film) formed on a Si wafer and kept in a refrigerator for 24 h. After removing the solution and rinsing with ultrapure water, the DNA-immobilized Au film was measured by X-ray photoelectron spectroscopy (XPS:ESCA Quantum 2000, 486.6 eV Al K α 1 radiation, ULVAC-PHI, Chigasaki, Japan) to observe the surface phosphorus peak. LSV curves were obtained with an ALS/CHI 720E electrochemical analyzer (CHI Instruments, Austin, TX, USA).

2.4 Construction of EC-SPR cell

An SPR chip was cleaned with Piranha solution for 15 min. After cleaning, it was rinsed with ultrapure water. A DNA probe was cast on the chip and left for 3, 6, 18, or 24 h. After immobilization, excess DNA on the chip surface was washed with ultrapure water and dried. A hole of 8 mm diameter was drilled in the flow path tape and PDMS. The spacer tape used to make a thin-layer flow cell and PDMS were attached and fixed on the chip to form a small electrochemical cell. A copper tape was sandwiched between the flow path tape and the SPR chip to use the Au pattern as a working electrode (Fig. 1). The EC-SPR cell was electrochemically evaluated by cyclic voltammetry with $K_3[Fe(CN)_6]$. A low-leakage reference electrode (Dri-Ref, World Precision Instruments, Inc., Sarasota, FL, USA) and a platinum (Pt) plate auxiliary electrode were used for measurement.

2.5 EC-SPR measurement

Before EC-SPR measurement, the EC-SPR cell fabricated by the above process was set on the prism of a portable SPR system (NTT Advanced Technology, Tokyo, Japan). The system had a width of 16 cm, a depth of 6 cm, a height of 9.5 cm, and a mass of 770 g. Index-matching oil ($n = 1.510$, Cargille Laboratories, Cedar Grove, NJ, USA) was used to obtain optical contact between the BK7 glass plate at the bottom of the EC-SPR cell and the BK7 prism on the SPR system. For EC-SPR measurement, approximately 150 μ L of aqueous KOH solution (0.5 M) was injected into the cell and degassed with Ar gas for 15 min. LSV measurement was performed by continuously monitoring the SPR angle change by scanning the potential in the positive-to-negative direction at 0.05 V/s.

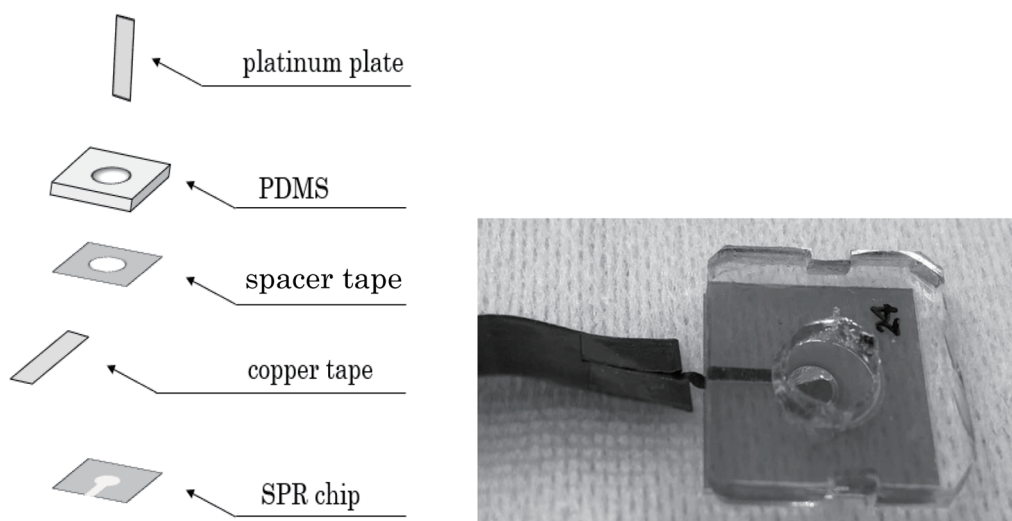


Fig. 1. Construction of an electrochemical SPR cell. The cell was constructed by stacking all parts in the left of the figure. The PDMS and spacer tape were perforated with an 8 mm hole to introduce the measurement solution.

2.6 Hybridization measurement with EC-SPR results

The 15-mer DNA was immobilized on the SPR chip for 0 or 24 h in the same manner as in the XPS sample preparation. The EC-SPR cell was constructed with the chip with the DNA probe immobilized for 0 or 24 h and PDMS, then set on the SPR system. The difference between the densities of immobilized DNA probes in the cases of 0 and 24 h immobilization was evaluated using the difference in SPR angle shift induced by electrochemical desorption in 0.5 M KOH solution.

The coverage of the surface was calculated from the SPR angle shift by using the relationship between the charge induced by reductive desorption and the SPR angle shift obtained as discussed in Sect. 2.5. The SPR angle shift upon introducing the target DNA (2.5 μM) was compared with that induced by probe immobilization.

3. Results and Discussion

3.1 Characterization of EC-SPR cell

A small-volume EC-SPR cell was studied to confirm the electrochemical response of the redox species. Figure 2 shows voltammograms of the $\text{K}_3[\text{Fe}(\text{CN})_6]$ solution. We observed reversible redox peaks for both 1 and 10 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$, indicating that our EC-SPR cell shows an acceptable voltammetric response.

3.2 Evaluation of DNA probe immobilization

We confirmed the immobilization of DNA probes by XPS and electrochemical measurements. Figure 3(a) shows XPS spectra of the Au film surface after applying the DNA probe solution for 24 h. A phosphorus peak assigned to DNA was observed. LSV measurement was performed with a Au film electrode after 12 h immobilization [Fig. 3(b)]. A low-leakage reference electrode

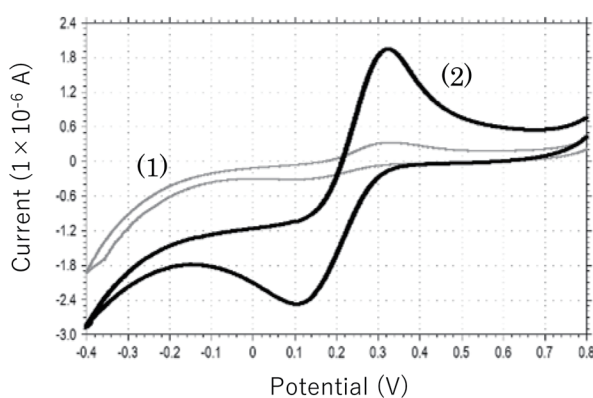


Fig. 2. Voltammograms of $\text{K}_3[\text{Fe}(\text{CN})_6]$ solution obtained using EC-SPR cell. The potential was scanned at 0.05 V/s. (1) 1 mM and (2) 10 mM.

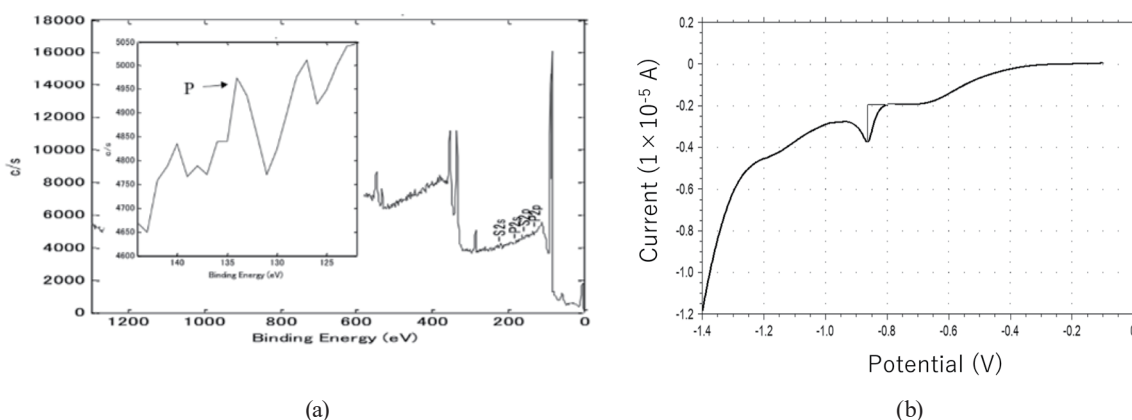


Fig. 3. (a) XPS spectra of Au film surface after 24 h DNA immobilization. (b) Linear sweep voltammogram of Au film electrode after 12 h DNA immobilization. The potential was scanned at 0.05 V/s. 0.5 M KOH solution was used as the measurement solution.

was used in each measurement to reduce the effect of chloride ions on the Au film surface. A platinum plate was used for the auxiliary electrode. A clear reduction peak induced by electrochemical reductive desorption was observed at -0.85 V. These results clearly indicate that the DNA probe was immobilized on the Au film surface.

3.3 EC-SPR measurement

EC-SPR measurement enables us to obtain the relationship between the absolute amount of immobilized DNA probe evaluated by electrochemical reduction and the SPR angle shift. Therefore, the amount of immobilized DNA can be accurately evaluated without electrochemical reductive desorption if we know the relationship between the charge obtained from the desorption peak and the SPR angle shift. Figures 4(a)–4(e) show the EC-SPR results obtained with DNA-immobilized Au films with the DNA probes immobilized for 0, 3, 6, 18, and 24 h, respectively. Reduction peaks at around -0.8 V assigned to DNA probe desorption were clearly observed, and the magnitude of the peak increased with increasing immobilization time. The peak angle of the SPR changed significantly in the same potential region regardless of the immobilization time, indicating that the desorption of the probe significantly changed the surface refractive index. However, a reductive peak was also observed below -1.0 V, which was due to the oxygen reduction because we did not perform deoxygenation before the measurement. The angle shift that gradually occurred in the negative potential region might be due to the change in the Au surface induced by the background current.

We calculated the surface coverage of the DNA probes with the charge obtained from the reductive desorption peak. Assuming that each thiolated probe occupies three Au atoms, as previously reported,^(2,13) the number of DNA probe molecules immobilized on the Au surface was calculated as follows. First, by calculating the number of Au atoms per cm^2 (n) from the Au lattice parameter, we obtained $n = 1.20 \times 10^{15}$ Au atoms/ cm^2 .

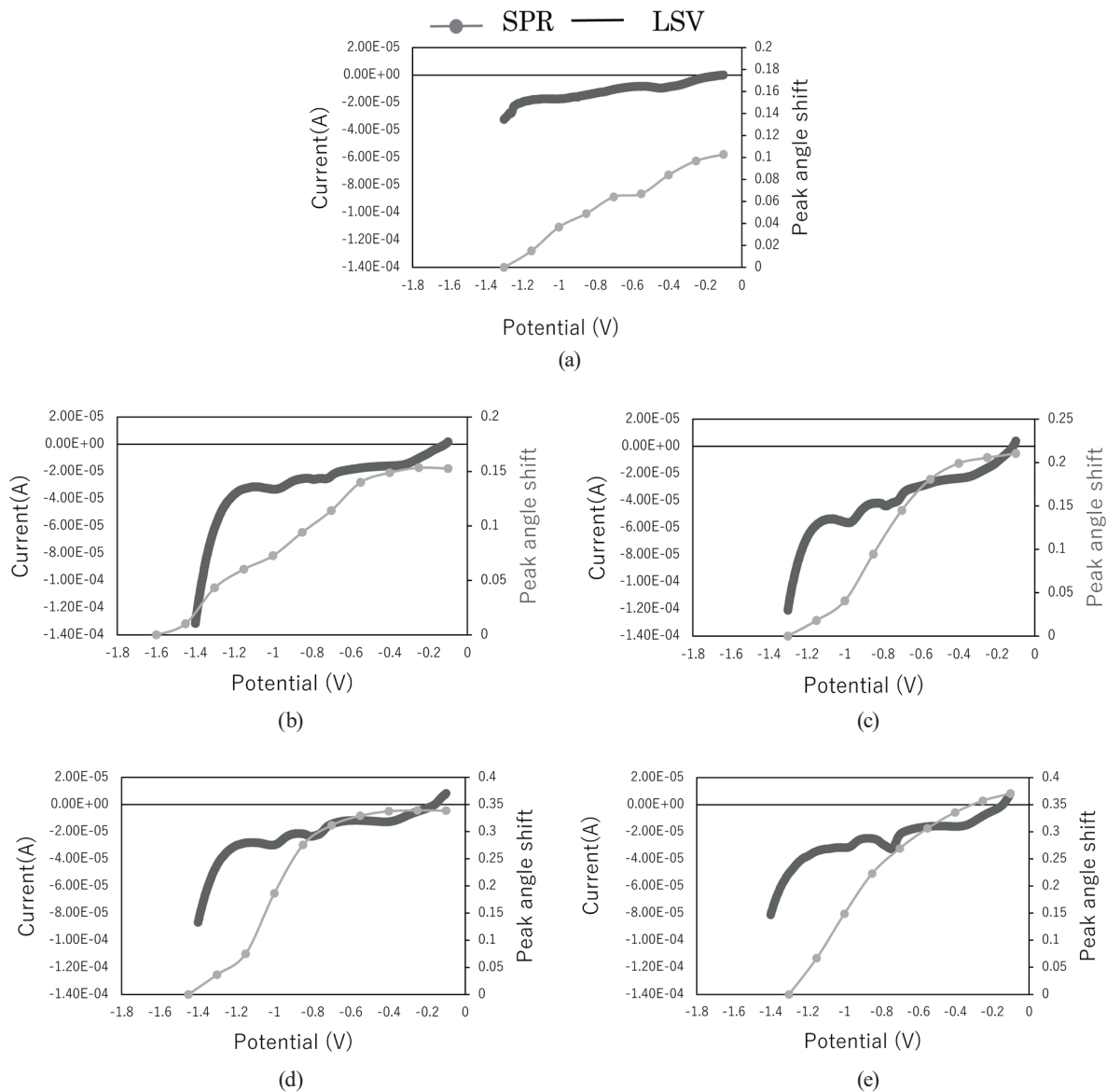


Fig. 4. Changes in LSV and SPR peak angles obtained by EC-SPR measurement. The potential was scanned in the positive-to-negative direction at 0.05 V/s after filling the cell with approximately 150 μL of aqueous KOH solution (0.5 M). (a) 0 h immobilization, (b) 3 h immobilization, (c) 6 h immobilization, (d) 18 h immobilization, and (e) 24 h immobilization.

Therefore, theoretical charges at full coverage (Q_{full}) were expressed using the formula below.

$$Q_{full} = (n/3) \times 1.6 \times 10^{-19} \text{ C } (n: \text{ number of Au atoms/cm}^2). \quad (1)$$

Then, the coverage (S) was calculated as $S = Q_{meas}/Q_{full}$, where Q_{meas} is the charge calculated from the reductive desorption peak of LSV.

Therefore, the charge in Fig. 4 can be converted to the coverage of the DNA probe on the surface. Although the coverage of the DNA probe is reasonably high, this might be due to the increased Au surface area owing to the non-flatness of the Au film. The variations of the angle shift and probe coverage with the immobilization time are shown in Figs. 5(a) and 5(b), respectively. Both curves had similar shapes, with a rapid increase between immobilization times of 3 and 6 h. We considered this rapid increase to be due to the following reason: When we applied the DNA probe solution to the SPR chip, the probe molecules dispersed homogeneously. Then, the DNA probes accumulated near the Au surface by diffusion and were immobilized on the Au surface.

From the results in Figs. 5(a) and 5(b), we obtained the relationship between the SPR angle shift and the probe coverage as shown in Fig. 6. Although a relatively large fluctuation was observed, there was a clear correlation between the probe coverage and the SPR peak angle shift. These results suggest that the probe coverage can be evaluated by SPR measurement without destroying the surface by electrochemical reduction, after a calibration curve between the probe coverage and the SPR angle shift for the same probe size has been obtained.

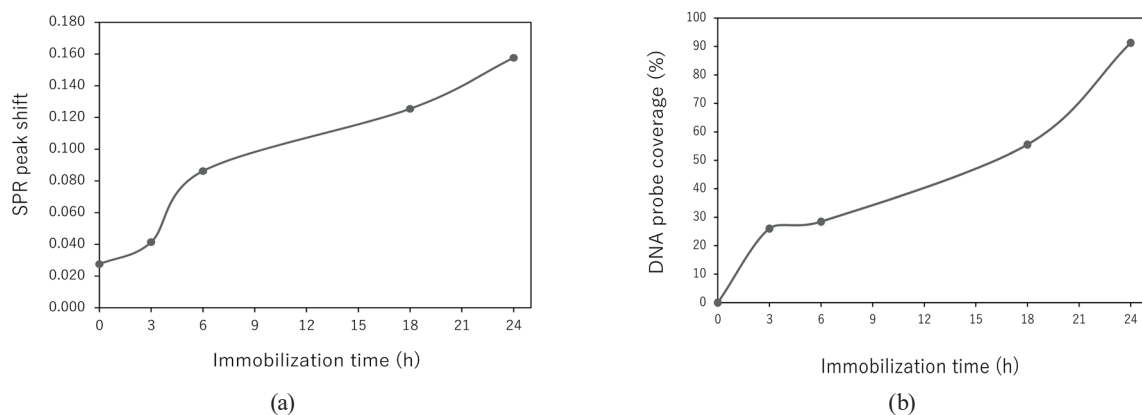


Fig. 5. (a) Relationship between immobilization time and SPR peak angle shift obtained from Fig. 4. (b) Relationship between immobilization time and surface coverage of DNA probe. The coverage was calculated using the charge obtained with the peak area of each LSV result.

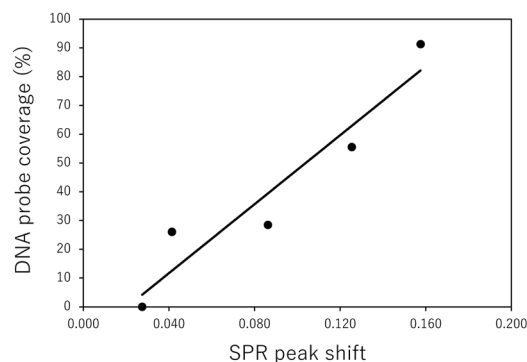


Fig. 6. Relationship between peak angle shift and DNA probe coverage in Figs. 5(a) and 5(b).

Table 1

Relationship between probe immobilization time and SPR angle shift ($\Delta\theta$) induced by electrochemical reduction of DNA probe (first line) and target DNA hybridization.

Probe immobilization time/h	0	24	$\Delta\theta(t_{24}-t_0)$
SPR peak shift by EC-SPR measurement ($\Delta\theta$)	0.027	0.16	0.133
SPR peak shift by hybridization ($\Delta\theta$)	0.024	0.25	0.226
Coverage (%)	0	91.2	

3.4 Evaluation of target DNA hybridization efficiency using EC-SPR results

In Sect. 3.3, we obtained the relationship between the SPR angle shift ($\Delta\theta$) and the coverage of the DNA probe by changing the immobilization time. Since the immobilization time is very long and it is difficult to evaluate the SPR angle shift during immobilization, we used the EC-SPR results obtained with the chips with 0 and 24 h probe immobilization (Table 1).

The first row in Table 1 shows the SPR angle shift ($\Delta\theta$) induced by the electrochemical desorption of DNA probes using the chips with 0 and 24 h immobilization. $\Delta\theta$ was 0.027° for the Au chip with 0 h immobilization, suggesting that the probe was not immobilized. In contrast, $\Delta\theta$ was 0.16° for the Au chip with 24 h immobilization. The difference in the angle shift of the two chips [$\Delta\theta(t_{24} - t_0)$] was 0.133° , which was caused by the immobilized DNA probe. The coverage of the chip (24 h immobilization) was 91.2%, which was estimated from Fig. 6.

In contrast, the second row in Table 1 shows $\Delta\theta$ of both Au chips when target DNA ($2.5 \mu\text{M}$) was applied. Since the $\Delta\theta(t_{24} - t_0)$ value for the hybridization of 0.226° is much larger than that for the DNA probe immobilization, we conclude that an almost complete hybridization occurred. However, the $\theta(t_{24} - t_0)$ value for hybridization is very large when the sizes of the probe and target DNAs are similar. This might be due to the structure of the double-stranded DNA, which becomes more compact and closer to the Au surface after hybridization.⁽¹⁴⁾

4. Conclusion

We constructed an EC-SPR cell that can perform electrochemical and SPR measurements at the same time to obtain the relationship between the SPR peak shift and the reductive current induced by the electrochemical desorption of the DNA probe on the Au film surface. The redox species of 1 and 10 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ show a sufficiently reversible electrochemical response in the EC-SPR cell, indicating that the cell can be used for EC-SPR measurements with a small sample volume. A good correlation between the DNA probe coverage and the difference in SPR peak shift was observed by EC-SPR measurement, indicating that the amount of immobilized DNA probe can be evaluated quantitatively by simply measuring the SPR peak before and after DNA probe immobilization. By performing EC-SPR measurement, the amounts of hybridized target DNA and DNA probes can be evaluated by comparing the SPR peak shifts induced by the electrochemical desorption of the DNA probe and target hybridization.

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