Fabrication and characterization of non-labeled IgA immunosensor

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The interdigitated electrode (IDE), that has a series of parallel micro-band electrodes with alternating micro-bands connected together, was utilized in electrochemical impedance spectroscopy (EIS) to build non-labeled human immunoglobulin A (IgA) immunosensor. Anti-human IgA was employed as a molecular receptor being covalently immobilized on the IDE through a self-assembled monolayer. EIS results exhibited that the adsorption induced by the antigen-antibody reaction between IgA and anti-IgA made an increase in resistance of the interfacial electron transfer (Rct). A linear relationship between the ΔRct and the logarithm of IgA concentration was confirmed for the IgA concentration range of 0.1 - 100 ng/mL. No modulation of Rct was detected by immersing in the solution of other proteins such as human immunoglobulin G, which indicates a high selectivity of this sensor for IgA. The surface images of sensor before and after the IgA binding were observed by atomic force microscopy.

Key words: electrochemical impedance spectroscopy, interdigitated microelectrode, immunosensor, human immunoglobulin A,

1. INTRODUCTION

The concentration of immunoglobulins in blood is known to be a good indicator of a variety of diseases for human body [1]. Consequently, an accurate monitoring of the concentration of immunoglobulins in blood allows early detection of the diseases. The antigen-antibody reaction, which is a selective binding interaction between specific antigen and antibody pair, provides a sensing principle of immunoglobulin sensor. However the antigen-antibody reaction is difficult to detect, since the reaction is simply a monolayer adsorption and does not create a final detectable product like enzymatic reaction. Accordingly, the labeled methods such as enzyme-linked immunosorbent assay (ELISA) have been generally used in diagnosis and in laboratory examinations. However ELISA requires the experience and skills, also takes time and effort, which makes difficult for non-specialists to apply for point-of-care diagnostics [2]. The electrochemical impedance spectroscopy (EIS) detects antibody-antigen reaction as electrical signals directly, which does not need the labeling process nor other pretreatment process. The easy and rapid detection aspect of EIS promises the application of easy diagnosis. EIS analyzes the resistance and capacitance at the electrode surface that are very sensitive to a biological binding event. This feature makes EIS suitable for measuring the antigen-antibody reaction. Up to the present, a number of immunosensors based on EIS has been developed [3-13].

In recent years, the great progress in nano-production technologies such as photo-electron beam lithography or micromachining techniques has opened new opportunities for assembling electrochemical analyzing systems. The technology allows fabricating the interdigitated electrode (IDE) that has a series of parallel micro-band electrodes in which alternating micro-bands are connected together. IDE, in particular, offers many promising advantages such as rapid reaction, high sensitivity, large aspect ratio and great signal-to-noise ratio. Because of the short distance of anodic and cathodic electrodes, the redox species in the soluction can enhance the redox cycle between electrodes effectively, which results in high collection efficiency. Furthermore, IDE’s effective surface is larger than that of conventional electrode, which increases sensitivity of the sensor. Up to the present, many applications using IDE have been reported extensively. [4-5, 7-8, 12, 14-15].

To the best of our knowledge, the IgA immunosensor employing EIS with IDE structure has not been reported. In this paper, non-labeled IgA immunosensor fabricated by combining those new and high effective techniques is described. Anti-body of anti-IgA was covalently immobilized on IDE surface through self-assembled monolayer (SAM). IgA sensing performance was characterized by EIS measurement and the spectra were analyzed by numerical fitting method based on an equivalent circuit. AFM measurements were performed to confirm the absorption of IgA.

2. EXPERIMENT

2.1 Chemicals
3-mercaptopropionic-acid (MPA), 1-Ethyl-3-carboxylic diimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), human immunoglobulin A (IgA), human immunoglobulin G (IgG), bovine serum albumin (BSA), and anti-human IgA (anti-IgA) were purchased from Sigma-Aldrich-Japan. The 50 mM phosphate buffer solutions (PBS, pH 7.4, 6.0 and 4.0) were prepared by adding 3.0 g NaH2PO4 and 3.55 g Na2HPO4 in 500 mL deionized water, then the pH was adjusted with 50 mM NaOH or 50 mM H3PO4. Both IgA and IgG solutions were prepared in the pH 6.0 PBS and stored at -20°C. The Anti-IgA and the BSA solutions were prepared in pH 4.0 PBS and stored at 4°C.

2.2 IDE fabrication

IDEs were fabricated on a SiO2 wafer using photolithography techniques. Firstly, Ti/Au (400/2000 Å) layers were deposited on SiO2 surface using sputtering method. After that, the interdigitated patterns were made through liftoff process. The IDE consists of 130 fingers of Au layer with 8 mm length, 20 µm width, and 20 µm gap on SiO2 chip that has dimensions of 1 cm × 2 cm × 0.05 cm.

2.3 Preparation of SAM modified electrodes

IDE was pretreated by the piranha solution (3:1 mixture of 98 % H2SO4 and 30 % of H2O2) for 10 minutes and was rinsed by deionized water. The treated chip was modified with MPA-SAM in a 30 mM ethanolic solution of MPA for 16 hours at room temperature. After modification, the chip surface was rinsed with ethanol and deionized water to remove any unattached species and contaminants.

2.4 Immobilization of anti-IgA

To activate carboxyl groups of the MPA, the SAM modified IDE was immersed in a solution containing 20 mg/mL of EDC and 10 mg/mL of NHS for 2 hours at room temperature. After washed by deionized water and dried by nitrogen gas, the chip was immersed in a PBS solution of 250 µg/mL anti-IgA for 16 hours at 4°C. Following this procedure, anti-IgA was immobilized on electrode through the covalent bond between an amino group in anti-IgA and the carboxyl group of MPA. Subsequently, unreacted anti-IgA was washed away by deionized water and a PBS solution. Finally, the modified electrode was reacted with 3 % BSA for 3 hours at room temperature in order to block nonspecific binding site and enhance selectivity as immunosensor.

The fabricated biosensors were stored in air at 4°C when they were not in use.

2.5 Measurement and apparatus

EIS measurements were carried out at equilibrium potential without external biasing in the frequency range of 0.1-105 Hz with a 10 mV amplitude using Autolab PGSTAT128N (Netherlands). EIS measurements were performed in PBS buffer (pH 7.4) containing a mixture 2 mM K4[Fe(CN)6]4-, 2 mM K3[Fe(CN)6] and 0.1 M KCl. AFM experiments were performed using scanning probe microscope (SPM 9700, Shimadzu, Japan).

3. RESULTS AND DISCUSSION

3.1 Performance of IgA sensor

In order to assess sensing performance, the sensor was exposed to various IgA concentrations 0.1-10000 ng/mL. For each concentration, the sensor chip was incubated for 30 min, followed by PBS (pH 6.0) washing and EIS measurement. The measurement procedure is shown in Fig. 1.

![Fig. 1 EIS measurement process](https://example.com/fig1_eis_measurement_process)

The Nyquist plots of the impedance spectra obtained for different IgA concentrations were presented in Fig. 2. It was observed that two semicircles appeared in the each spectrum. As the IgA concentration was increased, the diameter of semicircle at higher frequency range (left side in Nyquist plot) was increased. In general, change in the semicircle diameter is a result in a change in the interfacial charge transfer resistance Rct that is the resistance corresponding to carrier transfer between the modified electrode and the ferricyanide ions in the solution. Accordingly, the observed diameter increase is explained as the adsorption of IgA to anti-IgA following antigen-antibody reaction. In other words, the IgA adsorption effectively blocked the [Fe(CN)6]3-/4- and thus led to an enhancement of Rct. The reaction, however, was saturated at around 100 ng/mL and the semicircle diameter was stable even in much higher IgA concentrations.

![Fig. 2 Nyquist plots of impedance spectra of anti-IgA modified IDE immersed in different concentrations of IgA](https://example.com/fig2_nyquist_plots_impedance_spectra)

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3.2 Equivalent circuit analysis

In order to evaluate the $R_{ct}$, the EIS results were simulated with an equivalent circuit as shown in Fig. 3. Here, we assumed that the organic layer formed on the IDE (anti-IgA layer and SAM layer) was divided into two parts: interfacial part and inner part. The interfacial part was expressed by the parallel connection of $R_{ct}$ and $C_{dl}$ (the double layer capacitance), and the inner part was expressed by a constant resistance $R_\alpha$ and a constant phase element (CPE). Numerical fittings were performed for all the spectra, then we obtained all the parameters that well reproduces the experimental results (not shown). Fig. 4 shows a calibration curve of $\Delta R_{ct}$ vs. logarithmic IgA concentration. As seen in this figure, the $\Delta R_{ct}$ linearly increases with increasing logarithmic IgA concentration in the 0.1 - 100 ng/mL range, then the $\Delta R_{ct}$ stays around a constant value in much higher concentration range. From the linear range, we found that the measurable IgA concentration was 0.1 - 100 ng/mL. This value was almost same as that of the non-labeled IgA sensor reported previously [2]. Moreover, the present sensor has a potential of detecting much lower concentration of IgA, because we have observed the 0.01 ng/mL detection with other sensor chip (result not shown). It is notable that the linear dependence of $\Delta R_{ct}$ on the logarithmic IgA concentration can be explained by Temkin isotherm model, which has a heterogeneous binding energy between the probe-target interaction [16]. The constant value of $\Delta R_{ct}$ in the higher concentrations will correspond to the fully occupied state of anti-IgA.

3.3 Selectivity of the immunosensor

For a practical application as a real world sensor, it is necessary to detect IgA concentration in serum samples that consist of a mixture of various proteins. In this sense, the selectivity is quite important for sensor performance. In order to verify the selectivity of the sensor, the EIS measurements were performed by immersing the sensor in IgA and immunoglobulin G (IgG) solutions alternately. IgG has the similar protein structure to that of IgA, and is intermingled in human serum with the higher concentration than IgA. For the experiment, the EIS measurements were performed for the IgA concentrations 0.1-1000 ng/mL from the low to high concentration levels. Between the measurements of 1 ng/mL and 10 ng/mL IgA, the sample was immersed into the IgG solution (10 ng/mL) for 30 min., and the EIS data was recorded to assess the impact of IgG exposure. The obtained nyquist plots are shown in Fig. 5. When the sensor was immersed in IgA solutions, the diameter of the circle in high frequency side was increased. The diameter, however, was not changed by the IgG immersion: the plots of IgG (10 ng/mL) + IgA (1 ng/mL) trace over the same position of that of IgA (1 ng/mL). The result demonstrates that the sensor has a high selectivity for IgA, detecting only IgA concentration. In should be noted that the increase of $\Delta R_{ct}$ observed in this experiments was proportional to the logarithm of IgA concentration in the 0.1 - 100 ng/mL range and was saturated at around 100 ng/mL, which were almost the same as the spectra of other samples.

3.4 AFM measurement

To confirm immobilizing anti-IgA on the Au surface and adsorption of IgA onto anti-IgA, the AFM measurements were carried out for both anti-IgA/SAM and IgA/anti-IgA/SAM samples (Fig. 6). The anti-IgA/SAM surface was not smooth and planar; small particles were uniformly distributed on surface (Fig. 6(a)). These particles are supposed to be anti-IgA. After reacting with IgA, the particles with a diameter of 20-30 nm appeared on the sensor surface (Fig. 6(b)). The size of diameter was consistent with previously reported IgA dimer size [17]. Much larger particles (~100 nm) were also observed in the IgA immobilized AFM image. We assume that such larger clusters would be the aggregation of several IgA molecules. The results of AFM measurements suggest that anti-IgA was immobilized on Au surface and IgA was bound by antigen-antibody reaction certainly.
4. CONCLUSION

We immobilized anti-IgA on the IDE by using SAM method and EDC/NHS reaction and applied EIS measurement, and succeeded in fabrication of non-labeled IgA immunosensor that has measurable IgA concentration range between 0.1-100 ng/mL. The measurable concentration range of the sensor is wide enough for many applications. Furthermore, experiments proved that the sensor has high selectivity only to IgA. By observing morphology by AFM, immobilization of anti-IgA and adsorption of IgA were confirmed. This work indicated that the combination of IDE and EIS is very effective in detecting the antigen-antibody reaction without labeling.

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References


Fig. 6 anti-IgA/MPA/Au (a) and IgA/anti-IgA/MPA/Au (b) (500 nm × 500 nm)