Oriented antibody immobilization on self-assembled monolayers applied as impedance biosensors

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Abstract. Oriented immobilization of antibodies on a sensor chip is crucial for enhancing both the sensitivity and antigen-binding capacity of immunosensors. Here, we report a comparative study of the effect of oriented and random antibody immobilization on the binding efficiency by electrochemical impedance spectroscopy (EIS). Oriented immobilization of anti-myoglobin immunoglobulin G (anti-Myo IgG) was achieved by bonding to an Fc receptor of protein G (PrG) on a self-assembled monolayer (SAM), which results in the myoglobin (Myo) binding sites being exposed outside the sensing surface. Random immobilization of anti-Myo IgG was achieved by direct covalent attachment to the SAM surface. Both immobilizations were applied to interdigitated electrodes to enhance the electrochemical signal, and the Myo biosensor performance was then evaluated by a series of EIS measurements. We found that (i) the rate of the normalized charge transfer resistance for the oriented sample was 3 times higher than that for the random sample and (ii) the detection limit was 0.001 ng/mL, which is the lowest recorded detection limit among Myo immunosensors based on EIS. These findings indicate that oriented antibody immobilization is crucial for preparing highly sensitive EIS-based biosensors.

1. Introduction
Immunization of antibodies not only produces functionalized sensing surfaces but it also determines the sensitivity of immunosensors [1]. Covalent immunization is the preferred method for antibody immobilization. Owing to their abundance, the amino/carboxylic groups on the antibodies are mostly used for this process. However, antibodies have these groups almost uniformly over their surface, which leads to randomly oriented immobilization with respect to the sensing surface. Such randomness may hinder antibody–antigen binding, resulting in poor sensor performance [2]. This problem can be avoided by using protein A (PrA) or protein G (PrG), which are found on the surface of Staphylococcus aureus or Streptococcus, for oriented immobilization of the antibody [3]. These proteins specifically bind to the non-antigenic Fc region of the antibody, and the antigen binding sites of the antibody are then exposed outside the sensing surface, leading to better antigen binding capacity [4,5] and decreasing non-specific binding [6] (see figure 1). Compared with PrA, PrG possesses a
wide range of binding abilities to immunoglobulin G (IgG) subclasses, which comprise 70–80% of total serum antibodies and have high affinity constants [7]. These features make PrG very attractive as an oriented IgG binding support for immunosensor applications.

Electrochemical impedance spectroscopy (EIS) is a very powerful tool to analyze the interfacial properties related to bio-recognition events occurring at the sensing surface [8,9]. EIS directly analyzes the resistance and capacitance of the surface. Both parameters are sensitive to the occurrence of molecular adsorption, such as Myo and anti-Myo IgG binding, without any need for labelling or other pretreatment processes. In most cases, the resistance change is used as the analysis parameter because of its high sensitivity with ease of detection through a Nyquist plot (−Z′ vs. Z″ where Z′ and Z″ are the imaginary and real parts of the complex impedance, respectively). In this study, we used the faradic EIS technique with a [Fe(CN)₆]⁢₃⁻/⁴⁻ redox probe to detect the resistance, because this technique has received much attention for application to immunodetection [10], aptamer-based protein biosensing [11], and genosensing [12]. To enhance the EIS signal, we used a patterned substrate of interdigitated electrodes (IDEs) composed of a series of parallel microbands electrodes with alternating microbands connected together [13]. Because of the short distance between the anode and cathode electrodes, the speed of the oxidation and reduction cycle of the redox species is greatly enhanced, which results in highly efficient data collection [14].

In this work, we developed a label-free Myo biosensor based on the affinity binding between Myo and anti-Myo IgG. The sensing target Myo is the earliest marker molecule of acute myocardial infarction (AMI). The level of Myo significantly increases as early as 1 h after the onset of AMI and peaks within 3–6 h [15]. These characteristics facilitate early confirmation of AMI and help in clinical decision making. The oriented anti-Myo IgG surface was prepared by attaching anti-Myo IgG to a PrG layer covalently immobilized on a self-assembled monolayer (SAM). Random anti-Myo IgG was obtained by direct covalent attachment to the SAM surface. The effect of the IgG orientation and the Myo sensing performance were evaluated by EIS through a comparative study of oriented and random IgG immobilization surfaces.

Figure 1. Schematic diagrams of IgG immobilization on the electrode surface: (a) oriented IgG immobilization via PrG and (b) random immobilization with covalent attachment to the SAM surface.

2. Materials and methods

2.1. Materials

11-Mercaptoundecanoic acid (MUA), 6-mercaptophexanol (C6OH), bovine serum albumin (BSA), phosphate-buffered saline (PBS, pH 7.2), recombinant PrG, myoglobin from horse skeletal muscle (Myo), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Recombinant PrG was used because it shows better sensing performance than native PrG [16]. Anti-Myo IgG was purchased from Bethyl (Montgomery, TX, USA). The IDEs were fabricated on a quartz glass plate by photolithography techniques. Ti/Au (40/200 nm) layers were deposited on the glass plate. IDEs with
52 fingers with length 8.0 mm and width 50 μm with a 50 μm gap between adjacent fingers were patterned by the lift-off process.

2.2. Preparation of the mixed SAM
The IDE substrate was pretreated in piranha solution (3:1 mixture of H₂SO₄ and H₂O₂) for 10 min and rinsed with deionized water. The substrate was then immersed in a binary mixture of MUA and C6OH ethanolic solution with a concentration of 1.0 mM + 9.0 mM (10% MUA) (figure 2). The immersion period was 24 h and the samples were thoroughly rinsed with ethanol and deionized water, and then dried under N₂ flow. It should be noted that in a previous our study, the use of mixed SAMs with MUA and C₆OH was shown to improve the EIS-based biosensing performance [17].

2.3. Oriented immobilization of anti-Myo IgG
To immobilize the PrG layer on the mixed SAM through covalent bonding, the carboxyl group of MUA was activated in an aqueous solution containing 200 mM EDC and 50 mM NHS for 15 min. After washing with deionized water, the sample was exposed to 0.1 mg/mL PrG solution in PBS for 20 min. Following this procedure, PrG molecules were immobilized on the IDE surface by covalent bonds. Subsequently, unbound PrG was removed by washing with PBS. Finally, the IDE was immersed in PBS containing 1.5% BSA for 1 h to block the non-specific sites and then washed twice with PBS and deionized water. Freshly prepared 0.1 μg/mL anti-Myo IgG solution in PBS was applied to the PrG-modified IDE surface for 2 h. The electrodes were then thoroughly washed with PBS and deionized water to remove any unbound IgG fragments and dried under a N₂ stream.

2.4. Random immobilization of anti-Myo IgG
Random immobilization of IgG was performed by direct covalent attachment to the mixed SAM surface. Similar to the procedure for oriented immobilization (section 2.3), the carboxyl group on the mixed SAM was activated in a mixed solution of 200 mM EDC and 50 mM NHS for 2 h. After washing with deionized water, the sample was immersed in 0.1 μg/mL anti-Myo IgG solution for 2 h and washed with deionized water. Finally, the residual NHS esters were blocked with 1.5% BSA solution in PBS for 1 h. The electrode was washed with PBS and deionized water, and dried under a N₂ stream.

2.5. Measurements and apparatus
To characterize the Myo sensor performance, the prepared samples were immersed in PBS containing different concentrations of Myo at 25 °C for 30 min and carefully washed with PBS. EIS measurements were performed in PBS containing a mixture of 5 mM K₄[Fe(CN)₆] and 5 mM K₃[Fe(CN)₆]. The EIS spectra were recorded in the frequency range of 0.1 to 1 × 10⁵ Hz with a 10 mV amplitude using an AutoLab PGSTAT128N potentiostat (Metrohm, Netherlands).

![Figure 2. Structural formulas of MUA and C6OH that form the mixed SAM.](image)

3. Results and discussion

3.1. Results of EIS measurements
To characterize the sensor performance, successive cycles of immersion in a fixed Myo concentration and corresponding EIS measurements were performed for each Myo concentration from the lowest to the highest Myo concentration. Figure 3 shows Nyquist plots of the EIS spectra for the oriented IgG sample exposed to various Myo concentrations (0.001–1000 ng/mL). Each spectrum has a
semicircular profile. As the Myo concentration increases, the diameter of the semicircle gradually increases. In general, the diameter of the semicircle represents the interfacial charge transfer resistance $R_{ct}$, that is, the resistance corresponding to carrier transfer between the IDE and $[\text{Fe(CN)}_3]^{3−/4−}$ in solution. Accordingly, the observed diameter increase is attributed to formation of the antigen–antibody complex between Myo and anti-Myo-IgG, where the adsorbed Myo effectively blocks $[\text{Fe(CN)}_3]^{3−/4−}$ resulting in an increase in $R_{ct}$. An increase in $R_{ct}$ is observed in the concentration range 0.001–10 ng/mL. Above this range, very small increases in the diameter are observed for the spectra. We consider that this behaviour corresponds to saturation of the Myo binding sites.

Figure 4 shows the spectra for the random IgG sample. Similar to the oriented sample, each spectrum has a semicircular shape. The diameter increases with increasing Myo concentration, which indicates an increasing amount of Myo bound to the sensor surface. These observed behaviours are very similar to those observed for the oriented IgG sample. However, some different features are found for the random IgG sample. First, the semicircle diameter of the initial surface (0 ng/mL Myo) is three times larger than that for the oriented IgG sample. Such a large initial value of $R_{ct}$ is considered to originate from a large amount of IgG immobilization leading to a highly resistive surface. Second, the relative $R_{ct}$ change with respect to $R_{ct}$ (0 ng/mL) remains small compared with the oriented IgG sample, which will be discussed in detail in section 3.2.

![Figure 3](image1.png)

**Figure 3.** Nyquist plots of the impedance spectra for the oriented anti-Myo-IgG sample immersed in various concentrations of Myo. The solid lines are the fitted curves obtained using the equivalent circuit in figure 5.

![Figure 4](image2.png)

**Figure 4.** Nyquist plots of the impedance spectra for the random anti-Myo-IgG sample immersed in various concentrations of Myo. The solid lines are the fitted curves obtained using the equivalent circuit in figure 5.
3.2. Equivalent circuit analysis

To appropriately evaluate $R_{ct}$, the EIS results were simulated using the equivalent circuit shown in figure 5. Here, the solution–electrode interfacial part is expressed as a parallel circuit of $R_{ct}$ and $C_{dl}$ (double-layer capacitance). At low frequencies, where the charge transfer might be faster than the diffusion of the redox substance to the electrode, Warburg impedance $Z_W$ must be introduced in series to $R_{ct}$. The parameter $R_s$ represents the resistance of the bulk solution. We then performed numerical fittings of all of the spectra, which are shown as solid lines in figures 3 and 4. In figure 6, for both the oriented and random IgG immobilization samples, the calibration curves of calculated $R_{ct}$ versus logarithmic IgG concentration are plotted, where normalization is applied as $R_{ct}/R_{ct}(0$ ng/mL). The normalized $R_{ct}$ linearly increases with increasing logarithmic IgG concentration for both samples. This linear increase is commonly observed for many affinity biosensors [9] and it is well explained in terms of the Temkin isotherm adsorption model [18]. In figure 6, the important point is that the oriented sample shows a significantly higher rate of increase in $R_{ct}$ compared with the random sample. This behaviour indicates that the oriented sample exhibits better sensor performance than the random sample. For the Myo sensing performance, the oriented sample shows a linear increase of the normalized $R_{ct}$ in the concentration range 0.001–10 ng/mL, and the rate of increase decreases above this range. This shows that the possible Myo detection range for the oriented sample is 0.001–10 ng/mL. Conversely, the random sample shows a linear increase in the range 0.001–1000 ng/mL, although the rate of increase is comparatively small. The observed detection limit of 0.001 ng/mL for both samples is the lowest reported detection limit for an Myo immunosensor based on EIS [19, 20]. We believe that such high sensitivity is the result of signal enhancement by the IDEs.

![Figure 5. Equivalent circuit model used for the analysis.](image-url)
Figure 6. Calibration curves of Myo immunosensors with oriented and random immobilization of anti-Myo IgG on IDEs. The best-fit line for the linear range of the oriented plots is $R_{ct}/R_{ct(0 \ ng/mL)} = 0.438 \ log C_{Myo} + 2.83 \ (R^2 = 0.997)$.

3.3. Effect of oriented IgG immobilization

The high performance of the oriented IgG sample can be explained by the enhanced antigen-binding efficiency. As shown in figure 7, the amount of immobilized IgG on the oriented sample is smaller than that on the random sample. However, for the same sample, a large increase in the rate of normalized $R_{ct}$ with the Myo concentration is observed. This indicates that the oriented anti-Myo IgG sample has higher binding efficiency to Myo than the random sample. This is because most of antigen binding sites are directly exposed to the assay solution, which leads to higher binding efficiency with Myo. In contrast, for the random orientation, although a large amount of IgG is immobilized, there are only a limited number of binding sites exposed to the solution, which results in lower binding efficiency. Similar results of increased binding capacity by oriented antibody immobilization have been reported in previous studies with other experimental techniques [2, 21, 22]. In most of the previous studies, oriented antibody coupling techniques give an antigen-binding capacity that is 2–8 times higher than the efficiencies obtained with the random coupling method. In our study, we found that the oriented anti-Myo IgG sample shows a 3 times higher increase in the rate of normalized $R_{ct}$ than the random sample. Although no previous studies have been reported using the EIS technique for a similar PrG–IgG system, the increase in the rate of normalized $R_{ct}$ in this study is consistent with previous values.

For the Myo sensing performance, we found saturation of anti-Myo IgG binding sites in the high concentration range (>10 ng/mL) for the oriented sample. In contrast, no clear saturation behaviour is observed for the random sample. Even though a slight decrease of the increasing trend occurs at 0.1 ng/mL, the signal continuously increases at higher concentrations. The different saturation behaviours can be explained by different efficiencies of binding. In the oriented IgG configuration, the binding efficiency to the antigen is high and homogeneous for all of the IgG molecules immobilized on the surface, which leads to saturation in the low concentration range. However, in the random orientation configuration, IgG possesses its own binding efficiency with a large variety of the degree depending on the direction, which leads to saturation in a high concentration environment.
4. Conclusions
We have investigated the effect of the antibody orientation on EIS-based biosensors. Oriented anti-Myo IgG was prepared by immobilizing the Fɛ receptor PrG on the SAM surface, which resulted in the Fab binding domain pointing away from the surface. Random immobilized anti-Myo IgG was also prepared by direct covalent bonding to the SAM surface as a reference. EIS measurements revealed that the rate of increase of the normalized $R_\text{ct}$ for the oriented sample is 3 times higher than that for the random sample. In addition, saturation of IgG binding sites was observed for the oriented sample, but no clear saturation behaviour is observed for the random sample. These findings can be explained by the homogeneous and higher binding efficiency of the oriented IgG surface compared with the random surface. The results suggest that this strategy of orientated IgG immobilization using PrG will be applicable to a variety of EIS-based biosensors.

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