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Electrochemical Immunosensor Using a Gas Diffusion Layer as an Immobilization Matrix

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The modification of a gas diffusion layer (GDL), a vital component in polymer electrolyte fuel cells, is described here for use in the electrochemical detection of antibody-antigen biosensors. Compared to other substrates (gold foil and graphite), mouse *anti*-rHBsAg monoclonal antibody immobilized on gold-coated GDL (G-GDL) detected analytes of goat *anti*-mouse IgG antibody-ALP using a relatively low potential (−0.0021 V *vs*. Ag/AgCl 3 M NaCl), indicating that undesired by-reactions during electrochemical sensing should be avoided with G-GDL. The dependency of the signal against the concentration of analytes was observed, demonstrating the possibility of quantitative electrochemical biosensors based on G-GDL substrates. When a sandwich method was employed, target antigens of rHBsAg with a concentration as low as 500 ng/mL were clearly measured. The detection limit of rHBsAg was significantly improved to 10 ng/mL when higher concentrations of the 4-aminophenylphosphate monosodium salt (APP) acting on substrates were used for generating a redox-active product. Additionally, it was shown that a BSA blocking layer was essential in improving the detection limit in the G-GDL biosensor.

Key Words : Graphite, Electrochemical detection, Biosensors, Gas diffusion layer

Introduction

The demand for the fabrication of biosensors completely integrated with their own energy generators, such as fuel or solar cells, is expected to rise in the near future. The integration of these generators would especially be an attractive feature for the success of fully implantable biosensors because surgical operation would be required for the replacement of discharged energy sources in implanted biosensors lacking integrated energy generators. Along these lines, one platform substrate that shows exhibits dual functions as both biosensors and energy generators after minor modification would be very desirable for the development of integration.

The gas diffusion layer (GDL) is a vital component in polymer electrolyte fuel cells (PEFCs) and serves as a distributor for the reactant gas over the catalyst layer as well as a conductor for generated electrons to the outer circuit current collectors.[1,2] GDLs typically consist of carbon paper (or woven carbon cloth substrates) coated with a mixture of carbon black powder; a hydrophobic dispersion agent, such as polytetrafluoroethylene (PTFE); and solvents. Because the carbon substrate serves as a well-known conductive electrode for biosensors, and the hydrophobic dispersion agent can be used as a blocking layer against non-specific binding,[3-5] the GDL is inherently applicable for use in biosensors. Indeed, much research is already in process for GDL-based biosensors. Most work currently intends to develop amperometric enzyme biosensors that detect a gaseous analyte.[6] However, biosensors that could detect a specific antibody or antigen in blood are a much-needed application with respect to energy generator integration.

In this report, we describe electrochemical biosensors for the detection of antigen-antibody binding prepared on a modified GDL. The performance in the modified GDL biosensor is superior to that prepared on gold or graphite electrodes. Even though this work concerning the integration of biosensors and fuel cells is very preliminary, the results show that antigen-antibody can be reliably detected in modified GDL sensors.

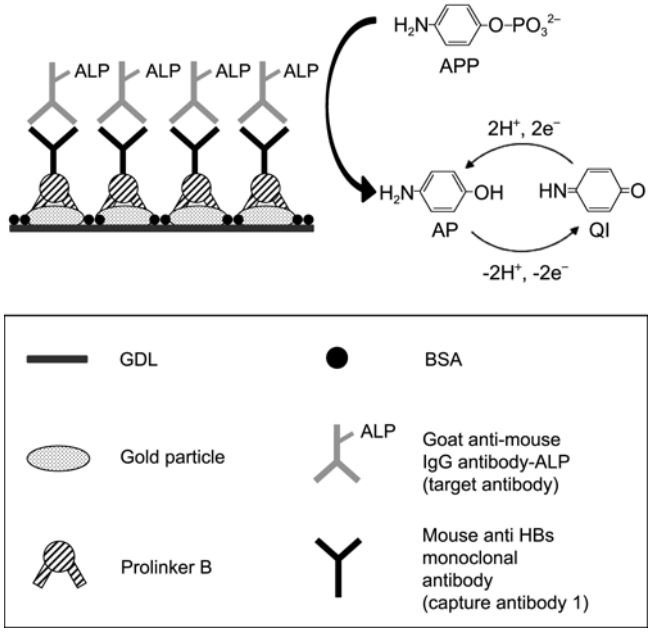
Experimental

Chemical Materials. Gold foil (0.127 mm, 99.99%) and Prolinker B ($C_{40}H_{47}O_7S_2$) were purchased from Aldrich (USA) and Proteogen Inc. (Korea), respectively. Phosphate buffered saline (PBS) at pH 7.4 was purchased from Bioneer (Korea). Alkaline phosphatase (ALP) was used as a tag in the target antibodies. The following antibodies, antigens and 10% bovine serum albumin (BSA) solution were provided from Biofocus Co. Ltd. (Korea): mouse *anti*-rHBsAg monoclonal antibody (capture antibody 1), goat IgG (capture antibody 2), goat *anti*-mouse IgG antibody-ALP (target antibody), and goat *anti*-rHBsAg antibody-ALP (secondary antibody). The 97% 4-aminophenylphosphate monosodium salt (APP) that acts on substrates for generating a redox-active product was purchased from LKT Laboratories Inc (Korea). The gold foil, graphite (rod, 99.995%, Aldrich,

USA) and GDL (GDL10BB, Sigracet, Germany, content of PTFE: 5 wt %) were each used as a platform electrode for the biosensor.

Preparation of Electrodes. For the gold electrode, the gold foil was cleaned with piranha solution for 5 min, which consisted of 1 part 28% H_2O_2 and 3 parts 95% H_2SO_4, and was rinsed thoroughly with deionized water and absolute ethanol. In the case of the graphite and GDL electrodes, the surface of the electrode was coated with a gold film of approximately 10 to 20 nm thick by a sputter coater (108 auto sputter coater, Cressington Scientific Instruments, USA). Islands like films were formed on the graphite and GDL due to surface roughness of the matrix, meaning that the gold films were partially deposited. The gold deposited area would be places for anchoring the SAM layer. The gold-coated graphite and GDL were abbreviated G-graphite and G-GDL, respectively. The same size of gold, G-graphite, and G-GDL (0.2 cm^2) was evaluated for the immunbiosensors by using home-made Teflon cells.

Immobilization of Antibody. As shown in Scheme 1, the pretreated electrode was immersed in a chloroform solution containing 1 mM Prolinker B at room temperature, which leads to the formation of a self-assembled monolayer (SAM) on the surface of the gold layer.[7] After 2 h, the electrode was washed with chloroform, ethanol, deionized water and acetone each in subsequent steps. Then, 100 μL of 0.1 mg/mL capture antibody 1 was deposited onto the surface of the electrode, and the electrode was incubated in a refrigerator overnight to allow the antibody to bind with the SAM. For the blocking process, the 1% BSA solution was prepared by dilution of 10% BSA solution with PBS buffer solution. The G-GDL was immersed in 100 μL of 1% bovine serum albumin (BSA) solution at 4 °C for 1 h before the target antibody was combined with the probe antibody. Finally, 100 μL of target antibody at different concentrations was deposited at room temperature for 30 min. After each step, the electrode was cleaned with PBS buffer solution.



Scheme 1. Immobilization of capture antibody 1 on the G-GDL *via* Prolinker B. Capture antibody 1 captures ALP-conjugated goat *anti*-mouse IgG antibody (target antibody).

Sandwich Method. Scheme 3 shows the sandwich method using the G-GDL. The G-GDL electrode that was modified with 0.1 mg/mL capture antibody 1 was incubated in 100 μL of various concentrations of rHBsAg solution at room temperature for 1 h. Following the incubation, the electrode was rinsed with PBS solution. Next, 100 μL of 0.02 mg/mL secondary antibody was deposited onto the top of the electrode and incubated at room temperature for 30 min. Note that capture antibody 1 binds with secondary antibody through rHBsAg. Afterward the electrode was washed with PBS buffer solution.

Electrochemical Measurements. All electrochemical measurements were carried out with potentiostat (autoLab PGSTAT12, Eco Chemie) interfaced to a computer. A three-electrode configuration was employed in this study. A platinum mesh and a Ag/AgCl (3 M NaCl) electrode were used as the counter and reference electrode, respectively. For cyclic voltammetry (CV), a scan rate of 50 mV/s was applied to 10 mL of varying concentrations of APP solution (1 mM-5 mM) at room temperature. The typical incubation time was within 30 min.

Results and Discussion

Because the GDL used in this experiment is very hydrophobic (contact angles ≈ 137.7) due to the PTFE,[8] the SAM

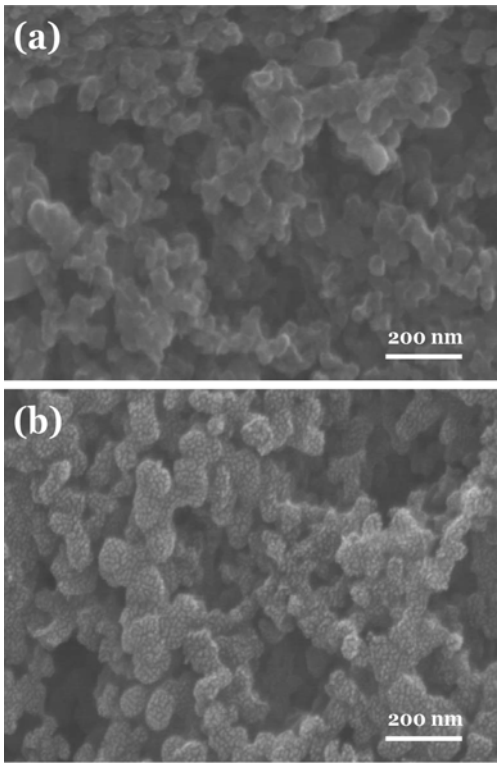


Figure 1. FE-SEM images of GDL (a) before and (b) after gold sputtering.

does not form to an appreciable extent. Thus, a gold layer, which serves as an anchoring site for the SAM on the PTFE, was deposited on the GDL surface by sputtering. Figure 1 shows field-emission scanning electron microscopy (FE-SEM) images of the GDL before and after gold sputtering. Gold films with a thickness of approximately 10 to 20 nm are formed on the entire surface of the G-GDL. As described in the experimental section, capture antibody 1 was immobilized on the sputtered gold through Prolinker B. Target antibody, which binds to capture antibody 1, was spotted onto the G-GDL as shown in Scheme 1. If target antibody is bound to capture antibody 1 after washing, then the ALP tag in target antibody will convert APP to *p*-aminophenol (AP), generating an electrochemical signal.[9-12] Figure 2 shows CV

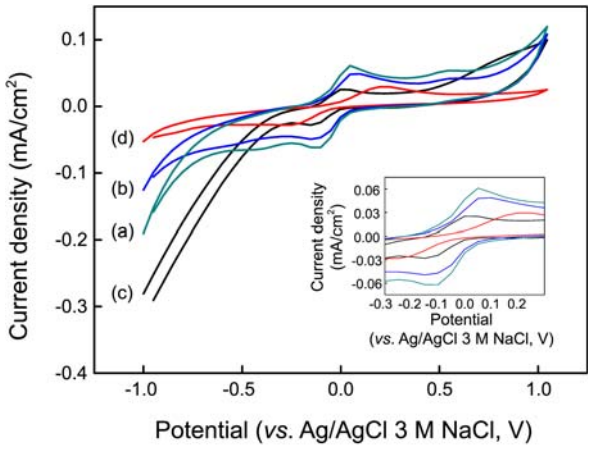


Figure 2. CV for electrochemical signal measurements of target-capture antibody binding at different electrodes: (a) bare graphite, (b) G-graphite, (c) G-GDL, and (d) gold foil. Note that the capture antibody was immobilized on G-graphite, G-GDL and gold foil *via* Prolinker B, but it was anchored to bare graphite without any linker. The detailed experimental procedures are described in Section 2.3. The inset shows the enlarged view of the CV data.

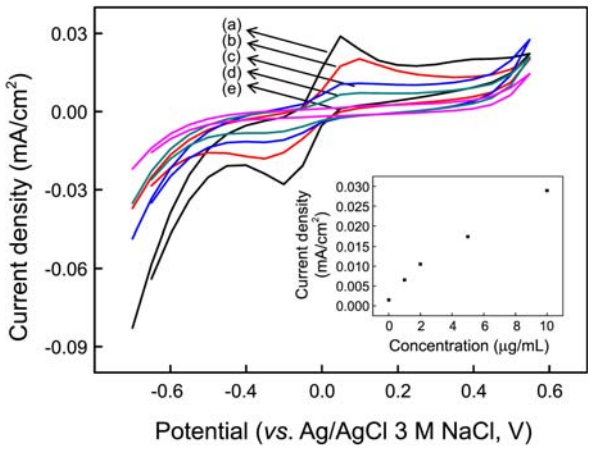
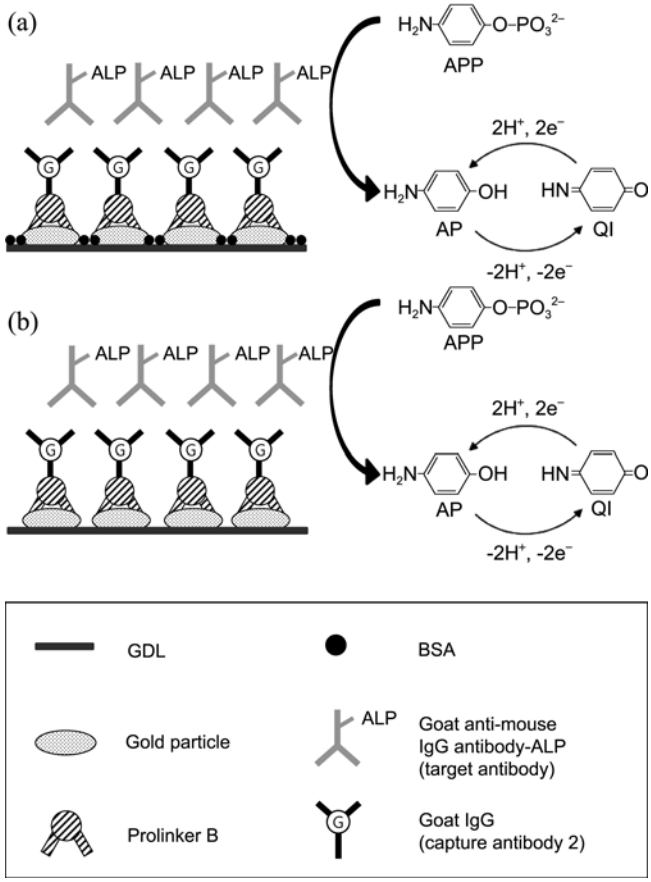


Figure 3. CV data for different concentrations of target antibody on the G-GDL, showing that the electrochemical signal increased as the concentrations of target antibody (Scheme 1): (a) 10 μg/mL, (b) 5, (c) 2, (d) 1, and (e) 0. Note that 0.1 mg/mL mouse *anti*-rHBsAg monoclonal antibody (capture antibody 1) was used as the capture antibody. The inset shows the anodic peak of CV *vs.* concentrations of target antibody.

data after carrying out the experiment of Scheme 1 for different substrates. The gold substrate shows redox peaks at higher positive (0.20 V) and negative potentials (−0.25 V) compared to G-GDL (−0.0021 V/−0.15 V). Because a high potential can produce undesired reactions, the redox peaks are best observed at low redox potential for biosensing. In this respect, G-GDL showed better performance than gold, bare graphite, and G-graphite. Because G-GDL contains hydrophobic PTFE, the intensity of the signal in G-GDL was lower than the others. Without hydrophobic PTFE, comparable signals were observed for both bare graphite and G-graphite. This indicates that the capture antibody was immobilized on both the gold and graphite surface, meaning that we could not control the immobilization site of capture antibody 1 on G-graphite, and the signal had low reproducibility because immobilization sites were different in each sample. Interestingly, G-graphite does not show any improved performance compared to bare graphite.

As shown in Figure 3, the electrochemical signals increased linearly as a function of target antibody concentration using the G-GDL biosensor, confirming that G-GDL should be applicable to electrochemical-based quantitative antibody sensors.

If non-specific binding of a target antibody to the G-GDL occurs, then an electrochemical signal will be produced due to the reaction of APP with the ALP tag. To prevent this



Scheme 2. Immobilization of capture antibody 2 on the G-GDL *via* Prolinker B (a) with and (b) without 1% BSA blocking layer. Note that capture antibody 2 cannot capture target antibody1.

reaction, we investigated the effects of a blocking layer on the G-GDL to prevent non-specific binding. Two types of capture antibodies were immobilized on gold film *via* Prolinker B. One was capture antibody 1, which binds to target antibody. The second was capture antibody 2, which cannot bind target antibody. Thus, in the case of capture antibody 2, target antibody should be completely removed after washing if target antibody was non-specifically bound to the G-GDL. In this case, electrochemical signal should not be detected. The effect of the blocking layer on the non-specific binding was investigated as shown in Scheme 2. The procedure for the formation of blocking layer was the same as that described in Section 2.3.

Because capture antibody 2 cannot bind with the target, no electrochemical signal should appear. When BSA was used as the blocking layer, peaks were not observed, which was what we expected (Figure 4(a) and Scheme 2(a)). Unfortunately, the amount of electrochemical signal generated when BSA was not employed could not be ignored (Figure 4(b) and Scheme 2(b)). This finding confirms that non-specific binding of the target antibody to G-GDL occurs, even though G-GDL contains a hydrophobic PTFE layer on its surface. For comparison, electrochemical signal from the binding of the capture antibody 1 and the target antibody was displayed in Figure 4(c). The above results strongly suggest that the formation of a blocking layer improves signal difference of positive and negative binding in G-GDL

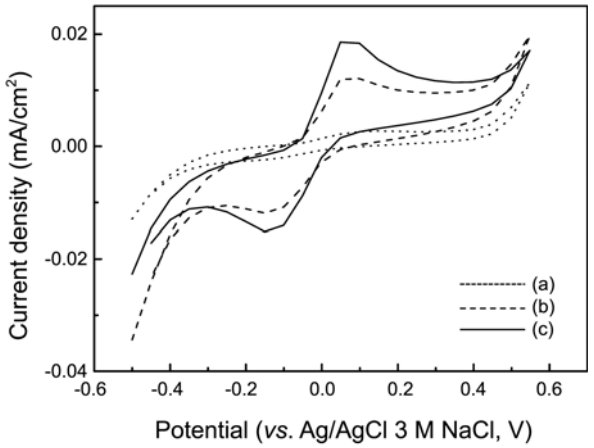
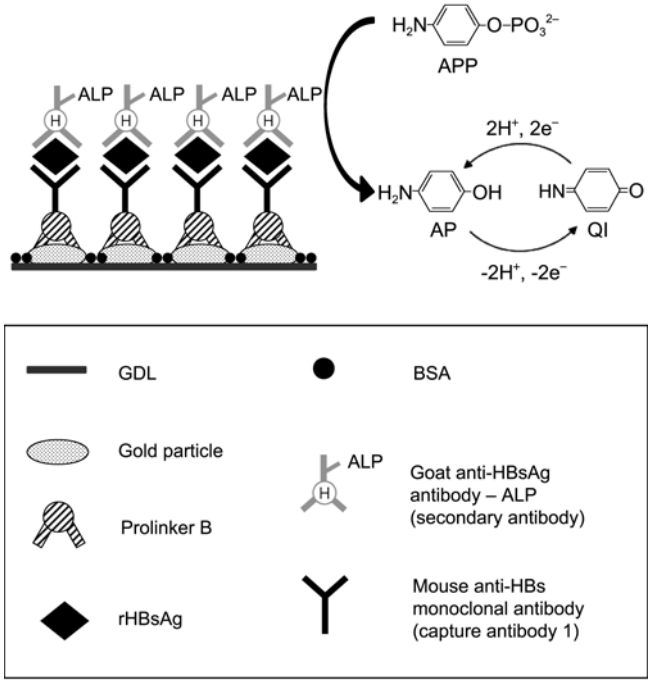


Figure 4. CV data for different types of capture antibodies with/without BSA blocking layer. (a) Goat IgG (capture antibody 2, 0.1 mg/mL) with 1% BSA blocking layer (Scheme 2(a)), (b) capture antibody 2 (0.1 mg/mL) without blocking layer (Scheme 2(b)), (c) capture antibody 1 (0.1 mg/mL) with 1% BSA blocking layer (Scheme 1).



Scheme 3. Sandwich assay for the detection of rHBsAg by using capture antibody 1 as a primary capture antibody and the secondary antibody.

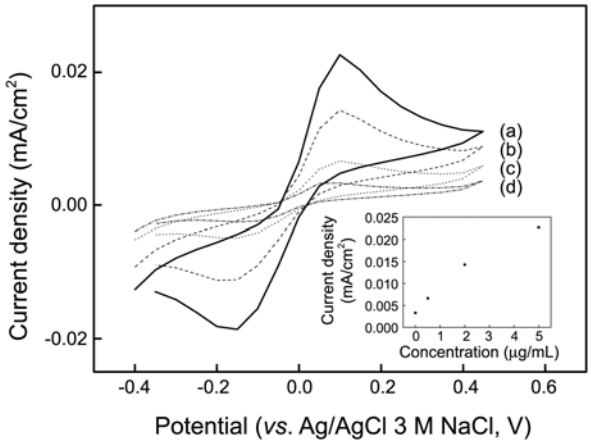


Figure 5. Electrochemical signals as a function of the concentration of target antigen (rHBsAg) in sandwich assay. Detailed experimental conditions are described in Section 2.4 and 2.5. The concentration of rHBsAg was (a) 5, (b) 2, (c) 0.5, or (d) 0 μg/mL. Note that the APP substrate was 1.5 mM for all cases. The inset shows the fairly acceptable linearity of the electrochemical signal against the concentration of target antigen.

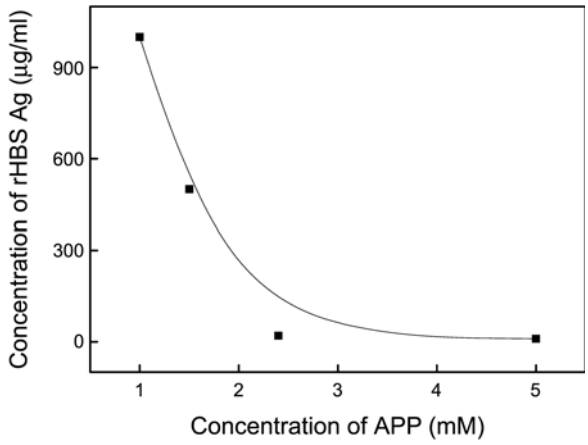


Figure 6. Detection limits in terms of APP concentration in sandwich assay. In the case of 5 mM APP, the detection limit was 10 ng/mL. Note that 0.1 mg/mL capture antibody 1 and 0.02 mg/mL ALP-conjugated goat *anti*-rHBsAg antibody were used as the primary capture antibody and the secondary antibody, respectively.

biosensors.

The G-GDL substrates were used as a platform for a sandwich assay (Scheme 3). As shown in Figure 5, the electrochemical signals were investigated in terms of the concentration of rHBsAg in 1.5 mM APP substrate. As the concentration of rHBsAg increased, the electrochemical signal increased linearly. The detection limit of rHBsAg was 500 ng/mL. Typically, by increasing the concentration of APP substrate while using the same ALP concentration, the corresponding responses will also increase.[13,14] Therefore, we increased the concentration of the APP substrate to increase the sensitivity of the biosensor (Figure 6). We found that 10 ng/mL could be reproducibly measured using the G-GDL methods.

Conclusions

We have demonstrated that the G-GDL substrate could possibly be applicable for use in quantitative electrochemical antibody-antigen biosensors. Compared to other platform substrates such as gold, graphite, and G-graphite, G-GDL showed electrochemical redox peaks at a relatively low potential, indicating that undesired reactions during the electrochemical measurements should be minimized. Even though G-GDL contained a PTFE layer which could theoretically be useful as a blocking layer due to its hydrophobicity, a blocking layer such as BSA significantly improved the difference between positive and negative binding. In the sandwich assay, electrochemical signal increased linearly as a function of antigen concentration, showing a detection limit of 500 ng/mL when the APP substrate was 1.5 mM. As the concentration of APP substrate was increased, the detection limit was significantly improved to 10 ng/mL.

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