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ORIGINAL PAPER

Electrochemical glucose biosensor by electrostatic binding of PQQ-glucose dehydrogenase onto self-assembled monolayers on gold

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Abstract Efficient binding of enzymes onto the electrode surface has been prerequisite for the construction of sensitive biosensors and biochips. Here, a simple and robust construction of electrochemical glucose biosensor based on pyrroloquinoline quinone-glucose dehydrogenase was demonstrated. The glucose biosensor was fabricated by binding the enzyme onto the anionic self-assembled monolayers on gold electrode via electrostatic interactions. The resulting glucose biosensor gave rise to twofold higher detection sensitivity than that by covalent conjugation under the same condition. Surface plasmon resonance and atomic force microscopy analyses revealed that electrostatic binding of the enzyme leads to much higher surface density of the enzyme. This approach will find wide applications to the development of robust enzyme-based biosensors and biochips.

Keywords PQQ · Glucose dehydrogenase · Glucose biosensor · Electrostatic binding · Self-assembled monolayers

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1 Introduction

An enzyme-based electrochemical biosensor is a class of the most widespread techniques to analyze the targets in many fields such as human healthcare, food safety, and environmental monitoring [1, 2]. With recent progress of electrochemical techniques, particular attention has been given to the electrochemical biosensors based on pyrroloquinoline quinone (PQQ)-containing enzymes, denoted as quinoproteins. The structure and function of such proteins has revealed their excellent properties with respect to catalytic and electron-transfer efficiencies, which leads to their extensive applications in the fields of biosensing and biocatalysis [3]. It was reported that the prosthetic group of the quinoproteins, PQQ, is the third redox cofactor following nicotinamide and flavin, serving as an important electron transporter in the periplasm of gram-negative bacteria [4]. Among other quinoproteins, a glucose sensitizer, PQQ-dependent glucose dehydrogenase (GDH) has been suggested as an alternative to glucose oxidase (GOD) or NAD-dependent GDH, due to its high catalytic activity for glucose (5,000 U mg^{-1}) and complete independence from dissolved oxygen [5, 6]. To this end, PQQ-GDH with improved properties has been preferentially harnessed for glucose measurement especially in blood.

Electrochemical applications of PQQ-GDH have been extensively accomplished by using various matrices such as conducting polymer [7], metal nanoparticles [8–10], and carbon gel [11] through sol–gel network or multiple layer on the electrode. Such biocomposite methods are capable

of improving the catalytic capability of PQQ-GDH, but the native functions and properties of the enzymes have been often hampered especially when they are covalently confined or cross-linked inside the composite, due to the surface modification. This problem could be critical for the construction of biochips or biosensors that require the surface immobilization of the enzymes. It was evident that many enzymes including PQQ-GDH lose their activities during the process of the covalent conjugation or cross-linking [12–14]. Over the years, the electrostatic binding of oppositely charged biomolecules on a charged surface has been alternatively used to overcome this problem in various biosensors. Charge-based layer-by-layer assembly was shown to be one of the most useful approaches to simply control the amount and space distribution of enzyme [15, 16]. Nonetheless, a labor-intensive multi-step procedure and difficulty in reproducible construction on the electrode have been a main bottleneck. In a simpler approach, self-assembled monolayers (SAMs) have been suggested as a promising interconnecting matrix between the electrode and the enzyme [17] because it can potentially provide a reproducible surface in a highly ordered way by modifying the chain length and functional group at their ends. Many approaches based on the Au/SAMs for the construction of biosensors have been established elsewhere [18–22], but most of them have been focused on the covalent binding of enzymes via chemical modifications, where inactivation of the GDH by successive coupling steps was also observed [23].

Here, we present a simple and robust fabrication of electrochemical glucose biosensor based on PQQ-GDH. The glucose biosensor was constructed by binding the enzyme onto the anionic SAMs made on gold electrode via electrostatic interaction. The resulting glucose biosensor was shown to give rise to a higher detection sensitivity compared with that by covalent conjugation under the same condition. SPR and AFM analyses were conducted to elucidate the difference in the detection sensitivities. Some critical factors, which enable the efficient electrostatic binding of the enzyme onto the Au/SAMs, were examined for fabricating the glucose biosensor in a very facile and robust way. Details are reported herein.

2 Experimental

2.1 Materials

PQQ-GDH was provided by Allmedicus Co., Ltd. (Korea), which had been purified from *Acinetobacter calcoaceticus*. The reagents used in the preparation of SAMs were all purchased from commercial sources: 99 % 11-mercaptoundecanoic acid (MUA, Aldrich); 99 %, 3-mercapto-1-propanol (MPOH, Aldrich); and 99 % 11-mercaptoundecylamine (MUAM, Dojindo). 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), D-(+)-glucose, ethanolamine and ferrocene methanol (FcMeOH) were purchased from Sigma-Aldrich. *N*-hydroxysulfosuccinimide (sulfo-NHS) and bis(sulfosuccinimidyl) suberate (BS^3) were from Pierce. All other chemicals used were of analytical grade.

2.2 Binding of PQQ-GDH on the anionic SAMs on Au electrode

A gold electrode was mechanically polished with aluminum oxide on a micro cloth, and subsequently with diamond paste on a nylon disk. Unless stated otherwise, this step was generally performed to reuse the gold working electrode. The cleaned gold electrode was immersed for 2 h in a 2 mM solution of one of self-assembly reagents (MUA, MPOH/MUA (9:1), and MUAM) in absolute ethanol, followed by a through rinsing with ethanol and distilled water. The resulting electrodes were then subjected to further modification in different ways. For covalent binding, carboxyl-functionalized electrodes with the MUA or MPOH/MUA SAMs were first modified by EDC (0.2 M)/NHS (50 mM) in distilled water for 30 min at RT and then immersed for 1 h in a 1 mg mL^{-1} solution of PQQ-GDH dissolved in 10 mM phosphate buffered saline (PBS) (pH 7.4). The amine-functionalized electrode with the MUAM SAMs was modified for 30 min by a 2 mM solution of BS^3 in distilled water and then is followed by the same process above. Then, NHS hydrolysis and passivation were performed by soaking the surface in 0.1 M PBS (pH 8.5) containing 1.0 M ethanolamine for 20 min. For electrostatic binding, all types of electrodes (MUA, MPOH/MUA, and MUAM-modified ones) were directly dipped for 1 h in a 1 mg mL^{-1} solution of PQQ-GDH without further treatment. After enzyme immobilization, all electrodes were washed with PBST (PBS containing 0.05 % Tween-20 and 1 M NaCl, pH 7.4) for 10 min to remove nonspecifically bound enzymes. Finally, the electrodes were thoroughly washed with 0.1 M PBS and water before use.

2.3 Electrochemical analysis

Electrochemical experiments were performed using a CV-50 W voltammetric analyzer (Bioanalytical Systems). A standard three-electrode configuration with a working electrode, a platinum counter electrode, and an external Ag/AgCl (3 M NaCl, BAS) reference electrode were used. Cyclic voltammetry was typically performed in 0.1 M PBS (pH 7.4) containing 0.1 mM FeMeOH at room temperature. For the measurement of glucose concentration, D-(+)-glucose stock solution (1 M, in 0.1 M PBS) was diluted to obtain a final concentration of 0.1–20 mM.

2.4 Surface plasmon resonance (SPR) analysis

SPR analysis was carried out using a BIAcore-X instrument and gold sensor chips (BIAcore). The chip surface was first cleaned with 0.1 M NaOH containing 0.1 % Triton-X for 5 min. For the formation of SAMs, the sensor chips were immersed for 2 h in a 2 mM ethanol solution of SAMs (MUA, MPOH/MUA, or MUAM), followed by a thorough rinsing with absolute grade ethanol and distilled water. After drying the gold surface with N_2 gas, the gold chip was docked into the instrument. Of two channels, one was used to deliver activating reagents, i.e., a mixed solution of EDC (0.2 M)/NHS (50 mM) to MUA SAM (or BS_3 to MUAM SAM) in distilled water for 20 min, whereas there was no treatment in the other channel. To compare the covalent (the first channel) and electrostatic (the second channel) binding at the same time, PQQ-GDH (1 mg mL^{-1} in 10 mM PBS) was injected for 30 min by flowing through both channels at a flow rate of 2 μL min^{-1}. Enzyme binding on gold chips modified with MPOH/MUA was also performed by the same protocol with that for the MUA-modified surface. For the surfaces modified with EDC/NHS or BS^3, NHS hydrolysis and passivation were executed by injecting 0.1 M PBS (pH 8.5) containing 1.0 M ethanolamine for 10 min after PQQ-GDH binding. All gold sensor chips were finally washed with 10 mM PBST containing 0.05 % Tween-20 (pH 7.4) by injecting the solution for 10 min to remove nonspecific adsorption. Following further washing with running buffer (0.1 M PBS), the amount of the bound enzyme was determined by comparing the initial and final values in the refractive index unit (RU).

2.5 Atomic force microscopy (AFM) imaging

Tapping-mode AFM imaging was performed with a Dimension-3100 AFM (Veeco Digital Instruments) equipped with a Nanoscope IIIa controller. Supersharp tips (SSS NCH-10, Nanoworld AG) with a nominal tip radius of <5 nm at resonance frequencies of 240–270 kHz were used in all image collections. All measurements were carried out at ambient temperature in air with 512 × 512 data acquisitions at a scan size of 1 × 1 μm^{-2}. Scan rate, set point, and proportional gain values were adjusted according to the condition of each sample during the scanning process. Surface roughness of the scanned images was determined after first-order flattening using the Nanoscope IIIa software (version 5.30r2).

3 Results and discussion

We first investigated the binding of PQQ-GDH onto the anionic SAMs on gold and the performance of the resulting electrode in the glucose sensing. GDH used in this study is a homodimer, and each subunit (54 kDa) possesses one PQQ molecule and three calcium ions [24]. To construct a glucose biosensor, PQQ-GDH was bound to the SAMs of 11-MUA on gold electrodes by two different ways, namely electrostatic and covalent methods as depicted in Fig. 1. Either electrostatic binding via simple adsorption (Fig. 1a) or covalent conjugation using EDC/NHS chemistry (Fig. 1b) was performed between the carboxyl groups of the SAMs and the amino groups of the enzyme. At neutral pH (7.4 in PBS), the net charge of PQQ-GDH (*p*I ∼ 9.5) is positive, which, in turn, confers a strong electrostatic attraction to the carboxyl groups of 11-MUA.

In the PQQ-GDH layer on gold electrodes, direct electron transfer can hardly occur because the active site of PQQ-GDH is deeply buried into the protein globule. An alternative to the direct electron transfer, thus, is to use electron mediators such as ferrocene and its derivatives [25, 26]. In such case, FcMeOH is exogenously added for the electrochemical reaction by PQQ-GDH. Figure 2 shows the cyclic voltammograms of electrostatically bound

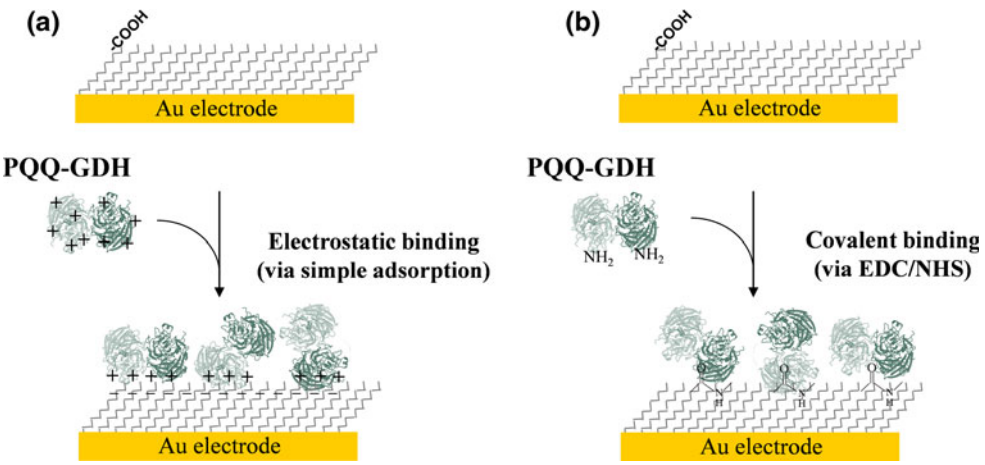

Fig. 1 Schematic for binding of PQQ-GDH onto the SAMs of 11-MUA: **a** electrostatic and **b** covalent binding via EDC/NHS chemistry

PQQ-GDH onto the Au/SAM electrode at different scan rates in the range of 1–500 mV s^{-1} in 0.1 mM FcMeOH. At a given scan rate, the anodic peak potential and the cathodic peak potential were observed at 260 and 190 mV, respectively, displaying a well-defined redox behavior at an equilibrium potential ($E_{1/2}$) of 225 mV vs. Ag/AgCl in 0.1 M PBS (pH 7.4) due to the electron-transfer reaction of Fc^{+}/Fc redox. The anodic peak currents increased linearly with the square root of scan rate (Fig. 2, inset), indicating that FcMeOH functions properly as a diffusional electron-transferring mediator, and the PQQ-GDH layer provides sufficient permeability for the diffusion of FcMeOH molecules.

To examine the electrochemical response to glucose, two types of PQQ-GDH electrodes were subjected to cyclic voltammetry in the presence of FcMeOH at different concentrations of glucose (0–20 mM). Intriguingly, the electrostatically modified PQQ-GDH electrode gave rise to higher signal in response to glucose (Fig. 3a), whereas a marginal increase in current signal was observed on the covalently modified PQQ-GDH electrode (Fig. 3b). As a result, PQQ-GDH sensor constructed by electrostatic binding of the enzyme enabled more sensitive detection of glucose, leading to almost a twofold higher current density than that by covalent conjugation over the tested range of glucose (Fig. 3c). In particular, there was a steep increase at the glucose concentration lower than 1 mM for the

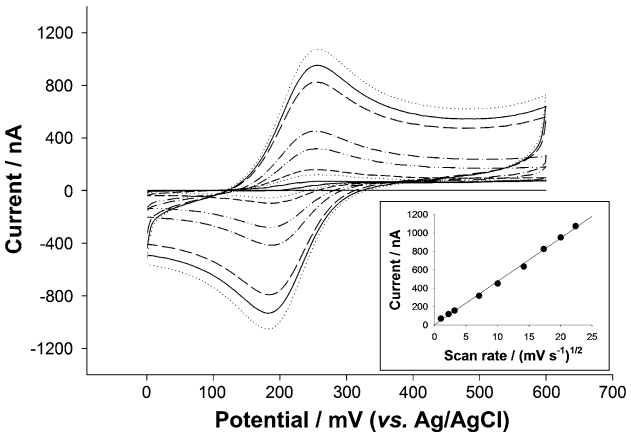

Fig. 2 Cyclic voltammograms showing the redox behavior of Au/SAM/PQQ-GDH (by electrostatic binding method) in 0.1 M phosphate buffer (pH 7.4) at different scan rates: 1, 2, 5, 10, 50, 100, 200, 300, 400, and 500 mV s^{-1} (from inside to outside). FcMeOH was added as a diffusional electron-transferring mediator at the concentration of 0.1 mM. The *inset* displays the correlation between anodic peak currents and the square root of scan rate

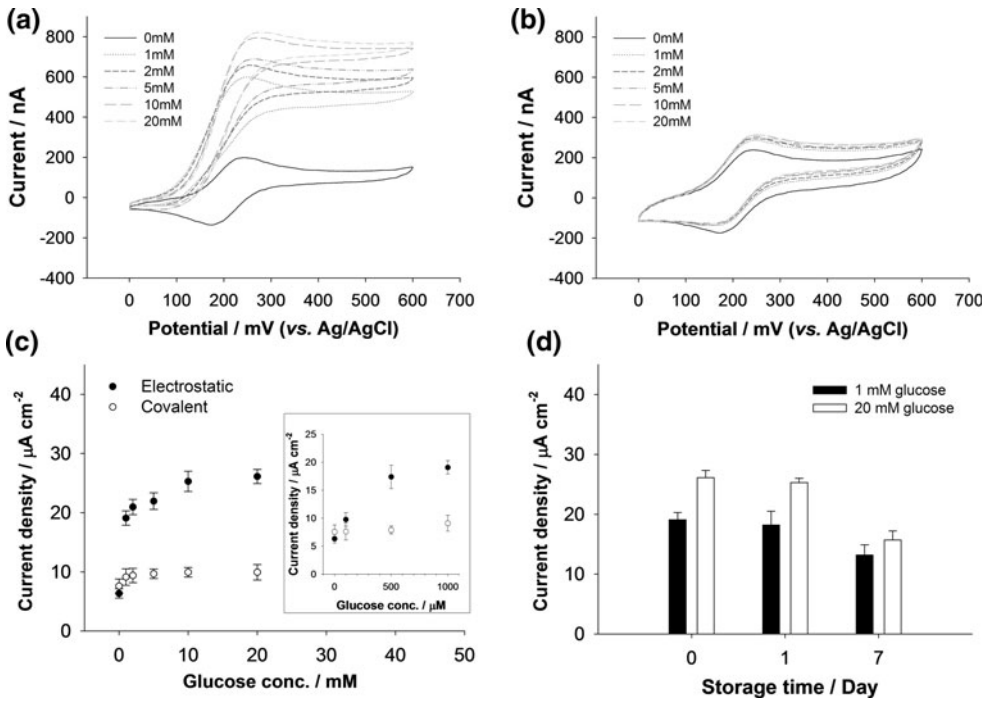

Fig. 3 Cyclic voltammograms for the electrodes constructed by **a** electrostatic and **b** covalent binding of PQQ-GDH onto SAMs at different glucose concentrations (0, 1, 2, 5, 10, and 20 mM). **c** Calibration plot for glucose based on the cyclic voltammetric current response (μA cm^{-2}) at $E = 260$ mV. The *inset* represented the current response in the micromolar range of glucose concentration. **d** Storage stability of the PQQ-GDH electrodes by the electrostatic binding method. The current response to 1 mM (*black bar*) or 20 mM (*white bar*) of glucose was monitored at different time points (0, 1, and 7 days) while the electrodes were stored at 4 °C in a 0.1 M phosphate buffer (pH 7.4). The cyclic voltammetry was performed at a scan rate of 10 mV s^{-1} in a 0.1 M phosphate buffer solution (pH 7.4) including 0.1 mM FcMeOH. The standard error was obtained from two independent electrodes under the same conditions

electrostatically modified electrode, even detecting the tested range of 100–500 μM in a hyperbolic fashion (inset in the Fig. 3c). Thus the detection sensitivity is likely to be determined in the micromolar range. Blood glucose level is maintained at a reference range between 3.6–5.6 mM as a part of metabolic homeostasis, and mean normal blood glucose level in humans is about 4 mM (equivalent to 72 mg dL^{-1}). When considering the blood glucose level, the present biosensing system has a potential to be implemented to monitoring the glucose level in human blood. To examine the storage stability, the PQQ-GDH electrodes via electrostatic conjugation were kept at 4 °C and were subjected to the cyclic voltammograms at different times (Fig. 3d). A relatively stable response to 1 or 20 mM glucose was observed for the initial 24 h, which was followed by a fast decrease of current response in a week, reaching ~69 % (1 mM glucose) and 60 % (20 mM glucose) of the respective initial value. Similar result was found even when the enzyme was electrostatically attached on the electrode 1 week after dissolving the dried enzyme in phosphate buffer at 4 °C (data not shown). This result strongly reveals that the signal decline might be caused by loss in PQQ-GDH activity, not by leakage of the enzyme on the electrode. Although this study is desirable for a single-use application, this enzyme stability could be elaborated for the long-term use through genetic engineering, as reported elsewhere [27, 28].

Apart from the stability issue, two different factors, namely surface density and enzyme activity, might account for the striking difference between electrostatic and covalent conjugation in signal generation. It was reported that the structure of an enzyme would be easily changed by covalent binding or cross-linking with chemical compounds such as glutaraldehyde [29], which causes a decrease in the enzyme activity. However, it is expected that a surface modification of PQQ-GDH for covalent binding is not critically detrimental to the enzyme activity because the active site is buried in the interior. Consequently, it is more likely that the low glucose response in covalently modified PQQ-GDH surface might be caused by low surface density of the enzyme on the SAMs.

To compare the surface densities of PQQ-GDH conjugated by two different methods, we performed SPR analysis for the respective surfaces. For this, an SPR sensor gold chip was firstly modified with the SAM of 11-MUA, and then the enzyme binding was monitored by tracing the refractive index change on the surface (Fig. 4). It has been generally accepted that an angle shift of 0.1° (=1,000 RU in Biacore instrument) is approximately equivalent to 1 ng mm^{-2} of mass increase on the SPR sensorgram [30]. Assuming three dimensional size (60 × 92 × 85 Å) [31] of PQQ-GDH and its smallest radius of 30 Å, the maximum binding amount (M) in a defined area (ng mm^{-2}) of a densely packed monolayer would be 6.3 ng mm^{-2} according to the following equation[32, 33].

$$M = \frac{10^{14}/r^2}{10^{-9}(N_A/\mathrm{MW})}$$

where r, MW, and N_A represent the radius (Å), molecular weight, and Avogadro's number, respectively. Thus, the surface coverage of PQQ-GDH was calculated by dividing the mass change on the SPR sensorgram by M.

Since the electrostatic binding of the enzyme resulted in the RU change of about 3,130, which was equivalent to 3.1 ng of bound enzyme per mm^2 (Fig. 4a), the surface coverage of PQQ-GDH was, thus, estimated to be 49 % on the basis of the above calculation. It was reported that the maximum surface coverage of protein on the SAMs would

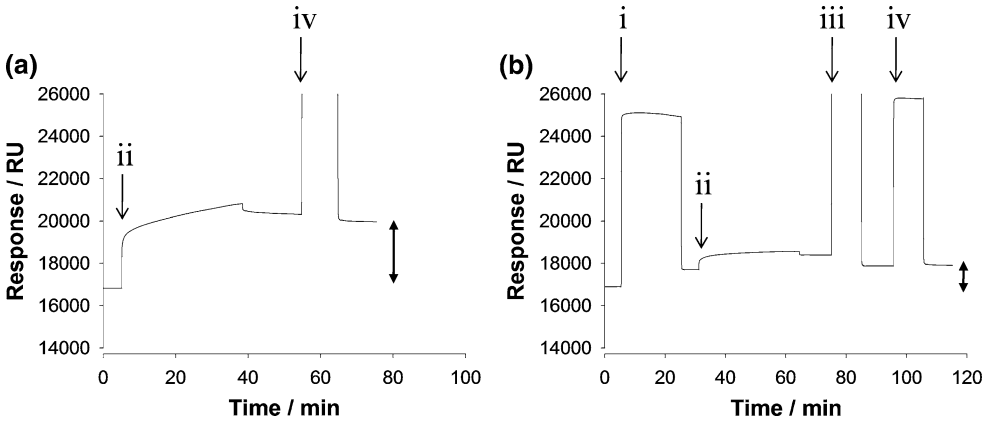

Fig. 4 SPR analysis for the binding of PQQ-GDH on the Au/SAM surface by two different methods: **a** electrostatic and **b** covalent binding. Sensograms were obtained with basal buffer (0.1 M PBS, pH 7.4), and each reagent was injected at the indicated time: (*i*) EDC/NHS in dH_2O, (*ii*) 1 mg mL^{-1} PQQ-GDH in PBS, (*iii*) 0.1 M PBS (pH 8.5) + 1.0 M ethanolamine, (*iv*) 0.1 M PBS (pH 7.4) + 0.05 % Tween20 + 1.0 M NaCl. The *bold arrow* indicates the amount of bound PQQ-GDH

be ~50 % by the random sequential adsorption [34]. Accordingly, the surface coverage of electrostatically bound PQQ-GDH is likely to reach a nearly maximum level on the SAMs. In addition, once the enzyme is electrostatically bound onto the SAMs, there was a negligible dissociation even after harsh treatment with 1 M NaCl, which indicates a strong binding of PQQ-GDH onto the carboxyl SAMs. On the other hand, in the case of covalent binding of PQQ-GDH, the change in RU was about 1,020 (Fig. 4b), which is equivalent to 1.0 ng enzyme per mm^2, and consequently a surface fractional coverage would be ~16 %. The ionic strength of binding buffer was also very critical for increasing the surface density and the stability of the enzyme onto the anionic SAMs. The relatively higher ionic strength in binding buffer (0.1 M PBS) reduced the surface coverage of the bound enzyme down to ~20 %, compared to the 1/10 diluted ion strength (10 mM PBS) (data not shown). This might be due to the fact that ions at a high concentration inhibit the electrostatic interaction between two differently charged molecules. Based on the result, it is evident that electrostatic binding of PQQ-GDH onto the anionic SAMs on gold electrode is an effective way of constructing a sensitive and robust glucose biosensor.

To further verify the role of charge interactions, we examined the effect of different SAMs on the surface density of PQQ-GDH (Table 1). For comparison, the SAMs mixed with 3-MPOH/11-MUA (9:1, mol/mol) and the amine SAMs were employed for binding of PQQ-GDH under the same condition. While covalent binding of the enzyme was carried out via chemical cross-linkers (EDC/NHS for MPOH/MPA and BS^3 for MAUM), electrostatic binding was performed by simple adsorption on each surface. In contrast to the Au/MUA, the Au/MUAM having cationic groups showed significantly decreased surface density of PQQ-GDH (1.3 ng mm^{-2}) under the same conditions, supporting that the carboxyl groups on the SAMs are very critical for the PQQ-GDH binding. The 3-MPOH/11-MUA surface with different alkyl chain length was expected to provide easy accessibility for the enzyme binding, but only resulted in weaker adsorption of PQQ-GDH (2.0 ng mm^{-2}) than that on MUA. Less dense distribution of negative charge, thus, could contribute to reduced binding of PQQ-GDH. Interestingly, the enzyme accessibility was more critical to the covalent binding than electrostatic one, which was supported by the result that the Au/MPOH/MUA led to twofold higher binding density (2.1 ng mm^{-2}) than that (1.0 ng mm^{-2}) on the Au/MUA. The binding density of the enzyme (2.2 ng mm^{-2}) on the Au/MUAM was shown to be similar to that on the mixed SAMs. This seems to be due to the fact that unlike the zero-length crosslinker (EDC/NHS), the homobifunctional cross-linker (BS^3) can endow the surface with long alkyl chains, allowing easy accessibility to the enzyme. In the covalent binding of PQQ-GDH, however, no surfaces exceeded the maximum surface density attained by the electrostatic binding of the enzyme onto the Au/MUA. It is noteworthy that a similar result was observed for streptavidin molecules, where the binding density of the protein on the SAMs via EDC/NHS reaction was much lower than that by the affinity-based reaction [35]. Other proteins such as immunoglobulin G and lysozyme also showed a notable binding on the polymerized film, compared to the covalent conjugation [36]. This result clearly indicates that the electrostatic interactions on the homogeneous anionic SAMs are mainly responsible for high surface density of PGG-GDH and this might bring higher detection sensitivity in the glucose sensing.

Table 1 Surface densities of PQQ-GDH measured by SPR analysis on different surfaces

Surface	Immobilization method	SPR response change (ΔRU)	Surface density[a] (ng mm^{-2})	Surface coverage[b] (%)
Au/MUA	Electrostatic	3,130	3.1	49
Au/MPOH/MUA	Electrostatic	1,980	2.0	31
Au/MUAM	Electrostatic	1,330	1.3	21
Au/MUA	Covalent	1,020	1.0	16
Au/MPOH/MUA	Covalent	2,100	2.1	33
Au/MUAM	Covalent	2,170	2.2	34

[a] 1 RU (resonance unit) corresponds to 1 pg protein mm^{-2}

[b] Surface coverage is a relative percentage divided by the theoretically maximal packing density (6.3 ng mm^{-2}) of PQQ-GDH

To gain insight into the surface morphology, we measured the surface roughness of the Au/SAM surfaces with differently bound PQQ-GDH. Characterization of surface topography provides an important clue not only for understanding the degree of homogeneity based on the height of the sample, but also for the quantitative analysis of the surface. The most popular parameter characterizing the morphology of surfaces is an RMS roughness (R_{RMS}), which represents the root mean square height of a surface around its mean value. Figure 5 shows the AFM images of PQQ-GDH on the SAMs on gold and R_{RMS} values. Compared to the control surfaces of bare Au (Fig. 5a) and Au/SAM (Fig. 5b), of which R_{RMS} values were 0.860 and 1.040, respectively, the R_{RMS} value in the electrostatic binding of the enzyme increased up to 1.517 (Fig. 5c). By contrast, the surface with covalently bound enzymes gave rise to the relatively low R_{RMS} value of 1.131 (Fig. 5d). Taking into consideration the SPR data regarding the binding of PQQ-GDH (Fig. 4), the difference in roughness between two binding methods may be attributed to the surface density. High roughness by electrostatic binding

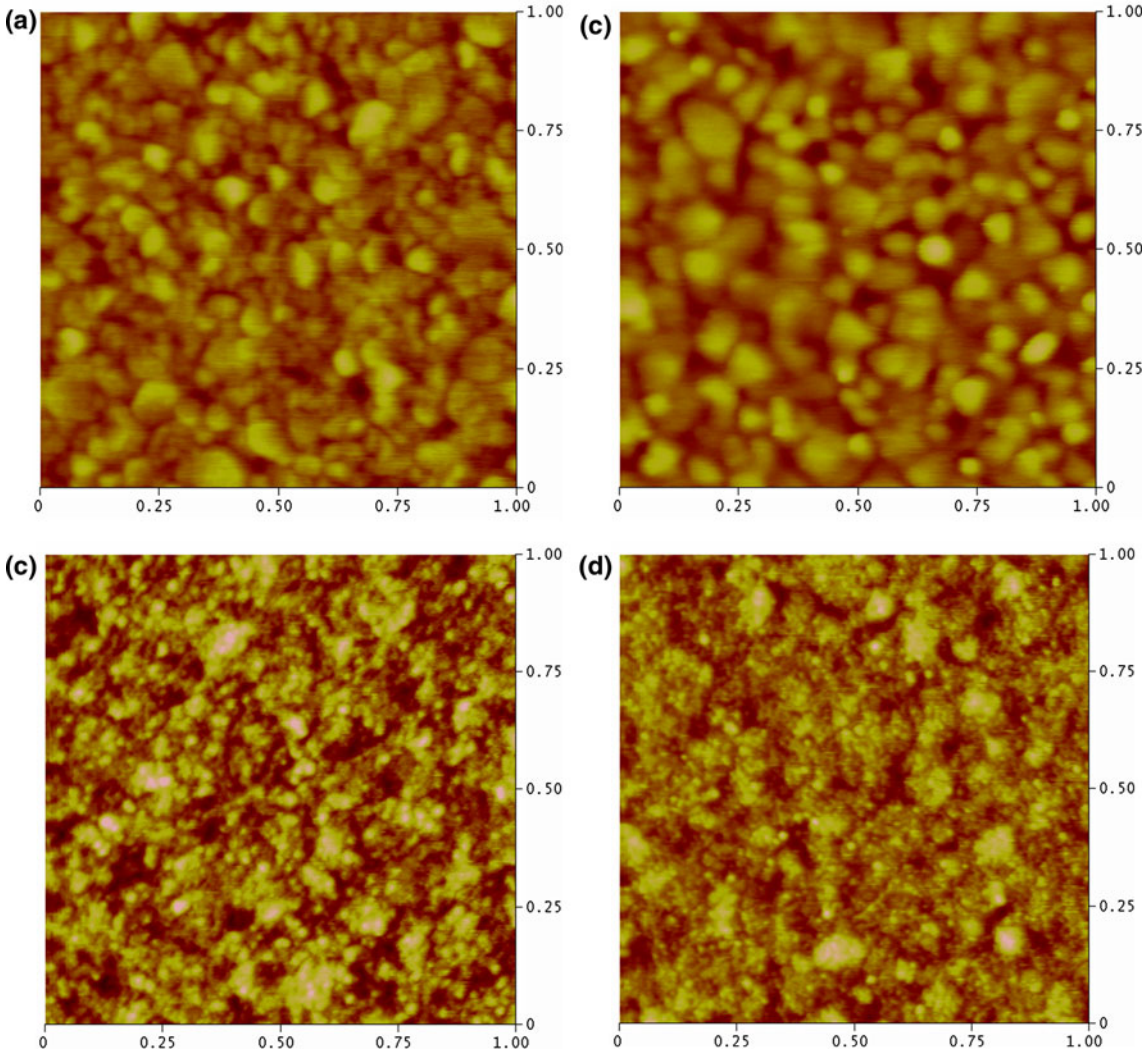

Fig. 5 AFM images of different surfaces: **a** bare Au, **b** Au/SAM, and PQQ-GDH attached surfaces on the Au/SAMs via **c** electrostatic and **d** covalent binding method. The values of root mean square (R_{RMS}) representing surface roughness were calculated to be 0.860 (**a**), 1.040 (**b**), 1.517 (**c**), and 1.131 (**d**)

may be related to the increasing heterogeneous adsorption both by lateral (side-on) and vertical (head-on) orientation of PQQ-GDH. Thus, the result implies high density of PQQ-GDH on the surface, due to a strong binding via electrostatic interactions.

4 Conclusions

In conclusion, we demonstrated that electrostatic association of PQQ-GDH on the gold electrode enabled a greater detection sensitivity of glucose than the covalent immobilization of the enzyme. SPR and AFM data supported that this result is mainly due to the high surface density of PQQ-GDH via the electrostatic method. Along with the genetic engeineering of PQQ-GDH, our strategy is anticipated to be broadly used for developing simple and robust biosensors or biochips with high sensitivity.

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