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Electrochemically oriented immobilization of antibody on poly-(2-cyano-ethylpyrrole)-coated gold electrode using a cyclic voltammetry

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ABSTRACT

Using quartz crystal microbalance (QCM) as an immunosensor, this work investigates the contribution of a cyclic voltammetry (CV) on the proper immobilization of antibodies with the aim of enhancing its target recognition and binding ability. Primarily, CV in the range of −0.1 to 0.9 V was applied to form a layer of poly-(2-cyano-ethylpyrrole) (PCEPy) on gold quartz crystal electrode. Then the efficiencies of antibodies (anti-IgG, AIgG) immobilized electrochemically with CV applied in 0–0.65 V were compared to those immobilized via physical adsorption, by observing relative affinity towards AIgG-Fab and AIgG-Fc fragments. The results showed antibody–AIgG-Fab interaction could be enhanced about 4 times when CV is applied (11.2 ± 1.3 vs 41.6 ± 3.4 relative fluorescence unit). On the contrary, physisorbed antibodies showed a higher degree of affinity towards AIgG-Fc indicating inappropriate orientations of physisorbed antibodies. AIgG immobilized PCEPy-gold QC electrode was characterized further for its sensitivity towards a new target bovine albumin with both a QCM and fluorescence measurement. Such electrode exhibited a good sensitivity as well as a large linear dynamic range, from 0.4 μg/ml to 1.0 μg/ml and from 0.5 μg/ml to 10.0 μg/ml, at QCM and fluorescence measurement, respectively.

1. Introduction

An immunosensor is a device comprising an antigen or antibody species coupled to a signal transducer, which detects the binding of the complementary species [1]. Its unique ability to detect and quantify biological targets like antigen proteins with extreme sensitivity and specificity [2,3] has allowed its application in a wide range of fields including biotechnology, diagnostics, and sensing applications [4]. As various devices and methods such as electrochemical quartz crystal microbalance (QCM) [5], surface plasmon resonance (SPR) [6], and fluorescence method [7] exist for diagnostic immunoassays, selecting an efficient immobilization method that can yield high binding capacity with a specific target is the key element in achieving great diagnostic results. The easiest method involving physical adsorption of antibodies onto the sensor surface will induce inconsistency in antibody immobilization, which will lead to heterogenic receptor surface. The covalent cross-linking of antibodies on surfaces treated with chemicals such as aldehyde [8], expoxy [9], N-hydroxysuccinimide (NHS) [10] does not provide a control over orientations of immobilized antibodies.

During the past several years, considerable attention has been focused on conducting polymers, which provide a great potential for the immobilization of antibodies [11,12]. Particularly, pyrrole and its derivatives have been widely used for the preparation of electroconductive polymeric coatings in biosensors and are showing highly attractive results in biomedical applications such as coating for electrodes and neural probes [13]. The ease of electropolymerization and modification with various functional groups make pyrrole suitable for electrocoating metal surfaces and further provides feasibility of antibody immobilization via electrostatic interaction between its polymer and the hydroxyl of antibody. Antibodies immobilized via electrostatic interaction have benefits over those immobilized alternatively that the former can have more proper orientation with their active site available for target binding. However, the major drawback of this immobilization method lies in

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the strength of the electrostatic interaction that is dependent on the environmental solution: changes in the ionic strength and/or pH can cause a release of antibodies from the electrode surface, especially during the regeneration step of the sensor by acid solution washing [12,13]. To overcome such weakness for the possible optimization of immunoassay results, this study makes a new approach to traditional immobilization methods. While the hydroxyl group of antibodies (anti-IgG) is immobilized electrostatically to the cyano group of poly-(2-cyano-ethylpyrrole) (PCEPy) deposited gold electrode, a cyclic voltammetry (CV) was applied to assign its receptor surface a charge that will attract the antibodies. The performance of the functionalized gold electrodes was studied and compared to that of the control and the orientations of antibodies were assured by observing relative differential fluorescence intensities.

2. Materials and methods

2.1. Preparation of poly-(2-cyano-ethylpyrrole) (PCEPy)-coated gold QC electrodes

The electrochemical quartz crystal microbalance (QCM) system was composed of three electrodes: a platinum plate as counter electrode; a silver chloride as reference electrode; and a gold coated quartz crystal electrode (gold QC electrode) as working electrode. The effective area of the working electrode was approximately 1.33 cm^2 and 0.2 μm thick. All three electrodes were connected with a potentiostat (H5000, Hokuto Denko, Japan) to induce electric fields, with the additional connection for the gold-QC electrode to a frequency counter (HQ101D, Hokuto Denko, Japan) to record weight changes. After each electrode was carefully cleaned using chloroform (Merck, Germany) solution for 3 min and thoroughly rinsed with ultrapure water, it was subsequently dried by nitrogen gas. PCEPy film on the working electrode was formed by cyclic voltammetry in a range of −0.1 to 0.9 V at 5 °C with a scan rate 5 mV/s. The electrochemical polymerization was carried out using 10 mM 2-cyano-ethylpyrrole (CEPy) in 0.1 M $NiClO_4$ acting as electrolyte at 25 °C. The PCEPy coated working electrode was thoroughly washed with ultrapure water and 0.1 M $NaClO_4$ (Merck, Kilsyth, Australia) for at least 30 min before AIgG antibody immobilization. The cross-sectional image of PCEPy film on gold QC electrode was confirmed using a field emission scanning electron microscope (FE-SEM) (U9320A 8500, Agilent, USA).

2.2. Immobilization of anti-IgG on the PCEPy-gold QC electrode

Anti-immunoglobulin G (rabbit anti-bovine albumin IgG, Sigma, USA; AIgG) is immobilized on the PCEPy-gold QC electrode via electrochemical and physical immobilization. For electrochemical immobilization of AIgG on PCEPy-gold QC electrode, a cyclic voltammetry (CV) was repeatedly used in a potential range of 0–0.65 V at 5 °C with a scan rate 10 mV/s. The electrochemical immobilization was carried out in a chamber containing 1 ml AIgG solution in 10 mM HEPES (1 mg/ml). Meanwhile, weight changes of the PCEPy-gold QC electrode during electrochemical immobilization of AIgG were monitored by the frequency counter. The AIgG immobilized electrode was then washed twice with ultrapure water and 10 mM HEPES to remove physisorbed AIgG protein. For physical immobilization of AIgG on PCEPy-gold QC electrode, PCEPy-gold QC electrode was incubated in the same chamber used for electrochemical immobilization for 12 h at 5 °C. Weight changes of the PCEPy-gold QC electrode during physical immobilization of AIgG were also measured by the frequency counter. After the experiment, the AIgG immobilized PCEPy-gold QC electrode was rinsed twice in HEPES and in ultrapure water.

2.3. Orientation analysis of AIgG immobilized on the PCEPy-gold QC electrode using Fab and Fc fragments

The AIgG-PCEPy gold QC electrodes, which were prepared by either electrochemical or physical immobilization methods, were incubated for surface coupling reactions in fluorescein isothiocyanate-conjugated goat anti-human IgG $F(ab')_2$ fragment (FITC-Fab; Thermo Scientific, USA) and fluorescein isothiocyanate-conjugated goat anti-human IgG Fc fragment (FITC-Fc; Antibodies, Germany) solutions (1 μg/ml in 10 mM HEPES) for 1 h at room temperature. The electrodes were then washed twice with 10 mM HEPES and twice with PBS, followed by one wash with ultrapure water to remove physisorbed Fab or Fc protein. The electrodes were dried under vacuum and kept at −20 °C before surface analysis. Fluorescence intensities created by the binding interaction between AIgG on PCEPy-gold QC electrode and FITC-Fab or FITC-Fc protein were measured using a PerkinElmer LS50B luminescence spectrometer with emission wavelength at 518 nm. The results were expressed as relative fluorescence unit (R.F.U.) values.

2.4. Detection limit analysis of AIgG-PCEPy gold QC electrode using bovine albumin

For the detection limit analysis, the AIgG-PCEPy gold QC electrodes were incubated in 1 ml bovine albumin protein (fluorescein conjugated bovine albumin protein, Sigma, USA) of various concentrations (0.01, 0.05, 0.1, 0.15, 0.2, 0.4, 0.6, 0.8, 1, 2, and 4 μg/ml for QCM measurements; 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 40, 60, 80, and 100 μg/ml for fluorescence measurements) in 10 mM HEPES solution for 10 min at room temperature. The electrodes were then washed twice with 10 mM HEPES, and once with ultrapure water to remove physisorbed protein. Subsequently, incubated plates were dried under vacuum. The frequency change of each concentration sample was measured by the frequency counter in QCM measuring cell, and the fluorescence intensities of the samples with the blank (prepared in a similar manner without protein) expressed as relative fluorescence unit (R.F.U.) values were measured at 495 nm, 20 °C.

3. Results and discussion

3.1. Electrochemical polymerization of 2-cyano-ethylpyrrole (CEPy) on gold QC electrodes

Cyclic voltammetry is commonly used to investigate kinetics of electrode processes for electrochemical polymerization and to characterize the resulting polymer film [14]. The electrochemical behavior of the CEPy-deposited gold electrode is thus investigated by cyclic voltammetry. The cyclic voltammogram was obtained using a cyclic potential from −0.1 to 0.85 V (vs Ag/AgCl in saturated KCl solution) at a scan rate of 10 mV/s (results not shown), during the electrochemical polymerization of 10 mM CEPy in 0.1 M $NiClO_4$ solution, the supporting electrolyte. The cyclic voltammogram of polymerized electrode exhibited a reversible oxidation wave around 0.4 V, which is in good agreement with the previously reported a half wave potential ($E_{1/2}$) values for poly-(2-cyano-ethylpyrroles) (PCEPy) [12]. In this study, the electropolymerization of the PCEPy on the QC electrode was achieved by controlled potential oxidation at 0.85 V for at least 20 min to avoid overoxidation of the PCEPy and thereby to preserve its conductivity. The resulting PCEPy-gold QC electrode was transferred, after thorough rinsing, to ultrapure water and 0.1 M $NaClO_4$ solution free of monomer. Fig. 1a shows the resulting frequency and mass changes of an aqueous PCEPy in $NiClO_4$ solution. While the mass of PCEPy was continuously increasing, the opposite pattern

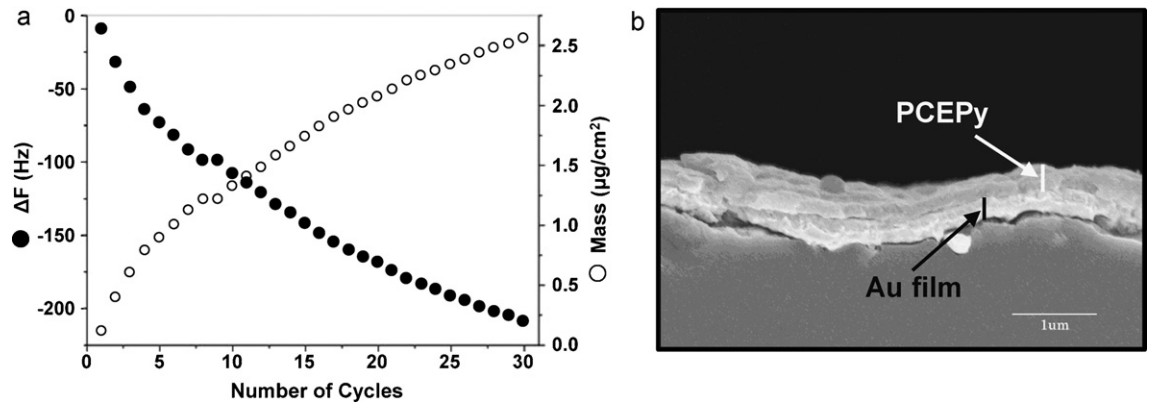

Fig. 1. (a) Representative plot of the frequency and mass changes caused by the 2-cyano-ethylpyrroles immobilization on gold-coated QC electrode using electrochemical polymerization: ●, frequency; ○, mass. (b) A cross-sectional field emission scanning electron microscope (FE-SEM) image of PCEPy-gold QC electrode.

was exhibited with frequency change during the 30 cycles. A total frequency change decrease of about 213 Hz, and a total mass change of 2.63 μg/cm^2 was observed as a result of PCEPy immobilization onto the gold electrode. The electro-formation of PCEPy layer on the gold QC is shown in a photomicrograph taken by FE-SEM (Fig. 1b). The thickness of PCEPy deposited onto a gold electrode was about 0.3 μm, while that of the gold layer adsorbed on bare QC electrode was determined to be about 0.2 μm. The formation of electropolymerized PCEPy film on the electrode surface as a consequence of cyano-pyrrole oxidation was hence assured.

3.2. Immobilization of AIgG on the PCEPy-gold QC electrode

It has been recognized previously that electrostatic interactions between protein–surface and protein–protein are generally accepted to influence the adsorption of proteins because the protein is composed of amino acids [15]. In this study, PCEPy-deposited gold electrode is to form electrostatic interaction between the cyano group of its polymer and the hydroxyl group of the antibody found in fragment crystallizable (Fc) region. Cyclic voltammetry applied in one direction to QCM that uses a PCEPy-gold QC electrode as the working electrode and a platinum plate as the counter electrode is likely to induce the binding between Fc region of the antibody carrying a negative charge and the working electrode at times when the surface electrical charge of the electrode becomes a positive. As a result, the charged antibody may be immobilized in one direction with the antigen-binding region being exposed for the specific target.

The electrochemical behavior of the AIgG antibody with a PCEPy-gold QC electrode was investigated by cyclic voltammetry in a voltage range of 0–0.65 V as shown in Fig. 2. In the positive region, an irreversible peak system recorded at 0.45 V (marked with an asterisk in Fig. 2) corresponds to the oxidation of the AIgG proteins. The successive attachment of AIgG on the PCEPy surface leads to an increase in coating mass and a decrease in the frequency change during the repeated cycles. Fig. 3 shows the resulting frequency and mass changes of the AIgG immobilized on a PCEPy-gold QC electrode by cyclic voltammetry (values taken after initializing to zero). Similar to the result obtained from the polymerization of CEPy on a gold QC (Fig. 1a), a progressive decrease in frequency change was recorded. As shown in Fig. 3 and in Table 1, the overall decrease in frequency observed from AIgG immobilized using cyclic voltammetry was approximately 146.6 Hz, which corresponds to a mass increase of 1.8 μg/cm^2. On the other hand, the frequency and mass change of AIgG immobilized by physical adsorption were estimated to be about −76.2 Hz and −0.96 μg/cm^2, respectively (Table 1). The difference in frequency and mass changes of two immobilization methods revealed electrochemical immobilization of AIgG using

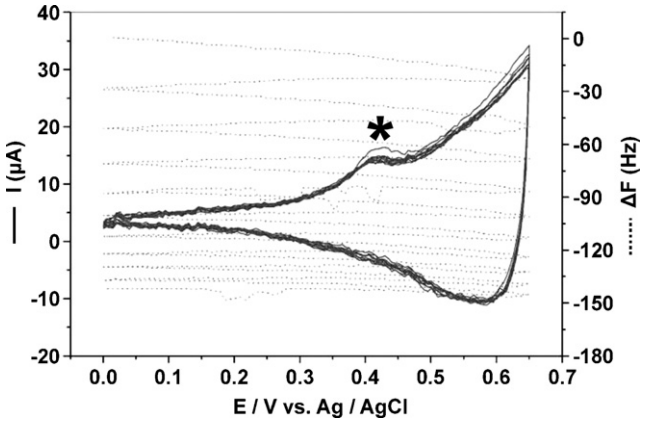

Fig. 2. The cyclic voltammogram of PCEPy-QC electrode in 10 mM HEPES solution containing 1 ml AIgG, at a scan rate 10 mV/s.

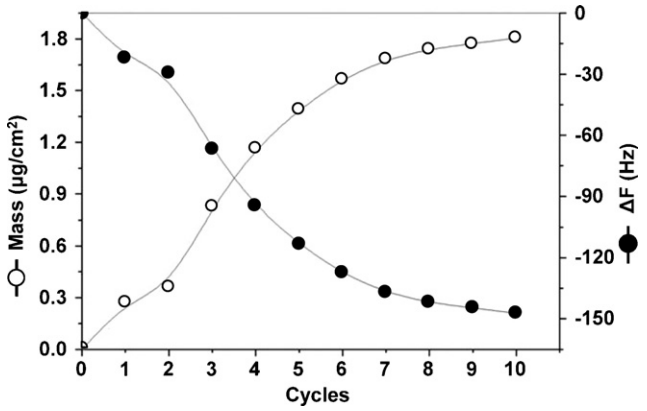

Fig. 3. Response plot of the frequency and mass changes caused by the AIgG immobilization on PCEPy-gold QC electrode using cyclic voltammetry: ●, frequency; ○, mass.

cyclic voltammetry caused a binding interaction that is twice as good as that of random immobilization which took place by physical adsorption. Moreover, the absence of any additional frequency variation from the circulation of HEPES was an indication of AIgG's a good fixation on PCEPy. The morphology of the AIgG immobilized on the PCEPy-gold QC electrode was also examined by FE-SEM, in which its micrograph showed a smooth and uniform coating of conducting polymer on metal plates. PCEPy-gold QC electrode used as a control surface is shown in Fig. 4a. Fig. 4b and c shows the images of AIgG immobilized on a PCEPy-gold QC electrode by the

Table 1
Frequency, mass, and relative florescence unit changes resulted from binding interaction between each electrode and AIgG, FITC-Fab, and FITC-Fc.

Electrode	Immobilization of AIgG			Binding interaction with FITC-Fab			Binding interaction with FITC-Fc		
	ΔF (Hz)	Mass density, Δwt (μg/cm^2)	Relative fluorescence unit (RFU)	ΔF (Hz)	Mass density, Δwt (μg/cm^2)	Relative fluorescence unit (RFU)	ΔF (Hz)	Mass density, Δwt (μg/cm^2)	Relative fluorescence unit (RFU)
Control PCEPy-gold QC electrode	0	0	1.1 ± 0.2	1.2	0.06	2.3 ± 2.1	1.3	0.05	2.4 ± 1.8
AIgG-PCEPy gold QC electrode prepared by physical adsorption	76.2	0.96	1.2 ± 0.2	96.7	1.21	11.2 ± 1.3	94.7	1.17	9.1 ± 0.5
AIgG-PCEPy gold QC electrode prepared by CV	146.6	1.81	1.2 ± 0.3	232.9	2.77	41.6 ± 3.4	154.3	1.91	3.7 ± 0.1

physical adsorption and the electrochemical immobilization using cyclic voltammetry, respectively. Globular lumpy form can be seen on both immobilization methods, yet the AIgG layer prepared by cyclic voltammetry was found to be more compact, uniform, and less cracked than the AIgG layer prepared by the physical adsorption. Consequently, this corroborates the formation of a specific interaction between AIgG and the PCEPy-gold QC electrode by the electrochemical immobilization of cyclic voltammetry.

3.3. Orientation analysis of AIgG immobilized on the PCEPy-gold QC electrode

An antibody is considered to be properly oriented and completely active when the Fc fragment of the antibody is immobilized on the sensor surface with the $F(ab')_2$, which contains antigen-binding sites, available for target binding. The immobilization of the AIgG on the PCEPy-gold electrode was ensured by the formation of electrostatic binding interaction between the terminal cyano groups of the PCEPy and the hydroxyl groups on the heavy chains (Fc) of the antibodies. Although antibodies as target capturing molecules can be adsorbed on gold directly by physical adsorptions and these methods are by far the easiest method of antibody immobilization, these methods produce a layer of randomly oriented antibodies on the modified surface, thereby generating conformational heterogeneity and inactive receptor molecules [16,17]. To further prove that AIgG molecules immobilized using CV are placed with the right orientation in a tight molecular monolayer of PCEPy-gold electrode, the characteristics of FITC-Fab and FITC-Fc proteins that specifically recognizes Fab or Fc domain of AIgG molecules were employed. If Fab (or Fc) region of immobilized AIgG is not readily exposing itself but rather immobilized on the electrode surface, FITC-Fab (or FITC-Fc) could not bind to the AIgG. The results of AIgG orientation analysis using FITC-Fab/Fc are summarized in Table 1. Compared to frequency and mass changes when only AIgG is immobilized in two ways on the PCEPy-gold electrode, the binding interaction of FITC-Fab and physically adsorbed AIgG resulted in a frequency and mass increases of 20.5 Hz (from 76.2 Hz to 96.7 Hz) and 0.25 μg/cm^2 (from 0.96 μg/cm^2 to 1.21 μg/cm^2), respectively, while that of FITC-Fab and electrochemically immobilized AIgG with CV resulted in frequency and mass increases of 86.3 Hz (146.6–232.9 Hz) and 0.96 μg/cm^2 (from 1.81 μg/cm^2 to 2.77 μg/cm^2), respectively. As seen from Table 1, the frequency change caused by the FITC-Fab (corresponding to the amount of FITC-Fab immobilized) is about 4 times greater for the electrochemical immobilization using CV (86.3 Hz) than for the physical adsorption (20.5 Hz). It is reasonable to presume that the electrochemical immobilization using cyclic voltammetry might allow for AIgG molecules to become attached with higher density on PCEPy-coated gold QC electrode. The quantification of FITC-Fab immobilization was performed by measuring its fluorescence intensity. Fluorescence intensities of FITC-Fab with physically and electrochemically immobilized AIgG on PCEPy-gold QC electrodes were 11.2 ± 1.3 RFU and 41.6 ± 3.4 RFU, respectively. The fluorescence intensity created by FITC-Fab with electrochemically immobilized AIgG was about 4 times greater than that of physical immobilization. While the results with FITC-Fab led to presumption that CV was making a contribution to the proper orientation of AIgG immobilization, an interesting results were shown with FITC-Fc fragments. FITC-Fc will only bind to AIgG if it is improperly immobilized onto electrode surface with Fc site available for binding. When FITC-Fc was binding with AIgG immobilized by both physical and electrochemical methods, physically immobilized AIgG showed a greater frequency and mass changes compared to the electrochemically immobilized group (18.5 Hz vs 7.7 Hz increase in frequency change and 0.21 μg/cm^2 vs 0.1 μg/cm^2 increase in mass change). RFU of FITC-Fc and physically immobilized AIgG was higher than that of FICT-Fc and electrochemically immobilized AIgG (9.1 ± 0.5 vs 3.7 ± 0.1 RFU). Considering that RFU of only AIgG immobilization was 1.2 ± 0.3 RFU, 3.7 ± 0.1 RFU created by FITC-Fc and electrochemically immobilized AIgG was a minor change. Therefore, it is reasonable to presume that covalent cross-linking of antibodies on chemically activated solid surfaces through amino groups were randomly oriented at physical adsorption compared with electrochemical immobilization. Consequently, our results suggested the

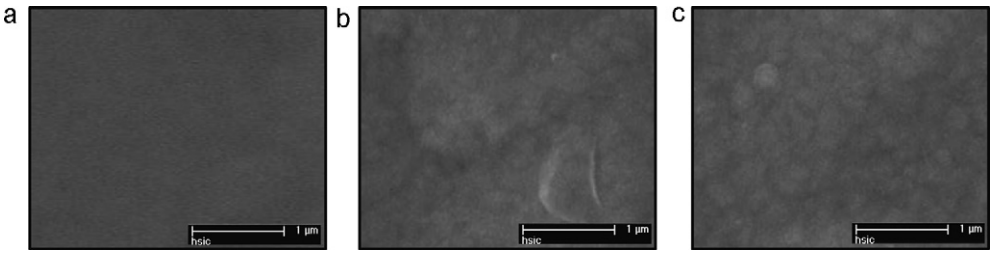

Fig. 4. Cross-sectional field emission scanning electron microscope (FE-SEM) images of (a) control PCEPy-gold QC electrode, (b) AIgG-PCEPy gold QC electrode prepared by physical adsorption, and (c) AIgG-PCEPy gold QC electrode prepared by cyclic voltammetry.

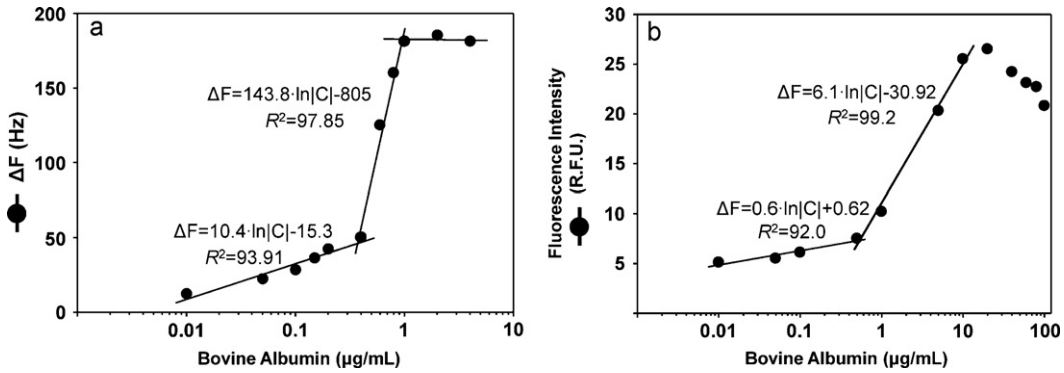

Fig. 5. Response curves for bovine albumin obtained for a PCEPy-gold QC electrode was investigated using (a) a frequency counter and (b) a luminescence.

advantage of the orientation-controlled immobilization occurred by the electrochemical immobilization procedure over the random immobilization occurred by the physical immobilization procedure.

3.4. Detection limit analysis of AIgG-PCEPy gold QC electrode using bovine albumin

The proper orientation of AIgG molecules on PCEPy-gold QC electrode could lead to not only a dense antibody immobilization in a unit area, but also a highly sensitive sensor chip. The frequency changes obtained from investigating the detection range of the AIgG-PCEPy gold QC electrode against various bovine albumin concentrations was plotted using QCM measurement. The value of steady-state frequency corresponded to the binding of the bovine albumin at certain concentration. Addition of bovine albumin resulted an increase in quartz crystal's oscillation frequency. Frequency change as a function of bovine albumin concentrations is shown in Fig. 5a with linear plot demonstrating the specific interaction between bovine albumin and AIgG. In the first region (concentrations < 0.4 μg/ml), the linear fitting curve can be expressed as $\Delta F = 10.4 \ln|C| - 15.31$ with a correlation coefficient $R^2 = 93.91$. In the second region (concentrations from 0.4 to 1 μg/ml), the linear curve can be expressed as $\Delta F = 143.8 \ln|C| - 805$ with a correlation coefficient $R^2 = 97.85$. In the third region, (concentration > 1 μg/ml), the saturation took place at approximately 1 μg/ml of bovine albumin and the saturation corresponds to the frequency changes of 180 Hz. This behavior at a high concentration may be due to the bovine albumin-AIgG complexes formed in the earlier additions hindering access for bovine albumin of later additions. Therefore, as is shown in Fig. 5a, bovine albumin detection range of AIgG-PCEPy gold QC electrode was suggested to the various bovine albumin concentrations in the range of 0.01–1 μg/ml. In this study, the detection limit of the AIgG-PCEPy gold QC electrode was also investigated by the measuring the fluorescence intensities as a function of bovine albumin concentrations as shown in Fig. 5b. The AIgG-PCEPy gold QC electrode exhibits an acceptable dynamic range with two linear zones: 0.01–0.5 μg/ml and 0.5–10 μg/ml. The response curve of low concentration is formed by a straight line with a slope of 0.62 Hz/μg/ml and a correlation coefficient of 0.92. The second linear region is also represented with a slope of 6.1 Hz/μg/ml and a correlation coefficient of 0.99. The detection limit for bovine albumin by fluorescence intensity measurements is 0.01–10 μg/ml. It was found that QCM allowed the detection of bovine albumin with relatively high sensitivity in low concentration range (0.01–1.0 μg/ml) compared to the fluorescence intensity measurement method. At higher concentrations (from 0.5 to 10 μg/ml), the fluorescence measurement exhibited more defined analysis.

4. Conclusion

For the successful construction of an antibody-based immunosensor, it is important to choose an efficient immobilization method that will reserve the proper orientation of the immobilized antibodies, thereby creating a compact biorecognition layer as it should lead to optimal immunoassay results. This study applied CV for the immobilization of AIgG antibody on a PCEPy-gold QC electrode, which was also prepared with CV. While electrochemically immobilized AIgG showed distinctively improved target recognition ability, physically immobilized AIgG exhibited random bindings. The results of this study helped to conclude that applying CV could provide a good control over the molecular orientation of the target capturing molecules such as AIgG. A novel immobilization procedure such as this may contribute to circumvent the limitations of traditional immobilization methods.

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