Non-invasive control of protein-surface interactions for repeated electrochemical immunosensor use

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ABSTRACT

Although most immunosensors which have high affinity antigen-antibody interactions can perform highly specific and sensitive detection, antibody-antigen bonds are typically too strong to allow for rapid reversibility. Therefore, creating a biosensor platform capable of non-invasive surface regeneration is important for reducing costs and being able to perform multiple measurements without losing sensor sensitivity. A reusable electrochemical immunosensor was developed on which a glassy carbon plate was passivated with a mixed layer of 4-nitroaniline/p-phenylenediamine, attached with gold nanoparticles, followed by surface-bound epitope (sulfo-NHS-biotin) to which the monoclonal anti-biotin IgG antibody was complexed. Free biotin was detected when the antibody dissociated from the surface-bound epitope in the presence of free biotin during a displacement assay. The electrochemical impedance spectroscopy technique was used to monitor the protein-surface interactions. To ensure that the immunosensor could be used repeatedly, electrode polarization was used to remove as much surface-bound antibody as possible by applying a negative potential to the working electrode. Therefore, the antibody could bind to the surface-bound epitope again after the electrode was reincubated in the antibody solution, and hence, the displacement assay could be performed again for repeated sensor use. Comparisons between disposable and reusable biosensors are discussed in this paper.

1. Introduction

Biosensors used for quantitative analysis require detailed calibration, and therefore, a series of measurements have to be performed for this purpose using disposable sensor units [1,2]. Nonetheless, variation arises from batch-to-batch irreproducibility and uncontrollable experimental conditions, generating unavoidable false-positive or -negative responses [1]. This has affected the accuracy and reliability of readings given by these novel devices (Table 1). Non-invasive surface regeneration of a biosensor platform is important for reducing costs and being able to perform multiple measurements without loss of sensor sensitivity. This has motivated many researchers to design a reusable type of biosensor for detecting analytes of interest [3].

The high affinity antigen-antibody interactions in immunosensors can lead to high specific, selective and sensitive detection [4]. In fact, an antibody-antigen bond is typically too strong to allow for rapid reversibility. Therefore, the development of immunobiosensors that are inherently reversible or that can be quickly

regenerated in situ to detect a continuous stream of analytes prompted this study. Several notable attempts to regenerate the affinity surfaces of sensors for repeated use have been reported [2]. Alternative approaches reported for the dissociation of bound antibody from the immuno-biosensor interface, including treatments such as extreme pH [1], temperature adjustments [5], chaotropic reagents [1] or additive assays [6], are available; however, these approaches suffer from significant losses of biospecific affinity activity [2]. Asonov et al. [2] reported using electric fields to enhance the dissociation of bound antibody from the biotin-coated antigen. A number of physical and chemical properties of the immunosensor interface are affected by electrochemical polarization [7]. Electrostatic properties are among the most obvious changes and play an important role in the interactions of biomolecules [7]. There is a strong correlation between surface charge and the adsorption affinity of proteins and small ions [8]. Varying electrode polarization (different potentials and different time intervals) results in changes in a number of surface properties and parameters in the electrical double layer. These surface properties and parameters include sign and surface densities of charges, variations in surface chemistry, intensities of the electric field in the Stern layer and diffuse layer of the electrical double layer, nonspecific and specific adsorption of proteins

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Table 1
Comparison between disposable and reusable biosensors.

	Disposable biosensor	Reusable biosensor
Definition	<ul style="list-style-type: none"> • A type of biosensor that is a single-use and non-regenerative device [17]. • It is very difficult to be regenerated [17]. 	<ul style="list-style-type: none"> • A type of biosensor that is a multiple-use and regenerative device [17]. • It can be used several times.
Examples	<ul style="list-style-type: none"> • A glucose biosensor based on co-dispersion of a diffusional polymeric mediator and glucose oxidase (GOX) in a nanoparticulate membrane on a screen-printed carbon electrode [18]. • A urea biosensor based on an ammonium-sensitive transducer [19]. • A DNA electrochemical biosensor used for the detection of metallic drugs such as titanium and platinum drugs, titanocene dichloride and cisplatin [20]. • A transcutaneous oxygen sensor based on the amperometric Clarke cell principle [21]. • A multi-walled carbon nanotube (MWNT)-based disposable biosensor for organo phosphorus detection using the amperometric method [22]. 	<ul style="list-style-type: none"> • A glucose biosensor developed by a robust radiofrequency-integrated passive device [23]. • A cholesterol biosensor based on cholesterol oxidase immobilized onto a thioglycolic acid self-assembled monolayer modified with smart bio-chips [24]. • A laccase-based biosensor for detection of ortho-substituted phenolic derivatives [25]. • A fiber optic biosensor for detection of drugs and toxicants [26]. • A potassium ion biosensor based on electrochemiluminescence resonance energy transfer [27]. • A mercury (II) biosensor based on “turn on” resonance light scattering [28].
Advantages	<ul style="list-style-type: none"> • Simple and time-saving because no regeneration steps are required. • Suitable for samples that contain infectious bacteria (especially clinical samples). 	<ul style="list-style-type: none"> • Multiple-use or multiple measurement [17]. • Regenerative. • Reproducible. • Low cost due to reusability of the device. • Environmentally friendly because less waste is being produced.
Disadvantages	<ul style="list-style-type: none"> • Non-regenerative. • Non-reusable. • High cost for long-term use. • Not environmentally friendly. 	<ul style="list-style-type: none"> • Loss of detection ability, sensitivity, and selectivity after multiple measurements if it is non-regenerative. • Limited sample types because it is not suitable to be used for samples that contain infectious bacteria.
Limitations	<ul style="list-style-type: none"> • Single-use or one-time measurement [17]. • Variation occurs if a disposable biosensor is used to perform a series of measurements for a detailed calibration purpose [1]. This may affect the accuracy and reliability of the results. 	<ul style="list-style-type: none"> • Not user-friendly due to required regeneration steps. • High technology is required for the sensor surface to be 100% regenerated so that it can be reused.
Challenges	The challenge faced by the disposable biosensor industry is the impact on the environment. It is difficult to replace the raw materials of disposable biosensors using bio-decomposable resources. Additionally, they have the challenge of maintaining cost-effectiveness without compromising quality in terms of accuracy, sensitivity and selectivity.	The industry has the challenge of developing reusable biosensors that are capable of performing with minimal detection loss while maintaining high sensitivity and selectivity after multiple uses.

(i.e., antibodies) or small ions at the surface and changes in the dielectric constants [7]. Choi et al. [9] have demonstrated an innovative way of producing a highly regenerative biosensor surface using electrochemical desorption of short-chain alkanethiols entirely from gold surfaces by applying a potential of -900 mV to the working electrode twice for 30 s. This attempt was successful; however, it increased the cost and time required for each regeneration step, with at least 1 h needed for the reformation of SAMs, followed by tedious immobilization steps of redepositing anti-fibrinogen antibody and fibrinogen onto the transducer surface.

In this paper, we present the use of time base pulse voltammetry to non-invasively control protein-surface interactions. A selected amount of constant potential was applied to a label-free electrochemical immunosensor interface for 10–15 min to regenerate the interface. Covalently surface-bound biotin was utilized as the biospecific recognition molecule that bound to the monoclonal anti-biotin IgG antibody, free biotin was used as the displacement agent, and the dissociation rate and the amount of antibody were controlled using voltage-induced stimulation. Biospecific association and the displacement assay between functionalized surface-bound epitope and antibody complexes were determined using electrochemical impedance spectroscopy (EIS) measurements tested in a potassium ferricyanide ($K_3Fe(CN)_6$) solution. The main objective of this study was to demonstrate that the electrode polarization method could exhibit better non-invasive control of antibody-antigen reversible interactions. This finding represents a potential strategy for effective biospecific association/disassociation reaction cycles for immunobiosensor regeneration that minimizes cross-reactive binding and/or non-specific protein binding, which cause false-positive signal interpretation, and control antigen-antibody binding events, thus showing promise as a reusable label-free electrochemical immuno-biosensor.

2. Research methodology

2.1. Sensor repeatability test using reversible association/dissociation interactions between the antibody and the surface-bound epitope

For the sensor repeatability test, the epitope-modified immunosensor interface was first exposed to the anti-biotin IgG antibody solution ($50 \mu\text{L}$, $0.5 \mu\text{g mL}^{-1}$, 30 min), followed by the free biotin solution (0.3 mg mL^{-1} , 20 min) to displace bound antibody. The original electrode was reincubated in the antibody solution so that the antibody could again bind to the epitope and the displacement assay could be performed again. This was conducted five times within 24 h.

2.2. Voltage-induced dissociation of antibody-epitope complexes for regeneration of the immunosensor interface (non-invasive protein-surface interactions)

To regenerate the immunosensor, the sensor was first exposed to the anti-biotin IgG antibody solution followed by the free biotin solution to displace bound antibody. Because not all of the bound antibody was eliminated from the immunosensor surface during the displacement step, even after prolonged incubation times in the analyte solution, the biorecognition interface was regenerated using electrode polarization to remove as much surface-bound antibody as possible. In this study, the biorecognition interface was regenerated using a series of electrode polarizations at different potentials and different time intervals [versus Ag/AgCl (3 M KCl)] to determine the optimal condition for maximum dissociation of antibody from the surface-bound epitope. Next, the electrode was reincubated in the antibody solution so that the antibody could again bind to the epitope and the displacement assay could be

performed again. This was continuously conducted five times within 24 h.

3. Results and discussion

3.1. An electrochemical immunosensor for direct detection of the small molecule biotin

Cyclic voltammetry was employed to investigate the various electrochemical characteristics of $\text{Fe}(\text{CN})_6^{3-/4-}$ redox species on the modified GC electrode. Fig. 1a shows the cyclic voltammograms of the bare GC electrode and the bottom-up stepwise attachment of mixed layers of *p*-phenylenediamine and 4-nitroaniline, AuNPs, 2-[2-(2-methoxyethoxy)ethoxy]acetic acid, *p*-phenylenediamine, sulfo-NHS-biotin, and monoclonal anti-biotin IgG antibody on the GC surfaces. As observed, well-defined peaks characteristic of $\text{Fe}(\text{CN})_6^{3-/4-}$ were noted on the bare GC electrode. However, Surface 1 (Scheme 1) prevented access of the $\text{Fe}(\text{CN})_6^{3-/4-}$ redox species to the electrode, and therefore, no Faradaic peaks for ferricyanide were observed between +0.6 V and −0.4 V. An increase in the peak current of the redox couple was observed after the attachment of AuNPs (Surface 2, Scheme 1) using the electrochemical method. This increase in the electron transfer rate upon attachment of the AuNPs was in line with the studies reported by Chen et al. [10] and Shein et al. [11] that showed that AuNPs can increase the electronic coupling to the underlying electrode. After the 2-[2-(2-methoxyethoxy)ethoxy]acetic acid was attached to the amino groups on Surface 3, the Faradaic peaks for $\text{Fe}(\text{CN})_6^{3-/4-}$ slightly decreased because the long chain molecules (2-[2-(2-methoxyethoxy)ethoxy]acetic acid) blocked the $\text{Fe}(\text{CN})_6^{3-/4-}$ redox species from reaching the electrode. Next, the *p*-phenylenediamine was attached to the AuNPs on the electrode surface to yield Surface 4. The $\text{Fe}(\text{CN})_6^{3-/4-}$ redox species were completely hindered from approaching the electrode surface. Thus, the Faradaic peaks disappeared [as shown in Fig. 1a(v)]. After incubation of Surface 5 with sulfo-NHS-biotin, the peak current of the redox couple decreased slightly, showing that the attachment of sulfo-NHS-biotin to the AuNPs slightly restricted the access of the $\text{Fe}(\text{CN})_6^{3-/4-}$ redox probe to the AuNP-modified GC surface. With the high binding affinity of the monoclonal anti-biotin IgG antibody to the surface-bound biotin, the peak current of the redox couple decreased significantly. This major decrease in the peak current was attributed to the monoclonal anti-biotin IgG antibody binding to the sulfo-NHS-biotin on the surfaces of the AuNPs, thus covering the AuNPs with protein and inhibiting access of the $\text{Fe}(\text{CN})_6^{3-/4-}$ redox species to the electroactive elements on the GC surface (Surface 6).

The characteristics of the modified electrode were further investigated using the electrochemical impedance spectroscopy (EIS) technique, and the obtained results were completely consistent with the cyclic voltammetry results. The Nyquist plots of these impedance results are shown in Fig. 1b, while the parameters derived from fitting these Nyquist plots with an equivalent circuit (inset of Fig. 1b) are shown in Table 2. As reported by Radi et al. [12], R_s represents the bulk properties of the electrolyte solution and diffusion features of the redox species in solution. R_s is the resistivity between the reference electrode and the working electrode and can be affected by the ion concentration, the type of ions, the temperature and the geometry of the area in which the current is carried [13]. Current flows between the working electrode and the reference electrode. When it passes through electrolyte, the current flow is usually non-uniform. As a result, the obtained R_s values might change. In other words, the different R_s values obtained from this study were probably not influenced by electrode surface modifications but were actually influenced by the uneven current distribution throughout the definite electrolyte area [13]. C_{dl} and R_{ct} are related to the dielectric and insulating features at the

electrode/electrolyte interface, and thus the changes at the electrode surface have a significant impact on these parameters [12]. However, this study showed that the capacitance changes (C_{dl}) were not as significant as the electron transfer resistance (R_{ct}) (Table 2). On the other hand, a constant phase element (Q, n) was used instead of a capacitor because the normal Randles equivalent circuit could not be fitted with the impedance semicircles obtained from the experiments. This observation might be attributed to the deformation or broadening of the impedance semicircle [14]. The deformation or broadening of the impedance semicircle was caused by the roughness of the electrode surface or the inhomogeneity in the local distribution of defects in the vicinity of the grain boundaries [14]. This problem was successfully solved by using a parallel connection of an ohmic resistor, R_{ct} , and a constant phase element (Eq. (1)) [14]. Q is the pre-factor of the constant phase element and n is the exponent of Q . For $n = 1$, Q is identified as the ideal C_{dl} . Taking into account the effect of the roughness of electrode surfaces ($n < 1$), the C_{dl} value was calculated from the formula below.

$$C_{dl} = R_{ct}^{\frac{1-n}{n}} \cdot Q^{\frac{1}{n}} \quad (1)$$

From the calculated results (Table 2), the capacitance changes throughout all of the bottom-up fabrication steps were not significant. Additionally, as shown in Table 2, all n values were close to 1, indicating that there were minimal defects in the modification layer on the electrode surface. In contrast, the changes in R_{ct} were much larger compared with the other impedance components. Therefore, R_{ct} was considered to be a suitable signal for understanding the interfacial properties of the fabricated immunosensor during all of the assembly steps and for monitoring the association/dissociation of antibodies to the electrode interface.

The modified GC electrode (Surface 6, Scheme 1) was used as the immunosensor interface for detection of the small molecule biotin during a displacement assay. The electrode bound with monoclonal anti-biotin IgG antibody was incubated in a phosphate buffered saline solution containing various concentrations of free biotin. After the exposure of Surface 6 (Scheme 1) to free biotin, the R_{ct} decreased because the antibody dissociated from the surface-bound biotin during the displacement assay (Surface 7). This resulted in increased access of the $\text{Fe}(\text{CN})_6^{3-/4-}$ redox species to the AuNP-modified GC surface. Different concentrations of free biotin were tested, and the corresponding impedance spectra were obtained. The observed decrease in the R_{ct} (Fig. 1c) was associated with an increase in the free biotin concentration, implying that more antibodies were being dissociated from the immunosensor interface. All of the impedance spectra for free biotin detection at different concentrations were fitted to the equivalent circuit to obtain the charge transfer resistance values, R_{ct} , at each concentration. Fig. 1c shows the derived calibration plot that corresponds to the electron transfer resistance at the immunosensor interface with different concentrations of free biotin. To compare the different electrode measurements made under equivalent conditions, the absolute relative values [$R_{ct(b)} - R_{ct(a)}$] were used. $R_{ct(b)}$ and $R_{ct(a)}$ are the charge transfer resistance values for Surface 6 (Scheme 1) recorded before and after a displacement assay with free biotin, respectively. A linear relationship between the electron transfer resistance and the free biotin concentration was found, ranging from 700 to 1500 $\mu\text{g mL}^{-1}$ (Fig. 1c). When the concentration of free biotin was increased to 1500 $\mu\text{g mL}^{-1}$, the impedance spectroscopy changes gradually leveled out, indicating that the maximum amount of antibody had been successfully dissociated from the surface-bound biotin during the displacement assay. The lowest concentration of free biotin that could be detected by this fabricated immunosensor was determined to be 100 ng mL^{-1} . To investigate the stability of the fabricated immunosensor, the biotin immunosensor (Surface 5, Scheme 1) was stored at 4 °C for 21 days.

Fig. 1. (a) Cyclic voltammograms recorded in phosphate buffer solution containing 0.05 M KCl and 1 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ with the scan rate of 100 mV s^{-1} after different steps of modification: (i) bare GC electrode (blue); (ii) Surface 1 (red); (iii) Surface 2 (orange); (iv) Surface 3 (light green); (v) Surface 4 (green); (vi) Surface 5 (purple); (vii) Surface 6 (grey); (viii) Surface 7 (pink). (b) Nyquist plots recorded in phosphate buffer solution containing 0.05 M KCl and 1 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ for impedance measurements for (i) bare GC electrode (blue); (ii) Surface 1 (red); (iii) Surface 2 (orange); (iv) Surface 3 (light green); (v) Surface 4 (green); (vi) Surface 5 (purple); (vii) Surface 6 (grey); (viii) Surface 7 (pink) with DC potential = 0.200 V, frequency range = 0.1–10000 Hz and amplitude = 0.01 V. (c) The calibration plot corresponding to the decrease of electron transfer resistance of the immunosensor with the concentrations of free biotin. Inset of Fig. 1b: Equivalent circuit for the impedance spectroscopy measurement.

Scheme 1. Schematic representation of fabricating an electrochemical immunosensor for direct detection of small molecule biotin via a displacement assay. Note the AuNP is not drawn to scale being many orders of magnitude larger than the molecules as drawn.

The sensor interface was still capable of detecting free biotin in a displacement assay without obvious loss of detection ability.

3.2. FE-SEM and FT-IR characterization of the immunosensor interface

Fig. 2a(i) shows the FE-SEM image for the bare GC plate. The clean and dark image indicates that the GC plate was

extremely clean and that no residue was present. The successful attachment of the AuNPs to create Surface 2 (Scheme 1) was confirmed by FE-SEM (Fig. 2a(ii)). The number of AuNPs in the FE-SEM images was counted manually. The density of the AuNPs on Surface 2 (Scheme 1) was calculated to be approximately $1.75 \times 10^7 \text{ AuNPs cm}^{-2}$. Most importantly, the FE-SEM image shows a homogeneous distribution of AuNPs on the 4-nitroaniline/p-phenylenediamine modified glassy carbon plate.

Table 2

Values of the equivalent circuit parameters of the fitting curves for the bottom-up stepwise fabrication of the electrochemical impedance immunosensor interface by z-view software.

Electrodes	$R_s (\Omega)$	$R_{ct} (\Omega)$	$Q (Mh_s)$	n	$C_{dl} (F)$
Bare GC	50.62	9.29×10^2	1.69×10^{-5}	0.802	6.095×10^{-6}
Surface 1	55.32	3.06×10^5	1.15×10^{-6}	0.884	1.001×10^{-6}
Surface 2	92.08	8.11×10^3	1.74×10^{-6}	0.852	8.309×10^{-7}
Surface 3	110.41	1.25×10^4	3.95×10^{-6}	0.818	2.022×10^{-6}
Surface 4	143.15	3.22×10^4	7.56×10^{-6}	0.827	5.630×10^{-6}
Surface 5	93.02	8.89×10^5	2.66×10^{-6}	0.808	3.266×10^{-6}
Surface 6	243.37	1.34×10^6	2.96×10^{-6}	0.787	4.296×10^{-6}
Surface 7	207.50	7.71×10^5	3.32×10^{-6}	0.781	4.316×10^{-6}

From the FE-SEM image, the sizes of the AuNPs were measured to be approximately 20 nm (Fig. 2a(ii)).

In addition to FE-SEM, FT-IR was used to characterize the fabricated immunosensor interface. Fig. 2b(ii) shows specific peaks for certain functional groups, for example, amino groups (at approximately 3700 cm^{-1}), aromatic rings (at 1548 cm^{-1}) and nitro groups (at 1209 cm^{-1}) in the spectra for Surface 1. This observation indicated that both 4-nitroaniline and p-phenylenediamine were successfully attached to the GC plate. In Fig. 2b(iii), the signal for the amino group on Surface 2 was significantly decreased because the amino groups were substituted with the AuNPs that covalently bound to the aromatic rings. After the electrochemical reduction of the nitro groups, the peak for the amino groups appeared again

in the spectra for Surface 3 [Fig. 2b(iv)]. Due to the attachment of 2-[2-(2-methoxyethoxy)ethoxy]acetic acid to the amino groups by the formation of amide bonds ($-\text{CONH}-$), the peak for the N-H stretching (at 3316 cm^{-1}) for amide groups, the peak for carbonyl groups (at 1626 cm^{-1}) and the broad band for ether groups ($1300\text{--}1000 \text{ cm}^{-1}$) were clearly visible in the spectra for Surface 4 [Fig. 2b(v)]. For AuNPs that had been further modified with p-phenylenediamine (Surface 5), the peak for the amino group appeared again at a frequency of 3641 cm^{-1} [Fig. 2b(vi)]. In this study, even though Surface 6 and Surface 7 were characterized using the FT-IR technique, however it was unable to provide clear and analyzable spectra for the complicated structures of proteins such as sulfo-NHS-biotin (Fig. 2b(vii)) and monoclonal anti-biotin IgG antibody (Fig. 2b(viii)) where any reasonable assignments of the FT-IR signals were not possible.

3.3. Regeneration of the immunosensor interface without the aid of voltage-induced dissociation of antibody-epitope complexes

Fig. 3a shows that when the same electrode was subjected to the repeated process of association of the monoclonal anti-biotin IgG antibody followed by the displacement assay with free biotin, the electrode did not yield similar responses for each repeated use of the sensor. In addition, complete regeneration of the surface was not evident even after a prolonged displacement assay with free biotin from 20 to 30 min. Fig. 3b and c shows the changes in R_{ct} when

Fig. 2. (a) FE-SEM image of GC for (i) bare surface and (ii) after the covalent attachment of AuNPs (Surface 2) with the magnification of 100K. Image was taken at an angle of 40° to the surface. (b) FT-IR surface characterization of (i) bare GC electrode; (ii) Surface 1; (iii) Surface 2; (iv) Surface 3; (v) Surface 4; (vi) Surface 5; (vii) Surface 6; (viii) Surface 7.

Fig. 3. The comparison of electrochemical signal (R_{ct}): (a) The repeating process of the association reaction of monoclonal anti-biotin IgG antibody and the displacement assay with free biotin. (b) The magnitude of R_{ct} increment after exposure to $0.5 \mu\text{g mL}^{-1}$ monoclonal anti-biotin solution. (c) The magnitude of R_{ct} decrease via a displacement assay in 0.3 mg mL^{-1} free biotin solution.

the biotinylated interface was exposed to $0.5 \mu\text{g mL}^{-1}$ monoclonal anti-biotin IgG antibody and 0.3 mg mL^{-1} free biotin, respectively, for five repeated uses. The increment of R_{ct} , as shown in Fig. 3b, indicated that the total amount of monoclonal anti-biotin IgG antibody bound to the surface-bound epitope increased after five repeated uses. After repeated exposure to the anti-biotin solution in the regeneration test without the aid of voltage-induced dissociation, the antibody was difficult to dissociate from the surface-bound epitope, even in the presence of free biotin. Therefore, no significant amount of antibody was dissociated from the sensor surface during the fourth and fifth regeneration tests (as shown in Fig. 3b). Because the bound anti-biotin IgG antibody was not fully removed from the biotinylated surface during the displacement assay, the remaining epitope-antibody complexes reduced the number of free surface-bound biotin binding sites for the next cycle of antibody complexation (Fig. 3a). This non-idealistic regenerative condition eventually caused “saturation,” and therefore, the immunosensor surface was not suitable for repeated use due to the degradation of the sensor sensitivity, resulting in unreliable readings for each replicate measurement (Fig. 3b and c).

3.4. Electric field-driven acceleration for reversible affinity interactions for repeated immunosensor use

When a negative potential was applied to the working electrode, the dissociation of the bound antibody from the surface-bound epitope was enhanced by electrostatic force [7]. Fig. 4a shows that the electrode polarization of -800 mV at the 10 min interval time was the optimum condition to yield the highest sensor sensitivity (the maximum decrease of R_{ct} value obtained after a displacement assay). However, at a less negative potential (-700 mV versus

Ag/AgCl, 3 M KCl), the bound anti-biotin antibodies were unable to be displaced by free biotin in 50 mM phosphate buffered saline (pH 7.4), and the obtained R_{ct} was even higher than the R_{ct} measured before the displacement assay. Additionally, the amount of antibodies dissociated from the surface-bound epitope during the displacement assay could not be improved by increasing the duration of the applied potential from 10 min to 20 min at -800 mV . Similarly, using a more negative potential (-900 mV , 10 min) did not improve the sensor response compared with the electrode polarization of -800 mV at a 10 min interval time (Fig. 4a).

For the electric field-driven strategy used in this study, the magnitude and the interval time employed for the electrode polarization were shown to have a strong impact on the amount of antibody that can be non-invasively dissociated from the surface-bound epitope and hence indirectly influence the sensor sensitivity (Fig. 4a). The ability to regenerate the electrochemical immunosensor interface (by enhancing the dissociation of antibody from the surface-bound epitope) was the essential criteria for repeated immunosensor use. All of these observations have important implications for the development of reusable biosensors. Control experiments were conducted without the presence of free biotin using the same experimental conditions, as shown in Fig. 4a. No significant R_{ct} decreases (ΔR_{ct} range between $0.3 \text{ k}\Omega$ and $1.03 \text{ k}\Omega$) were observed compared with the analytical signal decreases shown in Fig. 4a. These findings suggest that antibody displacement was essentially induced by the surface charge (e.g., electrode polarization) and the presence of small target analytes (e.g., free biotin) that inhibited the readsorption of the dissociated antibody onto the immunosensor surface. This is due to the higher affinity of free biotin for the dissociated antibody compared with the affinity of the surface-bound biotin to the dissociated antibody [16].

Fig. 4. (a) The conditions tested for the enhancement of immunosensor sensitivity in terms of larger decrease in R_{ct} value after a displacement assay via cathodic polarization at different potentials and different time intervals. (b) Five repeated measurements were made where the immunosensing interface was exposed repeatedly first to $0.5 \mu\text{g mL}^{-1}$ monoclonal anti-biotin IgG antibody followed by 0.3 mg mL^{-1} free biotin solution.

Scheme 2. The mechanism for the electrochemical immunosensor surface regeneration via a displacement assay in free biotin solution (0.3 mg mL^{-1}) enhanced by cathodic polarization at -800 mV for 10 min.

Based on the results obtained from the electric field-driven strategy used to enhance immunosensor sensitivity through the complete dissociation of antibody from the surface-bound epitope during a displacement assay, the repeatability assessment of the immunosensor in terms of regeneration ability and stability was next investigated (note: the detail mechanism of surface regeneration ability was shown in Scheme 2). To regenerate the immunosensor surface effectively, the sensor was first exposed to the monoclonal anti-biotin IgG antibody solution ($0.5 \mu\text{g mL}^{-1}$) followed by a displacement assay with aid of electrode polarization in free biotin solution (0.3 mg mL^{-1}) to enhance the dissociation of antibody from the surface bound epitope. In other words, while performing the displacement assay in free biotin solution, the bio-recognition interface was regenerated non-invasively by maintaining a potential of -800 mV for 10 min (versus Ag/AgCl, 3 M KCl) to remove as much surface-bound antibody as possible, so that there are free surface-bound biotin binding sites for the next cycle of antibody complexation. After the surface regeneration, the original electrode was re-incubated in the antibody solution so that the antibody could again bind with the epitope and a displacement

assay could be performed again. This was continuously conducted five times within the same day at ambient temperature (25°C). In this study, the antibody-epitope binding was found to be reversible with the aid of electrode polarization, and this observation was consistent with a previous study reported by Oldham et al. [7]. The displacement assay showed only little variation in the electrochemical signal after five replicate continuous measurements, with an RSD of 9.14% (Fig. 4b). The binding activity of the surface-bound biotin to the monoclonal anti-biotin IgG antibody was maintained at over 98.8% after five regeneration cycles (as compared with the initial value) (Fig. 4b), and no obvious signal degradation caused by the residual signal was observed. The residual signal was defined as the background signal caused by incomplete antibody removal after a displacement assay with the aid of electrode polarization.

This repeatability study also successfully demonstrated that a single immunosensor interface can be used multiple times for successive detection of large molecules (e.g., monoclonal anti-biotin IgG antibody) as well as small organic molecules (e.g., free biotin). This was a favorable result showing that a robust platform for electrochemical immunosensing applications was successfully

Fig. 5. The stability of the grafted mixed layer of in situ-generated aryl diazonium cations (p-phenylenediamine, 4-nitroaniline) upon multiple electrode polarization cycles (-800 mV, 10 min) was determined by the ability of the mixed layer formed to passivate the penetration of $\text{Fe}(\text{CN})_6^{3-/4-}$ (1 mM prepared in 50 mM phosphate buffer, pH 7) after different number of regeneration cycles.

developed. In terms of sensor sensitivity evaluation, the lowest detection limit of the immunosensor obtained was improved from 100 ng mL^{-1} to 1 ng mL^{-1} by using electric field-driven strategy. This value is lower than the detection limit reported by Liu et al. [15] for small molecule biotin detection.

3.5. Stability of the biorecognition interface upon multiple electrode polarization cycles

Fig. 5 shows the stability of the grafted mixed layer of in situ-generated aryl diazonium cations (p-phenylenediamine, 4-nitroaniline) after multiple electrode polarization cycles (-800 mV, 10 min), which was determined by the ability of the formed mixed layer to prevent penetration of $\text{Fe}(\text{CN})_6^{3-/4-}$ (1 mM prepared in 50 mM phosphate buffer, pH 7) after different numbers of regeneration cycles. The presence of CVs that successfully blocked penetration (after multiple electrode polarization cycles) indicates that the interface was not significantly changed (Fig. 5). This observation also indirectly indicated that the electrode polarization technique used to enhance the dissociation of bound antibody from the surface-bound epitope during a displacement assay could be considered as a non-invasive way to control protein-surface interactions. Therefore, with this stable platform for biospecific interactions, utilizing a negative polarization treatment provides a reproducible condition at the biotinylated immunosensor surface that is suitable for construction of a reusable immunosensor.

4. Conclusions

A stable reusable immunosensor for the detection of small organic molecules was successfully developed based on the concept of non-invasive control of protein-surface interactions using electrode polarization. The fabricated immuno-biosensor surface can be repeatedly used to detect small organic molecules (free biotin) because of the reversible interactions between the monoclonal anti-biotin IgG antibody and the surface-bound biotin, which was enhanced by voltage-induced dissociation of the antibody-epitope complexes. To regenerate the immunosensor interface of the immunosensor, electrode polarization was set at -800 mV (versus Ag/AgCl, 3 M KCl) for a 10 min interval time. This condition yielded the highest immunosensor sensitivity for free biotin detection with a dynamic range between $700 \mu\text{g mL}^{-1}$ and $1500 \mu\text{g mL}^{-1}$. After five regeneration cycles, no significant signal degradation was observed using this approach. This showed that a single immuno-biosensor surface could be used multiple times without

the need to re-modify the entire immunosensor interface starting at the bottom layer. The CVs that were successful at blocking the $\text{Fe}(\text{CN})_6^{3-/4-}$ testing solution indicated that the immunosensor interface was not physically damaged after multiple cycles of surface regeneration using electrode polarization. Thus, the immuno-biosensor surface was still stable for the detection of the small organic molecules. From all of the experimental results obtained, we can conclude that the immunosensor interfacial design as reported herein is stable, regenerable, highly sensitive and selective for the detection of small organic molecules. As for the impact of the immunosensor on society, the economy and the nation, the non-invasive control of protein-surface interactions using electrode polarization is a cost-effective and environmentally friendly surface regeneration approach because the fabricated immunosensor can be reused repeatedly without the need to re-modify the entire immunosensor interface.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.snb.2015.10.085>.

References

- [1] S. Choi, *Advancing Microfluidic-based Protein Biosensor Technology for Use in Clinical Diagnostics*, Arizona State University, 2011.
- [2] A.N. Asanov, W.W. Wilson, P.B. Oldham, Regenerable biosensor platform: a total internal reflection fluorescence cell with electrochemical control, *Anal. Chem.* 70 (6) (1998) 1156–1163.
- [3] J.A. Goode, J. Rushworth, P.A. Millner, Biosensor regeneration: a review of common techniques and outcomes, *Langmuir* (2014).
- [4] A.L. Ghindilis, et al., Immunosensors: electrochemical sensing and other engineering approaches, *Biosens. Bioelectron.* 13 (1) (1998) 113–131.
- [5] Y. Liu, et al., Novel renewable immunosensors based on temperature-sensitive PNIPAAm bioconjugates, *Biosens. Bioelectron.* 24 (4) (2008) 710–715.
- [6] J. Chung, et al., Additive assay for the repeated measurements of immunosensor without regeneration step, *Sens. Actuators B Chem.* 114 (2) (2006) 1007–1012.
- [7] Oldham, P.B. and A.N. Asanov, *Control of antibody-antigen binding or dissociation by electric field*, in *BIOS'99 International Biomedical Optics Symposium*, 1999. International Society for Optics and Photonics.
- [8] R.E. Holmlin, et al., Zwitterionic SAMs that resist nonspecific adsorption of protein from aqueous buffer, *Langmuir* 17 (9) (2001) 2841–2850.
- [9] S. Choi, J. Chae, A regenerative biosensing surface in microfluidics using electrochemical desorption of short-chain self-assembled monolayer, *Microfluid. Nanofluidics* 7 (6) (2009) 819–827.
- [10] S. Chen, R.W. Murray, S.W. Feldberg, Quantized capacitance charging of monolayer-protected Au clusters, *J. Phys. Chem. B* 102 (49) (1998) 9898–9907.
- [11] J.B. Shein, et al., Formation of efficient electron transfer pathways by adsorbing gold nanoparticles to self-assembled monolayer modified electrodes, *Langmuir* 25 (18) (2009) 11121–11128.
- [12] A.-E. Radi, et al., An electrochemical immunosensor for ochratoxin A based on immobilization of antibodies on diazonium-functionalized gold electrode, *Electrochim. Acta* 54 (8) (2009) 2180–2184.
- [13] Gheorghe-Constantin, I. and C. Petru, *Brief survey on electrochemical impedance spectroscopy evaluation and its application on nickel corrosion in chloride solutions*.
- [14] M.S. Abouzari, et al., On the physical interpretation of constant phase elements, *Solid State Ionics* 180 (14) (2009) 922–927.
- [15] G. Liu, N. Michael, J. Justin Gooding, Protein modulation of electrochemical signals: application to immunobiosensing, *Chem. Commun.* (33) (2008) 3870–3872.
- [16] T.T. Ngo, Ligand displacement immunoassay, *Anal. Lett.* 38 (7) (2005) 1057–1069.

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