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Technical Note

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Low-Interference Washing-Free Electrochemical Immunosensor Using Glycerol-3-phosphate Dehydrogenase as an Enzyme Label

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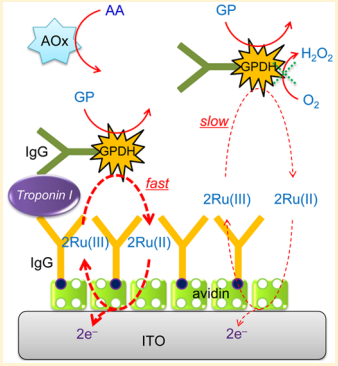
Supporting Information

ABSTRACT: In washing-free electrochemical detection, various redox and reactive species cause significant interference. To minimize this interference, we report a washing-free electrochemical immunosensor using flavin adenine dinucleotide (FAD)-dependent glycerol-3-phosphate dehydrogenase (GPDH) and glycerol-3-phosphate (GP) as an enzyme label and its substrate, respectively, because the reaction of FAD-dependent dehydrogenases with dissolved O_2 is slow and the level of GP preexisting in blood is low (<0.1 mM). A combination of a low electrocatalytic indium−tin oxide (ITO) electrode and fast electron-mediating $Ru(NH_3)_6^{3+}$ is employed to obtain a high signal-to-background ratio via proximity-dependent electron mediation of $Ru(NH_3)_6^{3+}$ between the ITO electrode and the GPDH label. Electrochemical oxidation of GPDH-generated $Ru(NH_3)_6^{2+}$ is performed at 0.05 V vs Ag/AgCl, at which point the electrochemical interference is very low. When a washing-free immunosensor is applied to cardiac troponin I detection in human serum, the calculated detection limit is approximately 10 pg/mL, indicating that the immunosensor is very sensitive in spite of the use of washing-free detection with a short detection period (10 min for incubation and 100 s for electrochemical measurement). The low-interference washing-free electrochemical immunosensor shows good promise for fast and simple point-of-care testing.



Miniaturized biosensors for health and environmental diagnostics have been widely developed.[1−9] Although there has been great progress in the miniaturization of catalytic biosensors, such as electrochemical glucose sensors,[10−12] it is still difficult to obtain miniaturized affinity biosensors, including immunosensors and DNA sensors. The main obstacle to miniaturization is that washing steps are required to obtain reproducible and sensitive sensor responses.[13] The purpose of washing is to remove (i) unbound signaling labels and (ii) interfering species in the sample solution. In particular, washing steps for the second purpose are more vital when complex samples such as serum and whole blood are electrochemically tested, which contain many interfering redox species and many species that react with the (bio)chemicals added during detection. Thus, to carry out electrochemical detection without washing steps, the influence of interfering redox and reactive species should be minimized.

Recently, we developed a noble washing-free electrochemical immunosensor using proximity-dependent electron mediation of ferrocenemethanol between an enzyme label (glucose oxidase) and an electrode, which enabled differentiation between a bound and an unbound label without the use of a washing step.[14] The problem of the most interfering redox species, L-ascorbic acid (AA), was avoided by using L-ascorbate oxidase (AOx). However, the previous immunosensor has three distinct limitations to its practical applications. First, the concentration of added enzyme substrate (200 mM glucose) was too high to be used in practical immunosensors. It takes a long time to dissolve the high concentration of glucose. The high concentration was employed to ensure that a wide variation of preexisting glucose concentration (<20 mM) in real physiological sample does not cause a significant change in the rate of the mediated oxidation of glucose by glucose oxidase. At a low concentration of added glucose, this variation significantly influences the rate. Second, reactive dissolved O_2, along with an electron mediator, participates competitively in glucose oxidation by glucose oxidase, which could cause lower electron-mediation currents and less reproducible data.[10] Third, an applied potential of 0.13 V that was used to oxidize ferrocenemethanol is high enough to cause little electro-oxidation of interfering species. The applied potential plays a crucial role in obtaining high and reproducible signal-to-background ratios, especially when electrochemical detection is performed in complex aqueous solution containing various interfering redox species.

Oxidoreductases [in particular, alcohol oxidoreductases (EC 1.1.x.x) such as glucose oxidase][15] can be used as enzyme labels to obtain proximity-dependent electron mediation.[14] However, many alcohol oxidoreductases can react with dissolved O_2 as well as electron mediators.[10] Furthermore, many substrates for

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alcohol oxidoreductases exist as metabolites in serum and blood, and their concentrations are often >0.1 mM. Undesirably, some alcohol oxidoreductases show low substrate specificity; consequently, they oxidize other alcohols as well as their substrates.[16] Therefore, alcohol-oxidoreductase labels that allow for low interference with dissolved O_2 and metabolites should be properly chosen.

Herein, we introduce a new enzyme label and its substrate that are very suitable for development of a low-interference washing-free electrochemical immunosensor. To overcome the limitations in our previous washing-free immunosensor, flavin adenine dinucleotide (FAD)-dependent glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.5.3) and glycerol-3-phosphate (GP) were used as an enzyme label and its substrate, respectively, because the reaction of FAD-dependent dehydrogenases with dissolved O_2 is slow[16] and the level of GP preexisting in blood is low (<0.1 mM).[17] Moreover, $Ru(NH_3)_6^{3+}$ was used as an electron mediator to apply a lower potential (0.05 V).

EXPERIMENTAL SECTION

Chemicals and Solutions. Avidin, AA, AOx, and all reagents for buffer solutions were purchased from Sigma-Aldrich, Co. Phosphate-buffered saline (PBS, pH 7.4) contained 10 mM phosphate, 0.138 M NaCl, and 2.7 mM KCl. PBSB contained all of the constituents of PBS in addition to 1% (w/v) bovine serum albumin. Human cardiac troponin I (30R-AT035), monoclonal mouse antitroponin-I IgG (capture probe, 10-T79A), monoclonal mouse antitroponin-I IgG (detection probe, 10-T79B), and troponin-I-free human serum (90R-106X) were obtained from Fitzgerald, Inc. (Acton, MA, USA). Indium–tin oxide (ITO) electrodes were obtained from Corning (Daegu, Korea). Sulfosuccinimidyl-4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC), *N*-succinimidyl-*S*-acetylthiopropionate (SATP), and EZ-link sulfo-NHS-LC-LC-biotin were obtained from Thermo Scientific Inc. GPDH-conjugated antitroponin-I IgG was prepared by cross-linking the amine group of IgG and the amine group of GPDH (see Supporting Information (SI)). The biotin-conjugated antitroponin-I IgG was synthesized by EZ-link sulfo-NHS-LC-LC-biotin (see SI).

Preparation of Immunosensing Electrodes and Immunosensing Procedure. The preparation and performance of immunosensing electrodes were reported previously.[14,18] In short, the immunosensing surface was prepared by passive avidin adsorption (10 μg/mL, for 2 h at room temperature) on ITO electrodes (1 cm × 2 cm each), followed by immobilization of biotinylated antitroponin-I IgG (10 μg/mL, for 30 min at 4 °C). The sample solution was prepared with 100 μL of PBSB buffer containing 20 μg/mL GPDH-conjugated antitroponin-I IgG, 100 μL of PBS buffer containing 50 mM GP, 100 μL of PBS buffer containing 1.0 mM $Ru(NH_3)_6^{3+}$, 100 μL of PBS buffer containing 50 U/mL AOx, and 100 μL of PBS buffer, which were mixed with 500 μL of troponin-I-free serum spiked with different concentrations of troponin I. The mixed solution (1 mL) was injected to the vessel of an electrochemical cell. The resulting concentrations of GPDH-conjugated antitroponin-I IgG, GP, $Ru(NH_3)_6^{3+}$, and AOx were 2 μg/mL, 5 mM, 0.1 mM, and 5 U/mL, respectively. Electrochemical measurement was carried out after an incubation period of 10 min.

Teflon electrochemical cells were assembled with a sensing electrode, an Ag/AgCl (3 M NaCl) reference electrode, and a platinum counter electrode. The exposed geometric area of the ITO electrodes was approximately 0.28 cm^2. The final incubation was performed at 25 °C, and electrochemical measurements were carried out using a CHI 708C system (CH Instruments, Austin, TX, U.S.A.).

RESULTS AND DISCUSSION

In electrochemical detection, the type of electrode, and the applied potential play crucial roles in obtaining high and reproducible signal-to-background ratios, especially when electrochemical detection is performed in complex aqueous solutions containing various interfering redox species. High electrocatalytic electrodes cause high and varying background levels due to the rapid oxidation of interfering redox species and the rapid reduction of dissolved O_2.[19,20] However, low electrocatalytic electrodes can kinetically minimize such unwanted reactions if appropriate potentials are applied. This effect is more evident when the applied potential is near 0 vs Ag/AgCl electrode.[14,19,20] Even in this case, high and reproducible signal levels can be obtained by using a signaling redox species that undergoes a fast outer-sphere electron-transfer reaction at low electrocatalytic electrodes.[14,21,22] Therefore, the combination of a low electrocatalytic ITO electrode and fast electron-mediating $Ru(NH_3)_6^{3+}$ was chosen to obtain a high and reproducible signal-to-background ratio in washing-free detection.

Figure 1 shows a schematic representation of a sandwich-type washing-free electrochemical immunosensor using GPDH as an enzyme label for detecting cardiac troponin I. The GPDH-labeled antibody was not washed away after the solution was incubated for 10 min for affinity binding, and it remained in the solution even when the electrochemical signal was measured. During incubation, troponin I is bound to the capture antibody and the GPDH-labeled antibody. As a result, the GPDH-labeled antibody becomes bound to the immunosensing electrode. The surface concentration of the bound GPDH-labeled antibody then increases with increasing concentration of troponin I. Signaling $Ru(NH_3)_6^{3+}$ acts as both a GPDH substrate (during incubation and electrochemical

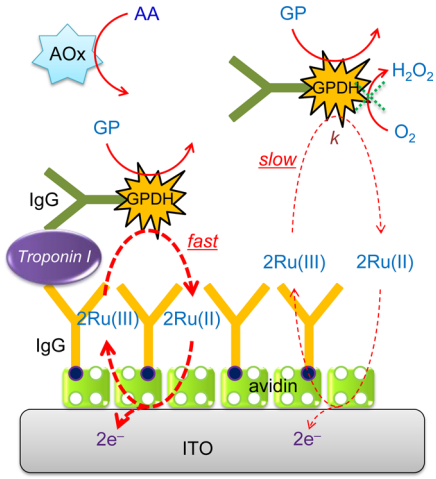

Figure 1. Schematic representation of a washing-free electrochemical immunosensor using GPDH and $Ru(NH_3)_6^{3+}$. GP, GPDH, AA, AOx, Ru(III), Ru(II), and ITO represent glycerol-3-phosphate, glycerol-3-phosphate dehydrogenase, L-ascorbic acid, L-ascorbate oxidase, $Ru(NH_3)_6^{3+}$, $Ru(NH_3)_6^{2+}$, and indium–tin oxide, respectively.

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measurement) and an electron mediator between the ITO electrode and GPDH label (during electrochemical measurement). During incubation, GPDH converts $Ru(NH_3)_6^{3+}$ into $Ru(NH_3)_6^{2+}$ in the presence of GP. The concentration of converted $Ru(NH_3)_6^{2+}$ near the electrode is much higher than that in solution when the surface concentration of bound GPDH-labeled antibody is substantial. During electrochemical measurement, $Ru(NH_3)_6^{2+}$ is converted into $Ru(NH_3)_6^{3+}$. In addition, electron mediation of $Ru(NH_3)_6^{3+}$ between the ITO electrode and GPDH label (i.e., electrochemical-enzymatic redox cycling)[22,23] occurs continuously. The mediation between the ITO electrode and the bound GPDH-labeled antibody is fast, but that between the electrode and unbound antibody is relatively slow, which allows for proximity-dependent electron mediation. In a GPDH concentration of 1 μg/mL, the mean distance between the unbound GPDH labels in solution is approximately 460 nm (see SI), indicating that the unbound GPDH label is far away from the ITO electrode. Consequently, the electrochemical signal increases with increasing surface concentration of the bound GPDH-labeled antibody.

For electrooxidation-based detection in serum, the most problematic interfering redox species are AA, uric acid, and acetaminophen,[19] whose formal potentials are −0.14 V in pH 7.2,[24] 0.29 V in pH 7,[25] and 0.40 V in pH 7,[26] respectively. Therefore, when the applied potential is near 0 V, oxidation of uric acid and acetaminophen is negligible. Therefore, only minimization of AA interference is important, which was obtained by oxidizing AA by AOx.[14] Figure 2a shows the cyclic voltammograms obtained in a potential range from −0.4 to 0.3 V in PBS (curve i) and serum (curve ii). Both voltammograms were similar, indicating that electrooxidation of interfering species was not significant and/or that the redox reactions of interfering species were slow at the ITO electrodes.

To obtain effective $Ru(NH_3)_6^{3+}$-mediated oxidation of GP by GPDH, the reaction should not occur in the absence of GPDH. The cyclic voltammogram in a solution containing $Ru(NH_3)_6^{3+}$ (curve i of Figure 2b) was almost similar to that obtained in a solution containing $Ru(NH_3)_6^{3+}$ and GP (curve ii of Figure 2b), indicating that $Ru(NH_3)_6^{3+}$ does not react with GP. In the presence of GPDH, the currents were higher all over the potentials due to $Ru(NH_3)_6^{3+}$-mediated oxidation of GP by GPDH (curve iii of Figure 2b).

To determine the optimum applied potential, chronocoulograms obtained at −0.05, 0.00, 0.05, and 0.10 V in a solution containing $Ru(NH_3)_6^{3+}$ and GP in the presence and absence of GPDH were compared (SI Figure S1). An applied potential of 0.05 V was found to be best for obtaining a high signal-to-background ratio. Accordingly, all chronocoulograms were obtained at 0.05 V. The chronocoulogram obtained in a solution containing $Ru(NH_3)_6^{3+}$ (curve i of Figure 3a) was similar to that obtained in a solution containing $Ru(NH_3)_6^{3+}$ and GP (curve ii of Figure 3a), reconfirming that $Ru(NH_3)_6^{3+}$ does not react with GP. In the presence of GPDH along with $Ru(NH_3)_6^{3+}$ and GP, the charge was significantly increased (curve iii of Figure 3a). This result further confirms that

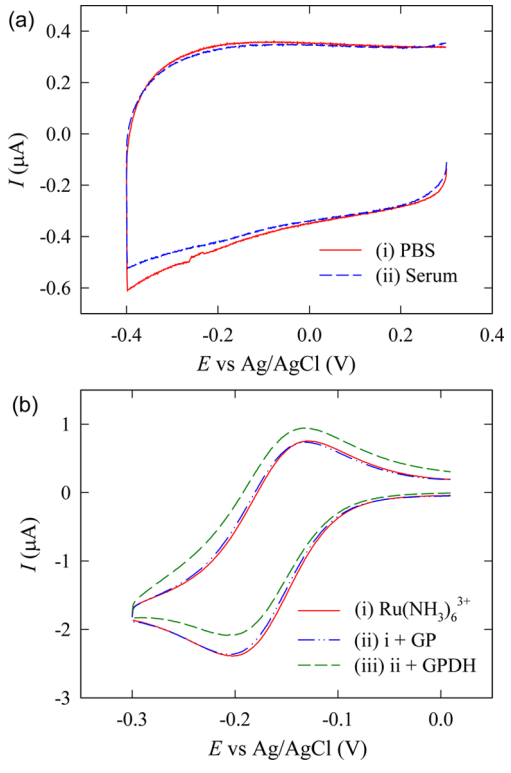

Figure 2. Cyclic voltammograms recorded (at a scan rate of 20 mV/s) using ITO electrodes (a) in (i) PBS (pH 7.4) and (ii) troponin-I-free human serum, and (b) in PBS containing (i) 100 μM $Ru(NH_3)_6^{3+}$; (ii) 100 μM $Ru(NH_3)_6^{3+}$ and 5.0 mM GP; and (iii) 100 μM $Ru(NH_3)_6^{3+}$, 5.0 mM GP, and 100 μg/mL GPDH.

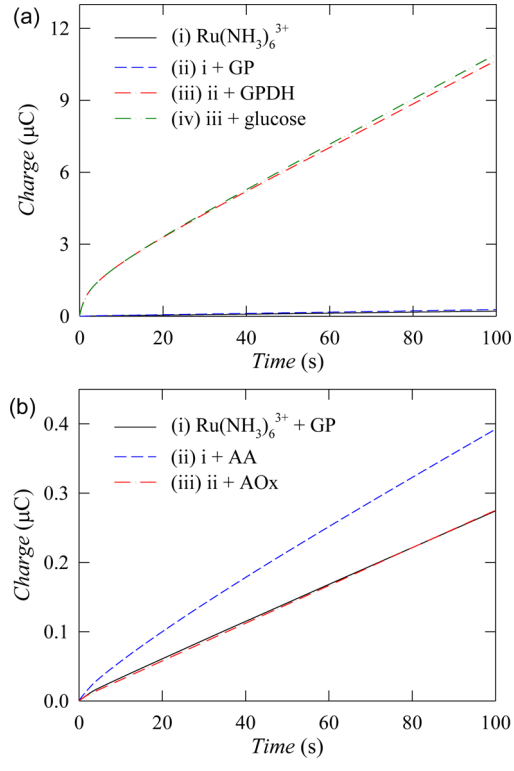

Figure 3. (a) Chronocoulograms recorded (at 0.05 V) using ITO electrodes in PBS containing (i) 100 μM $Ru(NH_3)_6^{3+}$; (ii) 100 μM $Ru(NH_3)_6^{3+}$ and 5.0 mM GP; (iii) 100 μM $Ru(NH_3)_6^{3+}$, 5.0 mM GP, and 100 μg/mL GPDH; and (iv) 100 μM $Ru(NH_3)_6^{3+}$, 5.0 mM GP, 100 μg/mL GPDH, and 5.0 mM glucose. (b) Chronocoulograms recorded (at 0.05 V) using ITO electrodes in PBS containing (i) 100 μM $Ru(NH_3)_6^{3+}$ and 5.0 mM GP; (ii) 100 μM $Ru(NH_3)_6^{3+}$, 5.0 mM GP, and 0.1 mM AA; and (iii) 100 μM $Ru(NH_3)_6^{3+}$, 5.0 mM GP, 0.1 mM AA, and 5 U/mL AOx.

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$Ru(NH_3)_6^{3+}$-mediated oxidation of GP by GPDH readily occurs. However, there is a possibility that GPDH shows low substrate specificity. To check this possibility, a chronocoulogram was obtained in the presence of glucose (curve iv of Figure 3a), because glucose is one of the most abundant and concentration-varying alcohol substrates for alcohol oxidoreductases in serum. Importantly, glucose did not cause a noticeable change in the chronocoulometric data.

It is important to obtain a high rate constant for the reaction between GPDH and $Ru(NH_3)_6^{3+}$. The rate constant (k in Figure 1) can be calculated from eq 1, when the initial concentration of $Ru(NH_3)_6^{3+}$ ($C_{Ru(NH_3)_6^{3+}}$) is much lower than that of GP. The limiting current (I_{lim}) measured from the chronocoulogram has the following relation:[27,28]

$$I_{lim} = FAC_{Ru(NH_3)_6^{3+}}\sqrt{2D_{Ru(NH_3)_6^{2+}}kC_{GPDH}} \quad (1)$$

where F is the Faraday constant (96,485 C/mol), A is the electrode area (0.28 cm^2), $D_{Ru(NH_3)_6^{2+}}$ is the diffusion coefficient of $Ru(NH_3)_6^{2+}$ (approximately 1×10^{-5} cm^2/s),[29] and C_{GPDH} is the concentration of GPDH. The background-corrected I_{lim} at 100 s was 90 nA (SI Figure S2). Considering that the concentrations of $Ru(NH_3)_6^{3+}$, GPDH (MW = 59 kDa), and GP are 0.1 mM, 100 μg/mL (1.7 μM), and 5 mM, respectively, the calculated k value was approximately 3.3×10^1 M^{-1} s^{-1}, which is lower than or comparable to the rate constant for the reaction between glucose oxidase and $Ru(NH_3)_6^{3+}$.[28,30] This result clearly demonstrates that k is considerable and that the reaction between GPDH and $Ru(NH_3)_6^{3+}$ is fast.

Chronocoulometric data were also compared in the absence and presence of AA and in the presence of AA and AOx (Figure 3b). Although AA caused higher charges (curve ii of Figure 3b) than those in the absence of AA (curve i of Figure 3b), AOx minimized the interference effect of AA (curve iii of Figure 3b). Interestingly, the chronocoulometric data obtained in PBS containing $Ru(NH_3)_6^{3+}$ and GP were similar to those obtained in serum containing $Ru(NH_3)_6^{3+}$, GP, and AOx (SI Figure S3). This result clearly shows that the interference effect of AA can be effectively removed by AOx and that the interfering effects of other serum components are not significant. Cyclic voltammograms of uric acid and acetaminophen (SI Figure S4) show that their oxidation currents were negligible at an applied potential of 0.05 V.

There is a further possibility that GPDH is affected by dissolved O_2. To investigate this possibility, chronocoulometric data were obtained without and with Ar purging (Figure 4). The charge data in both a solution containing $Ru(NH_3)_6^{3+}$ and GP and a solution containing $Ru(NH_3)_6^{3+}$, GP, and GPDH were similar, indicating that the charge variation caused by dissolved O_2 is not significant.

Finally, a new washing-free immunosensing scheme was applied for detection of cardiac troponin I in human serum. Figure 5a shows chronocoulograms obtained in various concentrations of troponin I after an incubation period of 10 min. The charge at zero concentration is related to the electron mediation between the ITO electrode and the unbound GPDH-labeled antibody, whereas the charges at nonzero concentrations are to the electron mediation between the ITO electrode and the unbound and bound GPDH-labeled antibodies. As the concentration of troponin I increases, the amount of the bound GPDH-labeled antibody increases. The charge data increased with increasing concentration of troponin I. This result clearly shows that proximity-dependent electron

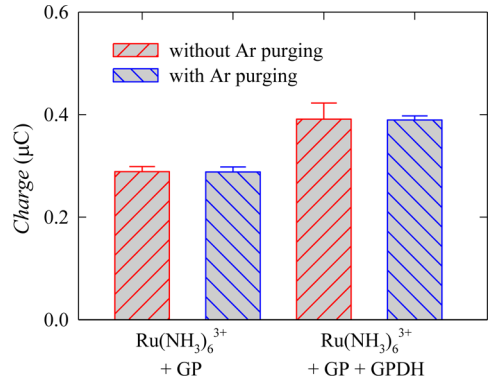

Figure 4. Histogram for the charge at 100 s in the chronocoulograms recorded (at 0.05 V) using ITO electrodes in PBS containing 100 μM $Ru(NH_3)_6^{3+}$ and 5.0 mM GP; and 100 μM $Ru(NH_3)_6^{3+}$, 5.0 mM GP, and 10 μg/mL GPDH without and with Ar purging.

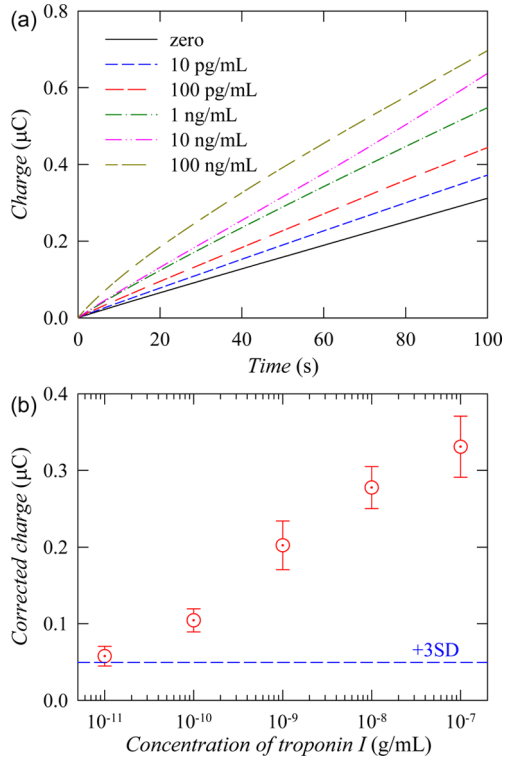

Figure 5. (a) Chronocoulogram recorded (at 0.05 V) after an incubation period of 10 min at immunosensing electrodes for detecting various concentrations of troponin I spiked in troponin I-free human serum. (b) Calibration plot: the concentration dependence of the charge at 100 s in panel (a). Each concentration experiment was carried out with three different sensing electrodes for the assay of the same sample. The data were subtracted by the mean value obtained from seven measurements at zero concentration. The dashed line corresponds to 3 times the charge standard deviation (SD) at zero concentration.

mediation occurs: the mediation between the ITO electrode and the bound GPDH-labeled antibody is fast, whereas that between the electrode and unbound antibody is relatively slow. A calibration plot for troponin I detection (Figure 5b) was drawn using the charge values recorded at 100 s shown in Figure 5a. The calculated detection limit was approximately 10 pg/mL, which is very low in spite of the use of washing-free detection with a short detection period (10 min for incubation

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and 100 s for electrochemical measurement). This result indicates that low-interference electrochemical detection can be obtained even in sandwich-type immunosensors without the use of a washing step. The normal level of troponin I in healthy human serum is <60 pg/mL, and the level of troponin I consistent with myocardial necrosis is >500 pg/mL.[31] Therefore, our immunosensor could differentiate between healthy persons and patients with myocardial infarction.

CONCLUSIONS

A new low-interference washing-free electrochemical immunosensor using GPDH and GP as an enzyme label and its substrate, respectively, was developed. GPDH and GP allow for low interference from redox and reactive species preexisting in serum. Proximity-dependent electron mediation of $Ru(NH_3)_6^{3+}$ between the ITO electrode and GPDH label enables differentiation between a bound GPDH label and an unbound GPDH label without the use of a washing step. Electrochemical oxidation of GPDH-generated $Ru(NH_3)_6^{2+}$ is performed at 0.05 V vs Ag/AgCl, at which point the electrochemical interference is very low. The immunosensor enabled us to detect cardiac troponin I in human serum with a detection limit of approximately 10 pg/mL. The immunosensing scheme could be applied to a disposable miniaturized immunosensor using micropatterned electrodes and dried reagents for fast and simple point-of-care testing, in which the only thing to do is to drop a sample solution on the immunosensor.

ASSOCIATED CONTENT

Supporting Information

More supporting data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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