Supporting Information

Optimization of Phosphatase- and Redox Cycling-Based Immunosensors and Its Application to Ultrasensitive Detection of Troponin I

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Preparation of Immunosensing Layers and Immunosensing Procedure. To obtain avidin-modified ITO electrodes, 70 μL of a carbonate buffer solution (pH 9.6) or a PBS buffer solution (pH 7.4) containing 100 μg/mL avidin was dropped onto the pretreated ITO electrodes, the dropped state was maintained for 2 h at 20 °C, and the electrodes were washed twice with rinsing buffer. Subsequently, 70 μL of a PBSB buffer solution was dropped onto the avidin-modified ITO electrodes, the dropped state was maintained for 30 min at 4 °C, and the electrodes were washed twice with rinsing buffer. To immobilize biotinylated IgG on avidin, 70 μL of a TBS buffer solution containing 10 μg/mL biotinylated anti-troponin-I IgG (or a PBSB buffer solution containing 10 μg/mL biotinylated anti-mouse IgG) was dropped onto the avidin- and BSA-modified ITO electrodes, the dropped state was maintained for 30 min at 4 °C, and the electrodes were washed twice with rinsing buffer. The resulting electrodes were stored at 4 °C before their use.

For the binding of target protein to the immunosensing electrodes, 70 μL of human-serum solutions containing different concentrations of troponin I (or PBSB buffer solutions containing different concentrations of mouse IgG) were dropped onto the immunosensing electrodes, the dropped state was maintained for 30 min at 4 °C, and the electrodes were washed twice with rinsing buffer. Afterward, 70 μL of a TBS buffer solution containing 10 μg/mL ALP-conjugated anti-troponin-I IgG (or a PBSB buffer solution containing 10 μg/mL ALP-conjugated anti-mouse IgG) were dropped on the target-treated electrodes, and the dropped state was maintained for 30 min at 4 °C, followed by washing twice with rinsing buffer.

The same procedure was used for preparation of the immunosensing layers on micropatterned ITO electrodes and for immunosensing with them. The glass substrates with micropatterned ITO were diced into smaller sections (2.2 cm × 1.2 cm each). The ITO and glass surface were simultaneously immobilized with avidin, followed by BSA. To immobilize biotinylated IgG, 1 μL of a TBS buffer solution containing 10 μg/mL

biotinylated anti-troponin-I IgG was dropped over an area larger than the micropatterned disk ITO electrode (diameter = 500 μm).

Measurement of the Surface Concentration of Avidin. Avidin-modified ITO electrodes were prepared with the same procedure as described above. Ten avidin-modified ITO electrodes were used for each experiment. To bind HABA to the avidin, 100 μL of a phosphate buffer solution (50 mM, pH 7.0) containing 1.0 mM HABA was dropped onto the avidin-modified electrodes and the dropped state was maintained for 1 h at room temperature. Afterward, the electrodes were washed thoroughly five times with PBS buffer. To replace HABA with biotin, 100 μL of a phosphate buffer solution (50 mM, pH 7.0) containing 1.0 mM biotin was dropped onto the HABA-bound electrodes, and the dropped state was maintained for 1 h at room temperature. The solution containing the detached HABA was then collected. In a control experiment, avidin-modified ITO electrodes were pretreated with 100 μL of a phosphate buffer solution (50 mM, pH 7.0) containing 1.0 mM biotin for 1 h, and the same procedure was then carried out. UV/visible absorption spectrum was obtained with a UV-1650 PC (UV-visible spectrophotometer, SHIMADZU).

Inhibition Effect of TCEP on ALP. To check the inhibition effects of TECP on ALP activities, hydrolysis of 4-nitrophenyl phosphate was measured spectrophotometrically in the absence and presence of TCEP (Figure S5 in Supporting Information). Because the ALP product (4-nitrophenol) and the ALP substrate (4-nitrophenyl phosphate) show high absorption and no absorption, respectively, at a wavelength of 400 nm,[S1] the absorbance at 400 nm increases as the enzymatic reaction proceeds. The time-course behavior in the absence of TCEP (curve i of Figure S5 in Supporting Information) was well consistent with that in the presence of TCEP (curve ii of Figure S5 in Supporting Information). This consistency indicates that TCEP did not cause inhibitory effects upon the enzymatic reaction. When 2.0 mM TCEP hydrochloride was added to a tris buffer solution of pH 9.6, the pH decreased to 8.9. At this pH, the time-course behavior (curve iii of Figure S5 in Supporting Information) was also similar to that at pH 9.6 (curve ii of Figure S5 in Supporting Information). This result shows that the rate of the enzymatic reaction at pH 8.9 is similar to that at pH 9.6. Enzyme activities can also be inhibited by the enzyme product. To verify this possibility, time-course data were obtained in the presence of AA (curve iv of Figure S5 in Supporting Information). The time-course behavior clearly reveals that AA does not act as an inhibitor against the enzymatic reaction.

Optimization of the concentration of ALP-conjugated IgG. If the concentration is too high, high levels of nonspecific binding of ALP-conjugated IgG occur. Conversely, if the concentration is too low, the binding of ALP-conjugated IgG to target is slow and a long saturation time is required. To examine the dependence of nonspecific binding

upon the concentration of ALP-conjugated IgG, electrochemical signals were obtained at a target concentration of zero (Figures S6c and S8a in Supporting Information). The data clearly show that the nonspecific binding of ALP-conjugated IgG increased with increasing its concentration and that a concentration of 1 or 10 μg/mL is better for low nonspecific binding than a concentration of 100 μg/mL. To investigate the dependence of the amount of biospecifically bound ALP-conjugated IgG on the concentration of ALP-conjugated IgG, electrochemical signals were obtained at a target concentration of 1 pg/mL (Figures S6d and S8b in Supporting Information). A concentration of 10 μg/mL allowed much higher signals. From these results, a concentration of 10 μg/mL was chosen for low nonspecific binding and high signal amplification.

Additional Explanation on Concentration Data. In cyclic voltammograms obtained at a concentration of zero (Figure S9a in Supporting Information and Figure 5a), little shoulders were observed during the anodic scans, indicating a low level of nonspecific binding. When the nonspecific binding was tested with avidin- and BSA-modified electrodes (curve ii in Figure 4d), it was nearly absent. In actual immunosensing experiments, however, some low level of nonspecific binding occurred. Nevertheless, the current at 0.035 V at a mouse-IgG concentration of 10 fg/mL ($4.5 \pm 0.4 \times 10^{-7}$ A) were clearly higher than at a concentration of zero ($2.3 \pm 0.4 \times 10^{-7}$ A) (Figure S9a in Supporting Information).

REFERENCES

(S1) Wunder, S.; Polzer, F.; Lu, Y.; Mei, Y.; Ballauff, M. *J. Phys. Chem. C* **2010**, *114*, 8814–8820.

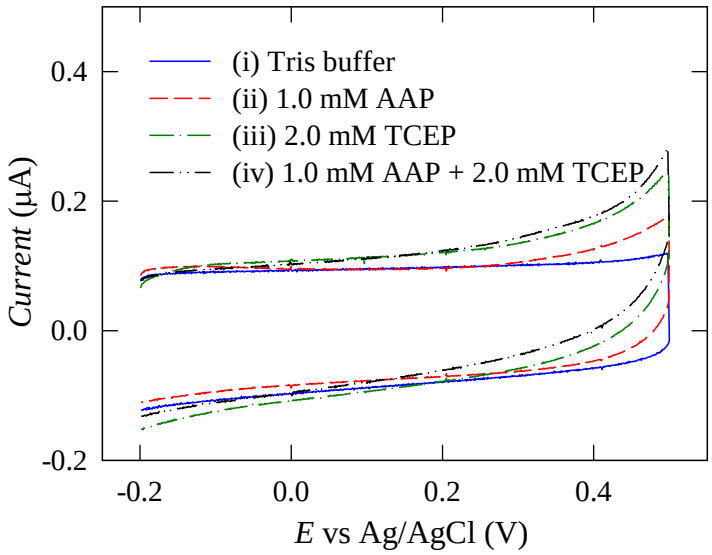

Figure S1. Cyclic voltammograms obtained at bare ITO electrodes (at a scan rate of 20 mV/s) in (i) a tris buffer solution (pH 9.6), (ii) a tris buffer solution containing 1.0 mM AAP (pH 9.6), (iii) a tris buffer solution containing 2.0 mM TCEP (pH 8.9), and (iv) a tris buffer solution containing 1.0 mM AAP and 2.0 mM TCEP (pH 8.9).

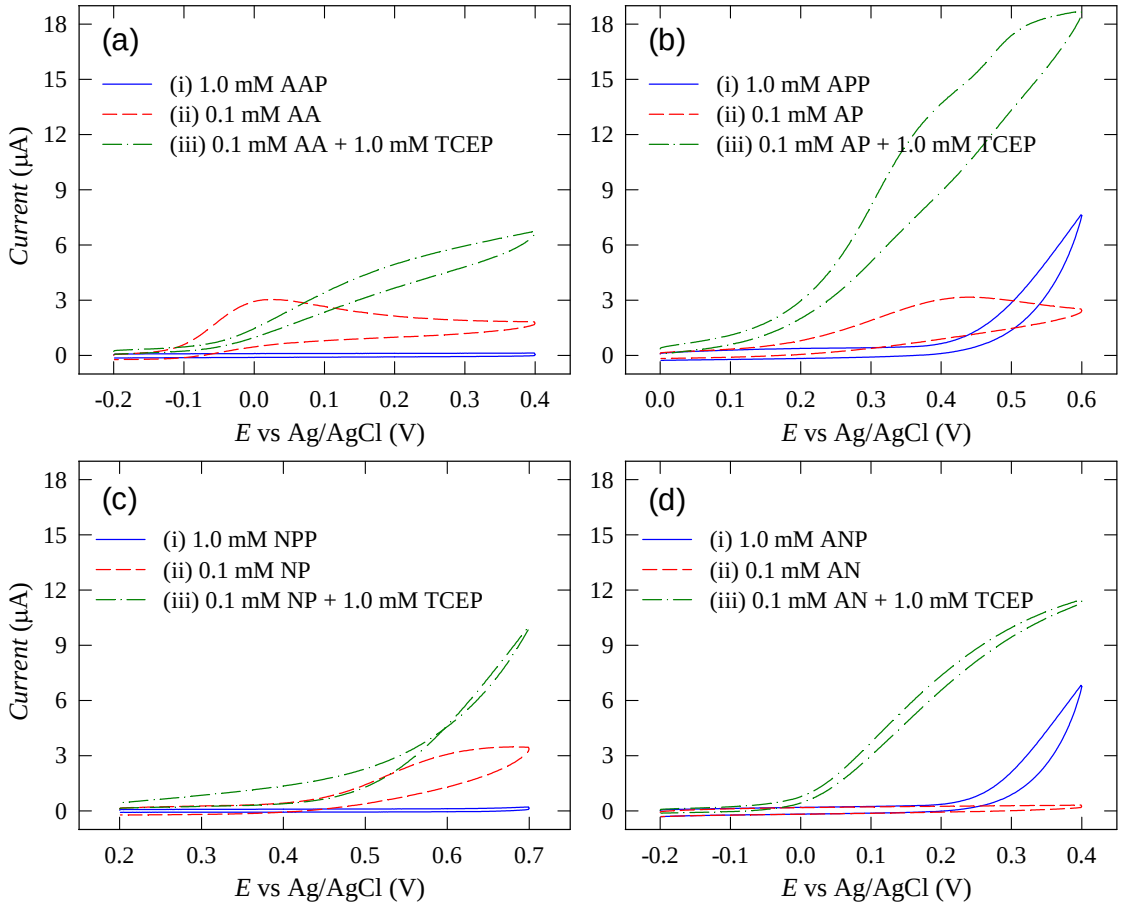

Figure S2. Cyclic voltammograms obtained at avidin-modified ITO electrodes (at a scan rate of 20 mV/s) in (i) a tris buffer solution containing 1.0 mM ALP substrate (pH 9.6), (ii) a tris buffer solution containing 0.1 mM ALP product (pH 9.6), and a tris buffer solution containing 0.1 mM ALP product and 1.0 mM TCEP (pH 8.9). ALP substrates are (a) AAP, (b) APP, (c) NPP, and (d) ANP; ALP products are (a) AA, (b) AP, (c) NP, and (d) AN.

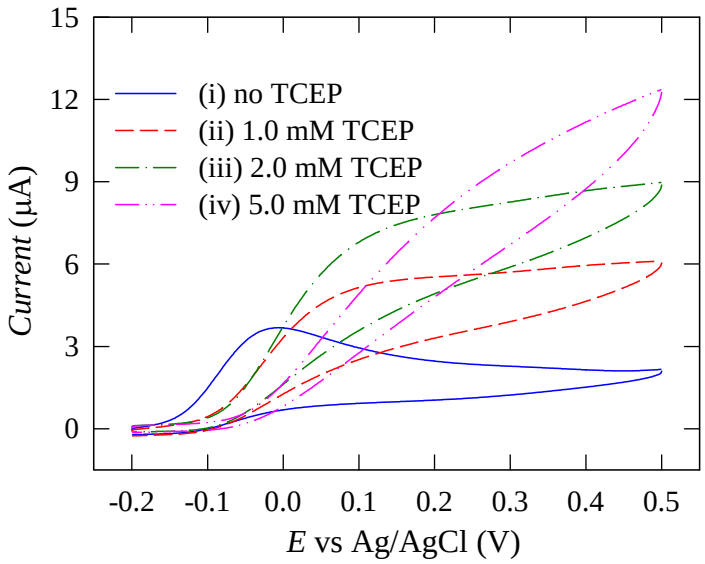

Figure S3. Cyclic voltammograms obtained at bare ITO electrodes (at a scan rate of 20 mV/s) in a tris buffer solution containing TCEP and 0.1 mM AA.

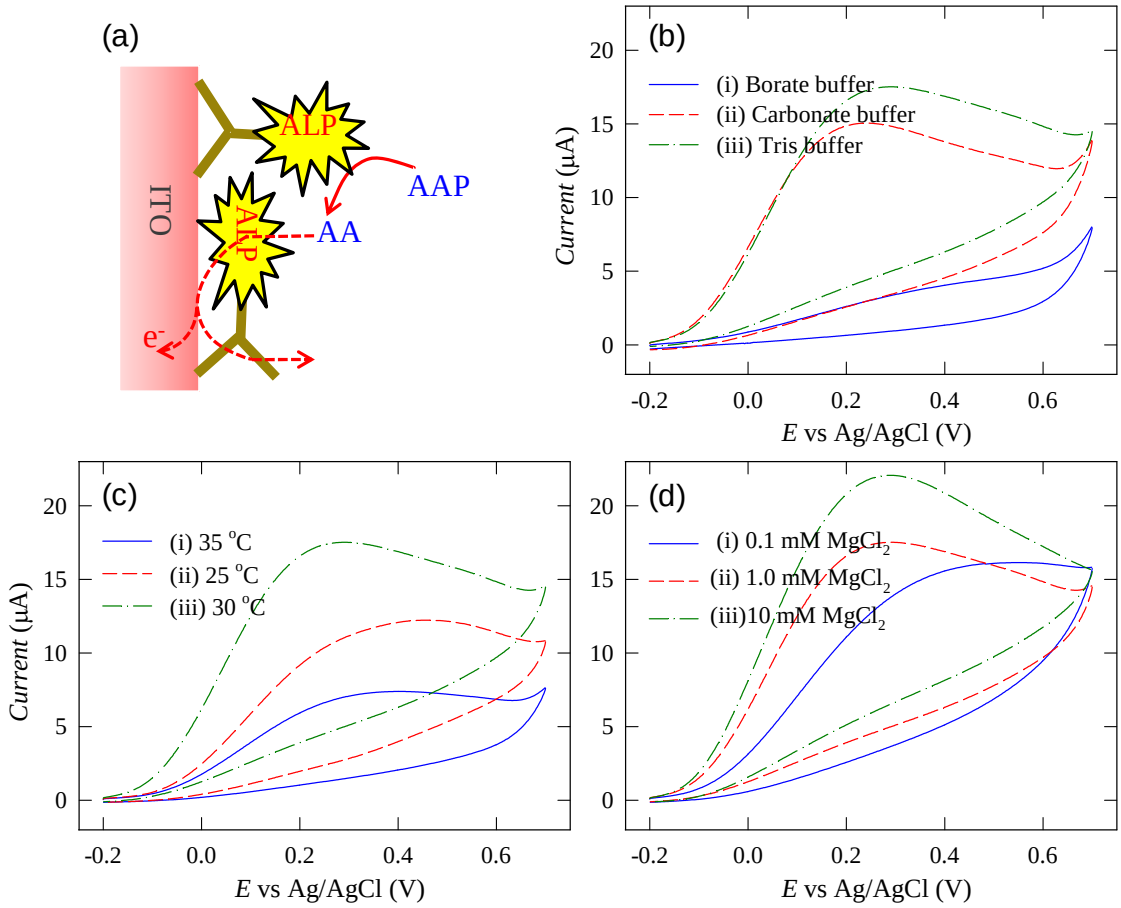

Figure S4. (a) Schematic of the electrodes used to determine optimum enzymatic conditions. Cyclic voltammograms were obtained at ALP-conjugated anti-mouse IgG-modified ITO electrodes (at a scan rate of 20 mV/s) under conditions of (b) different buffer solutions, (c) different temperatures, and (d) different concentrations of $MgCl_2$ after 10-min incubation. Standard conditions are tris buffer (pH 9.6), 30 °C, and 1.0 mM $MgCl_2$. The pH of borate buffer and carbonate buffer was 9.6.

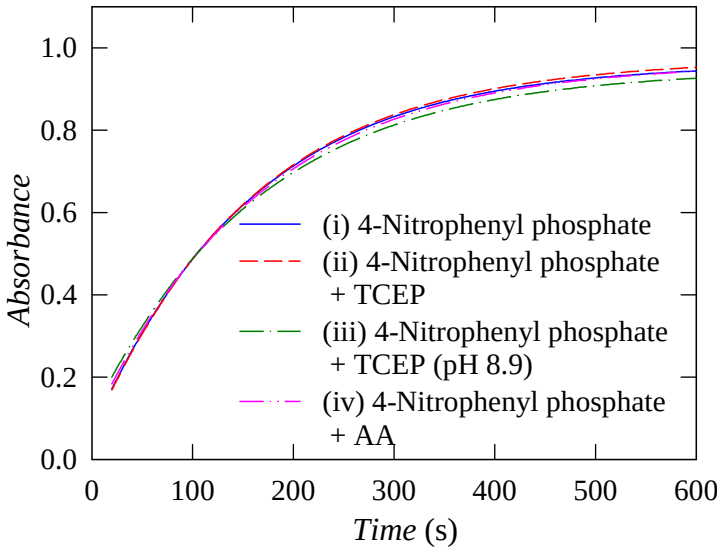

Figure S5. Time-course spectra observed at 400 nm at 25 °C in (i) a tris buffer solution containing 0.05 mM 4-nitrophenyl phosphate and 0.5 μg/mL ALP-conjugated anti-mouse IgG, (ii) a tris buffer solution containing 0.05 mM 4-nitrophenyl phosphate, 0.5 μg/mL ALP-conjugated anti-mouse IgG, and 2.0 mM TCEP (pH 9.6), (iii) a tris solution containing 0.05 mM 4-nitrophenyl phosphate, 0.5 μg/mL ALP-conjugated anti-mouse IgG, and 2.0 mM TCEP (pH 8.9), and (iv) a tris buffer solution containing 0.05 mM 4-nitrophenyl phosphate, 0.5 μg/mL ALP-conjugated anti-mouse IgG, and 1.0 mM AA.

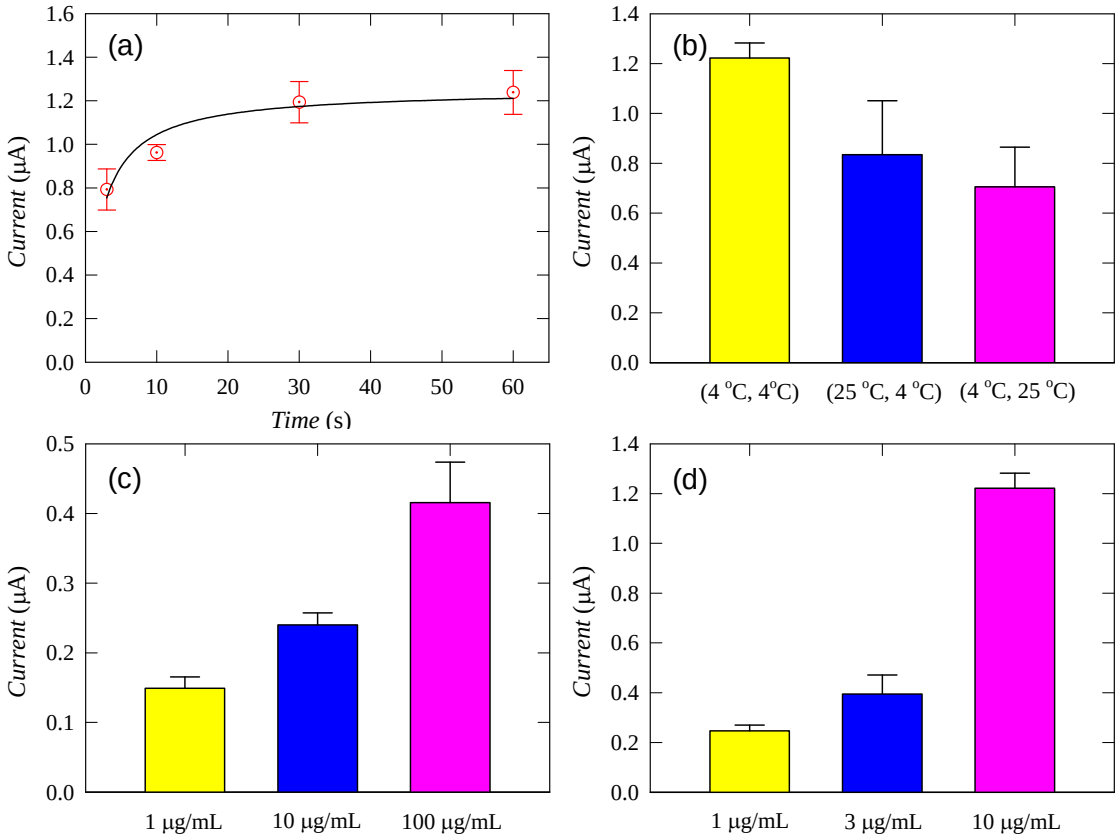

Figure S6. Results of the immunosensing experiments (without redox cycling by TCEP) of Figure 2 that were carried out with (c) 0 or (a, b, d) 1 pg/mL mouse IgG, to determine optimum conditions for antigen-antibody binding in terms of (a) incubation time, (b) temperature, and (c, d) concentration of ALP-conjugated IgG. Currents at 0.035 V in cyclic voltammograms were measured. In Figure b, the first temperature and the second temperature correspond to the temperature for the binding between biotinylated anti-mouse IgG and mouse IgG and the temperature for the binding between mouse IgG and ALP-conjugated anti-mouse IgG, respectively.

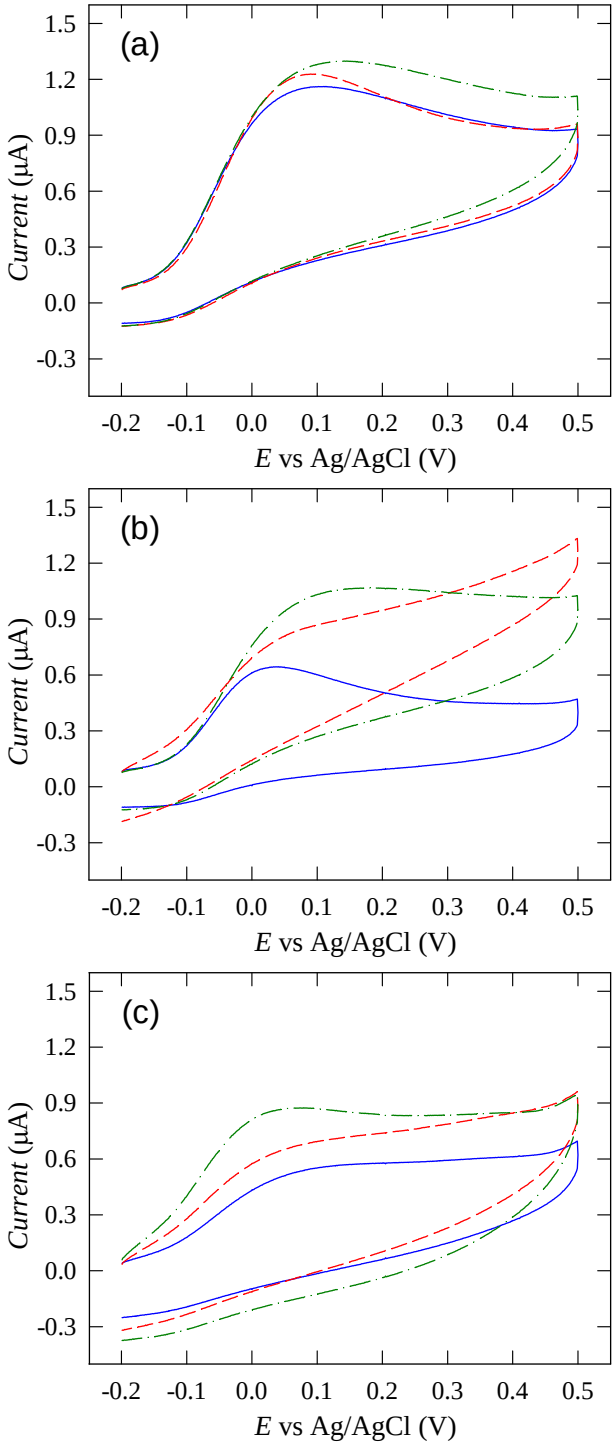

Figure S7. Results of the immunosensing experiments (without redox cycling by TCEP) of Figure 2 that were carried out with 1 pg/mL mouse IgG, to determine optimum conditions for antigen-antibody binding in terms of temperature. The temperature for the binding between biotinylated IgG and target protein was (a) 4, (b) 25, and (c) 4 °C; the temperature for the binding between target protein and ALP-conjugated IgG was (a) 4, (b) 4, and (c) 25 °C. Cyclic voltammograms were obtained (at a scan rate of 20 mV/s) in a tris buffer solution containing 1.0 mM AAP (pH 9.6) after 10-min incubation.

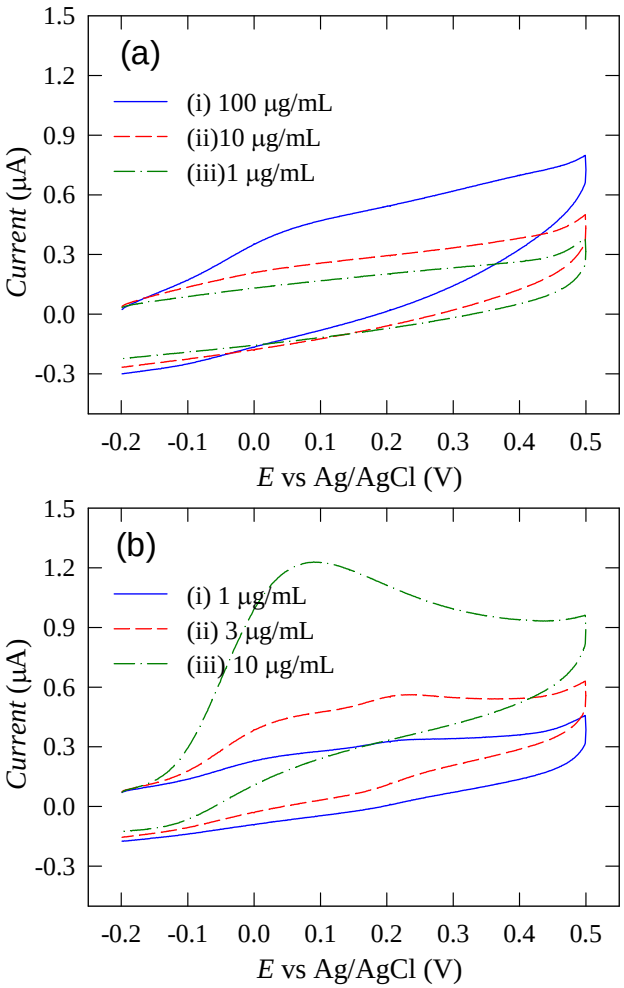

Figure S8. Results of the immunosensing experiments (without redox cycling by TCEP) of Figure 2 that were carried out with (a) 0 or (b) 1 pg/mL mouse IgG, to determine optimum conditions for antigen-antibody binding in terms of concentration of ALP-conjugated anti-mouse IgG. Cyclic voltammograms were obtained (at a scan rate of 20 mV/s) in a tris buffer solution containing 1.0 mM AAP (pH 9.6) after 10-min incubation.

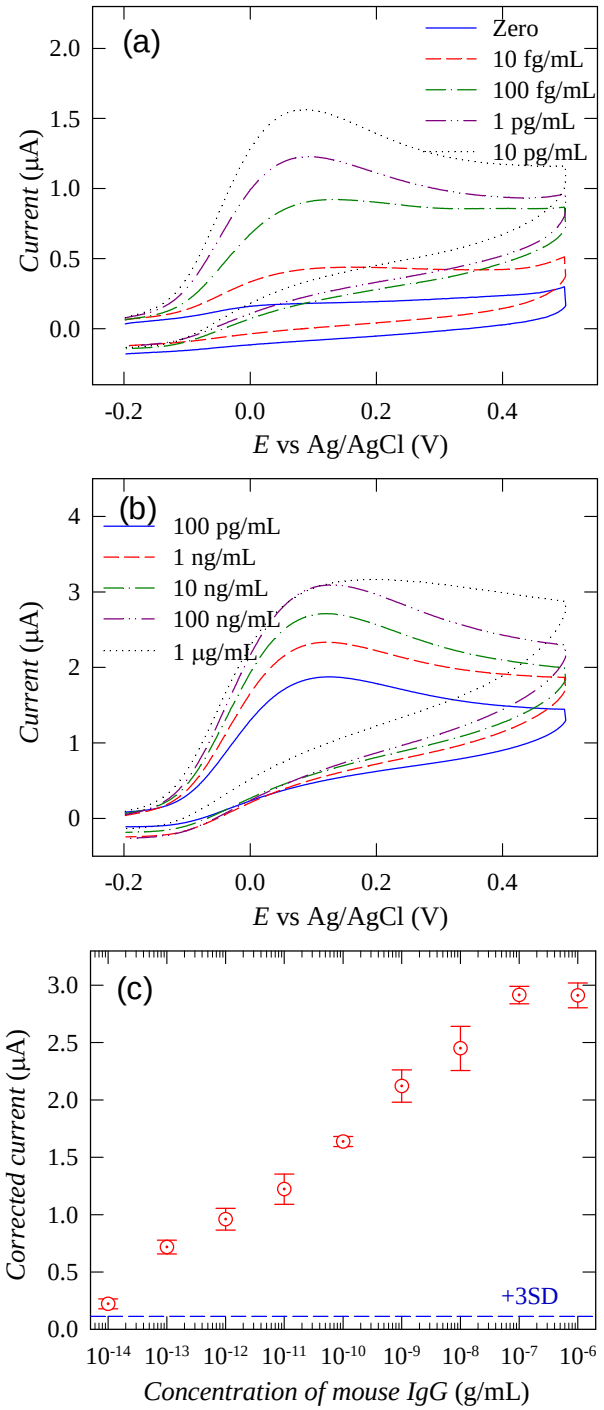

Figure S9. Results of the immunosensing experiments (without redox cycling by TCEP) of Figure 2 that were carried out with PBS buffer containing different concentrations of mouse IgG. (a, b) Cyclic voltammograms obtained (at a scan rate of 20 mV/s) in a tris buffer solution containing 1.0 mM AAP (pH 9.6) after 10-min incubation. (c) Calibration plot for the detection of mouse IgG: concentration dependence of the current at 0.035 V in cyclic voltammograms. All data were subtracted by the mean current at a concentration of zero determined by 7 measurements. The dashed line corresponds to 3 times the standard deviation (SD) of the current at a concentration of zero. The error bars represent the SD of three measurements.

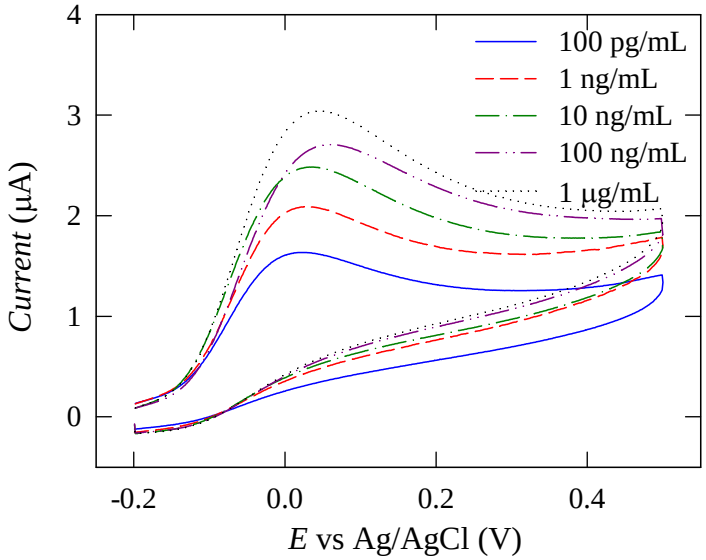

Figure S10. Results of the immunosensing experiments (without redox cycling by TCEP) of Figure 2 that were carried out with human serum containing different concentrations of troponin I. Cyclic voltammograms obtained (at a scan rate of 20 mV/s) in a tris buffer solution containing 1.0 mM AAP (pH 9.6) after 10-min incubation.

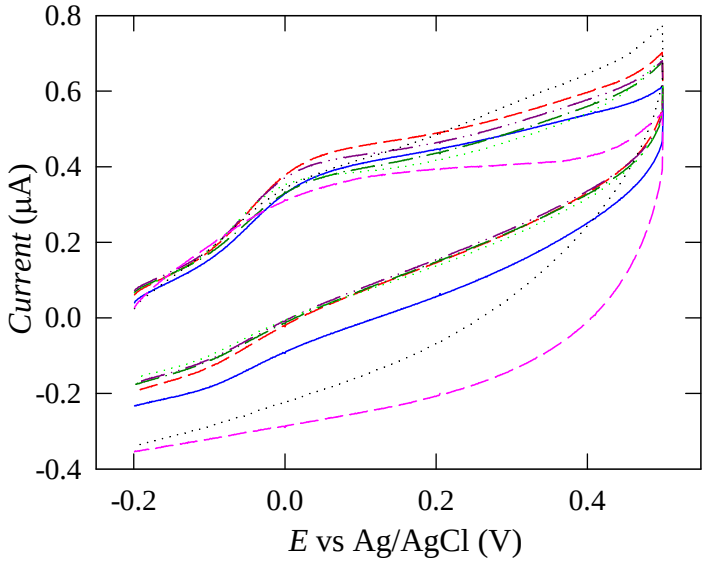

Figure S11. Results of the immunosensing experiments (without redox cycling by TCEP) of Figure 2 that were carried out with human serum containing no troponin I. Seven cyclic voltammograms obtained (at a scan rate of 20 mV/s) in a tris buffer solution containing 1.0 mM AAP (pH 9.6) after 10-min incubation. The anodic current behaviors were very reproducible below 0.2 V, although the anodic current behaviors at higher potentials and the cathodic current behaviors were less reproducible.

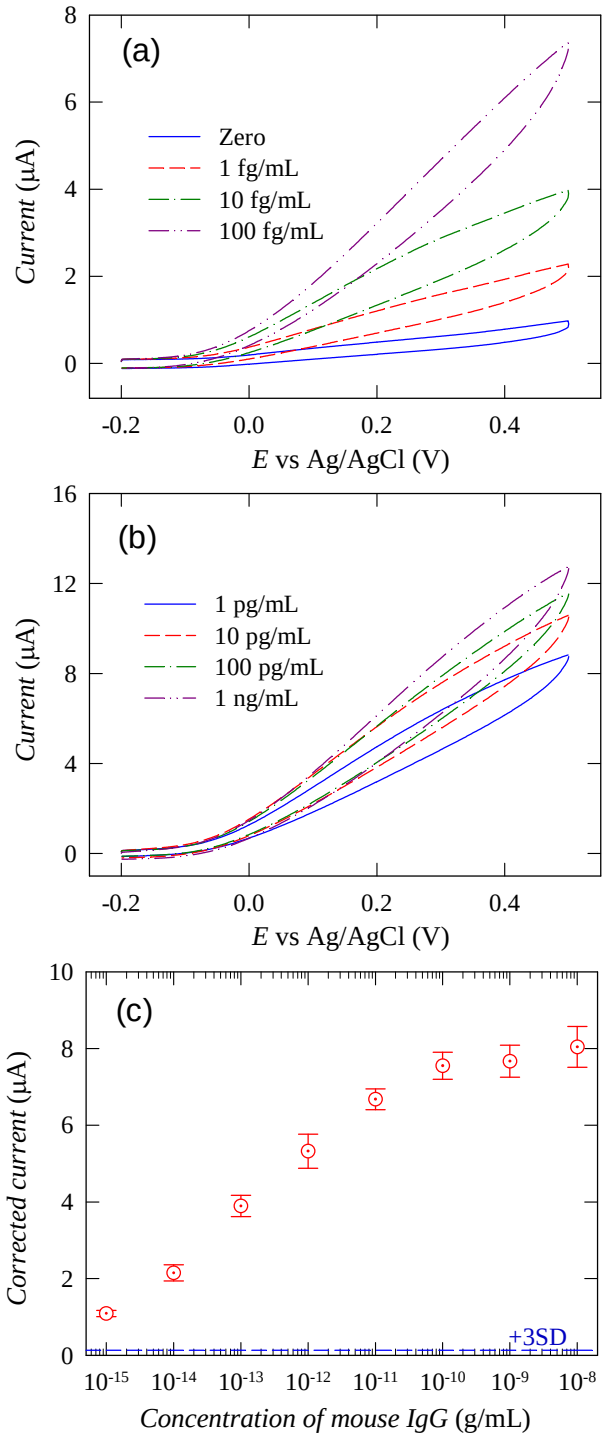

Figure S12. Results of the immunosensing experiments (with redox cycling by TCEP) of Figure 2 that were carried out with PBS buffer containing different concentrations of mouse IgG. (a, b) Cyclic voltammograms obtained (at a scan rate of 20 mV/s) in a tris buffer solution containing 1.0 mM AAP and 2.0 TCEP (pH 8.9) after 10-min incubation. (c) Calibration plot for the detection of mouse IgG: concentration dependence of the current at 0.20 V in cyclic voltammograms. All data were subtracted by the mean current at a concentration of zero determined by 7 measurements. The dashed line corresponds to 3 times the standard deviation (SD) of the current at a concentration of zero. The error bars represent the SD of three measurements.

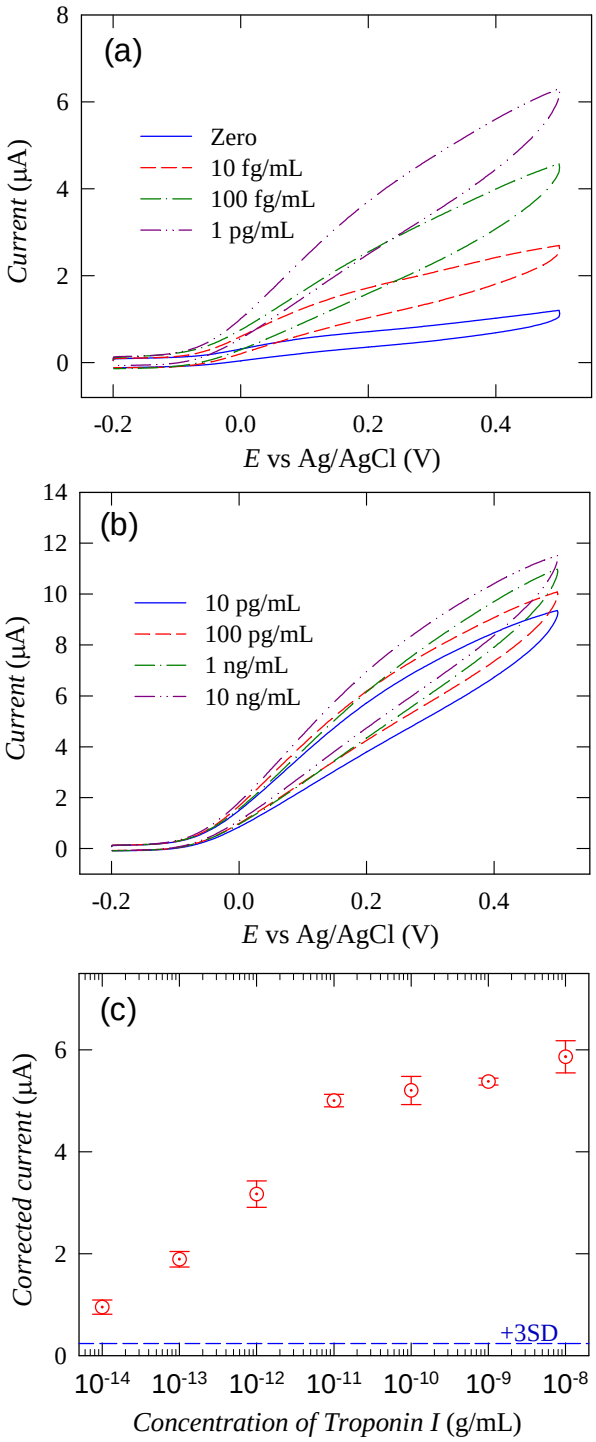

Figure S13. Results of the immunosensing experiments (with redox cycling by TCEP) of Figure 2 that were carried out with human serum containing different concentrations of troponin I. (a, b) Cyclic voltammograms obtained (at a scan rate of 20 mV/s) in a tris buffer solution containing 1.0 mM AAP and 2.0 TCEP (pH 8.9) after 10-min incubation. (c) Calibration plot for the detection of mouse IgG: concentration dependence of the current at 0.20 V in cyclic voltammograms. All data were subtracted by the mean current at a concentration of zero determined by 7 measurements. The dashed line corresponds to 3 times the standard deviation (SD) of the current at a concentration of zero. The error bars represent the SD of three measurements.

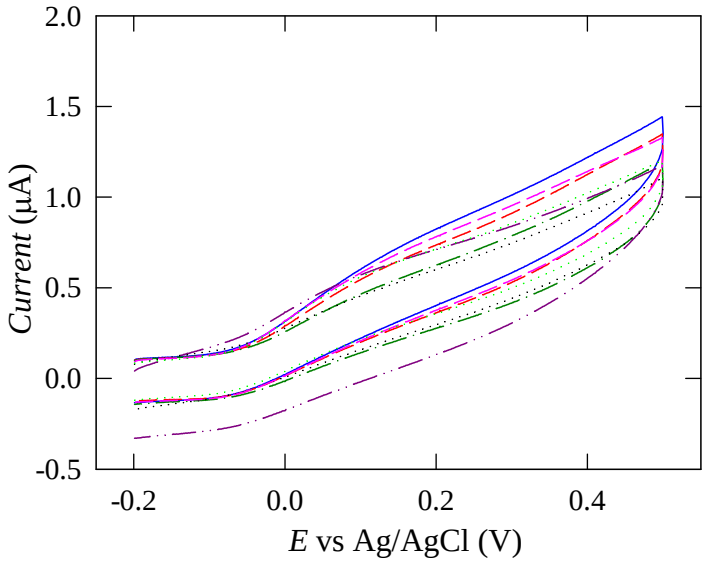

Figure S14. Results of the immunosensing experiments (with redox cycling by TCEP) of Figure 2 that were carried out with human serum containing no troponin I. Seven cyclic voltammograms obtained (at a scan rate of 20 mV/s) in a tris buffer solution containing 1.0 mM AAP and 2.0 mM TCEP (pH 8.9) after 10-min incubation.

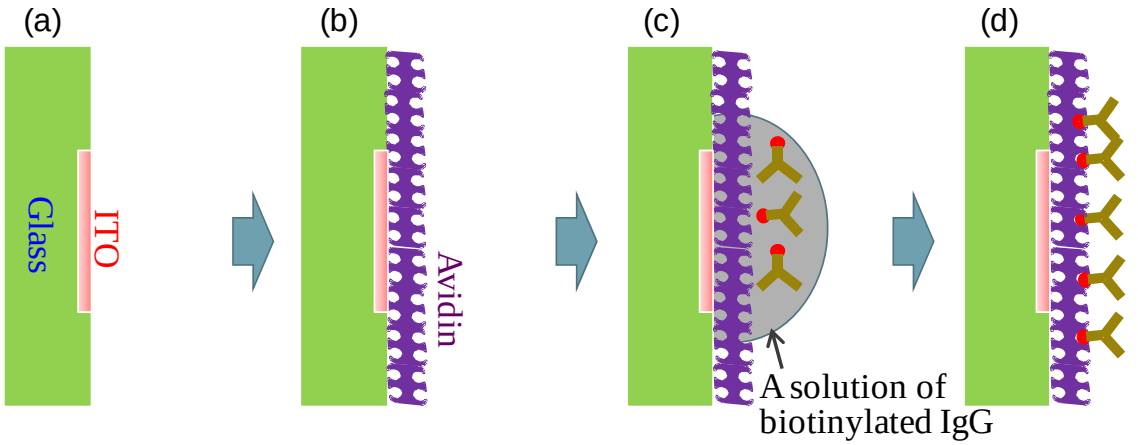

Figure S15. Schematic of preparation of an immunosensing electrode on a micropatterned ITO electrode.

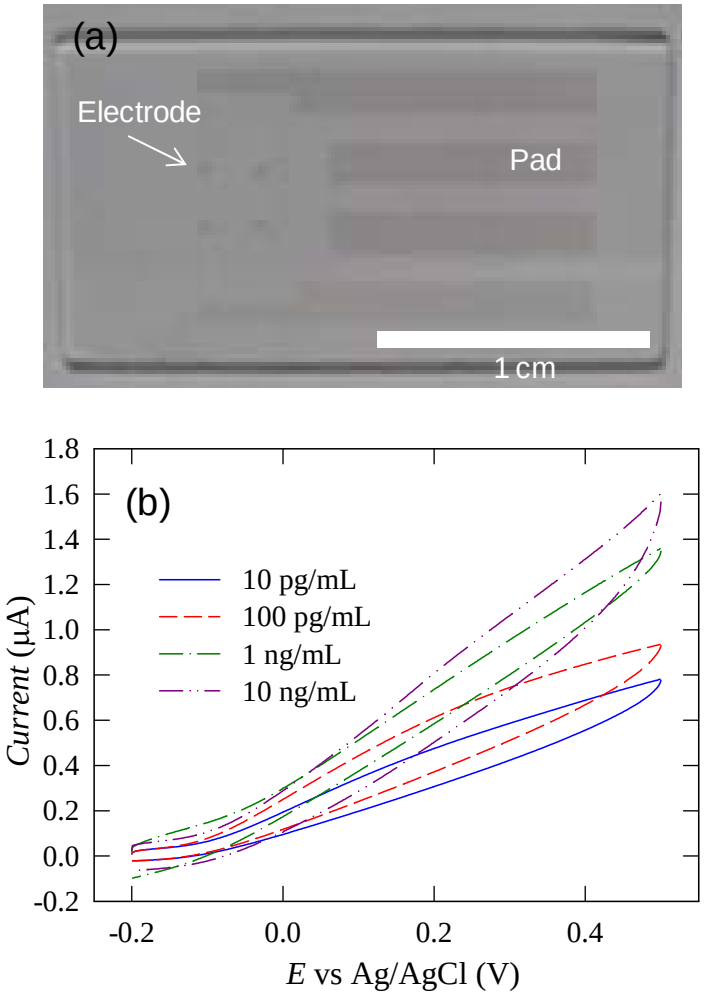

Figure S16. (a) Photograph of a micropatterned ITO electrode. (b) Results of the immunosensing experiments (with redox cycling by TCEP) of Figure 2 that were carried out with microfabricated ITO electrodes with human serum containing different concentrations of troponin I. Cyclic voltammograms obtained (at a scan rate of 20 mV/s) in a tris buffer solution containing 1.0 mM AAP and 2.0 mM TCEP (pH 8.9) after 10-min incubation.