Electronic Supplementary Information

Redox cycling-amplified enzymatic Ag deposition and its application in the highly sensitive detection of creatine kinase-MB

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Chemicals and Materials. Avidin, biotinylated polyclonal goat antimouse IgG, mouse IgG, and ALP-conjugated polyclonal goat antimouse IgG were obtained from Sigma-Aldrich. Mouse monoclonal anti-CK-MB antibody (10-1363), anti-CK-MB antibody (10-1364), CK-MB protein (30-1082), troponin I protein (30R-AT034), and troponin I-free human serum (90R-106) were purchased from Fitzgerald, Inc. (Acton, MA, U.S.A.). CK-MB concentration (0.6 ng/mL) in troponin I-free serum was measured with UniCell DxI 800 Immunoassay analyzer (Beckman Coulter). The ALP labeling kit-NH$_2$ (LK12) was purchased from Dojindo Laboratories (Tabaru, Kumamoto, Japan). ALP-conjugated anti-CK-MB antibody was prepared using anti-CK-MB antibody (10-1363) and the ALP labeling kit according to the manufacturer’s procedure. EZ-link sulfo-NHS-LC-LC-biotin was obtained from Thermo Fisher Scientific Inc. (Meridian, Rockford, USA). 4-Aminophenyl phosphate (APP) monosodium salt hydrate was obtained from Biosynth (Staad, Switzerland). 4-Aminophenol (AP), β-nicotinamide adenine dinucleotide reduced dipotassium salt (NADH), AgNO$_3$, and KNO$_3$ were obtained from Sigma-Aldrich. All reagents for buffer solutions were received from Sigma-Aldrich.

The phosphate-buffered saline (PBS buffer, pH 7.4) contained 10 mM phosphate, 0.138 M NaCl, and 2.7 mM KCl. The PBSB buffer contained all of the ingredients of the PBS buffer plus 1% (w/v) BSA. The rinsing buffer (pH 7.6) contained 50 mM tris(hydroxymethyl)aminomethane (Tris), 40 mM HCl, 0.05% (w/v) BSA, 0.05 % Tween 20, and 0.5 M NaCl. The Tris-HNO$_3$ buffer (pH 9.0) for enzymatic reaction contained 50 mM Tris and 10 mM Mg(NO$_3$)$_2$, and the pH was adjusted by adding concentrated HNO$_3$. All buffer and other aqueous solutions were
prepared using double distilled water. ITO electrodes were obtained from Samsung Corning (Daegu, Korea).

**Preparation of Biotinylated Anti-CK-MB Antibody.** Anti-CK-MB antibody (10-1364) was biotinylated by EZ-link sulfo-NHS-LC-LC-biotin. 1.35 µL of sulfo-NHS-LC-LC-biotin (10 mM in water) solution was added to 1 mL of anti-CK-MB antibody (100 µg/mL in PBS) solution and incubated for 30 min at 25 °C. The mixture solution was then filtered by centrifugation at 12,000 rpm for 20 min. The filtrate was dissolved and collected with 1 mL of PBSB.

**Preparation of CK-MB-Spiked Serum.** At first a small aliquot of an original solution of CK-MB protein (11.8 mg/mL) was diluted with troponin I-free human serum to prepare a stock solution of CK-MB with a concentration of 100 µg/mL. Then serial dilutions of the stock solution were carried out with the human serum to prepare the samples for various concentrations.

**Preparation of Immunosensing Layers and the Immunosensing Procedure.** ITO electrodes (1 cm × 2 cm each) were pretreated in a mixture containing H₂O, H₂O₂ (30%) and NH₄OH (30%) in a ratio of 5:1:1 at 70 °C for 1 h. The electrodes were then washed with copious amounts of water and dried under a stream of nitrogen gas.

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 10 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 PBSB buffer solution containing 10 µg/mL biotinylated anti-CK-MB antibody (or a PBSB buffer solution containing 10 µg/mL biotinylated antimouse 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 PBSB solutions (or human serum) containing different concentrations of CK-MB protein (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 PBSB buffer solution containing 10 μg/mL ALP-conjugated anti-CK-MB antibody (or a PBSB buffer solution containing 10 μg/mL ALP-conjugated antimouse 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 and water.

Ag Deposition and Electrochemical Measurements. Bare or avidin- and BSA-modified ITO electrodes were incubated with 70 μL of Tris-HNO₃ buffer (pH 9.0) containing 1.0 mM AgNO₃, 0.1 M AP or 1.0 mM APP, and 1.0 mM NADH for 10 min at 25 °C. The electrodes were then washed with Tris-HNO₃ buffer and water. The deposited silver was then measured by anodic stripping analysis using cyclic voltammetry or chronocoulometry. In the case of electrochemical immunosensors, after forming the sandwich-type immunocomplex, the sensing electrodes were incubated with 70 μL of Tris-HNO₃ buffer (pH 9.0) containing 1.0 mM AgNO₃, 1.0 mM APP, and 1.0 mM NADH for 10 min at 30 °C. The electrodes were then washed with Tris-HNO₃ buffer and water. The deposited Ag was then measured by anodic stripping analysis using cyclic voltammetry or chronocoulometry in a three electrode system. The Ag-deposited ITO electrodes were used as working electrodes. A Pt wire and an Ag/AgCl electrode were used as a counter and a reference electrode, respectively. Teflon electrochemical cells were assembled with the resulting sensing electrodes and 1.0 mL of an aqueous solution containing 0.1 M HNO₃ and 0.6 M KNO₃ was injected into the cell and the electrochemical measurement was then carried out using a CHI 708C (CH Instruments, Austin, TX, U.S.A.). The exposed area of the sensing electrodes was 0.28 cm².

Scanning Electron Microscope (SEM) Images. The SEM images were obtained at avidin- and BSA-modified ITO electrodes using a VEGA3 TESCAN microscope at 20 kV.
**Figure S1.** SEM images obtained at avidin- and BSA-modified ITO electrodes after incubation for 10 min at 25 °C in Tris-HNO₃ buffer (pH 9.0) containing (a) 1.0 mM AgNO₃ and 0.1 mM AP; (b) 1.0 mM AgNO₃, 0.1 mM AP, and 1.0 mM NADH; (c) 1.0 mM AgNO₃, 1.0 mM APP, and 1.0 mM NADH, and (d) without incubation in Tris-HNO₃ buffer.
Figure S2. Cyclic voltammograms obtained (at a scan rate of 50 mV/s) at the sensing electrodes in an aqueous solution containing 0.1 M HNO₃ and 0.6 M KNO₃, after the detection of mouse IgG at concentrations of zero and 1 ng/mL and incubation for 10 min at 25 °C in Tris-HNO₃ buffer (pH 9.0) containing 1.0 mM AgNO₃, 1.0 mM APP, and 1.0 mM NADH.
Figure S3. Chronocoulograms obtained at different potentials at bare ITO electrodes in an aqueous solution containing 0.1 M HNO$_3$ and 0.6 M KNO$_3$, after incubation for 10 min at 25 °C in Tris-HNO$_3$ buffer (pH 9.0) containing (a) 1.0 mM AgNO$_3$, 1.0 mM APP, and 1.0 mM NADH and (b) 1.0 mM AgNO$_3$, 0.1 mM AP, and 1.0 mM NADH.
**Figure S4.** Chronocoulograms obtained at 0.35 V at the sensing electrodes in an aqueous solution containing 0.1 M HNO$_3$ and 0.6 M KNO$_3$, after the detection of CK-MB at concentrations of (a) zero and (b) 1 ng/mL and incubation for 10 min at different temperatures in Tris-HNO$_3$ buffer (pH 9.0) containing 1.0 mM AgNO$_3$, 1.0 mM APP, and 1.0 mM NADH.
Figure S5. Chronocoulograms obtained at 0.35 V at the sensing electrodes in an aqueous solution containing 0.1 M HNO_3 and 0.6 M KNO_3, after the detection of CK-MB at concentrations of (i) zero and (ii) 1 ng/mL, and troponin I at concentration of (iii) 1 ng/mL and incubation for 10 min at 30 °C in Tris-HNO_3 buffer (pH 9.0) containing 1.0 mM AgNO_3, 1.0 mM APP, and 1.0 mM NADH.