Supporting Information

Electroreduction-Based Electrochemical-Enzymatic Redox Cycling for the Detection of Cancer Antigen 15-3 Using Graphene Oxide-Modified Indium–Tin Oxide Electrodes

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Selection of a Redox Enzyme and a Substrate for the Electroreduction-Based Two-Enzyme Scheme. The two enzymes in Scheme S1c and S1d [the enzyme label (E) and the enzyme for EN redox cycling (E_R)] need to meet the following requirements: (i) the optimal pH ranges of E and E_R should be similar in order to obtain fast enzymatic amplification and redox cycling, (ii) E should ideally be a non-redox enzyme, so as to avoid unwanted redox reactions between E and the reductant (R) or oxidant (O), and (iii) E_R should not react with the substrate (S). Therefore, judicious selection of E and E_R so that they fulfill these requirements is of the utmost importance.

To obtain low background levels in electroreduction-based two-enzyme scheme, enzyme substrate for enzymatic amplification should not be oxidized by enzyme for redox cycling (β-galactosidase, Gal), meaning that the reaction depicted in Scheme S1e should be very slow. This requirement can be verified by checking whether or not the reaction between S and O in the presence of E_R occurs. For this experiment, 4-aminophenyl β-D-galactopyranoside (AP-GP) was chosen as S because 4-aminophenol (AP), the most common enzyme product in electrochemical ELISAs, is generated after enzymatic reaction. Dissolved O_2 was used for O in the enzymatic reaction of laccase and Tyr, whereas H_2O_2 was used for O in the enzymatic reaction of HRP. In Figure S3, the cyclic voltammograms obtained in the presence of S and O were compared to those obtained in the presence of S, O, and E_R. Much higher cathodic currents observed in curves iii and iv compared to curves i and ii of Figure S3 are due to H_2O_2 electroreduction in H_2O_2-containing solutions. Cathodic currents in the presence of laccase (curve ii of Figure S3) were much higher than those in the absence of it (curve i of Figure S3). Moreover, cathodic currents in the presence of HRP (curve iv of Figure S3) were much higher than those in the absence of it (curve iii of Figure S3). The higher cathodic currents in the presence of E_R are due to electroreduction of the oxidized species (S_{ox}) generated by the enzymatic reaction of laccase or HRP. This result clearly shows that AP-GP can be readily oxidized in the presence of laccase or HRP that has high oxidizing power. Therefore, it was difficult to obtain the electroreduction-based EN redox cycling of Scheme S1d, when laccase and HRP were used as E_R. In the presence of Tyr, there was no increase in cathodic currents (curve ii of Figure S4) compared to those in the absence of Tyr (curve i of Figure S4). It seems that AP-GP is not readily oxidized in the presence of Tyr because AP-GP does not contain hydroxyl groups required for the oxidation by Tyr. However, cathodic currents

were not high even in the presence of Gal and Tyr (curve iii of Figure S4). This result indicates that, although AP is generated by Gal, high electrochemical signals cannot be obtained when AP-GP is used as S. If an enzyme substrate has a higher formal potential than AP-GP, its oxidation by redox enzyme can be more effectively minimized. P-GP has a higher formal potential than AP-GP. Moreover, phenol, generated from P-GP by Gal, is known to be a good substrate for Tyr. Therefore, Tyr and P-GP were chosen as a redox enzyme and an enzyme substrate, respectively, for the two-enzyme scheme.

Reason for Higher Signal Levels at Low Target Concentrations in the Two-Enzyme Scheme than in the One-Enzyme Scheme. At a very low target concentration, a low number of enzyme-labeled antibody is attached to the immunosensing electrode. In Figure S5, let's assume that only one enzyme-labeled antibody is attached. In the two-enzyme scheme (Figure S5b), many Tyr enzymes can exist near the electrode and as a result the EN redox cycling can occur at many places. On the contrary, in the one-enzyme scheme (Figure S5a), the EN redox cycling by Tyr can occur only at one place (enzyme label). Therefore, at low target concentrations, the signal amplification in the two-enzyme scheme is higher than that in the one-enzyme scheme. It seems that the presence of a high concentration of Tyr in solution in the two-enzyme scheme allows higher signal levels at low target concentrations. On the other hand, at high target concentrations, the number of attached enzyme-labeled antibody is high. In this case, the EN redox cycling in the one-enzyme scheme occurs at many places that are very near the electrode, whereas the EN redox cycling in the two-enzyme scheme occurs at many places that are more distant from the electrode. As a result, at high target concentrations, the signal amplification in the one-enzyme scheme is higher than that in the two-enzyme scheme.

Scheme S1. Schematic representation of (a) an electrochemical immunosensor using the electrooxidation-based EN redox cycling combined with simultaneous enzymatic amplification, (b) an electrochemical immunosensor using the electroreduction-based EN redox cycling combined with simultaneous enzymatic amplification, (c) an electrochemical immunosensor using the electrooxidation-based EN redox cycling combined with preceding enzymatic amplification, and (d) an electrochemical immunosensor using the electroreduction-based EN redox cycling combined with preceding enzymatic amplification. (e) Schematic representation of an unwanted reaction in scheme d. (f) Schematic representation of an immunosensor using enzymatic amplification, enzymatic oxidation, and electroreduction-based EN redox cycling including further enzymatic oxidation. E, E_R, S, P, Q, R, O, S_{ox}, and P′ refer to enzyme label, enzyme for EN redox cycling, substrate, product, oxidized (or reduced) form of P (or P′), reductant (or reduced form of O), oxidant (or oxidized form of R), oxidized form of S, and oxidized form of P.
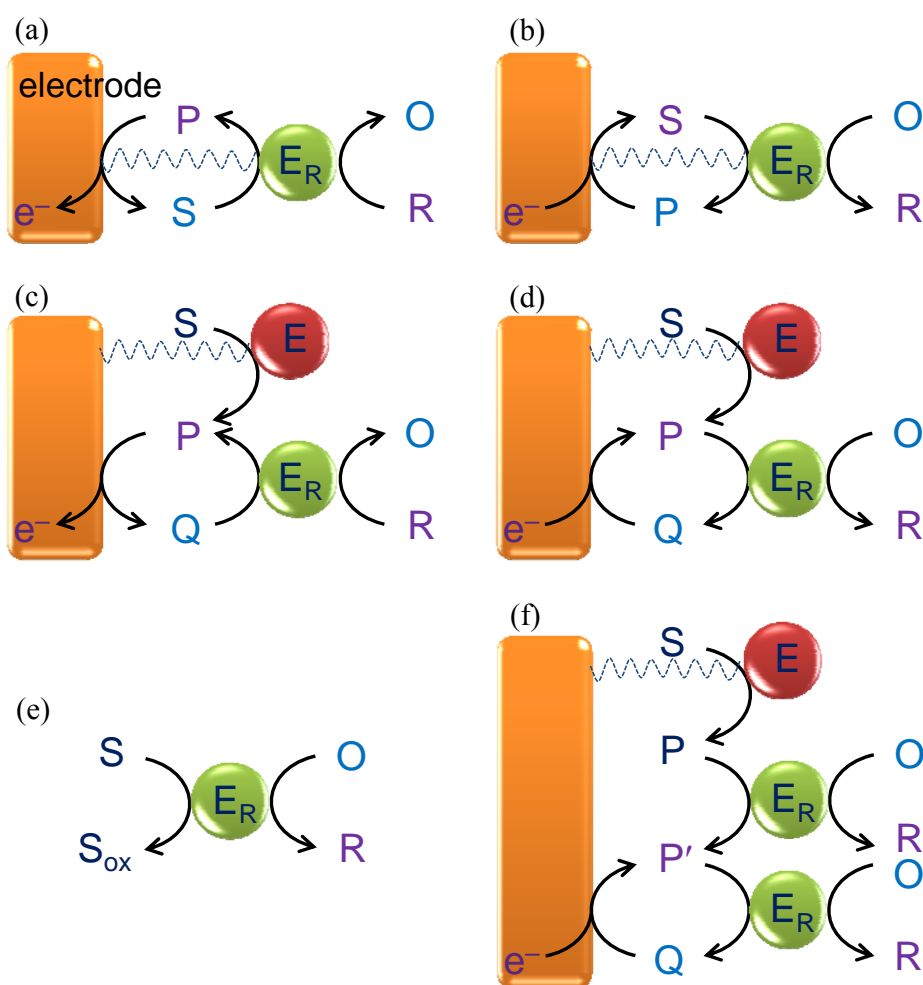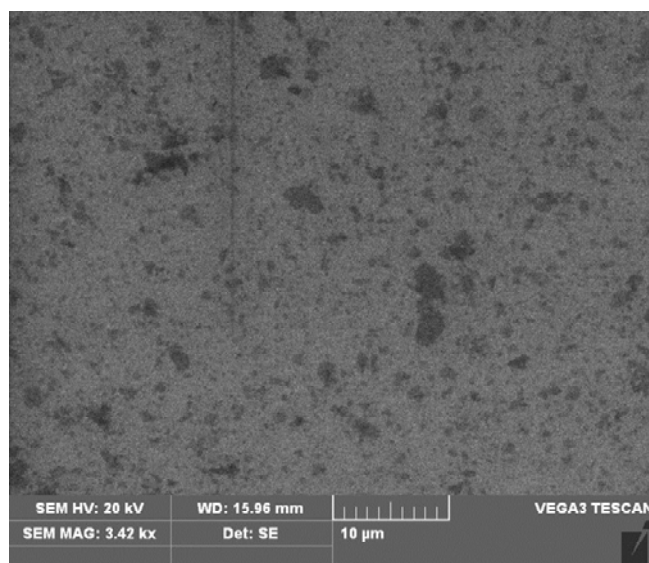
Figure S1. Scanning electron microscopic image of an ITO electrode obtained after it was dipped in a GO-dispersed aqueous solution for 2 h at 25 °C.
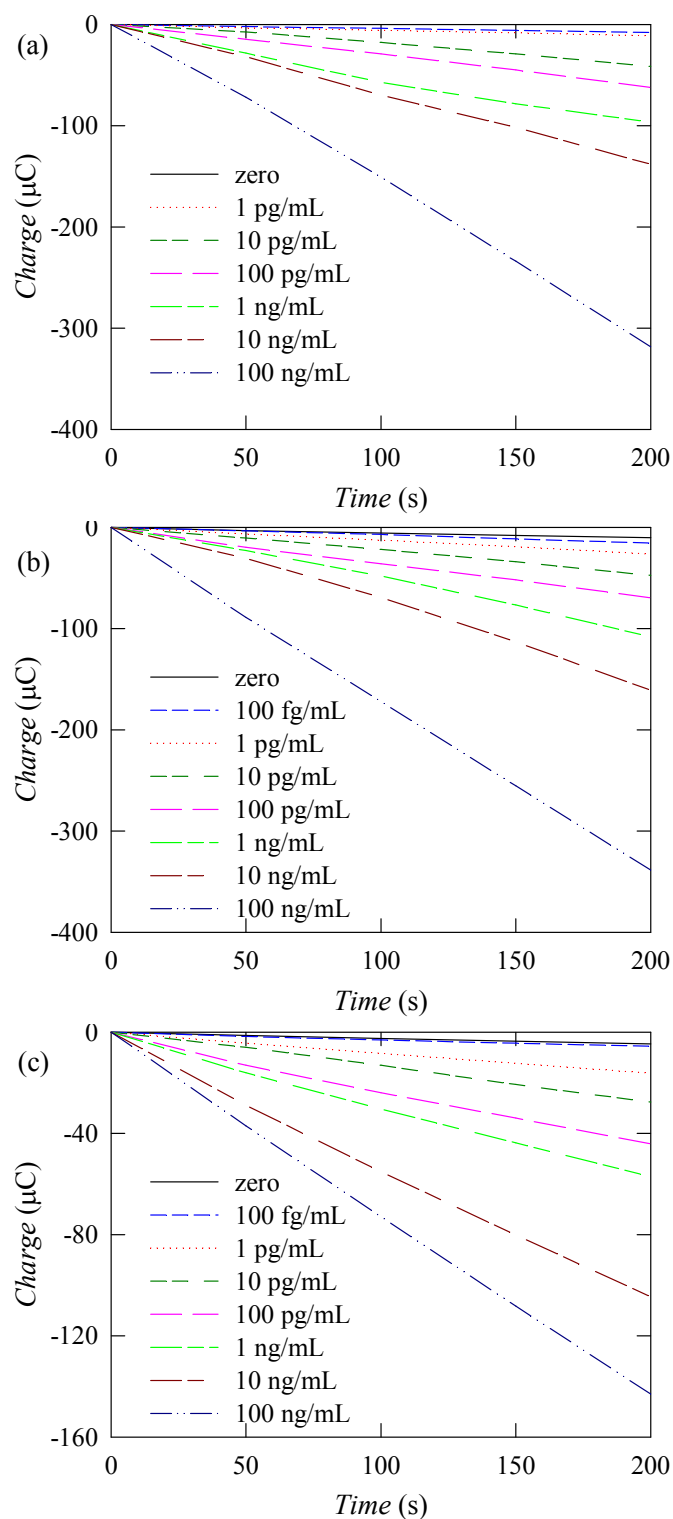
Figure S2. Chronocoulograms obtained at −0.20 V using either (a, b) one-enzyme scheme or (c) two-enzyme scheme and either (a, c) ITO or (b) GO/ITO electrodes for various concentrations of mouse IgG in PBS after the final immunosensing electrodes were incubated for 10 min in air-saturated phosphate buffer (50 mM, pH 6.4) containing either (a, b) 0.1 mM catechol or (c) 0.1 mM P-GP and 50 µg/mL Tyr.
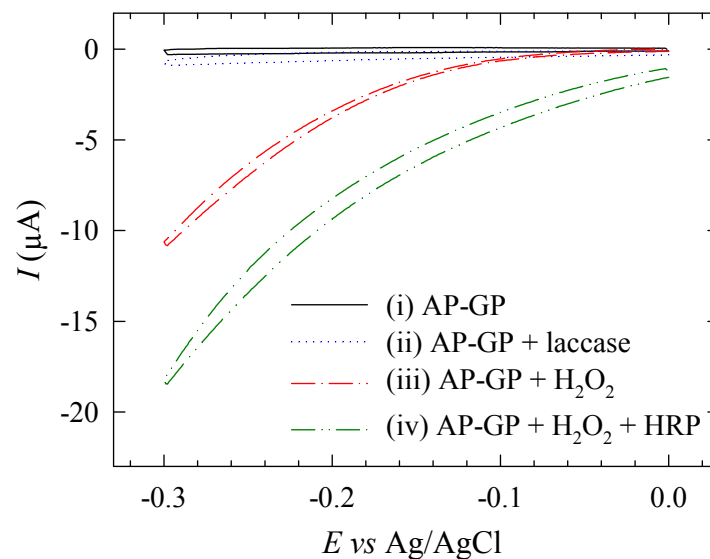
Figure S3. Cyclic voltammograms obtained using ITO electrodes (at a scan rate of 20 mV/s) after incubating for 10 min at 25 °C in air-saturated phosphate-buffered saline (PBS) buffer (50 mM, pH 6.4) containing either (i) 0.1 mM 4-aminophenyl β-D-galactopyranoside (AP-GP), (ii) 0.1 mM AP-GP and 50 µg/mL laccase, (iii) 0.1 mM AP-GP and 0.1% H_2O_2, and (iv) 0.1 mM AP-GP, 0.1% H_2O_2, and 50 µg/mL horseradish peroxidase (HRP).
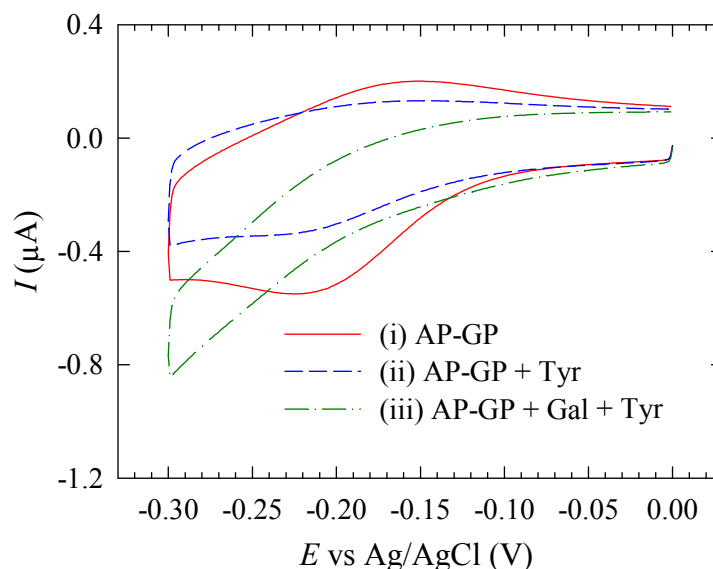
Figure S4. Cyclic voltammograms obtained using ITO electrodes (at a scan rate of 20 mV/s) after incubating for 10 min at 25 °C in air-saturated PBS buffer (50 mM, pH 6.4) containing either (i) 0.1 mM AP-GP, (ii) 0.1 mM AP-GP and 50 µg/mL Tyr, or (iii) 0.1 mM AP-GP, 50 µg/mL Gal, and 50 µg/mL Tyr.
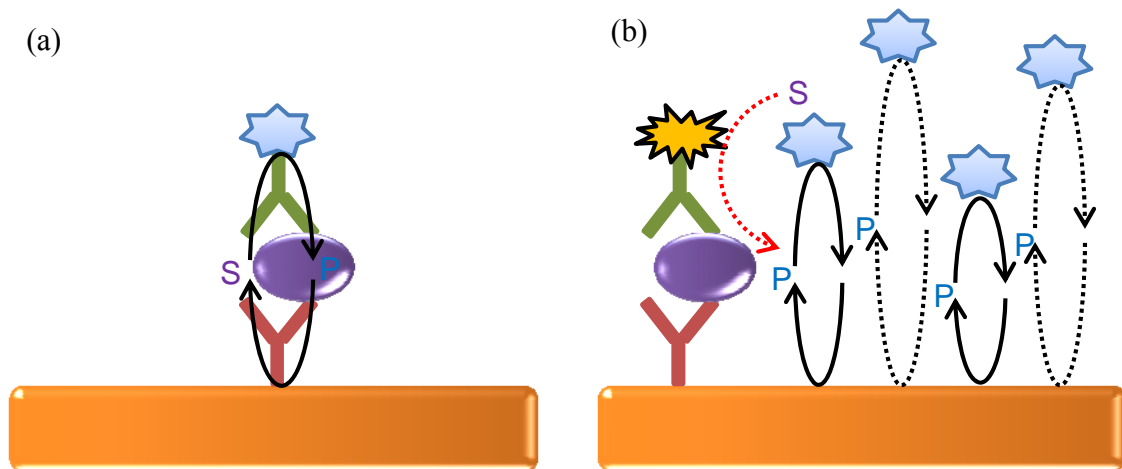
Figure S5. Schematic representation of the EN redox cycling at a very low target concentration in (a) the one-enzyme scheme and (b) the two-enzyme scheme.