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

ARTICLE *in* ANALYTICAL CHEMISTRY · JANUARY 2014

Impact Factor: 5.64

1 AUTHOR:

Amardeep Singh

IndianOil

14 PUBLICATIONS **145** CITATIONS

SEE PROFILE

analytical chemistry

Article

pubs.acs.org/ac

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

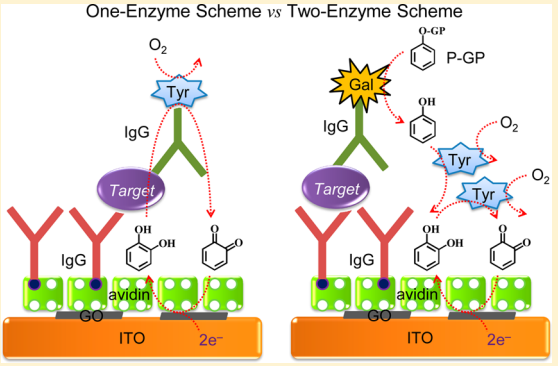
Seonhwa Park,[†,§] Amardeep Singh,[†,§] Sinyoung Kim,[‡] and Haesik Yang*[,†]

[†]Department of Chemistry and Chemistry Institute of Functional Materials, Pusan National University, Busan 609-735, Korea

[‡]Department of Laboratory Medicine, Yonsei University College of Medicine, Seoul 135-720, Korea

Supporting Information

ABSTRACT: We compare herein biosensing performance of two electroreduction-based electrochemical-enzymatic (EN) redox-cycling schemes [the redox cycling combined with simultaneous enzymatic amplification (one-enzyme scheme) and the redox cycling combined with preceding enzymatic amplification (two-enzyme scheme)]. To minimize unwanted side reactions in the two-enzyme scheme, β-galactosidase (Gal) and tyrosinase (Tyr) are selected as an enzyme label and a redox enzyme, respectively, and Tyr is selected as a redox enzyme label in the one-enzyme scheme. The signal amplification in the one-enzyme scheme consists of (i) enzymatic oxidation of catechol into *o*-benzoquinone by Tyr and (ii) electroreduction-based EN redox cycling of *o*-benzoquinone. The signal amplification in the two-enzyme scheme consists of (i) enzymatic conversion of phenyl β-D-galactopyranoside into phenol by Gal, (ii) enzymatic oxidation of phenol into catechol by Tyr, and (iii) electroreduction-based EN redox cycling of *o*-benzoquinone including further enzymatic oxidation of catechol to *o*-benzoquinone by Tyr. Graphene oxide-modified indium–tin oxide (GO/ITO) electrodes, simply prepared by immersing ITO electrodes in a GO-dispersed aqueous solution, are used to obtain better electrocatalytic activities toward *o*-benzoquinone reduction than bare ITO electrodes. The detection limits for mouse IgG, measured with GO/ITO electrodes, are lower than when measured with bare ITO electrodes. Importantly, the detection of mouse IgG using the two-enzyme scheme allows lower detection limits than that using the one-enzyme scheme, because the former gives higher signal levels at low target concentrations although the former gives lower signal levels at high concentrations. The detection limit for cancer antigen (CA) 15-3, a biomarker of breast cancer, measured using the two-enzyme scheme and GO/ITO electrodes is ca. 0.1 U/mL, indicating that the immunosensor is highly sensitive.



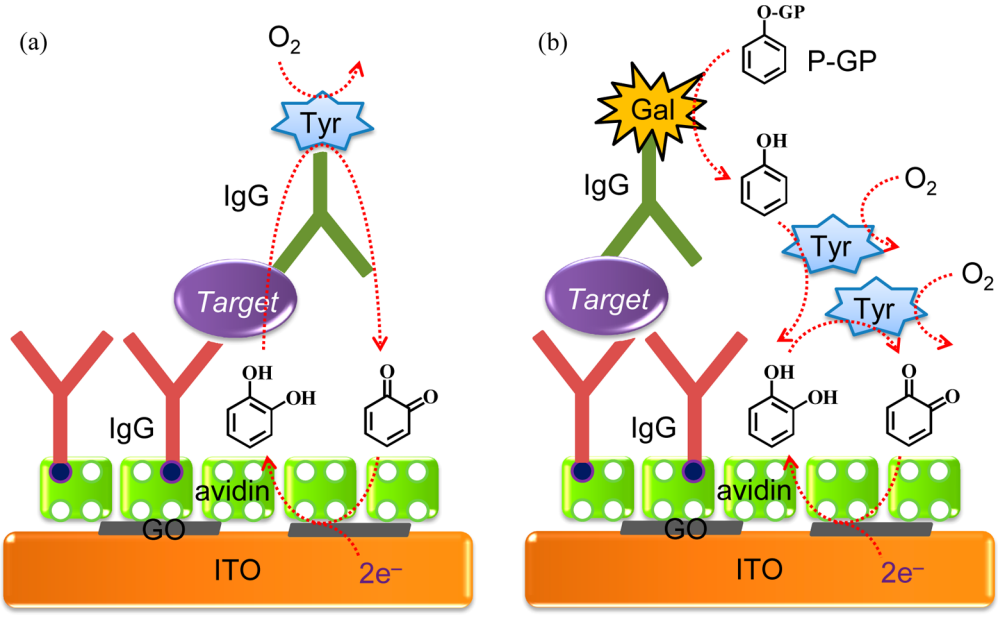
Rapid and sensitive detection of biomolecules is of great importance because it allows rapid and early diagnosis of diseases, as well as permits prompt bioanalysis. Many signal amplification schemes based upon chemical and/or physical amplification have been developed to achieve high signal amplification in a short period.[1−4] In particular, signal amplification schemes using nanomaterials have been an area of intense study in recent years.[5−7] However, enzyme-linked immunosorbent assays (ELISAs) still have been most commonly employed for biomolecular detection.[8−10] If a high signal-amplification scheme could be fused within conventional ELISAs, it could be readily adopted for biomolecular detection. For this reason, signal amplification schemes based on the redox cycling combined with enzymatic amplification are very promising, as they do not require changes in the detection procedure of conventional electrochemical ELISAs (the only requirement being the addition of more chemicals and/or enzymes to final signal-measuring solutions).[10−15]

Both electrooxidation and electroreduction are used to obtain electrochemical signals. In electrochemical biosensors using the electrochemical-enzymatic (EN) redox cycling combined with enzymatic amplification (Schemes S1a–S1d in Supporting Information (SI)), both electrooxidation- and electroreduction-based redox cycling can also be considered. In the case of the EN redox cycling combined with simultaneous enzymatic amplification (SI Scheme S1a and b), one enzyme participates. In the case of the EN redox cycling combined with preceding enzymatic amplification (SI Scheme S1c and d), two enzymes are involved. Although several schemes for the electrooxidation-based[15−17] and electroreduction-based[18−21] EN redox cycling combined with enzymatic amplification have been developed, the comparison in biosensing performance between the redox cycling combined with simultaneous enzymatic amplification (one-enzyme scheme) and the redox cycling combined with preceding enzymatic amplification (two-enzyme scheme) has never been reported. Moreover, in the electroreduction-based one-enzyme

Received: October 1, 2013
Accepted: January 3, 2014

   dx.doi.org/10.1021/ac403912d | *Anal. Chem.* XXXX, XXX, XXX–XXX

Scheme 1. Schematic Representation of (a) an Electrochemical Immunosensor Using the Electroreduction-Based Electrochemical-Enzymatic (EN) Redox Cycling Combined with Simultaneous Enzymatic Amplification (One-Enzyme Scheme) and (b) an Electrochemical Immunosensor Using the Electroreduction-Based EN Redox Cycling Combined with Preceding Enzymatic Amplification (Two-Enzyme Scheme)[a]



[a]The electrodes are graphene oxide-modified indium−tin oxide (GO/ITO) electrodes. Tyr, P-GP, and Gal refer to tyrosinase, phenyl β-D-galactopyranoside, and β-galactosidase.

scheme, horseradish peroxidase (HRP) has been mainly used as a redox enzyme label,[18−20] but tyrosinase (Tyr) has never been used.

In the electroreduction-based two-enzyme scheme (SI Scheme S1d), the following conditions must be satisfied in order to obtain high signal-to-background ratios: (i) electrochemical signal-generating species should be readily electroreduced at the electrode, (ii) other species should not be readily electroreduced, and (iii) dissolved O_2 should not be readily electroreduced. To meet these requirements, the electrode should be moderately electrocatalytic. This can be accomplished in two ways: (i) surface modification of a highly electrocatalytic electrode with an insulating or less electrocatalytic material (e.g., an Au electrode modified with a self-assembled monolayer),[22] and (ii) partial surface modification of a low electrocatalytic electrode with a fairly electrocatalytic material (e.g., an indium−tin oxide (ITO) electrode modified with carbon nanotubes[23] and Au nanoparticles).[20] We have previously demonstrated that the latter method is very effective in obtaining facile surface modification along with low signal-to-background ratios.[23,24] In recent years, graphene oxide (GO)-modified electrodes have been widely employed in order to obtain high electrocatalytic activities.[19,25,26] Although numerous approaches for the preparation of GO-modified electrodes have been attempted, many of the preparation methods are not trivial.

In this study, we compared two redox-cycling schemes [the electroreduction-based one-enzyme scheme using Tyr (Scheme 1a) and the electroreduction-based two-enzyme scheme using β-galactosidase (Gal) and Tyr (Scheme 1b)] in terms of biosensing performance. The redox cycling schemes were applied to the sensitive detection of mouse IgG and cancer antigen (CA) 15-3, a biomarker of breast cancer.[27] The detection limits for mouse IgG measured using the one-enzyme scheme were compared with those measured using the two-enzyme scheme in the absence and presence of GO on ITO electrodes.

EXPERIMENTAL SECTION

Chemicals and Solutions. Biotinylated polyclonal goat antimouse IgG, mouse IgG, polyclonal goat antimouse IgG, human-serum albumin, avidin, bovine-serum albumin (BSA), Gal from *E. coli*, Tyr from mushroom, phenol, catechol, 4-aminophenyl β-D-galactopyranoside, and all reagents used for the preparation of buffer solutions were obtained from Sigma-Aldrich, Co. Anti-CA-15-3 capture IgG (10-CA15A), anti-CA-15-3 detection IgG (10-CA15B), and CA 15-3 (30-AC17) were obtained from Fitzgerald, Inc. (Acton, MA, USA). Phenyl β-D-galactopyranoside (P-GP) was obtained from Tokyo Chemical Industry Co. Ltd. GO-dispersed aqueous solution was obtained from Graphene Laboratories Inc. (Calverton, NY, USA). Sulfosuccinimidyl-4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC) and *N*-succinimidyl-S-acetylthiopropionate (SATP) were obtained from Thermo Scientific Inc. The phosphate buffer (50 mM, pH 6.4) was prepared using disodium phosphate dibasic and sodium phosphate monobasic. The phosphate-buffered saline (PBS) buffer (pH 7.4) contained 10 mM phosphate, 0.138 M NaCl, and 2.7 mM KCl. The PBS buffer containing BSA (PBSB buffer) consisted of all of the ingredients of the PBS buffer and 1% (w/v) BSA. The rinsing buffer (pH 7.6) contained 50 mM tris-(hydroxymethyl)amino methane, 40 mM HCl, 0.05% (w/v) BSA, and 0.5 M NaCl.

Tyr-conjugated antimouse IgG was prepared by cross-linking the amine group of IgG and the amine group of Tyr. One mL of PBS buffer containing 100 μg/mL Tyr and 50 μL of PBS buffer containing 1 mg/mL sulfo-SMCC were mixed and incubated for 30 min at room temperature. Sulfo-SMCC-

dx.doi.org/10.1021/ac403912d | *Anal. Chem.* XXXX, XXX, XXX−XXX

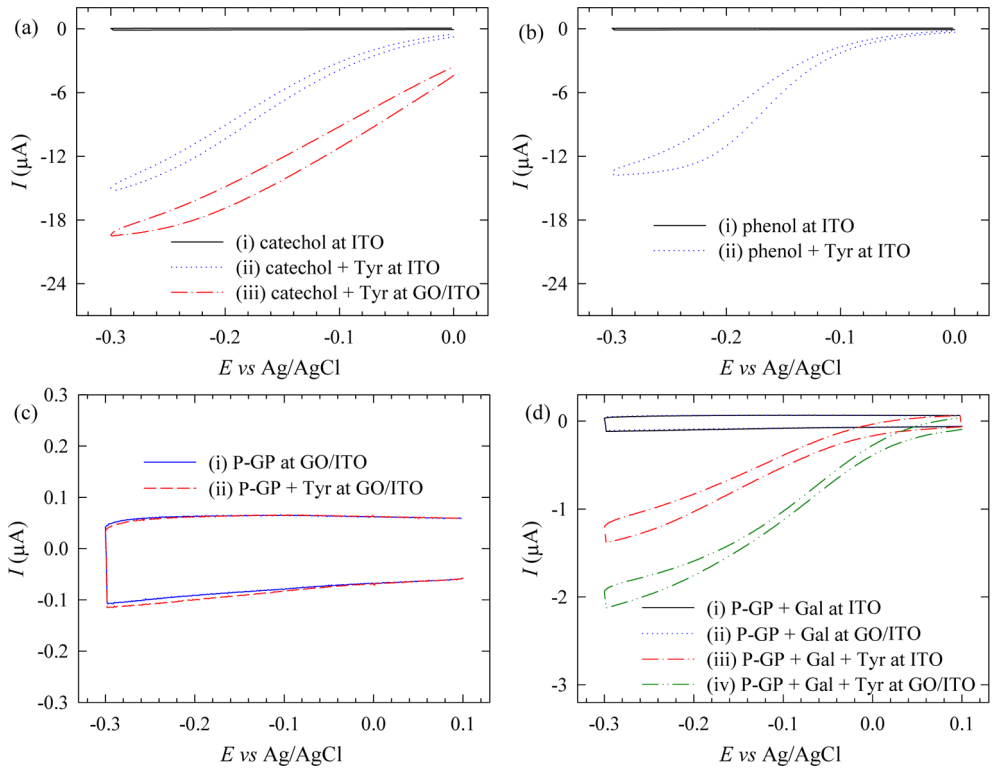


Figure 1. (a) Cyclic voltammograms obtained using either (i, ii) bare ITO or (iii) GO/ITO electrodes in air-saturated phosphate buffer (50 mM, pH 6.4) containing either (i) 0.1 mM catechol or (ii, iii) 0.1 mM catechol and 50 μg/mL Tyr. (b) Cyclic voltammograms obtained using ITO electrodes in air-saturated phosphate buffer containing either (i) 0.1 mM phenol or (ii) 0.1 mM phenol and 50 μg/mL Tyr. (c) Cyclic voltammograms obtained using GO/ITO electrodes in air-saturated phosphate buffer containing either (i) 0.1 mM P-GP or (ii) 0.1 mM P-GP and 50 μg/mL Tyr. (d) Cyclic voltammograms obtained using either (i, iii) bare ITO or (ii, iv) GO/ITO electrodes in air-saturated phosphate buffer containing either (i, ii) 0.1 mM P-GP and 10 μg/mL Gal or (iii, iv) 0.1 mM P-GP, 10 μg/mL Gal, and 50 μg/mL Tyr. The scan rate was 20 mV/s. All solutions were incubated for 10 min before measurement.

conjugated Tyr was filtered by centrifugation for 30 min at 10 000 rpm. Afterward, the filtrate was dissolved in 1 mL of PBS buffer. One mL of PBS buffer containing 100 μg/mL IgG and 10 μL of PBS buffer containing 2 mg/mL SATP were mixed, and the mixture was then incubated for 30 min at room temperature. The resulting solution was mixed with 20 μL of a deacetylation solution (PBS buffer, pH 7.4) containing 0.012 g/mL ethylenediaminetetraacetic acid and 0.044 g/mL hydroxylamine·HCl, for 2 h at room temperature, and the mixture was then filtered by centrifugation for 30 min at 10 000 rpm. The filtrate was dissolved in 1 mL of PBS. The solution containing sulfo-SMCC-conjugated Tyr and the solution containing SATP-conjugated IgG were mixed at a molar ratio of 1:1 for 2 h at room temperature. To filter Tyr-conjugated IgG, the final mixture was centrifuged for 30 min at 10 000 rpm. The filtrate was dissolved in 1 mL of PBSB.

Gal-conjugated IgGs were prepared by cross-linking the amine group of IgG and the thiol group of Gal. One mg/mL antimouse IgG (or anti-CA-15-3 detection IgG) was dissolved in 1 mL of phosphate buffer (50 mM, pH 7.4) containing 150 mM NaCl and 5 mM ethylenediaminetetraacetic acid, and 40 μL of phosphate buffer containing 4.8 mg/mL sulfo-SMCC was then added. The reaction mixture was incubated for 2 h at 4 °C or for 30 min at room temperature. To remove excess sulfo-SMCC, the mixture was centrifuged for 30 min at 10 000 rpm. Afterward, 1 mL of PBS buffer containing 100 μg/mL Gal was added and incubated for 2 h at 4 °C. Biotinylated anti-CA-15-3 capture IgG was prepared by using the biotin-labeling kit.

Preparation of Immunosensing Surface and Procedure for Mouse IgG and CA 15-3 Detection. ITO electrodes were obtained from Samsung Corning (Daegu, Korea). ITO electrodes (1 cm × 2 cm each) were cleaned and pretreated by dipping in 1 M HCl for 10 min.[28] To prepare GO-modified ITO (GO/ITO) electrodes, each pretreated ITO electrode was immersed into 400 μL of an aqueous solution containing 0.5 mg/mL GO for 2 h at 25 °C followed by washing with water. Avidin- and BSA-modified ITO and GO/ITO electrodes were obtained as reported previously.[12] To immobilize biotinylated antimouse IgG (or biotinylated anti-CA-15-3 IgG), 80 μL of a PBSB buffer solution containing 10 μg/mL biotinylated antimouse IgG (or biotinylated anti-CA-15-3 IgG) was dropped onto the avidin- and BSA-modified ITO electrodes. This state was maintained for 30 min at 4 °C, and the electrodes were then washed twice with rinsing buffer. The resulting electrodes were stored at 4 °C before use. For the binding of target mouse IgG (or CA 15-3) to the immunosensing electrodes, 80 μL of PBS containing different concentrations of mouse IgG (or PBS containing CA 15-3 and 5% human-serum albumin) was dropped onto the immunosensing electrodes. This state was maintained for 30 min at 4 °C, and the electrodes were then washed twice with rinsing buffer. Afterward, 80 μL of 10 μg/mL Gal-conjugated antimouse IgG (or Gal-conjugated anti-CA-15-3 IgG) was dropped onto the target-treated electrodes, and the dropped state was maintained for 30 min at 4 °C, followed by washing twice with PBS buffer. In the one-enzyme scheme, 80 μL of 10 μg/mL Tyr-conjugated antimouse IgG was dropped.

dx.doi.org/10.1021/ac403912d | Anal. Chem. XXXX, XXX, XXX−XXX

Teflon electrochemical cells were assembled with an ITO electrode, an Ag/AgCl (3 M NaCl) reference electrode, and a platinum counter electrode. In the two-enzyme scheme, separately prepared phosphate buffer solutions containing 0.1 mM P-GP and 50 μg/mL Tyr were injected into a vessel of the cell for measuring electrochemical signals. In the one-enzyme scheme, phosphate buffer containing 0.1 mM catechol was injected. The exposed area of the ITO electrode was ca. 0.28 cm^2. Incubation for enzymatic reactions was performed at 25 °C for 10 min, and electrochemical measurements were carried out using a CHI 405A or CHI 708C (CH Instruments, Austin, TX, USA).

RESULTS AND DISCUSSION

To compare biosensing performance of two redox cycling schemes [(i) the electroreduction-based EN redox cycling combined with simultaneous enzymatic amplification (one-enzyme scheme: SI Scheme S1b) and (ii) the electroreduction-based EN redox cycling combined with preceding enzymatic amplification (two-enzyme scheme: SI Scheme S1d)], enzymes suitable for fast redox cycling and enzymatic amplification were chosen on the preferential basis. In electroreduction-based EN redox cycling, Cu ion-containing redox enzymes such as laccase and Tyr[29] and Fe ion-containing redox enzymes such as HRP[30] can be used as a redox enzyme. Common nonredox enzyme labels such as alkaline phosphatase and Gal can be used as an enzyme for enzymatic amplification[31] in the electroreduction-based two-enzyme scheme. To achieve fast enzymatic amplification and redox cycling, the optimum pH ranges of two enzymes for EN redox cycling and enzymatic amplification should be similar. The optimum pH ranges of HRP, laccase, and Tyr are neutral or acidic. The optimum pH range of alkaline phosphate is basic, whereas that of Gal is neutral. Therefore, Gal was chosen as an enzyme for enzymatic amplification in the two-enzyme scheme.

To obtain low background levels in the two-enzyme scheme (SI Scheme S1d), an enzyme substrate for enzymatic amplification should not be oxidized by a redox enzyme for redox cycling. To meet this requirement, Tyr and P-GP were chosen as a redox enzyme and an enzyme substrate, respectively, for the two-enzyme scheme (Scheme 1b) (see SI). Due to unwanted side reactions, it was difficult to apply HRP and laccase to the two-enzyme scheme (see SI). Because Tyr was chosen in Scheme 1b, Tyr and catechol (oxidized product of phenol by Tyr) were selected as a redox enzyme and an enzyme substrate for the one-enzyme scheme (Scheme 1a). Although HRP has been commonly used as a redox enzyme label for the electroreduction-based one-enzyme scheme,[18−20] Tyr has never been used. Moreover, although the EN redox cycling using Tyr was previously used for the detection of phenol,[32] Gal activity,[33] and alkaline phosphatase activity,[34,35] Scheme 1b was never used. It is important to note that, when Tyr is used as a redox enzyme the addition of oxidant such as H_2O_2 is not required because dissolved O_2 is used as an oxidant. The overall signal amplification in Scheme 1b consists of (i) enzymatic amplification by Gal, (ii) enzymatic oxidation by Tyr, and (iii) electroreduction-based EN redox cycling including further enzymatic oxidation by Tyr. Gal converts P-GP into phenol, which is initially oxidized to catechol by Tyr, then further oxidized to *o*-benzoquinone by Tyr. When *o*-benzoquinone is electroreduced, electroreduction-based EN redox cycling is induced. Although the detection scheme looks complex, the detection procedure is almost similar to that in the one-enzyme scheme.

o-Benzoquinone electroreduction occurs in a region of potentials less than 0 V at ITO electrodes. In this region, cyclic voltammograms were obtained in order to investigate catechol and phenol oxidization by Tyr (Figure 1a and b). In a solution containing catechol and phenol (curve i of Figure 1a and curve i of Figure 1b, respectively), only capacitive background currents were observed. However, in a solution containing catechol and Tyr (curve ii of Figure 1a), high cathodic currents were observed. Moreover, in a solution containing phenol and Tyr (curve ii of Figure 1b), similar high currents were also observed. These results indicate that the conversion of phenol to catechol to *o*-benzoquinone by Tyr occurs efficiently. To obtain low background levels, P-GP should not be oxidized by Tyr. In Figure 1c, the cyclic voltammogram obtained in a PBS solution containing P-GP (curve i of Figure 1c) was similar to that obtained in a solution containing P-GP and Tyr (curve ii of Figure 1c), indicating that the oxidation of P-GP by Tyr did not occur or was negligible.

In electroreduction-based sensors, electroreduction of dissolved O_2 significantly affects background levels. ITO electrodes show low electrocatalytic activities toward electroreduction of O_2 as well as low and reproducible capacitive current.[28] In particular, HCl-treated ITO electrodes show lower electrocatalytic activities toward electroreduction of O_2.[28] Therefore, ITO electrodes were chosen as working electrodes. However, ITO electrodes also show low electrocatalytic activities toward electroreduction of *o*-benzoquinone, which makes it difficult to obtain fast EN redox cycling of *o*-benzoquinone. To improve their electrocatalytic activities, GO was immobilized on ITO electrodes by immersing ITO electrodes in a GO-dispersed solution. SI Figure S1 shows a scanning electron microscopic image for a GO/ITO electrode. The image indicates that GO was randomly adsorbed on an ITO electrode. Cyclic voltammograms (Figure 1d) were obtained in order to check the effects of GO modification on signal-to-background ratio. Importantly, the background level of a GO/ITO electrode (curve ii of Figure 1d) in a solution of P-GP and Gal was similar to that of a bare ITO electrode (curve i of Figure 1d). Because ITO electrodes are partially modified with GO, the increase in capacitive current was not considerable and the electroreduction of dissolved O_2 was negligible. Nevertheless, cathodic currents of *o*-benzoquinone at a GO/ITO electrode (curve iv of Figure 1d) were much higher than those at a bare ITO electrode (curve iii of Figure 1d), since GO improves electrocatalytic activities toward electroreduction of *o*-benzoquinone. For the same reason, curve iii of Figure 1a showed higher cathodic currents than curve ii of Figure 1a. Consequently, GO/ITO electrodes were better than bare ITO electrodes in obtaining high signal-to-background ratios.

To compare biosensing performance of two redox-cycling schemes and more clearly observe the effects of GO modification, the detection of mouse IgG was carried out with four different schemes [(i) the one-enzyme scheme using ITO electrode, (ii) the one-enzyme scheme using GO/ITO electrode (Scheme 1a), (iii) the two-enzyme scheme using ITO electrode, and (iv) the two-enzyme scheme using GO/ITO electrode (Scheme 1b)]. The chronocoulograms, obtained at −0.20 V after the final immunosensing electrodes were incubated for 10 min in air-saturated phosphate buffer (50 mM, pH 6.4) containing either 0.1 mM catechol (in the one-

dx.doi.org/10.1021/ac403912d | Anal. Chem. XXXX, XXX, XXX−XXX

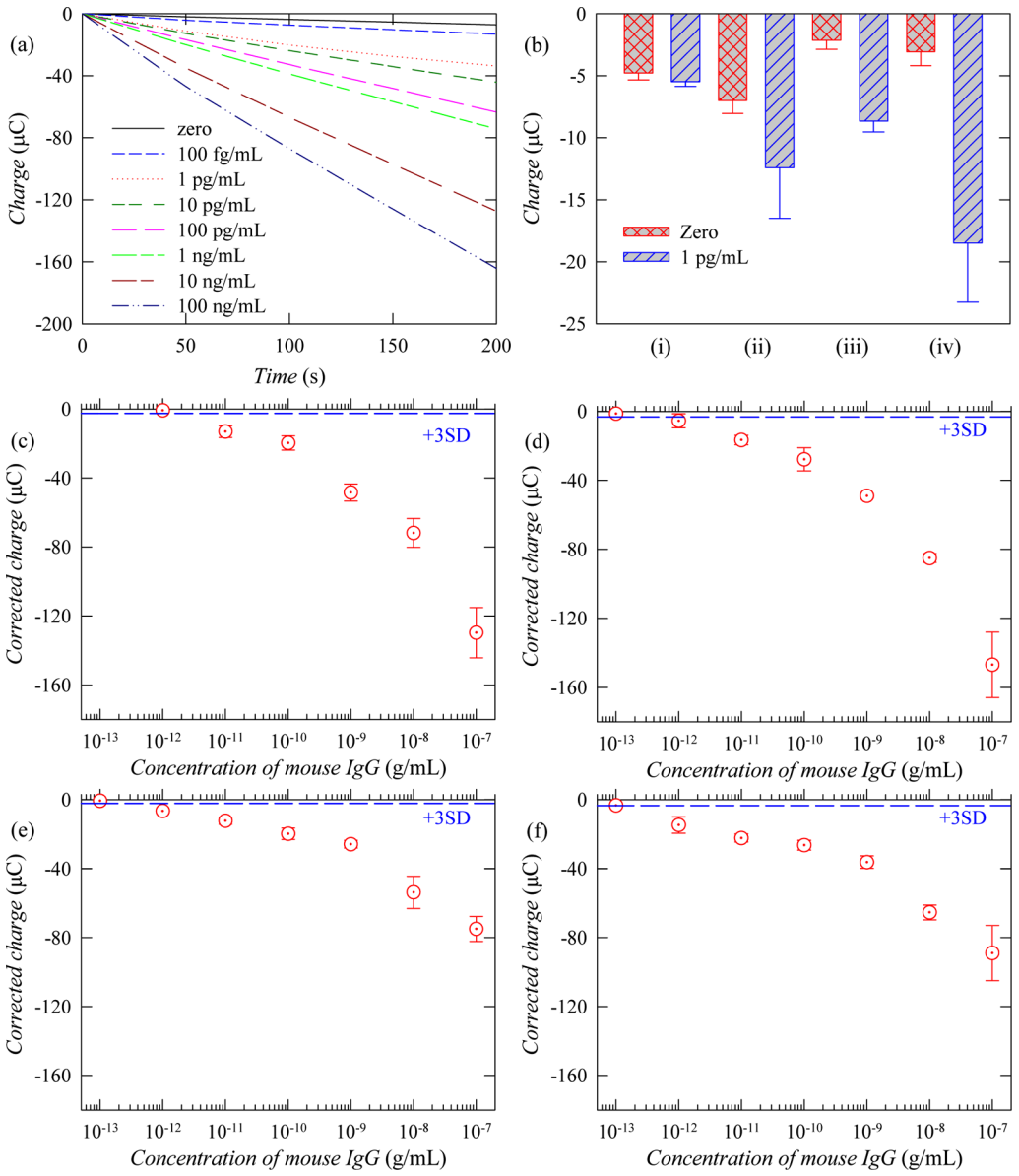


Figure 2. (a) Chronocoulograms obtained at −0.20 V using the two-enzyme scheme and GO/ITO electrode for various concentrations of mouse IgG in PBS after the final immunosensing electrodes were incubated for 10 min in air-saturated phosphate buffer (50 mM, pH 6.4) containing 0.1 mM P-GP and 50 μg/mL Tyr. (b) The charges measured at 100 s in the chronocoulograms obtained at a concentration of zero and at a concentration of 1 pg/mL in the immunosensors using four different schemes [(i) the one-enzyme scheme using ITO electrode, (ii) the one-enzyme scheme using GO/ITO electrode (Scheme 1a), (iii) the two-enzyme scheme using ITO electrode, and (iv) the two-enzyme scheme using GO/ITO electrode (Scheme 1b)]. (c–f) Calibration plots [for the immunosensors using (c) the one-enzyme scheme using ITO electrode, (d) the one-enzyme scheme using GO/ITO electrode, (e) the two-enzyme scheme using ITO electrode, and (f) the two-enzyme scheme using GO/ITO electrode] obtained from the charges at 100 s in chronocoulograms in SI Figure S2 and panel a. Each concentration experiment was carried out using 3 different sensing electrodes for the assay of the same sample. All of the data were corrected by the mean value of seven measurements determined at a concentration of zero. The dashed line corresponds to three times the standard deviation (SD) of the charge at a concentration of zero. The error bars represent the SD of the three measurements.

enzyme scheme) or 0.1 mM P-GP and 50 μg/mL Tyr (in the two-enzyme scheme), are shown in SI Figure S2 and Figure 2a. The absolute charges increased with increasing the concentration of mouse IgG. To investigate relative background levels and signal levels in the four schemes, the charges measured at 100 s in the chronocoulograms obtained at a concentration of zero and at a concentration of 1 pg/mL were compared (Figure 2b). The absolute background levels in the two two-enzyme schemes (2.2 ± 0.7 and 3.1 ± 1.4 μC) were lower than those in the two one-enzyme schemes (4.8 ± 0.6 and 7.1 ± 0.9 μC). However, the absolute signal levels (at a concentration 1 pg/mL) in the two-enzyme schemes using ITO and GO/ITO electrode (8.8 ± 0.8 and 18.4 ± 5.4 μC) were higher than those in the corresponding one-enzyme schemes using ITO and GO/ITO electrode (5.6 ± 0.3 and 12.4 ± 3.8 μC, respectively). Overall, the signal-to-background ratio increased in the sequence of the one-enzyme scheme using ITO electrode, the one-enzyme scheme using GO/ITO electrode, the two-enzyme scheme using ITO electrode, and the two-enzyme scheme using GO/ITO electrode (1.1, 1.7, 4.0, and 6.0, respectively). This result clearly shows that the two-enzyme

dx.doi.org/10.1021/ac403912d | Anal. Chem. XXXX, XXX, XXX–XXX

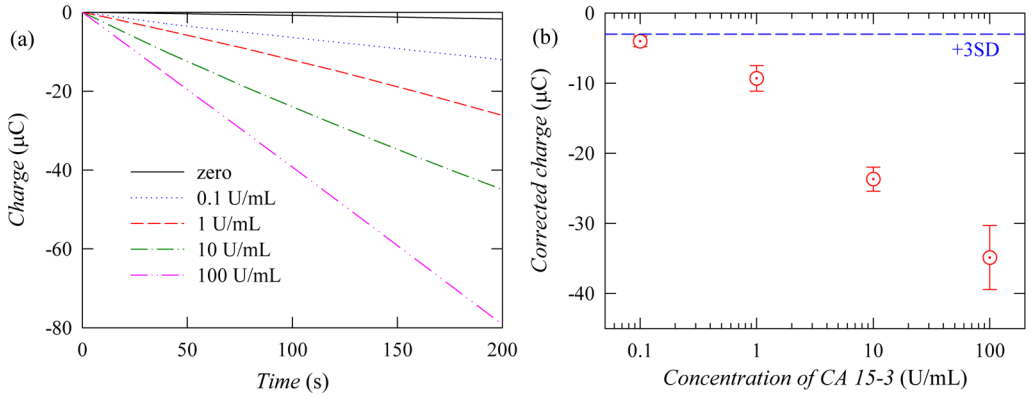


Figure 3. (a) Chronocoulograms obtained at −0.20 V using GO/ITO electrodes for various concentrations of CA 15-3 in PBS containing 5% human-serum albumin after the final immunosensing electrodes were incubated for 10 min in air-saturated phosphate buffer (50 mM, pH 6.4) containing 0.1 mM P-GP and 50 μg/mL Tyr. (b) Calibration plots calculated from the charges at 100 s in panel a.

scheme and GO modification are effective for obtaining both low background levels and high signal levels.

Figure 2c−f represent calibration plots calculated from the charges at 100 s in SI Figure S2 and Figure 2a. The detection limits for the four schemes, calculated after regression curves were fitted, were 2, 0.3, 0.2, and 0.1 pg/mL. The two-enzyme scheme using GO/ITO electrode allowed the lowest detection limit, because it showed the highest signal-to-background ratio. The detection limit of 0.1 pg/mL indicates that the immunosensor using the redox cycling is highly sensitive. The absolute signal levels at each concentration in the two schemes using GO/ITO electrode (Figure 2d and f) were higher than those in the two schemes using bare ITO electrode (Figure 2b and e, respectively). Interestingly, the absolute signal levels at concentrations higher than ca. 10 pg/mL in the two-enzyme scheme using ITO electrode (Figure 2e) were lower than those in the one-enzyme scheme using ITO electrode (Figure 2c), and the absolute signal levels at concentrations higher than ca. 100 pg/mL in the two-enzyme scheme using GO/ITO electrode (Figure 2f) were lower than those in the one-enzyme scheme using GO/ITO electrode (Figure 2d). Very importantly, this trend was opposite to that obtained at a concentration of 1 pg/mL as shown in Figure 2b. Overall results indicate that the two-enzyme schemes allowed higher signal levels at low concentrations but lower signal levels at high concentrations than the one-enzyme schemes. The possible reason for this is explained in the SI. 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.

Scheme 1b was also applied to the detection of CA 15-3. The concentration-dependent chronocoulometric data (Figure 3a) were obtained with serum-like solutions (PBS solutions containing human-serum albumin spiked with CA 15-3). The detection limit for CA 15-3 calculated from Figure 3b was 0.1 U/mL, which is low enough to differentiate between a normal level and an increased, abnormal level because the normal level of CA 15-3 is less than 30 U/mL.[36] The immunosensor result clearly shows that the developed immunosensing scheme is efficient for highly sensitive detection of proteins.

CONCLUSIONS

This paper demonstrates that the electroreduction-based EN redox cycling is effective for obtaining highly sensitive biomolecular detection. In particular, the electroreduction-based two-enzyme scheme allowed lower detection limits than the one-enzyme scheme. Moreover, the detection procedure in the two-enzyme scheme was almost similar to that in the one-enzyme scheme. Due to unwanted side reactions, it was difficult to apply HRP and laccase to a redox enzyme in the two-enzyme scheme. Accordingly, Tyr was applied to a redox enzyme in the two-enzyme scheme and the one-enzyme scheme. Simply prepared GO/ITO electrodes allowed better electrocatalytic activities toward *o*-benzoquinone reduction and lower detection limits for mouse IgG than bare ITO electrodes. The detection limit for CA 15-3 measured using the two-enzyme scheme GO/ITO electrodes was ca. 0.1 U/mL, indicating that the immunosensor employing is highly sensitive.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: hyang@pusan.ac.kr.

Author Contributions

[§]Seonhwa Park and Amardeep Singh contributed equally.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was supported by the National Research Foundation of Korea (2010-0020772, 2012R1A1A2006478, 2012R1A2A2A06045327, and 2012-M3C1A1-048860).

REFERENCES

(1) Zhu, L.; Anslyn, E. V. *Angew. Chem., Int. Ed.* **2006**, *45*, 1190−1196.
(2) Dhawan, S. *Expert Rev. Mol. Diagn.* **2006**, *6*, 749−760.
(3) Andras, S. C.; Power, J. B.; Cocking, E. C.; Davey, M. R. *Mol. Biotechnol.* **2001**, *19*, 29−44.
(4) Brakmann, S. *Angew. Chem., Int. Ed.* **2004**, *43*, 5730−5734.
(5) Rosi, N. L.; Mirkin, C. A. *Chem. Rev.* **2005**, *105*, 1547−1562.
(6) Tansil, N. C.; Gao, Z. *Nano Today* **2006**, *1*, 28−37.
(7) Das, J.; Aziz, M. A.; Yang, H. *J. Am. Chem. Soc.* **2006**, *128*, 16022−16023.
(8) Scheller, F. W.; Bauer, C. G.; Makower, A.; Wollenberger, U.; Warsinke, A.; Bier, F. F. Immunoassays Using Enzymatic Amplification

Electrodes. In *Biomolecular Sensors*; Gizeli, E., Lowe, C. R., Eds.; Taylor & Francis Ltd.: New York, 2002, pp 207−238.
(9) Warsinke, A. *Adv. Biochem. Eng./Biotechnol.* **2008**, *109*, 155−193.
(10) Yang, H. *Curr. Opin. Chem. Biol.* **2012**, *16*, 422−428.
(11) Das, J.; Jo, K.; Lee, J. W.; Yang, H. *Anal. Chem.* **2007**, *79*, 2790−2796.
(12) Akanda, M. R.; Aziz, M. A.; Jo, K.; Tamilavan, V.; Hyun, M. H.; Kim, S.; Yang, H. *Anal. Chem.* **2011**, *83*, 3926−3933.
(13) Akanda, M. R.; Choe, Y. L.; Yang, H. *Anal. Chem.* **2012**, *84*, 1049−1055.
(14) Akanda, M. R.; Tamilavan, V.; Park, S.; Jo, K.; Hyun, M. H.; Yang, H. *Anal. Chem.* **2013**, *85*, 1631−1636.
(15) Singh, A.; Park, S.; Yang, H. *Anal. Chem.* **2013**, *85*, 4863−4868.
(16) Limoges, B.; Marchal, D.; Mavre, F.; Saveant, J. *J. Am. Chem. Soc.* **2006**, *128*, 6014−6015.
(17) Limoges, B.; Marchal, D.; Mavre, F.; Saveant, J. *J. Am. Chem. Soc.* **2008**, *130*, 7276−7285.
(18) Liu, G.; Wan, Y.; Gau, V.; Zhang, J.; Wang, L.; Song, S.; Fan, C. *J. Am. Chem. Soc.* **2008**, *130*, 6820−6825.
(19) Haque, A.-M. J.; Park, H.; Sung, D.; Jon, S.; Choi, S.-Y.; Kim, K. *Anal. Chem.* **2012**, *84*, 1871−1878.
(20) Kang, H. J.; Aziz, M. A.; Jeon, B.; Jo, K.; Yang, H. *Electroanalysis* **2009**, *21*, 2647−2652.
(21) Caruana, D. J.; Heller, A. *J. Am. Chem. Soc.* **1999**, *121*, 769−774.
(22) Kwon, S. J.; Yang, H.; Jo, K.; Kwak, J. *Analyst* **2008**, *133*, 1599−1604.
(23) Aziz, M. A.; Park, S.; Jon, S.; Yang, H. *Chem. Commun.* **2007**, 2610−2612.
(24) Aziz, M. A.; Patra, S.; Yang, H. *Chem. Commun.* **2008**, 4607−4609.
(25) Chen, D.; Feng, H.; Li, J. *Chem. Rev.* **2012**, *112*, 6027−6053.
(26) Wu, S.; He, Q.; Tan, C.; Wang, Y.; Zhang, H. *Small* **2013**, *9*, 1160−1172.
(27) Duffy, M. J.; Evoy, D.; McDermott, E. W. *Clin. Chim. Acta* **2010**, *411*, 1869−1874.
(28) Choi, M.; Jo, K.; Yang, H. *Bull. Korean Chem. Soc.* **2013**, *34*, 421−425.
(29) Durán, N.; Rosa, M. A.; D'Annibale, A.; Gianfreda, L. *Enzym. Microb. Technol.* **2002**, *31*, 907−931.
(30) Ruzgas, T.; Csöregi, E.; Emnéus, J.; Gorton, L.; Marko-Varga, G. *Anal. Chim. Acta* **1996**, *330*, 123−128.
(31) Savage, M. D.; Mattson, G.; Desai, S.; Nielander, G. W.; Morgensen, S.; Conklin, E. J. *Avidin-Biotin Chemistry: A Handbook*, 2nd ed.; Pierce: Rockford, IL, 1994; pp 155−164.
(32) Yildiz, H. B.; Castillo, J.; Guschin, D. A.; Toppare, L.; Schuhmann, W. *Microchim. Acta* **2007**, *159*, 27−34.
(33) Serra, B.; Morales, M. D.; Zhang, J.; Reviejo, A. J.; Hall, E. H.; Pingarron, J. M. *Anal. Chem.* **2005**, *77*, 8115−8121.
(34) Ito, S.; Yamazaki, S.-I.; Kano, K.; Ikeda, T. *Anal. Chim. Acta* **2000**, *424*, 57−63.
(35) Bauer, C. G.; Eremenko, A. V.; Ehrentreich-Förster, E.; Bier, F. F.; Makower, A.; Halsall, H. B.; Heineman, W. R.; Scheller, F. W. *Anal. Chem.* **1996**, *68*, 2453−2458.
(36) Wojtacki, J.; Dziewulska-Bokiniec, A.; Skokowski, J.; Ciesielski, D. *Neoplasma* **1994**, *41*, 213−216.