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Immunosensor Employing Stable, Solid 1-Amino-2-naphthyl Phosphate and Ammonia-Borane toward Ultrasensitive and Simple Point-of-Care Testing

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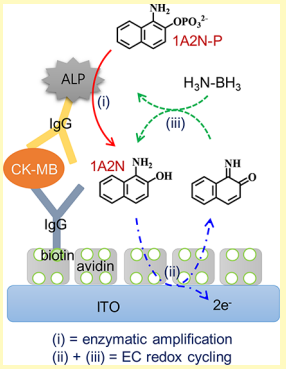
Supporting Information

ABSTRACT: Biosensors for ultrasensitive point-of-care testing require dried reagents with long-term stability and a high signal-to-background ratio. Although ortho-substituted diaromatic dihydroxy and aminohydroxy compounds undergo fast redox reactions, they are not used as electrochemical signaling species because they are readily oxidized and polymerized by dissolved oxygen. In this report, stable, solid 1-amino-2-naphthyl phosphate (1A2N-P) and ammonia-borane (H_3N-BH_3) are respectively employed as a substrate for alkaline phosphatase (ALP) and a reductant for electrochemical-chemical (EC) redox cycling. ALP converts 1A2N-P to 1-amino-2-naphthol (1A2N), which is then employed in EC redox cycling using H_3N-BH_3. The oxidation and polymerization of 1A2N by dissolved oxygen is significantly prevented in the presence of H_3N-BH_3. The electrochemical measurement is performed without modification of indium−tin oxide (ITO) electrodes with electrocatalytic materials. For comparison, nine aromatic dihydroxy and aminohydroxy compounds, including 1A2N, are evaluated to achieve fast EC redox cycling, and four strong reductants, including H_3N-BH_3, are evaluated to achieve a low background level. The combination of 1A2N and H_3N-BH_3 allows the achievement of a very high signal-to-background ratio. When the newly developed combination is applied to the detection of creatine kinase-MB (CK-MB), the detection limit for CK-MB is ~80 fg/mL, indicating that the combination allows ultrasensitive detection. The concentrations of CK-MB in clinical serum samples, determined using the developed system, are in good agreement with the concentrations obtained using a commercial instrument. Thus, the use of stable, solid 1A2N-P and H_3N-BH_3 along with bare ITO electrodes is highly promising for ultrasensitive and simple point-of-care testing.



KEYWORDS: *electrochemical immunosensor, biosensor, redox cycling, alkaline phosphatase, point-of-care testing, ITO electrode*

Electrochemical detection is commonly employed in biosensors for ultrasensitive point-of-care testing, because it allows for miniaturized and sensitive instruments.[1,2] Such electrochemical biosensors require (i) dried reagents with long-term stability and (ii) a high electrochemical signal-to-background ratio. The dried reagents should be readily dissolved for simple detection when a sample solution is dropped. In general, high electrocatalytic electrodes facilitate high signal levels, whereas low electrocatalytic electrodes give rise to low background levels. A good way to achieve a high signal-to-background ratio is to employ a low electrocatalytic electrode along with an electroactive signaling species that undergoes a fast redox reaction even at low electrocatalytic electrodes.[3]

Aromatic dihydroxy compounds (hydroquinones) play crucial roles as electron mediators, electron shuttles, and redox catalysts in biological systems due to their fast and reversible redox reactions.[4−7] Ortho-substituted hydroquinones, such as catechol derivatives, play an important role in producing polymerized natural pigments (e.g., melanin) via oxidation and subsequent nucleophilic addition.[8−10] These unique properties have inspired the use of hydroquinones as electron mediators for (bio)catalytic redox reactions,[11] energy storage materials for redox flow batteries,[12] and interfacing materials between solid surfaces and (bio)molecules.[13] Aromatic aminohydroxy compounds such as aminophenols and aminonaphthols have also been utilized for the same purpose because their redox properties and reactivities are comparable to those of hydroquinones.[14]

Aromatic dihydroxy and aminohydroxy compounds have been commonly employed as electroactive signaling species for obtaining high electrochemical signals from electrochemical biosensors employing enzyme or nanoparticle labels, where these hydroxy compounds are produced via enzymatic or catalytic reactions[15−21] and their redox reactions are fast and allow for two-electron transfer. These compounds include monoaromatic compounds [hydroquinone[15,21] and 4-aminophenol (4AP)][17,20] and diaromatic compounds [1,4-dihydrox-

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ynaphthalene (14DHN)[18] and 4-amino-1-naphthol (4A1N)].[19] In general, electrochemical oxidation of the diaromatic compounds is faster than the corresponding process for the monoaromatic congeners. For this reason, fast electrochemical oxidation of the diaromatic compounds can be achieved even with indium−tin oxide (ITO) electrodes having low electrocatalytic activity,[22,23] whereas fast electrochemical oxidation of the monoaromatic counterparts requires modification of the ITO electrodes with electrocatalytic materials such as carbon nanotubes.[24] Nevertheless, the diaromatic compounds are not commonly used in electrochemical biosensors because the diaromatic compounds generated via enzymatic or catalytic reactions during the incubation period (10−30 min) are rapidly oxidized by dissolved oxygen.[18,19] Electrochemical reduction of the oxidized diaromatic compounds is required to obtain electrochemical signals. In this case, both direct and mediated electrochemical reductions of dissolved oxygen interfere with reproducible electrochemical detection. On the other hand, oxidation of the monoaromatic compounds by dissolved oxygen is slow. Therefore, electrochemical oxidation of the monoaromatic compounds has been commonly used to obtain electrochemical signals with no or little interference from dissolved oxygen.

In many cases, ortho-substituted aromatic dihydroxy and aminohydroxy compounds undergo faster electrochemical and catalytic reactions than the para-substituted congeners.[25] However, the ortho-substituted compounds have been of limited use in electrochemical biosensors that employ alkaline phosphatase (ALP) and galactosidase as an enzyme label.[26] This is because the ortho-substituted compounds are susceptible to polymerization via oxidation by dissolved oxygen and subsequent nucleophilic addition.[10]

The electron-mediating properties of aromatic dihydroxy and aminohydroxy compounds have been applied in electrochemical redox cycling to obtain highly amplified electrochemical signals.[3,17,20−22,27−31] Such redox cycling includes electrochemical−chemical (EC) redox cycling using an aromatic dihydroxy or aminohydroxy compound and a reductant[17,20,28−31] and electrochemical−chemical−chemical redox cycling using an oxidant, an aromatic dihydroxy or aminohydroxy compound, and a reductant.[21,22] For this purpose, hydrazine (H_2NNH_2),[20] sodium borohydride,[17] tris(2-carboxyethyl)phosphine (TCEP),[28,29] reduced β-nicotinamide adenine dinucleotide (NADH),[30] and dithiothreitol[31] have been used as strong reductants. Importantly, when an excess amount of a reductant is used, it is possible to achieve electrochemical oxidation of the diaromatic dihydroxy and aminohydroxy compounds even in the presence of dissolved oxygen.[23] Although strong reductants can be readily oxidized at metal and carbon electrodes, their electrooxidation is very slow at ITO electrodes, resulting in low background currents. Ammonia borane (H_3N-BH_3) is a promising hydrogen storage material that exists stably as a solid form in air,[32,33] and is readily dissolved in water. These properties are essential requirements for dried reagents that are used in biosensors for point-of-care testing. To date, the use of H_3N-BH_3 in redox cycling has been never attempted.

Herein, we report an ultrasensitive electrochemical immunosensor that uses stable, solid 1-amino-2-naphthyl phosphate (1A2N-P) and H_3N-BH_3 as a new substrate for alkaline phosphatase and a new reductant for redox cycling. For comparison, four strong reductants are evaluated to achieve a low background level, and nine aromatic dihydroxy and aminohydroxy compounds (Figure 1a) are evaluated to achieve fast EC redox cycling. The combination of H_3N-BH_3 and 1-amino-2-naphthol (1A2N) produces a very high signal-to-background ratio and is applied to the detection of creatine kinase-MB (CK-MB), an important cardiac marker[34] (Figure 1b).

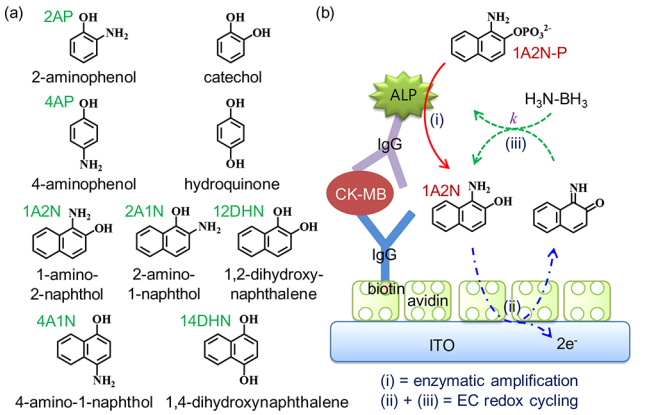

Figure 1. (a) Chemical structures of alkaline phosphatase (ALP) products and (b) schematic of an electrochemical immunosensor using (i) enzymatic amplification and (ii)+(iii) electrochemical-chemical (EC) redox cycling.

EXPERIMENTAL SECTION

Chemicals and Materials. Mouse monoclonal anti-CK-MB IgG (10−1363), anti-CK-MB IgG (10−1364), and CK-MB protein (30−1082) were obtained from Fitzgerald, Inc. (Acton, MA, USA). An ALP-labeling kit-NH_2 (LK12) was obtained from Dojindo Laboratories (Kumamoto, Japan). EZ-link sulfo-NHS-LC-LC-biotin was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). 1A2N was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 1A2N-P was synthesized by modification of a reported method for the synthesis of a similar product[16] [see Supporting Information (SI)]. Avidin (from egg white), ALP-conjugated polyclonal antimouse IgG (from goat), 2-aminophenol (2AP), 4AP, catechol, hydroquinone, 2-amino-1-naphthol (2A1N) hydrochloride, 4A1N hydrochloride, 1,2-dihydroxynaphthalene (12DHN), 14DHN, 4-nitrophenyl phosphate, H_3N-BH_3, H_2NNH_2 monohydrate, NADH dipotassium salt, and TCEP hydrochloride were obtained from Sigma-Aldrich Co. All reagents for the buffer solutions were obtained from Sigma-Aldrich Co. The ITO electrodes were obtained from Corning Co. (Daegu, Korea). The concentrations of CK-MB in the clinical serum samples were measured with a Cobas 6000/e601 analyzer (Roche Diagnostics, Indianapolis, IN, USA). The study protocol using real clinical samples was approved by the Institutional Review Board of Gangnam Severance Hospitals (#3−2011−0284).

The phosphate-buffered saline buffer (PBS buffer, pH 7.4) contained 10 mM phosphate-buffered saline, 138 mM NaCl, and 2.7 mM KCl. The PBSB buffer contained all of the ingredients of the PBS buffer and 1% (w/v) bovine serum albumin (BSA). Tris buffer (pH 8.0) contained 50 mM tris(hydroxymethyl)aminomethane and 10 mM $MgCl_2$, and the pH was adjusted by adding concentrated HCl. The rinsing buffer (pH 7.6) contained 50 mM tris(hydroxymethyl)-aminomethane, 40 mM HCl, 500 mM NaCl, 0.05% (w/v) BSA, and 0.05% Tween 20.

Immunosensing Procedure. ITO electrodes were pretreated with a 5:1:1 solution of H_2O, H_2O_2 (30%), and NH_4OH (30%) at 70 °C for 1 h. To prepare the avidin-modified ITO (avidin/ITO) electrodes, 75 μL of carbonate buffer (20 mM, pH 9.6) containing 10 μg/mL avidin was dropped onto the ITO electrodes and allowed to stand for 2 h at 20 °C. Subsequently, 75 μL of PBSB buffer was dropped onto the avidin/ITO electrodes and allowed to stand for 30

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min at 4 °C to prepare the BSA/avidin/ITO electrodes. To immobilize biotinylated anti-CK-MB IgG, 75 μL of PBSB buffer containing 10 μg/mL biotinylated anti-CK-MB IgG was dropped onto the BSA/avidin/ITO electrodes and allowed to stand for 30 min at 4 °C; the electrodes were then washed with rinsing buffer. To bind CK-MB to the immunosensing electrodes, 75 μL of PBSB buffer (or human serum) containing different concentrations of CK-MB was dropped onto the immunosensing electrodes and allowed to stand for 30 min at 4 °C; the electrodes were then washed with rinsing buffer. Subsequently, 75 μL of PBSB buffer containing 10 μg/mL ALP-conjugated anti-CK-MB IgG was dropped onto the target-treated electrodes and allowed to stand for 30 min at 4 °C, followed by washing with rinsing buffer twice.

For CK-MB detection, 10 μL of tris buffer containing 5.0 mM 1A2N-P and 20 μL of tris buffer containing 100 mM H_3N-BH_3 were mixed with 970 μL of tris buffer. The mixed solution was injected into an electrochemical cell containing the immunosensing electrode. The electrochemical cells were incubated at 25 °C for 10 min. The final concentrations of 1A2N-P and H_3N-BH_3 were 50 μM and 2.0 mM, respectively.

Electrochemical Measurements. Teflon electrochemical cells were assembled using the immunosensing electrode, a Ag/AgCl (3 M NaCl) reference electrode, and a Pt counter electrode. The exposed geometric area of the immunosensing electrode was approximately 0.28 cm^2. Chronocoulometry and cyclic voltammetry were performed at 25 °C using a CHI 1040C apparatus (CH Instruments, Austin, TX, USA).

RESULTS AND DISCUSSION

Selection of a Reductant and an ALP Substrate. In our previous studies, H_2NNH_2, NADH, and TCEP were used as reductants to achieve fast EC redox cycling.[3,17,20−22,27−31] Although these compounds are strong reductants, their background current levels are low at the ITO electrodes. To compare the background current levels obtained with H_3N-BH_3 versus H_2NNH_2, NADH, and TCEP, cyclic voltammograms were acquired in the absence and the presence of each reductant (Figure 2). The cyclic voltammogram obtained

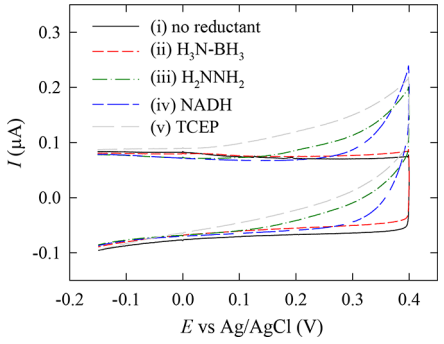

Figure 2. (a) Cyclic voltammograms recorded (at a scan rate of 20 mV/s) using bare ITO electrodes in tris buffer (pH 8.0) containing (i) no reductant, (ii) 2.0 mM H_3N-BH_3, (iii) 2.0 mM H_2NNH_2, (iv) 2.0 mM NADH, and (v) 2.0 mM TCEP.

without any reductant (curve i in Figure 2) was in good agreement with that obtained with H_3N-BH_3 (curve ii in Figure 2), implying that oxidation of H_3N-BH_3 at the ITO electrodes is very slow in the potential range where most electrooxidation-based detection is performed. In the case of H_2NNH_2, NADH, and TCEP, noticeable oxidation currents were observed in the given potential range, although the currents were not high. These results indicate that H_3N-BH_3 is better than the other reductants for obtaining low background levels. Therefore, H_3N-BH_3 was chosen as the new reductant for EC redox cycling.

To select an ALP product that adequately matches H_3N-BH_3, nine aromatic dihydroxy and aminohydroxy compounds (Figure 1a) were evaluated with the objective to achieve high signal amplification and a low background level during EC redox cycling. Figure 3 shows the cyclic voltammograms obtained at the bare ITO electrodes in tris buffer (pH 8.0) containing only the ALP product and tris buffer containing the ALP product and H_3N-BH_3. In general, the formal potentials of the diaromatic compounds (1A2N, 2A1N, 4A1N, 12DHN, and 14DHN) are lower than those of the monoaromatic compounds (2AP, catechol, 4AP, and hydroquinone). Oxidation of the monoaromatic compounds at the ITO electrodes was slow and required considerable overpotentials. Moreover, signal amplification via EC redox cycling using H_3N-BH_3 was not observed in the cases of 2AP, 4AP, and hydroquinone. Because it is better to apply a lower potential to obtain a low background level in electrooxidation-based EC redox cycling, the monoaromatic compounds were excluded as an ALP product.

It is known that para-substituted diaromatic compounds (4A1N and 14DHN) are readily oxidized by dissolved oxygen.[18,19] For this reason, in the cyclic voltammograms (Figure 3h,i), no anodic currents were observed for the para-substituted compounds. Moreover, the increase in the current due to EC redox cycling in the presence of H_3N-BH_3 was not significant. However, the ortho-substituted diaromatic compounds (1A2N, 2A1N, and 12DHN) produced significant anodic currents (Figure 3e,f,g). The increase in the current due to EC redox cycling was considerable for 1A2N and 2A1N. In particular, 1A2N exhibited good redox behavior and fast EC redox cycling. Therefore, 1A2N was chosen as the ALP product.

Signal-to-Background Ratio and Amplification Factor. To further evaluate the performance of H_3N-BH_3 and 1A2N, chronocoulograms were recorded under various conditions. Figure 4a shows the chronocoulograms obtained at 0.1 V in the presence of H_3N-BH_3 only, 1A2N only, and both H_3N-BH_3 and 1A2N. The slope obtained with the solution containing both H_3N-BH_3 and 1A2N was much steeper than that for the solutions containing H_3N-BH_3 only and 1A2N only, indicating that the EC redox cycling involving ITO electrode, 1A2N, and H_3N-BH_3 was very fast. The charge values measured from the chronocoulograms at 100 s for the four reductants were compared (Figure 4b). The charge value obtained in the presence of the reductant only corresponds to the background level, and the charge value obtained in the presence of both the reductant and 1A2N corresponds to the signal level. The calculated signal-to-background ratios for EC redox cycling were 2500, 1100, 2100, and 85 for H_3N-BH_3, H_2NNH_2, NADH, and TCEP, respectively. H_3N-BH_3 gave rise to the highest signal-to-background ratio because its signal level was very high but its background level was very low.

Figure 4c shows the charge values for the three ALP products (1A2N, 2A1N, and 4A1N). The charge value obtained in the presence of the ALP product only is mainly due to electrochemical oxidation of the same, whereas the charge value obtained in the presence of both the ALP product and H_3N-BH_3 is mainly due to EC redox cycling. Therefore, the ratio of the latter to the former corresponds to the amplification factor of the electrochemical signal due to EC redox cycling. The calculated amplification factors were 22, 16, and 5.8 for

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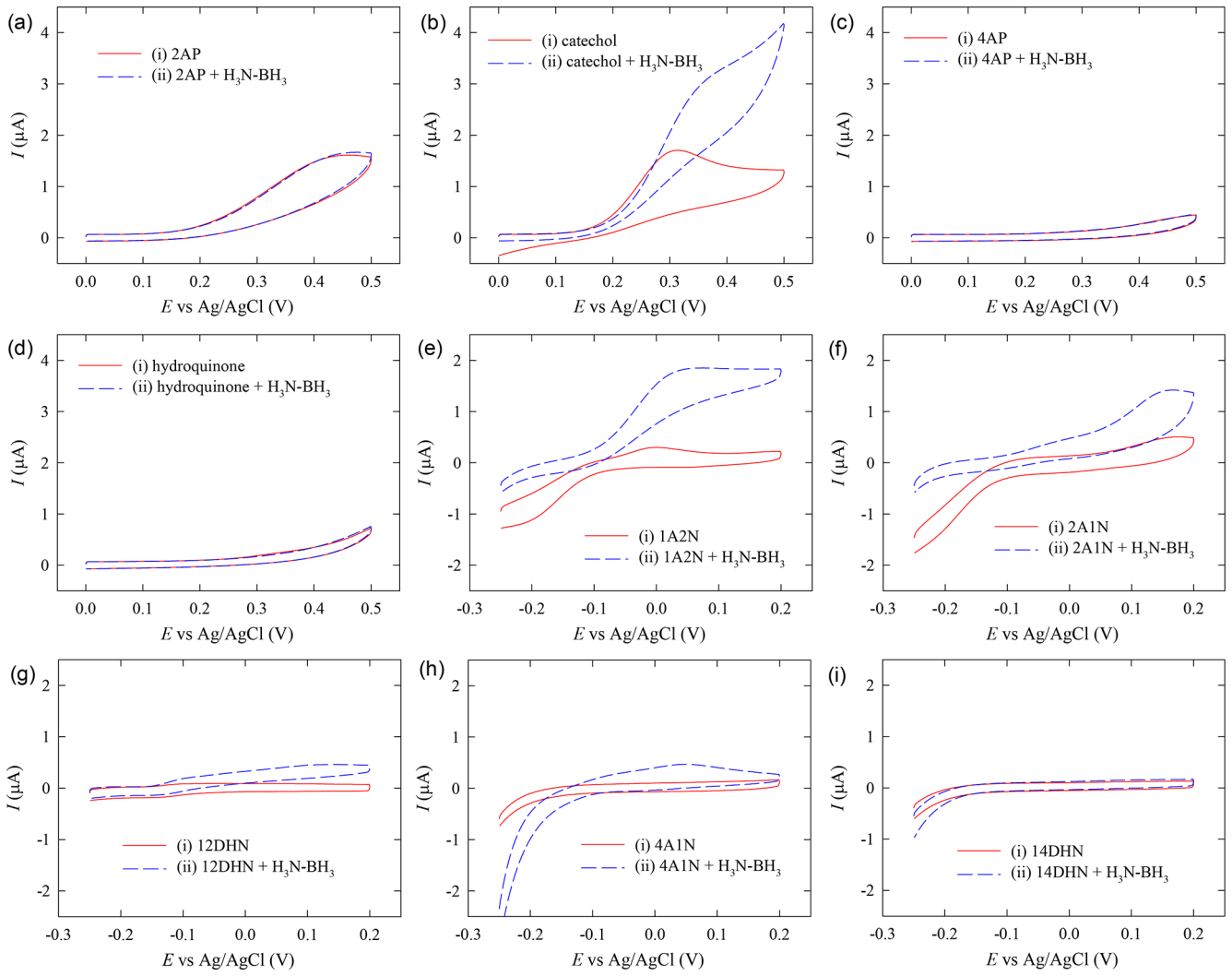

Figure 3. Cyclic voltammograms obtained (at a scan rate of 20 mV/s) using bare ITO electrodes in tris buffer (pH 8.0) containing (i) 50 μM ALP product and (ii) 50 μM ALP product and 5.0 mM H_3N-BH_3. The ALP product was (a) 2AP, (b) catechol, (c) 4AP, (d) hydroquinone, (e) 1A2N, (f) 2A1N, (g) 12DHN, (h) 4A1N, and (i) 14DHN.

1A2N, 2A1N, and 4A1N, respectively. 1A2N had the highest amplification factor as well as the highest charge value for EC redox cycling, as expected from the cyclic voltammograms in Figure 3.

The final solution for electrochemical detection in Figure 1b contained H_3N-BH_3 and 1A2N-P (ALP substrate). The charge value obtained using this solution corresponds to another background level, whereas the charge value obtained with the solution containing H_3N-BH_3, 1A2N-P, and ALP-conjugated IgG (ALP-IgG conjugate) corresponds to another signal level. The ratio of the latter to the former is related to the signal-to-background ratio for the enzymatic amplification plus the EC redox cycling. The ratios were measured at five different pH values (pH 7.5, 8.0, 8.5, 9.0, and 9.5) using three concentrations of H_3N-BH_3 (2.0, 5.0, and 10.0 mM) (SI Figure S-1). Because the signal-to-background ratio was the highest at pH 8.0, tris buffer (pH 8.0) was used for the ensuing experiments. Although the highest signal-to-background ratio was obtained with a concentration of 10.0 mM H_3N-BH_3 at pH 8.0 (SI Figure S-1b), a concentration of 2.0 mM H_3N-BH_3 was used for the ensuing experiments because it is economically and environmentally better to use lower concentrations of reagents.

The strong reductant H_3N-BH_3 may influence the enzymatic activity of ALP via reduction reactions. To check this possibility, the generation of colored 4-nitrophenol from uncolored 4-nitrophenyl phosphate by ALP was monitored in the absence and presence of H_3N-BH_3 (Figure 5a). The absorbance increased with time in both cases. The similar increasing trend shows that H_3N-BH_3 did not affect the ALP activity.

Some of the ALP substrates are slowly converted into dephosphorylated forms in air,[15] which causes high background levels. 1A2N-P may also be dephosphorylated into 1A2N. If this occurs, the background level would gradually increase with time. However, the charge values obtained six months after the synthesis of 1A2N-P were similar to those obtained just after the synthesis (Figure 5b). Moreover, the signal-to-background ratio for the enzymatic amplification plus EC redox cycling did not decrease with time (SI Figure S-2). These results clearly show that 1A2N-P was stable in air for six months. In SI Figure S-3, the color of tris buffer containing 1A2N and H_3N-BH_3 did not significantly change after 30 min and 24 h incubation, although the color of tris buffer containing 1A2N only becomes dark yellow. This result indicates that H_3N-BH_3 prevents the oxidation and polymerization of 1A2N by dissolved oxygen.

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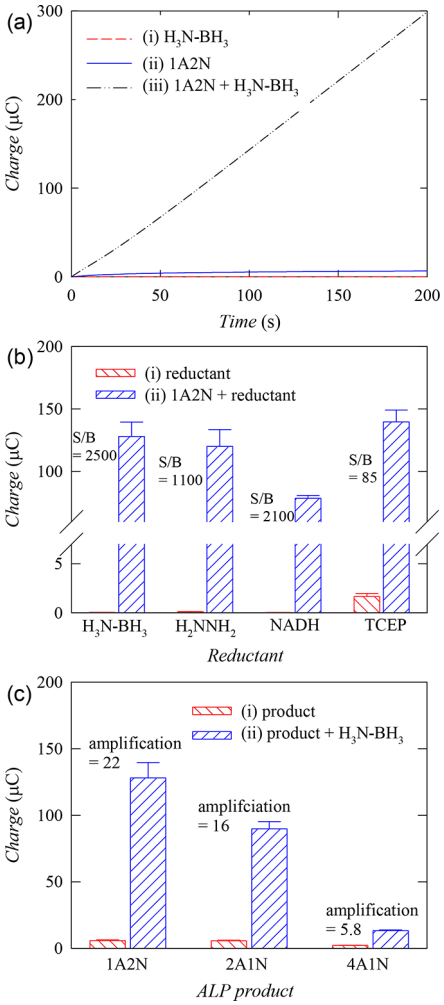

Figure 4. (a) Chronocoulograms obtained at 0.10 V using bare ITO electrodes in tris buffer (pH 8.0) containing (i) 5.0 mM H_3N-BH_3, (ii) 50 μM 1A2N, and (iii) 50 μM 1A2N and 5.0 mM H_3N-BH_3. (b) Histograms of the signal-to-background ratios (for EC redox cycling) calculated from the charge values measured at 100 s from chronocoulograms obtained at 0.10 V using bare ITO electrodes at 25 °C in tris buffer (pH 8.0) containing (i) 5.0 mM reductant and (ii) 50 μM 1A2N and 5.0 mM reductant. (c) Histograms of the amplification factors calculated from the charge values measured at 100 s from chronocoulograms obtained at 0.10 V using bare ITO electrodes at 25 °C in tris buffer (pH 8.0) containing (i) 50 μM ALP product and (ii) 50 μM ALP product and 5.0 mM H_3N-BH_3.

The rate constant for the reduction of the ALP product by H_3N-BH_3 (k in Figure 1b) can be calculated from the background-subtracted limiting current in the chronoamperogram (or chronocoulogram in Figure 4a) under the condition where the concentration of H_3N-BH_3 is much larger than the concentration of the ALP product. The limiting current is represented by[20,35]

$$I_{lim} = nFAC_{1A2N}\sqrt{DkC_{H3N\text{-}BH3}} \quad (1)$$

where C_{1A2N} and $C_{H3N\text{-}BH3}$ are the concentrations of 1A2N and H_3N-BH_3 (50 μM and 5.0 mM), respectively, and D is the diffusion coefficient of 1A2N (6.43×10^{-6} cm^2/s).[36] It is assumed that two electrons are involved in reactions ii and iii in Figure 1b. The apparent k value for the EC redox cycling using 1A2N and H_3N-BH_3 was 7.4 M^{-1} s^{-1}. Although the apparent k value was lower than the k values for the EC redox cycling using

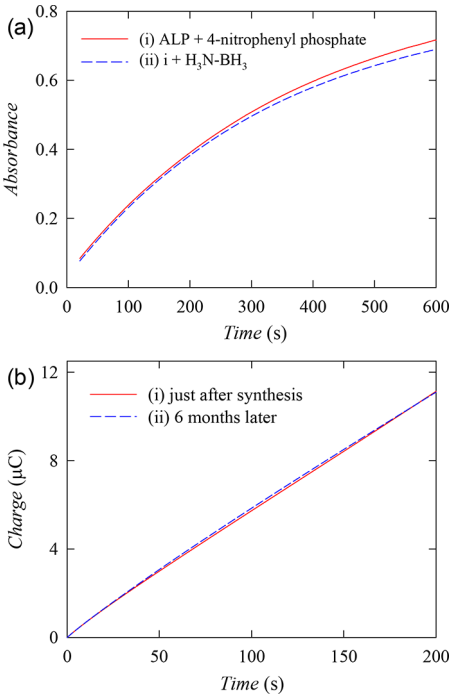

Figure 5. (a) Time-course spectra of absorbance at 400 nm at 25 °C in tris buffer (pH 8.0) containing (i) 50 μM 4-nitrophenyl phosphate and 1 μg/mL ALP-conjugated antimouse IgG and (ii) 50 μM 4-nitrophenyl phosphate, 1 μg/mL ALP-conjugated antimouse IgG, and 5.0 mM H_3N-BH_3. (b) Chronocoulograms obtained at 0.10 V using bare ITO electrodes in tris buffer (pH 8.0) containing 5.0 mM H_3N-BH_3 and 50 μM 1A2N-P. 1A2N-P was used (i) just after its synthesis and (ii) 6 months after its synthesis.

4AP and H_2NNH_2[20] and the EC redox cycling using 4AP and NADH[30] (71 and 37 M^{-1} s^{-1}, respectively), it was large enough to give high signal amplification. Because 1A2N is readily oxidized by dissolved oxygen, the real k value would be much higher than the apparent one.

CK-MB Detection. EC redox cycling using 1A2N and H_3N-BH_3 was applied to a sandwich-type electrochemical immunosensor for CK-MB detection (Figure 1b). CK-MB was captured by an anti-CK-MB IgG probe and then attached by using an ALP-IgG conjugate probe. ALP converted 1A2N-P into 1A2N over an incubation period (reaction i in Figure 1b). Finally, 1A2N was electrochemically oxidized at the ITO electrode, and EC redox cycling was induced (reactions ii and iii of Figure 1b) when 1A2N was electrochemically oxidized. Redox cycling combined with enzymatic amplification allows high electrochemical signals to be obtained. In our previous studies, the nonspecific binding of the ALP-IgG conjugate on avidin- and BSA-modified ITO electrodes was low enough to achieve ultrasensitive detection.[3,28]

Figure 6a shows the chronocoulograms obtained at the immunosensing electrodes for various concentrations of CK-MB. The charge slope increased with an increase in the concentration of CK-MB. Figure 6b shows the calibration curve obtained from the charge values measured at 100 s. CK-MB was detected over a very wide range of concentrations. The detection limit from the calibration curve was ~80 fg/mL, indicating that the immunosensor is ultrasensitive. Importantly, when the detection was carried out without EC redox cycling using H_3N-BH_3, the detection limit was ~40 pg/mL (SI Figure S-4), which was much higher than that with EC redox cycling.

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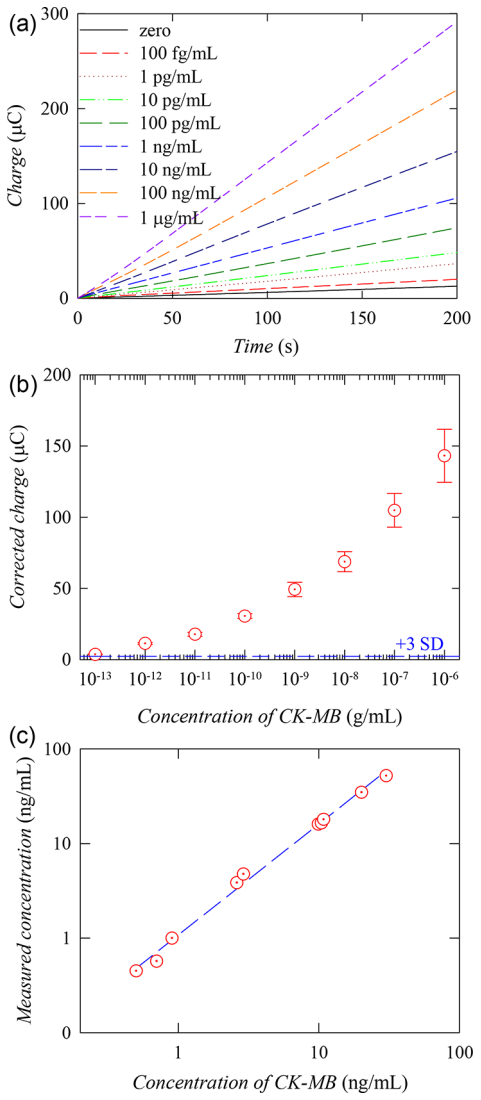

Figure 6. (a) Chronocoulograms obtained at 0.10 V using immunosensing electrodes and various concentrations of CK-MB in PBSB after an incubation period of 10 min at 25 °C in tris buffer (pH 8.0) containing 50 μM 1A2N-P and 2.0 mM H_3N-BH_3. (b) Calibration curve for the charge values measured at 100 s from chronocoulograms in panel a. All experiments were conducted using three different electrodes for each sample. All data were subtracted from the mean value at a concentration of zero, determined from seven measurements. The dashed line corresponds to three times the standard deviation (SD) of the charge value at a concentration of zero. The error bars represent the SD of three measurements. (c) Comparison of the concentration of CK-MB measured with the immunosensor and the concentration of CK-MB measured with a commercial instrument for 10 actual clinical serum samples.

To evaluate the performance of the immunosensor based on the new EC redox cycling, the concentration of CK-MB in clinical serum samples was assayed. The chronocoulograms for ten clinical serum samples are presented in SI Figure S-5. A comparison between the actual concentration of CK-MB and the concentration of CK-MB calculated using the calibration curve in Figure 6b is shown in Figure 6c. Both concentrations were in good agreement. Overall, the developed immunosensor was accurate as well as ultrasensitive.

CONCLUSIONS

We have shown that stable, solid 1A2N-P and H_3N-BH_3 can be used as an ALP substrate and a strong reductant for EC redox cycling, respectively. Among the four reductants (H_3N-BH_3, H_2NNH_2, NADH, and TCEP), H_3N-BH_3 gave rise to the lowest background currents. EC redox cycling involving 1A2N and H_3N-BH_3 produced very high signal amplification. The oxidation and polymerization of 1A2N by dissolved oxygen is significantly prevented in the presence of H_3N-BH_3. The electrochemical measurement was performed without modification of the ITO electrodes with electrocatalytic materials. When the newly developed combination was applied to the detection of CK-MB, the detection limit for CK-MB was ~80 fg/mL, indicating that the combination allows ultrasensitive detection. Assay of the concentration of CK-MB in clinical serum samples produced values that were in good agreement with the concentrations obtained using a commercial instrument. Thus, the use of stable, solid 1A2N-P and H_3N-BH_3 along with bare ITO electrodes is highly promising for ultrasensitive and simple point-of-care testing.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.7b00407.

Dependence of signal and background levels on pH, air stability of 1A2N-P, solution stability of 1A2N, and CK-MB detection without using EC redox cycling (PDF)

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Notes

The authors declare no competing financial interest.

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