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Signal Amplification in Enzyme-Based Amperometric Biosensors

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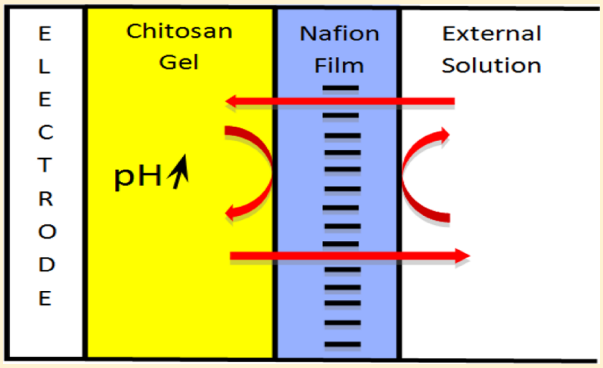
Supporting Information

ABSTRACT: A unique mode of current amplification was investigated in reagentless biosensors based on the clinically significant enzymes including alcohol dehydrogenase, glucose 6-phosphate dehydrogenase, glycerol 3-phosphate dehydrogenase, and glucose oxidase. The biosensors were designed by sandwiching the enzyme–polymer film between an electrode and Nafion film. In particular, each enzyme and its cofactor were covalently attached to the chains of polysaccharide chitosan and mixed with carbon nanotubes on the electrode surface. The coating of such biosensors with Nafion resulted in the current increase by up to 1000%, depending on the enzyme. The results were analyzed considering the interplay between the enzyme activity–pH profiles and the Nafion-induced pH increase in the underlying chitosan film. The data were collected by using the rapid (<5 min) amperometric enzyme assays and pH-sensitive iridium oxide films. The increase in the biosensor current was attributed to the pH-driven increase in the enzyme activity inside the two-film interface. Such signal amplification should also be feasible in other biosensors based on the polyelectrolytes and sandwiched enzymes providing that a proper match is made between the enzyme activity–pH profiles and the pH of buffer solutions.



Recently, a large Nafion-induced current amplification has been observed in dehydrogenase-based biosensors for the hydrophilic neutral analytes.[1−3] This unique phenomenon has the potential to benefit the development of sensitive analytical devices. However, the mechanism of signal amplification has been uncertain. It could not be explained by the well-known ability of Nafion to augment sensor's current via the analyte preconcentration.[4] In the absence of preconcentration, the polymeric films such as Nafion typically attenuate, rather than amplify, sensors' current by impeding the transport of species participating in the electrode processes.

It has been proposed that the signal amplification was due to the Nafion-induced changes around a sandwiched enzyme, which stimulated its enzymatic activity resulting in the increase in biosensor's current.[1] This hypothesis is tested in the present paper by studying the reagentless biosensors based on the alcohol dehydrogenase (ADH), glucose 6-phosphate dehydrogenase (G6pDH), glycerol 3-phosphate dehydrogenase (G3pDH), and glucose oxidase (GOx). These are important enzymes because they serve as biomarkers (ADH),[5] participate in the prevention of oxidative damage (G6pDH),[6] play a role in lipid metabolism (G3pDH),[7] and act as food preservatives (GOx).[8] The substrates of these enzymes are also of interest because they are involved in several major metabolic pathways.

The present studies focused on the role of enzyme activity–pH profile in the amplification of current flowing through a two-film chitosan/Nafion interface containing an enzyme. The activity–pH profiles of the four enzymes were determined by using a newly developed internally calibrated electrochemical continuous enzyme assay (ICECEA). The ICECEA allowed one to rapidly quantify the enzyme activity with no need for the enzyme-based calibration. The Nafion-induced changes in the underlying chitosan film were probed by using a pH-sensitive iridium oxide electrode.

EXPERIMENTAL SECTION

Reagents. Several chemicals were purchased from Sigma-Aldrich including chitosan (CHIT, MW ∼ 1×10^6 Da; ∼80% deacetylation), nicotinamide adenine dinucleotide (NAD^+, λ = 259 nm), dihydronicotinamide adenine dinucleotide (NADH), glutaric dialdehyde (GDI, 25 wt % solution), nicotinamide adenine dinucleotide phosphate ($NADP^+$) and its reduced form NADPH, iodoacetic acid, *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), Nafion (5.0 wt %, acid form), glucose 6-phosphate, glycerol 3-phosphate, D(+)-glucose, ethanol, alcohol dehydrogenase (ADH, from *Saccharomyces cerevisiae*, EC 1.1.1.1, G7141, 285 units mg^{-1}), glucose 6-phosphate dehydrogenase (G6pDH, from *Leuconostoc mesenteroides*, EC 1.1.1.49, G5885, 143 units mg^{-1}), *sn*-glycerol-3-phosphate dehydrogenase (G3pDH, from *rabbit muscle*, EC 1.1.1.8, G6880, 175 units mg^{-1}), and glucose oxidase (GOx, from *Aspergillus niger*, EC 1.1.3.4, G7141, 138.8 U mg^{-1}). The multiwalled carbon nanotubes (CNT, ∼95% nominal purity) were purchased from Nanolab (Brighton, MA).

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The Na_3IrCl_6 (Ir 31.9%) was purchased from Alfa Aesar. The $NaH_2PO_4{\cdot}H_2O$, Na_2HPO_4, HCl, NaOH, acetic acid, boric acid, sodium citrate, L-tartaric acid, sodium phthalate, and phosphoric acid were from Fisher. All solutions were prepared by using water that was purified with a Barnstead NANOpure cartridge system.

Electrochemical Measurements. The electrochemical data were collected using a CHI 832B workstation (CH Instruments, Inc.). The experiments were conducted in a conventional three-electrode system with a 3.0 mm-diameter glassy carbon (GC) or 1.6 mm platinum (Pt) disk working electrode (Bioanalytical Systems, Inc.), platinum wire auxiliary electrode, and Ag/AgCl/3 M NaCl (BAS) reference electrode. Prior to use, the working electrodes were wet polished on an Alpha A polishing cloth (Mark V Lab) with successively smaller particles (0.3 and 0.05 μm diameter) of alumina. The slurry that accumulated on the electrode surface was removed by a 30 s ultrasonication in deionized water and methanol.

Experiments were repeated at least three times, and results are presented with the relative standard deviation (RSD). All experiments were performed in 0.050 M buffer solutions unless stated otherwise. All experiments were performed at room temperature (20 ± 1 °C).

Preparation of CHIT, CHIT−CNT, CHIT−GDI, and CHIT−NAD$^+$ Solutions. The CHIT solutions were prepared by dissolving chitosan flakes in a hot 0.10 M HCl solution (90 °C), cooling to room temperature, adjusting their pH to 4.1 with a NaOH solution, and filtering with a 0.45 μm Millex-HA syringe filter unit (Millipore). The CHIT−CNT colloidal suspensions were prepared by the 15 min sonication of CNT (1.0 mg mL^{-1}) in 0.10 wt % CHIT solution.[9]

The CHIT−GDI solutions were synthesized by reacting 0.10 wt % CHIT solution with the excess of GDI (200:1 molar ratio of GDI to chitosan glucosamine units) and purifying the CHIT−GDI product by dialysis.[10] Such conditions favored the reaction of only one of the aldehyde groups of GDI with amino groups of CHIT leaving the other aldehyde group available for the subsequent reaction with amino groups of enzymes.[3] The latter resulted in the covalent attachment of enzyme molecules to CHIT chains.

The CHIT−NAD$^+$ solutions were prepared in two steps following our previous synthetic protocol.[3,11] The *N*′-carboxymethyl−NAD$^+$ was synthesized first by reacting the aqueous solutions of NAD$^+$ (2.0 g in 15 mL) and iodoacetic acid (4.0 g in 20 mL). The pH of the mixture was adjusted to 6.5 with a 3.0 M NaOH solution and monitored and kept constant during the first few hours. Then, the reaction was allowed to continue in the dark for 24 days at room temperature. The initially colorless solution turned orange red during that time. Next, the mixture was acidified to pH 3.0 with a 3.0 M HCl solution and poured into 10-fold excess of chilled ethanol (−20 °C) to form a precipitate overnight. The precipitate was filtered, washed three times with ethanol, and vacuum-dried to produce a pink powder. In step two, 0.20 g of *N*′-carboxymethyl−NAD$^+$ pink powder was dissolved in 10 mL of water and mixed with 10 mL of 0.20 wt % CHIT solution, which was followed by the slow addition of 0.20 g of EDC catalyst. The catalyst facilitated the reaction between the carboxylic group of modified NAD$^+$ and amino group of CHIT leading to the covalent attachment of NAD$^+$ to CHIT chains. During the first 3 h of the reaction, the pH was monitored and kept at 4.7 by using a 0.50 M HCl solution. After 12 h at room temperature, the product was purified by dialysis using four 1 L batches of pH 4.5 HCl solution (12 h each). The removal of unreacted species was monitored by following a decrease in the intensity of adenine absorption band of the cofactor at 259 nm in the dialysate.

All biosensors were made by using freshly prepared solutions of chemically modified chitosan CHIT−GDI and CHIT−NAD$^+$. It should be mentioned however that such solutions were usable even after 8 years of storage in a refrigerator. For example, the CHIT−GDI and CHIT−NAD$^+$ solutions from 2005 to 2006 yielded active amperometric biosensors. All solutions were stored at 4 °C in a refrigerator when not in use.

Amperometric Enzyme Assay. The internally calibrated electrochemical continuous enzyme assay (ICECEA) was used to determine the activity of enzymes.[12] The assay relied on the measurement of current flowing through a working electrode that was immersed in a stirred solution containing enzyme's substrate and cofactor. Spiking the solution with an aliquot of enzyme triggered an enzymatic reaction, which produced redox active species. The oxidation of these species at a working electrode generated a current−time trace with a slope that was proportional to the activity of enzyme. The known aliquots of the same redox active species were spiked into the solution to calibrate the measurements of current. The ICECEA of dehydrogenase enzymes was performed at the GC electrodes that were coated with CNT by casting 20.0 μL of CHIT−CNT colloidal suspensions and evaporating water for 1 h. The assays of glucose oxidase were performed at a platinum electrode. The assays were performed in the pH range of 6.0−9.0 and 4.0−8.0 for the dehydrogenases and glucose oxidase, respectively. The assay validity was ensured by using an enzyme as a limiting reagent, working within the linear range of a calibration plot, and measuring the current−time slope only at short times (<60 s).

Preparation of Biosensors. The dehydrogenase-based biosensors were prepared by mixing the aliquots of CHIT−NAD$^+$, enzyme, CHIT−GDI, and CHIT−CNT solutions on the surface of GC electrodes and evaporating water to form well-adhering surface films. The CHIT−NAD$^+$ solution was used for all three dehydrogenases, including the G6pDH, because this enzyme is able to utilize both the NAD$^+$ and NADP$^+$ cofactors.[13] The composition of the films was optimized in order to generate the maximum of faradaic current. The CNT were incorporated in the films in order to facilitate the electrooxidation of NADH, which is produced in the enzymatic reactions of dehydrogenases.

The ADH- and G6pDH-based biosensors were prepared by first mixing 30.0 μL of CHIT−NAD$^+$ solution with 5.0 μL of ADH (23.6 mg mL^{-1}) or G6pDH (23.7 mg mL^{-1}) solution in water. The mixture was allowed to equilibrate for 30 min in order to establish the enzyme-cofactor bioaffinity interactions, which were subsequently secured by cross-linking with 20.0 μL of 0.10 wt % CHIT−GDI solution. The mixture was kept in liquid state by adding a 20 μL aliquot of water, if necessary. This was followed by admixing 10.0 μL of CNT suspension (1.0 mg mL^{-1}) in 0.10 wt % CHIT solution. The surface films were cured at room temperature for 4 h and soaked in a stirred pH 7.40 phosphate buffer solution for 1 h in order to remove any unreacted components.

The G3pDH-based biosensors were prepared differently because the mixing of G3pDH and CHIT−NAD$^+$ solutions resulted in the precipitation of enzyme. Such ionotropic gelation of enzyme by chitosan has been observed before.[14,15] In order to maximize the dispersion of all components, the 30.0

μL of CHIT−NAD^+ solution and 10.0 μL of CNT suspension (1.0 mg mL^{-1}) in 0.10 wt % CHIT solution were mixed first. Then, the mixture was blended with 5.0 μL of G3pDH aqueous solution (19.4 mg mL^{-1}). The cross-linking solution CHIT−GDI was not used because the enzyme was firmly encapsulated in the surface film by gelation with chitosan. After curing the films at room temperature for 4 h, they were soaked in a stirred pH 7.40 phosphate buffer solution for 30 min in order to remove any unreacted components.

The GOx-based films were prepared on platinum electrodes, which facilitate the electrooxidation of H_2O_2 that is produced by the enzymatic reaction of GOx. They were made by mixing 15.0 μL of CHIT−GDI, 10.0 μL of CNT suspension (1.0 mg mL^{-1}) in 0.10 wt % CHIT solution, and 5.0 μL of GOx solution (500 U mL^{-1}) on the electrode surface. The films were cured at room temperature for 3 h and soaked in a stirred pH 7.40 phosphate buffer solution for 30 min.

All of the biosensors contained CNT unless stated otherwise. The biosensors without the CNT were prepared by substituting a CNT suspension in CHIT solution with a CHIT solution.

The film electrodes had to be soaked in a buffer solution before coating them with a Nafion film because the coating of air-dried film electrodes with Nafion did not yield any signal amplification. The same buffer solution was used to soak a film electrode and record voltammograms before and after coating it with Nafion. In a typical experiment, a film electrode was soaked in a selected buffer solution for 30 min and its amperometric response (I) to 10.0 mM enzyme substrate was recorded. Next, the electrode was removed from the solution and rinsed with water for 5 s to remove drops of glucose solution. Briefly touching a tissue to a side of the electrode drained the excess of water. Then, the film electrode was turned upside down, coated with a 20 μL aliquot of 5.0 wt % Nafion solution, and left to dry at room temperature for 1 h. The Nafion-coated electrode was subsequently soaked in the same buffer solution as before (30 min), and its current response (I_{Nafion}) to 10.0 mM enzyme substrate was recorded.

Measurements of Nafion-Induced pH Change in Chitosan Films. The pH was measured by casting a CHIT−CNT film on the surface of pH-sensitive iridium oxide (IrOx) electrode and measuring its open circuit potential E_{OCP}. The IrOx electrode was made by electroplating an IrOx film on the surface of GC electrode. The IrOx film was formed by a constant-potential electrodeposition (1.20 V, 15 min) from an aqueous solution of 0.20 mM Na_3IrCl_6. The latter was aged for 2 h at 80 °C in a closed vial before use. This was done to advance the aquation and hydrolysis of $IrCl_6^{3-}$ ions, which facilitated the electrodeposition of IrOx.[16]

The CHIT−CNT film was cast on the IrOx electrode by applying 20.0 μL of CNT suspension (1.0 mg mL^{-1}) in 0.10 wt % CHIT solution and evaporating water for 2 h at room temperature. The effect of Nafion overcoat on the pH inside the CHIT−CNT film was determined by performing the sequence of measurements. First, a calibration plot of E_{OCP} vs pH was prepared by measuring the E_{OCP} of IrOx/CHIT−CNT film electrode in a stirred buffer solution in the pH range of 6.0−8.0 or 4.0−6.0. Several E_{OCP}−pH data points were collected by titrating the solution with a 1.0 M HCl solution. Next, ten 5 min long E_{OCP}−time traces were recorded at the same electrode in a stirred phosphate buffer solution at a fixed pH of 4.00, 6.00, 7.00, 7.40, 8.00, and 9.00. The multiple traces were collected in order to assess the extent of potential drift and determine the value of E_{OCP} before the application of Nafion overcoat. The same wet film electrode was then coated with Nafion film and ten 5 min E_{OCP}−time traces were recorded again. The values of E_{OCP} before and after the application of Nafion yielded the ΔE_{OCP}, which was then recalculated into the ΔpH by using the slope of E_{OCP}−pH calibration plot.

RESULTS AND DISCUSSION

Nafion-Induced Signal Amplification in Reagentless Biosensors. The investigated biosensors can be represented by two interfaces

$$\text{electrode/chitosan} - \text{enzyme} - \text{cofactor} - \text{CNT/solution} \quad (1)$$

$$\text{electrode/chitosan} - \text{enzyme} - \text{cofactor} - \text{CNT/Nafion/solution} \quad (2)$$

The interface 1 represents an electrode (glassy carbon or platinum) that was coated with a hybrid film composed of chitosan with covalently attached enzyme and its cofactor (NAD^+) and dispersed carbon nanotubes (CNT). The interface 2 is the interface 1 that was covered with a Nafion film. For a given enzyme−cofactor pair, the only difference between the two interfaces was the presence or absence of Nafion. The interfaces were made by using the NAD^+-dependent dehydrogenase enzymes (alcohol dehydrogenase (ADH), glucose 6-phosphate dehydrogenase (G6pDH), and glycerol 3-phosphate dehydrogenase (G3pDH)) and glucose oxidase (GOx).

Figure 1 shows the amperometric traces that were recorded at four biosensors responding to the addition of relevant enzyme substrate to a pH 7.40 phosphate buffer solution before

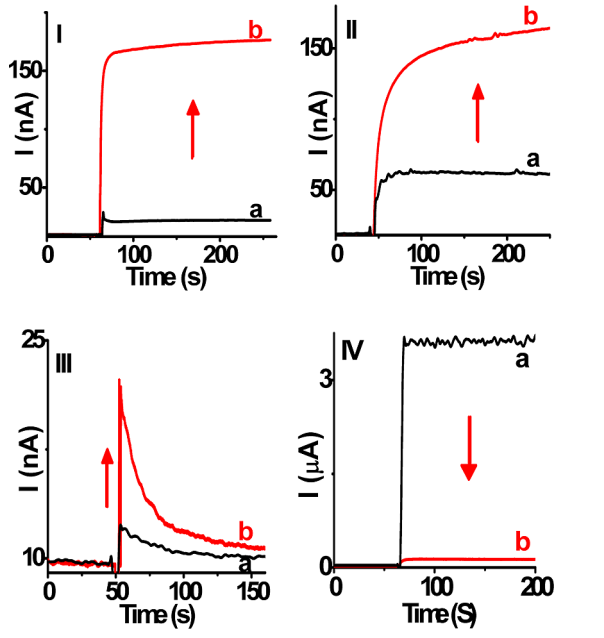

Figure 1. Amperometric traces recorded at the biosensors based on enzymes (I) ADH, (II) G6pDH, (III) G3pDH, and (IV) GOx: (a) before and (b) after coating a biosensor with a Nafion film. The current steps are due to the addition of 10.0 mM enzyme substrate (I) ethanol, (II) D-glucose 6-phosphate, (III) glycerol 3-phosphate, and (IV) β-D-glucose to a stirred pH 7.40 phosphate buffer solution (0.050 M). Potential, 0.40 V for panels I−III and 0.50 V for panel IV.

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(interface 1, traces a) and after (interface 2, traces b) the application of Nafion film. The Nafion dramatically increased the amount of current flowing through the interface 2 containing dehydrogenase enzymes. The ratio of current in the presence and absence of Nafion I_{Nafion}/I was equal to 11, 3, and 4 (±15%), which constituted a 1000%, 200%, and 300% signal increase in the case of ADH-, G6pDH-, and G3pDH-based biosensors, respectively (Figure 1, panels I–III). The G3pDH-based biosensor generated the smallest current that decayed in time, which may be related to the charge transport limitations in the only biosensor that was prepared by enzyme precipitation. The Nafion-induced signal amplification could not be explained by the preconcentration of enzyme substrates in the biosensors. For example, the anodic stripping voltammetry showed that the neutral ethanol molecules did not preconcentrate in the ADH-based biosensor (Figure S1, Supporting Information).

The two other substrates, glucose 6-phosphate (pK_{a2} 6.1)[17] and glycerol 3-phosphate (pK_a 6.7),[17] exist mostly in the anionic form at pH 7.40 and should be partially rejected, rather then preconcentrated, by the negatively charged Nafion film. Such rejection probably contributed to the lower values of current ratio I_{Nafion}/I for the G6pDH- and G3pDH-based biosensors. In contrast to three dehydrogenase-based biosensors, the application of Nafion film to a GOx-based biosensor resulted in a dramatic signal decrease by 97% (I_{Nafion}/I = 0.03, Figure 1, panel IV).

One can hypothesize that the Nafion-induced changes in the biosensor current shown in Figure 1 are due to a difference in the activity of enzyme between the interfaces 1 and 2. The activity of enzymes is known to be sensitive to the properties of their environment including the pH. In order to test this hypothesis, the two sets of data were collected: (i) the enzyme activity–pH profiles and (ii) Nafion effect on the pH of underlying chitosan film. The expectation was that the Nafion-induced shift in pH toward the apex of the activity–pH volcano profile would increase the enzyme activity in the interface 2 and, thus, amplify the biosensor current.

Enzyme Activity–pH Profiles. The enzyme activity was determined by measuring the initial rate of a relevant enzymatic reaction by using the ICECEA. The activity was quantified by determining the activity unit (U), which was defined as the amount of enzyme's substrate that was converted to a product per unit time (1 U = 1 μmole min^{-1}). The ICECEA involved the use of three solutions: (A) a buffer solution containing enzyme's substrate and cofactor (if necessary), (B) a solution of redox active species that were generated in an enzymatic reaction, and (C) a solution of assayed enzyme. The assay was composed of two phases that were performed successively in the same solution and at the same working electrode, which greatly improved the accuracy and precision of activity determination (RSD < 3%). The electrode was held at a potential that was suitable for the oxidation of species that were present in the solution B. In the first phase, the three known aliquots of solution B were added to a stirred solution A in order to record the three current steps that were used to calculate a calibration slope (CS). This was followed by the second phase in which a solution C was added to the mixture A + B in order to trigger the enzymatic reaction and record a linear ascending current–time segment. The slope of this segment was a measure of the initial reaction rate and was called an assay slope (AS). The enzyme activity unit per liter of solution (U L^{-1} = μmole min^{-1} L^{-1} = μM min^{-1}) was calculated by substituting the slopes AS and CS into the equation

$$\text{activity unit } (\mu\text{M min}^{-1}) = \frac{\text{AS } (\mu\text{A s}^{-1}) \times 60 \text{ (s min}^{-1})}{\text{CS } (\mu\text{A } \mu\text{M}^{-1})} \quad (3)$$

The enzyme activity–pH profiles for ADH, G6pDH, G3pDH, and GOx were determined by measuring the initial rates of their enzymatic reactions

$$\text{ethanol} + \text{NAD}^+ \xrightarrow{\text{ADH}} \text{acetylaldehyde} + \text{NADH} + \text{H}^+ \quad (4)$$

$$\text{D-glucose 6-phosphate} + \text{NAD}^+ \xrightarrow{\text{G6pDH}} \text{6-phospho-1,5-lactone} + \text{NADH} + \text{H}^+ \quad (5)$$

$$\text{glycerol 3-phosphate} + \text{NAD}^+ \xrightarrow{\text{G3pdH}} \text{dihydroxyacetonephosphate} + \text{NADH} + \text{H}^+ \quad (6)$$

$$\beta\text{-D-glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{GOx}} \text{glucono-1,5-lactone} + \text{H}_2\text{O}_2 \quad (7)$$

at different pH and using solutions A, B, and C, which contained the relevant reactants and products of reactions 4–7. The composition and concentration of these solutions (Table 1) were such that the enzyme was always a limiting reagent and the current measurements were within the linear range of the calibration plot for each assay.

In a typical experiment, a stirred solution A (10.0 mL) was spiked with three 10 μL aliquots of solution B and one 5.0 μL or 10.0 μL aliquot of solution C in succession. This generated the characteristic ICECEA current–time traces (Figure 2). The current steps in Figure 2 are due to the oxidation of known amounts of either NADH or H_2O_2 that were added with the aliquots of solution B. The current steps were followed by the ascending current–time segment, which was due to the oxidation of NADH or H_2O_2 that was generated in the enzymatic reactions 4–7. The latter was triggered by the addition of the enzyme-containing aliquots of solution C to the mixture. The numerical values of slopes AC and CS and enzyme activity units U are shown in Table S1, Supporting Information.

Figure 3 shows the enzyme activity–pH plots for the enzymes ADH, G6pDH, G3pDH, and GOx. The plots reveal a large pH effect on the enzymatic activity of all four enzymes. The activity–pH profiles have a volcano shape showing the maximum of enzyme activity at pH 8.0 and 5.0 for dehydrogenases and GOx, respectively.

Nafion Effect on the pH of Underlying Chitosan Film. The pH-sensitive IrOx electrode was used to measure the difference in a local pH inside the CHIT–CNT film before and after the application of Nafion overcoat (ΔpH). The CHIT–CNT film was used instead of a whole film in order to avoid interferences from the mixed potential generated by the NAD^+/NADH cofactor couple. Figure 4 shows that the open circuit potential E_{OCP} of the IrOx/CHIT–CNT film electrode shifted toward the less positive values, irrespective of the solution pH, after the application of Nafion overcoat. Such shift in E_{OCP} indicated the increase in the pH of underlying chitosan film because the calibration plots E_{OCP} vs pH had a negative slope.

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Table 1. Solutions A, B, and C Used in the ICECEA of Four Enzymes[a]

enzyme	solution A	solution B	solution C	electrode reaction
alcohol dehydrogenase (ADH)	0.56 M ethanol + 7.50 mM NAD^+ in 0.050 M BR (pH 6.0–9.0)	1.0 mM NADH in water	7.50 U mL^{-1} ADH in water	oxidation of NADH at CNT
glucose 6-phosphate dehydrogenase (G6pDH)	3.0 mM D-glucose 6-phosphate + 1.0 mM $NADP^+$ in 0.050 M BR (pH 6.0–9.0)	5.0 mM NADPH in water	250 U mL^{-1} G6pDH in water	oxidation of NADPH at CNT
glycerol 3-phosphate dehydrogenase (G3pDH)	10 mM glycerol 3-phosphate + 0.50 mM NAD^+ in 0.050 M BR (pH 6.0–9.0)	1.0 mM NADH in water	27.39 U mL^{-1} G3pDH in water	oxidation of NADH at CNT
glucose oxidase (GOx)	0.10 M glucose in 0.050 M BR (pH 4.0–8.0)	2.0 mM H_2O_2 in water	40.0 U mL^{-1} GOx in water	oxidation of H_2O_2 at Pt

[a]BR, Britton Robinson buffer.

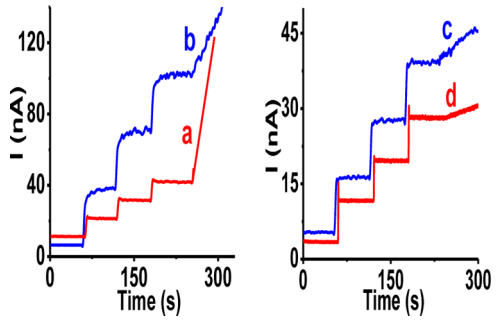

Figure 2. ICECEA traces for enzymes (a) ADH, (b) G6pDH, (c) G3pDH, and (d) GOx. The traces a–c were recorded at a GC/CHIT–CNT film electrode at 0.40 V. The trace d was recorded at a Pt electrode at 0.50 V. Background electrolyte, stirred pH 7.40 Britton Robinson buffer (0.050 M). Solutions A, B, and C (Table 1) were used in these assays.

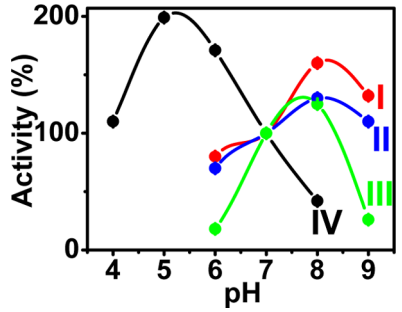

Figure 3. Enzyme activity–pH profiles for (I) ADH, (II) G6pDH, (III) G3-pDH, and (IV) GOx. Background electrolyte, 0.050 M Britton Robinson buffer. The error bars (±1 RSD) are smaller than the symbols. The plots are normalized by setting the activity unit at pH 7.00 as 100%.

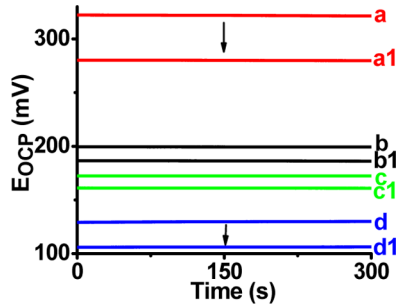

Figure 4. Open circuit potential E_{OCP} vs time traces measured at an IrOx/CHIT–CNT film electrode (a–d) before and (a1–d1) after coating it with a Nafion film. Background electrolyte, stirred 0.050 M phosphate buffer solution at pH (a, a1: red) 6.00, (b, b1: black) 7.00, (c, c1: green) 8.00, and (d, d1: blue) 9.00.

The increase in pH was observed even though the acidic form of Nafion was used to coat the film electrodes. The values of ΔpH were calculated by using the shift in E_{OCP} (Figure 4) and the slope of a calibration plot E_{OCP} vs pH (Table S2, Supporting Information). The ΔpH was equal to 0.50, 0.40, 0.30, and 0.30 pH unit (±20%) at pH 6.00, 7.00. 8.00, and 9.00, respectively.

The increase in pH beneath the Nafion film can be explained by a better retention of basic (HPO_4^{2-}) and then acidic ($H_2PO_4^-$) buffering species in the chitosan film. One can hypothesize that the Nafion overcoat precludes the escape of

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dianion more than that of monoanion from the underlying chitosan via the Donnan exclusion principle. This should lead to the increase in the concentration ratio $[HPO_4^{2-}]/[H_2PO_4^-]$ in the underlying chitosan film and, thus, to its higher pH. Such pH increase can be generally expected in the case of buffers based on the polyprotic acids such as the phosphoric acid. This is because the basic species of such buffers are always more negatively charged than the conjugate acids and, therefore, they should experience stronger electrostatic rejection by the anionic Nafion overcoat. The control experiment with a pH 7.40 Tris buffer trapped beneath the Nafion overcoat showed no Nafion effect on the internal pH and biosensor current.[1] This implied the importance of negative charge on both the basic and acidic buffering species in the observed phenomena.

The signal amplification was further tested by using a concentrated 1.0 M instead of diluted 0.050 M phosphate buffer solution. With high ionic strength on both sides of Nafion film, the Donnan exclusion principle should break resulting in the cancellation of signal amplification. However, the experiments with the ADH-based biosensor in a 1.0 M phosphate buffer solution at pH 7.40 showed that this was not the case. Under such conditions, the pH beneath the Nafion film increased by 0.19 pH unit and, accordingly, the signal amplification persisted although at a lower level of 350% (Figure S2, Supporting Information) instead of 1000% that was observed in the diluted buffer solution. Such persistence of a Nafion-induced increase in pH may be ascribed to a difference in the ionic strength on both sides of Nafion film. The internal phase beneath Nafion is made of a polycationic chitosan gel with embedded carbon nanotubes, polyanionic enzyme molecules, and enzyme cofactor that are all covalently cross-linked and cannot cross the Nafion barrier. The actual ionic strength of such cross-linked gel is not known. However, one can speculate that the mobile ions in such gel experience a dielectric permittivity that is lower than that of the outer solution. The lower dielectric permittivity of gel should promote the formation of ion pairs, which decrease the net ionic charge leading to lower ionic strength. Under such condition, the Donnan dialysis transferring protons into the outer solution could also contribute to the increase in internal pH and corresponding biosensor signal.

The CNT were a necessary component of the surface films in order to observe the Nafion effect on the pH inside the biosensors. Without the CNT, the Nafion-induced ΔpH was very small (Figure S3, Supporting Information). Interestingly, this correlated with small signal amplification in a CNT-free ADH-based biosensor, which was only 80% (Figure S4, Supporting Information) vs 1000% amplification seen in the ADH-based biosensor containing CNT (Figure 1, panel I). The role of CNT in determining the ΔpH and signal amplification is not clear at present, but they may be preventing chitosan films from collapsing under a Nafion overcoat and, thus, facilitating the charge transport in the biosensors.

Amplification Factor. The amperograms shown in Figure 1 were collected in a diluted pH 7.40 phosphate buffer solution (0.050 M) in which the Nafion-induced pH increase in the underlying chitosan film was equal to 0.40 pH unit. Thus, the changes in these amperograms were associated with the increase in a local pH inside the biosensors from 7.40 to 7.80. According to the activity−pH profiles in Figure 3, the change in pH from 7.40 to 7.80 resulted in the increase in the activity of dehydrogenases (profiles I−III) and decrease in the activity of GOx (profile IV). Figure 1 shows that this correlated with the Nafion-induced signal amplification in dehydrogenase-based biosensors (panels I−III) and signal attenuation in a GOx-based biosensor (panel IV).

The more quantitative correlation between the signal amplification in dehydrogenase-based biosensors and enzyme activity is shown in Figure 5 by plotting the current ratio I_{Nafion}/I vs the enzyme activity ratio $U_{pH\,=\,7.80}/U_{pH\,=\,7.40}$. The latter was calculated by the linear interpolation of plots U vs pH in the pH range from 7.00 to 8.00 for the three enzymes ADH, G6pDH, and G3pDH. The fourth data point for the glucose dehydrogenase (GDH) was taken from ref 1. For the sake of internal consistency, all the data in Figure 5 were collected by using the same batch of CNT and the same loading of CNT in chitosan films. This is important because the source of CNT and the amount of CNT in the biosensors influenced the current ratio I_{Nafion}/I and ΔpH (Figure S3, Supporting Information). The variability in the current ratio could be ascribed to differences in the linear range of calibration plots for the determination of NADH at CNT that were from different batches and manufacturers.

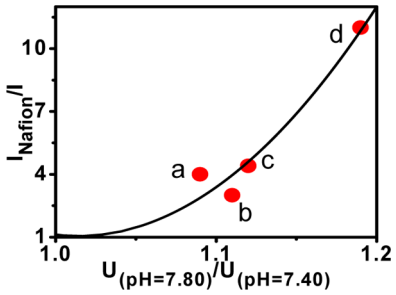

Figure 5. Relationship between the current ratio I_{Nafion}/I and the enzyme activity ratio $U_{pH\,=\,7.80}/U_{pH\,=\,7.40}$ for dehydrogenase enzymes (a) G3pDH, (b) G6pDH, (c) GDH, and (d) ADH. Background electrolyte, pH 7.40 phosphate buffer solution (0.050 M).

The plot in Figure 5 shows that the Nafion-induced signal amplification was a nonlinear function of the pH-induced increase in enzyme activity. The slope of the plot increased with the extent of enzyme activation and reached ∼80 indicating that the biosensor signal was becoming a very sensitive function of enzyme activity at higher unit ratios.

Signal Amplification at Different pHs. The above analysis of Nafion-induced signal amplification was focused on the performance of four different dehydrogenase-based biosensors in a buffer solution at one pH (7.40). The analysis was broadened by studying the signal amplification in one dehydrogenase-based biosensor but at four different pH values. Figure 6 shows a current ratio I_{Nafion}/I for the ADH-based biosensor in phosphate solutions at pH 6.00, 7.00, 8.00, and 9.00. The plot I_{Nafion}/I vs pH had a volcano shape similar to that of enzyme activity−pH profile for ADH (Figure 3, profile I). This further underlined the importance of pH in the signal amplification.

Figure 3 (profile I) shows that the pH values 6.00 and 7.00 are on the ascending slope of the activity−pH profile for ADH. Under such conditions, the Nafion-induced increase in pH inside the biosensor should increase the activity of ADH leading to $I_{Nafion}/I > 1$, which is indeed observed in Figure 6. The pH values 8.00 and 9.00 are near or on the descending slope of the ADH activity−pH profile. By the same logic, at these pH values, the Nafion-induced increase in pH inside the

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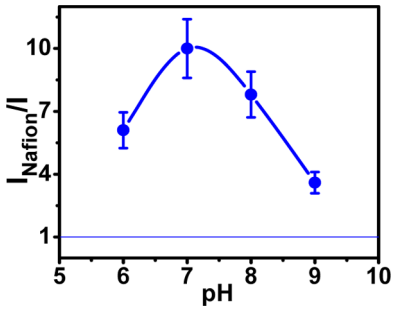

Figure 6. Current ratio I_{Nafion}/I vs pH plot for an ADH-based biosensor responding to 10.0 mM ethanol in a stirred 0.050 M phosphate buffer solution at a selected pH. Potential, 0.40 V.

biosensor should decrease the activity of ADH leading to the decrease in current ratio I_{Nafion}/I, which is also shown in Figure 6. However, such decrease should be much more pronounced ($I_{Nafion}/I < 1$) based on the pH effect alone. Apparently, a biocompatible matrix of chitosan in the biosensor protected the ADH and extended its activity at basic pH better than the matrix of an aqueous buffer solution.

The current flowing through an ADH-based biosensor was generated by the electrooxidation of NADH that was produced in the enzymatic reaction 4 inside the biosensor. Therefore, the sensitivity of biosensor signal to pH could be due to the pH effect on the electrooxidation of NADH at CNT. In order to test this option, the amperometric traces for the electrooxidation of 10 μM NADH at a GC/CHIT−CNT film electrode were recorded in solutions at four different pH values. The amperometric traces were not affected by the solution pH (Figure S5, Supporting Information), which eliminated this option as an explanation for the signal amplification.

Amplifying the Signal of GOx-Based Biosensor. Figure 1 (panel IV) showed deep signal attenuation by Nafion in the case of GOx-based biosensor at pH 7.40. This pH is on the descending slope of the activity−pH plot for GOx (Figure 3, profile IV). Using the pH on the ascending slope of this plot was a strategy to induce signal amplification by the Nafion film. The expectation was that the application of Nafion to the biosensor at such pH would increase the pH around GOx leading to the increase in enzyme activity and the consequent amplification of signal.

This strategy was tested in pH 4.00 tartrate and citrate buffers, which are based on the polyprotic acids with the pK_{a2} values close to that pH. Figure 7 shows the amperometric traces that were recorded at a GOx-based biosensor responding to the addition of glucose to pH 4.00 buffer solutions before and after the application of Nafion film. As predicted, the Nafion overcoat increased the amount of current flowing through the GOx-based biosensor. The increase in current was small, which correlated with a small Nafion-induced pH change in the underlying chitosan ($\Delta pH \approx 0$) under such conditions. The small Nafion effect on ΔpH could be ascribed to the protonation of chitosan ($pK_a = 6.3$)[18] at pH 4.00. Apparently, the highly protonated chitosan matrix retained strongly both the basic and acidic buffering anions, which minimized the effect of Nafion on the ionic fluxes inside the biosensor. It should be mentioned that a slow dissolution of chitosan film in the acidic buffers was a complicating factor. Nevertheless, the amplification of biosensor signal by 20% and 5% (Figure 7) in pH 4.00 tartrate and citrate buffer solutions, respectively, contrasted sharply with the 97% current decrease in pH 7.40 phosphate buffer solution (Figure 1, panel IV).

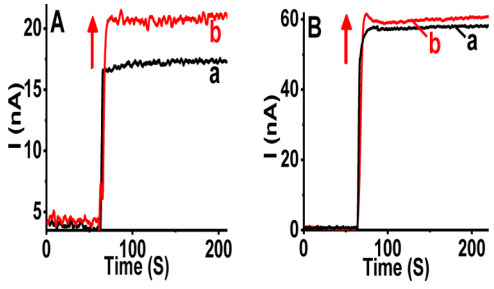

Figure 7. Amperometric traces recorded at a GOx-based biosensor responding to 10.0 mM glucose in stirred pH 4.00 (A) tartrate and (B) citrate buffer solutions (0.050 M): (a) before and (b) after coating the biosensor with a Nafion film. Potential, 0.50 V.

CONCLUSIONS

The two-film interface made of cationic (chitosan) and anionic (Nafion) polyelectrolytes amplified the signal of enzyme-based biosensors. The amplification was due to the Nafion-induced pH increase in the underlying chitosan film, which contained an enzyme. This affected the pH-sensitive activity of enzyme and allowed one to either amplify or attenuate the biosensor signal by selecting a solution pH on the ascending or descending slope of the enzyme activity−pH profile, respectively. The necessary condition for signal amplification was the presence of carbon nanotubes and polyprotic acid-based buffer in the chitosan film. Under such conditions, the two-film interface amplified the current even in the case of negatively charged enzyme substrates, which were prone to the rejection by the negatively charged Nafion film. The biocompatible matrix of chitosan protected an enzyme and extended its activity at basic pH better than the matrix of an aqueous buffer solution. The combination of two-film interface with enzyme engineering to modify enzyme activity−pH profiles can lead to the enzyme-based devices with highly amplified current output.

ASSOCIATED CONTENT

Supporting Information

Additional data to demonstrate the lack of ethanol preconcentration in ADH-based biosensors (Figure S1), amplification of biosensor signal in a concentrated buffer solution (S2), role of CNT in potentiometric and amperometric measurements (Figures S3 and S4), pH effect on the electrooxidation of diluted NADH at CNT (Figure S5), amperometric traces and calibration plots for an ADH-based biosensor (Figure S6), amperometric traces illustrating the selectivity of ADH-based biosensor (Figure S7), numerical data for the four amperometric enzyme assays (Table S1), numerical data for the measurements of Nafion-induced pH changes in surface films (Table S2), and analytical figures of merit for an ADH-based amperometric biosensor with and without Nafion overcoat (Table S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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