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Rapid Electrochemical Enzyme Assay with Enzyme-Free Calibration

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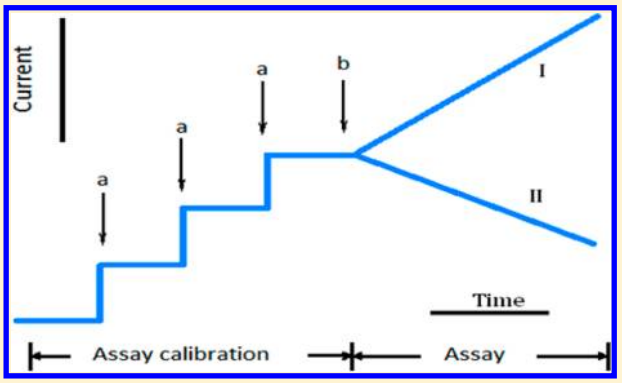
Supporting Information

ABSTRACT: The internally calibrated electrochemical continuous enzyme assay (ICECEA, patent pending) was developed for the fast determination of enzyme activity unit (U). The assay depends on the integration of enzyme-free preassay calibration with the actual enzyme assay in one continuous experiment. Such integration resulted in a uniquely shaped amperometric trace that allowed for the selective picomolar determination of redox enzymes. The ICECEA worked because the preassay calibration did not interfere with the enzyme assay allowing both measurements to be performed in succession in the same solution and at the same electrode. The method displayed a good accuracy (relative error, <3%) and precision (relative standard deviation (RSD), <3%) when tested with different working electrodes (carbon nanotubes/chitosan, glassy carbon, platinum) and enzymes (alcohol dehydrogenase, ADH; lactate dehydrogenase, LDH; xanthine oxidase, XOx; glucose oxidase, GOx). The limit of detection for the ADH, LDH, XOx, and GOx was equal to 0.18, 0.14, 0.0031, and 0.11 U L^{-1} (or 4.2, 0.72, 89, and 6.0 pM), respectively. The simplicity, reliability, and short analysis time make the ICECEA competitive with the optical enzyme assays currently in use.



Enzymes are biological catalysts of great scientific and economic importance. They are one of the best-established products in biotechnology with the billion-dollar market.[1] Therefore, there is a high demand for simple, reliable, and cost-effective assays for the rapid evaluation of their catalytic activity.

The majority of existing enzyme assays relies on changes in the optical properties of enzyme solution.[2−9] Usually, such changes are due to the interaction of dyes with hydrogen peroxide that is produced by oxidase enzymes or the reduction of β-nicotinamide adenine dinucleotide (NAD^+ or $NADP^+$) cofactor by dehydrogenase enzymes. The optical enzyme assays have limitations because they often involve toxic chromogenic agents, auxiliary enzymes, multiple liquid-handling steps, and time-consuming incubation and have a limited utility in turbid solutions.

The enzyme assays can also be performed by monitoring changes in the electrochemical properties of enzyme solution.[10−18] Such changes are typically due to the formation or consumption of redox active species in the course of enzymatic reaction. The contemporary electrochemical enzyme assays also have their problems including limited selectivity, sensitivity that is critically dependent on the activity of electrode surface, and the use of enzymes (sometimes fairly expensive) to calibrate the measurements. All of these limit the precision and accuracy of the electrochemical determination of enzyme activity.

The proposed internally calibrated electrochemical continuous enzyme assay (ICECEA) avoids most of the problems listed above. The new assay requires only a small amount of enzyme to quickly determine its activity with no need for the reactivation of electrode surface or enzyme-based calibration. In the present paper, the merits of ICECEA are investigated by performing the case study of four enzyme systems made of key dehydrogenases and oxidases.

The selected enzymes included the alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), xanthine oxidase (XOx), and glucose oxidase (GOx). These are important enzymes. The ADH has been used as a biomarker of graft function after liver transplantation.[19] The LDH is a biomarker of tissue breakdown,[20] and the alleviated levels of LDH have also been linked to the myocardial infarction,[21] meningitis,[22] encephalitis,[23] acute pancreatitis,[24] and histoplasmosis.[25,26] The XOx is a biomarker of oxidative stress and plays a role in tissue and vascular injuries including liver damage, inflammatory diseases, and chronic heart failure.[27] Finally, the GOx is commonly used as a medical diagnostic reagent and component of glucose sensors for monitoring diabetes and fermentation processes.[28] It is also used as a food preservative.[29]

EXPERIMENTAL SECTION

Reagents. Chitosan (CHIT, MW ~ 1 × 10^6 Da; ~80% deacetylation), nicotinamide adenine dinucleotide (NAD^+, λ = 259 nm), dihydronicotinamide adenine dinucleotide (NADH), D(+)-glucose, alcohol dehydrogenase (ADH, from *Saccharomyces cerevisiae*, EC 1.1.1.1, cat.# G7141, 285 U mg^{-1}, 150 kDa[30]), lactate dehydrogenase (LDH, from *rabbit muscle*, EC

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1.1.1.27, Type XI, cat.# L1254, 661 U mg^{-1}, 300 kDa[31]), xanthine oxidase (XOx, from *bovine milk*, EC 1.1.3.22, cat.# X4376, 0.12 U mg^{-1}, 290 kDa[32]), and glucose oxidase (GOx, from *Aspergillus niger*, EC 1.1.3.4, cat.#G7141, 138.8 U mg^{-1}, 133 KDa[33]) were purchased from Sigma-Aldrich. Multiwalled carbon nanotubes (CNT, ~95% nominal purity) were purchased from Nanolab (Brighton, MA). The $NaH_2PO_4 \cdot H_2O$, Na_2HPO_4, HCl, and NaOH were from Fisher. All solutions were prepared using deionized water that was purified with a Barnstead NANOpure cartridge system.

The chitosan solutions (0.10 wt %) were prepared by dissolving chitosan flakes in a hot 0.10 M HCl solution (90 °C), cooling to room temperature, adjusting their pH to 4.5 with a NaOH solution, and filtering with a 0.45 μm Millex-HA syringe filter unit (Millipore).[14] They were stored in a refrigerator (4 °C) when not in use. The suspensions of carbon nanotubes in chitosan solutions (1.0 mg mL^{-1}) were prepared by a 15 min sonication.

Electrochemical Measurements. The electrochemical assays were performed in the three-electrode system with a bare or CNT-modified 3.0 mm-diameter glassy carbon disk or bare 1.6 mm-diameter platinum disk working electrode (BAS), platinum wire auxiliary electrode, and Ag/AgCl/3 M NaCl (BAS) reference electrode using a CHI 832B workstation (CH Instruments, Inc.). Prior to use or modification, 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 sonication in deionized water and methanol. The modification of glassy carbon electrodes was done by casting 20.0 μL of suspension of CNT (1.0 mg mL^{-1}) in 0.10 wt % chitosan solution on the electrode surface and evaporating water and drying the remaining CNT-CHIT film for 2 h at room temperature. The experiments were repeated at least three times, and results are presented with the relative standard deviation (RSD).

Electrochemical Enzyme Assays. The ICECEA was performed in the constant-potential amperometric mode in a stirred solution of enzyme's substrate. After recording a baseline current, the known aliquots of a product (or reactant) of enzymatic reaction were added to the solution in order to record the current steps that were used to calibrate the assay. This was followed by the addition of assayed enzyme in order to trigger an enzymatic reaction and record an angled current–time (I–t) segment, which was used to calculate the enzyme activity unit. Typically, such a "current steps–current slope" amperometric trace was recorded in a short 5 min experiment. For the proof-of-concept purposes, the assayed enzymes included the alcohol dehydrogenase, lactate dehydrogenase, xanthine oxidase, and glucose oxidase.

The validity of enzyme assays was assured by (a) measuring the I–t slope (initial reaction rate) only at a short reaction time, <60 s, (b) conducting an enzymatic reaction under the condition of zero order with respect to the enzyme's substrate, (c) using optimized pH and temperature, and (d) working within the linear range of a calibration plot for amperometrically monitored species. The accuracy of ICECEA was determined by calculating the relative error based on the known number of enzyme units that were added to a solution and enzyme units that were actually measured.

RESULTS AND DISCUSSION

Principle of ICECEA. The ICECEA measures the activity unit (U) of an enzyme via the direct amperometric determination of an initial rate of enzymatic reaction

$$\text{enzyme substrate} + \text{reactants} \xrightarrow{\text{enzyme}} \text{products} \quad (1)$$

The initial rate reflects the enzyme activity, which by definition is expressed as the amount of enzyme's substrate that is converted to a product per unit time (1 U = 1 micromole min^{-1}). The determination of U is based on measuring either the formation of a product or consumption of a reactant of the enzymatic reaction 1 over time. The unique feature of the ICECEA is that the preassay calibration and actual enzyme assay are performed consecutively in the same solution and at the same electrode. The assay requires only three solutions to quantify the enzyme activity: (A) a solution of enzyme's substrate in a background electrolyte, (B) a solution of reactant or product of reaction 1, and (C) a solution of assayed enzyme. The assay is performed at a working electrode that is poised at a potential E, which is adequate for either the oxidation or reduction of species that are present in the solution B. The experiment is done by spiking three known aliquots of a solution B followed by one aliquot of a solution C into a stirred solution A and measuring the current flowing through the working electrode.

Figure 1 shows a schematic amperometric trace, which is characteristic for the ICECEA. The trace is composed of two

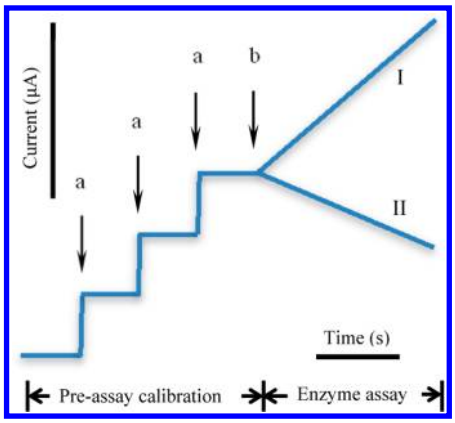

Figure 1. Schematic ICECEA current–time trace recorded at a working electrode in a stirred solution A. The arrows "a" indicate the moment of addition of known aliquots of solution B (preassay calibration phase). The arrow "b" shows the moment of spiking the solution with an aliquot of solution C containing the assayed enzyme (assay phase). The segments I and II are due to the redox of a product generated or reactant consumed, respectively, by the enzymatic reaction 1.

parts. The first part (preassay calibration phase) is made of three current steps "a" that are due to the oxidation (or reduction) of species added with known aliquots of solution B. These current steps are used to calculate the calibration slope (CS). The second part of the amperometric trace (assay phase) is made of either an ascending (I) or descending (II) I–t segment, which is recorded after the assayed enzyme is added to the solution at a moment indicated by arrow b in Figure 1. The I–t segments I and II are due to the oxidation (or reduction) of a product that is generated, or a reactant that is consumed, respectively, by the enzymatic reaction 1. The slope

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of the segments I and II reflects the initial rate of reaction 1 and will be called an assay slope (AS).

The calibration slope CS and assay slope AS are used to calculate the activity unit of an enzyme (U L^{-1} = micromole min^{-1} L^{-1} = μM min^{-1}) according to the equation

$$\text{units measured } (\mu\text{M min}^{-1}) = \frac{\text{AS } (\mu\text{A s}^{-1}) \times 60 \text{ (s min}^{-1})}{\text{CS } (\mu\text{A } \mu\text{M}^{-1})} \quad (2)$$

The ICECEA was studied by using enzymes alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), xanthine oxidase (XOx), and glucose oxidase (GOx). These enzymes allowed testing the ICECEA at different working electrodes (carbon nanotubes, glassy carbon, and platinum) and exploring various strategies for the preassay calibration phase and assay phase of the measurement. The preassay calibration was performed by recording the currents due to the electrooxidation of known aliquots of either the products (NADH, uric acid, hydrogen peroxide) or reactants (NADH) of the enzymatic reactions 3–6. The assay phase was conducted by measuring the currents due to the electrooxidation of the same species that were used in the calibration phase. The ICECEA was performed under the same conditions of pH and temperature as those used in the standard optical Sigma assays of the selected enzymes in order to assess the merits of a new method.

ICECEA of Alcohol Dehydrogenase. The activity of ADH was determined by measuring the initial rate of enzymatic reaction

$$\text{ethanol} + \text{NAD}^+ \xrightarrow{\text{ADH}} \text{acetylaldehyde} + \text{NADH} \quad (3)$$

using three solutions: (A) 20.0 mL of 0.56 M ethanol and 7.50 mM NAD^+ in pH 8.80 pyrophosphate buffer solution (0.050 M), (B) 1.0 mM NADH solution, and (C) ADH solution (7.50 U mL^{-1}). The ICECEA was performed at a glassy carbon electrode (E = 0.40 V, 25 °C) that was coated by a thin film of CNT that was dispersed in the inert matrix of chitosan.[34] The CNT were used because they facilitate the oxidation of NADH.[34,35] In a typical experiment, the three 20 μL aliquots of solution B and one 10 μL aliquot of solution C were added in succession to the 20.0 mL of stirred solution A. This generated the characteristic ICECEA amperometric trace (Figure 2).

The current steps in Figure 2 are due to the oxidation of NADH present in the solution B that was added in the preassay calibration phase of the experiment. The subsequent assay phase features an ascending I–t segment, which is due to the oxidation of NADH that is continuously generated in the enzymatic reaction 3. The latter was triggered by the addition of enzyme ADH (solution C) to the assay solution. The height of current steps and the initial angle of ascending I–t segment were used to determine the calibration slope CS and assay slope AS, respectively. The activity unit of ADH was calculated by substituting the CS and AS into eq 2. Table 1 presents the results of such calculations based on the three amperometric traces shown in Figure 2. They demonstrate a good precision of the determination of ADH activity unit (RSD, 2.1%, N = 3) based on the oxidation of NADH at CNT.

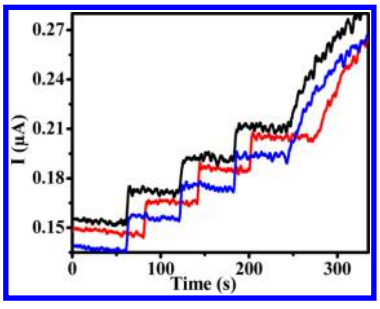

Figure 2. ICECEA current–time traces (E = 0.40 V) recorded at a glassy carbon/CNT film electrode in a stirred solution of 0.56 M ethanol in pH 8.80 pyrophosphate buffer solution (25 °C). The calibration I–t steps are due to the additions of 1.0 μM aliquots of NADH. The angled I–t segment was recorded after the addition of 3.74 U L^{-1} alcohol dehydrogenase.

Table 1. ICECEA of 3.74 U L^{-1} Alcohol Dehydrogenase (pH 8.80, 25 °C)

run #	CS (μA μM^{-1})	AS (μA s^{-1})	units measured (U L^{-1})
1	0.0200	1.19×10^{-03}	3.57
2	0.0201	1.20×10^{-03}	3.58
3	0.0204	1.26×10^{-03}	3.71
avg	0.0202	1.22×10^{-03}	3.62
RSD	1.0%	3.1%	2.1%

Table 1 also shows a small relative error (−3.2%) between the average activity unit measured in three runs (3.62 U L^{-1}) and the unit added (3.74 U L^{-1}), which demonstrates a good accuracy of the ICECEA. Apparently, the addition of a small amount of NADH (3 μM total) to the solution during the preassay calibration phase had a negligible effect on the subsequent assay phase where the rate of the enzymatic reaction 3 was measured. This indicated that both the preassay calibration with NADH and assaying of ADH with NADH could be conducted in succession in the same solution and at the same electrode. The optical assay of alcohol dehydrogenase (Table S1, Supporting Information) was much more elaborate than the ICECEA and required seven different solutions and multiple liquid-handling steps.

ICECEA of Lactate Dehydrogenase. The assay of LDH was based on the enzymatic reaction

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+ \quad (4)$$

and three solutions: (A) 20.0 mL of 2.30 mM sodium pyruvate in pH 7.50 phosphate buffer solution (0.10 M), (B) 50.0 mM NADH solution, and (C) LDH solution (20.0 U mL^{-1}). The assay was performed at 37 °C using a glassy carbon electrode (E = 0.40 V) that was modified by a thin film of carbon nanotubes in chitosan. The stirred solution A was spiked with three 10 μL aliquots of solution B and one 10 μL aliquot of solution C. Figure 3 shows the three representative ICECEA amperometric traces for the determination of LDH activity.

The current steps in Figure 3 are due to the oxidation of NADH, which was added to the solution in the preassay calibration phase of the experiment. The subsequent assay phase features a descending I–t segment that is due to the oxidation of NADH, which is consumed in the enzymatic reaction 4. The latter was triggered by the addition of LDH to the solution. The current steps and the descending slope of I–t segment were used to determine the calibration slope CS and assay slope AS, respectively, and calculate the activity of LDH based on the eq 2. Table 2 shows a good precision (RSD, 2.4%, N = 3) and accuracy (relative error, 2.0%) of the determination of LDH activity unit by ICECEA. This demonstrated that the

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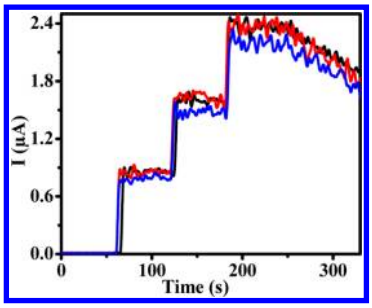

Figure 3. ICECEA amperometric traces (E = 0.40 V) recorded at a glassy carbon/carbon nanotubes film electrode in a stirred solution of 2.30 mM sodium pyruvate in pH 7.50 phosphate buffer solution (37 °C). The calibration I–t steps are due to the additions of 25.0 μM aliquots of NADH. The angled descending I–t segment was recorded after the addition of 10.0 U L^{-1} lactate dehydrogenase.

Table 2. ICECEA of 10.0 U L^{-1} Lactate Dehydrogenase (pH 7.50, 37 °C)

run #	CS (μA μM^{-1})	AS (μA s^{-1})	units measured (U L^{-1})
1	0.0320	5.49×10^{-03}	10.3
2	0.0320	5.55×10^{-03}	10.4
3	0.0298	4.94×10^{-03}	9.95
avg	0.0313	5.33×10^{-03}	10.2
RSD	4.1%	6.3%	2.4%

preassay calibration and assaying of LDH did not interfere with each other and can be conducted sequentially in one experiment.

The important distinction from the earlier assay of ADH is that in the LDH case the preassay calibration phase played a dual role. It provided data for the calibration and, at the same time, introduced a necessary reactant (NADH) for the reaction 4. Therefore, the calibration had to be done with a higher concentration of NADH (75 μM total) in order to conduct the reaction 4 under the enzyme control. The good precision and accuracy of the determination of LDH demonstrated the universality of the ICECEA concept. The optical assay of lactate dehydrogenase (Table S2, Supporting Information) was more elaborate and required five different solutions and much more time compared to the ICECEA.

ICECEA of Xantine Oxidase. The activity of XOx was determined by measuring the initial rate of reaction

$$\text{xanthine} + O_2 + H_2O \xrightarrow{\text{XOx}} \text{uric acid} + H_2O_2 \qquad (5)$$

The three solutions needed for the assay included (A) 20.0 mL of 0.50 mM xanthine in pH 7.50 phosphate buffer solution (0.10 M), (B) 2.50 mM solution of uric acid solution, and (C) XOx solution (8.06 U mL^{-1}). The enzyme XOx is different from the previous two enzymes because its reaction involves three species (O_2, uric acid, and H_2O_2) that can be either reduced or oxidized at conventional electrodes. In principle, any of them could be used to monitor the kinetics of reaction 5. However, in order to simplify the measurements and monitor only one type of species, a bare glassy carbon electrode was selected as a working electrode. At such electrode, only the uric acid can be easily oxidized (E = 0.35 V). The stirred solution A was spiked with the three 20 μL aliquots of solution B and one 20 μL aliquot of solution C. Figure 4 shows the representative ICECEA amperometric traces for the XOx assay.

The current steps in Figure 4 are due to the oxidation of known aliquots of uric acid (solution B) that were added to the

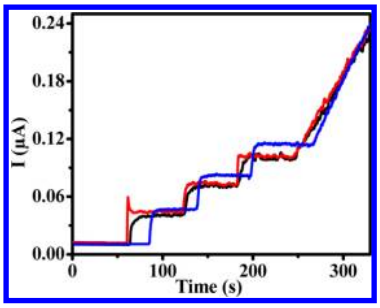

Figure 4. ICECEA amperometric traces (0.35 V) recorded at glassy carbon electrode in a stirred solution of 0.50 mM xanthine in pH 7.50 phosphate buffer (25 °C). The calibration I–t steps are due to the additions of 2.5 μM aliquots of uric acid. The angled I–t segment was recorded after the addition of 8.03 U L^{-1} xanthine oxidase.

solution A. The following ascending I–t segment is due to the oxidation of uric acid, which is generated by the enzymatic reaction 5 that was triggered by the addition of XOx to the solution at ~250 s. The current steps and the ascending I–t segment were used to determine the CS and AS, respectively, and calculate the activity of XOx using the eq 2. Table 3 shows

Table 3. ICECEA of 8.03 U L^{-1} Xanthine Oxidase (pH 7.50, 25 °C)

run #	CS (μA μM^{-1})	AS (μA s^{-1})	units measured (U L^{-1})
1	0.0121	1.61×10^{-03}	7.98
2	0.0123	1.61×10^{-03}	7.85
3	0.0141	1.95×10^{-03}	8.30
avg	0.0128	1.72×10^{-03}	8.04
RSD	8.6%	11%	2.8%

the results for the three typical runs presented in Figure 4. They demonstrate a good precision (RSD, 2.8%, N = 3) of the determination of XOx by the ICECEA. They also show a small relative error (0.12%) between the average activity unit measured in three runs (8.04 U L^{-1}) and the unit added (8.03 U L^{-1}). This illustrates that the preassay calibration with uric acid did not interfere with the XOx assay.

The good precision and accuracy was achieved regardless of a much larger standard deviation for the CS and AS (8.6% and 11%, respectively). Apparently, the difference in slopes between different runs did not impact the quality of unit determination. The reason for this was that the experiment did not require transferring the working electrode between the separate calibration and assay solutions, which provided an internal consistency between the calibration and assay phases of an individual run. This represents the inherent advantage of the ICECEA method, which provides reliable results without the need for repolishing of working electrode. The optical assay of xanthine oxidase (Table S3, Supporting Information) was similar to the ICECEA in that it required the same number of solutions and comparable amount of time to determine the enzyme activity.

ICECEA of Glucose Oxidase. The assay of enzyme GOx was based on the enzymatic reaction

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{GOx}} \text{glucono-1,5-lactone} + H_2O_2 \qquad (6)$$

and three solutions: (A) 20.0 mL of 0.10 M β-D-glucose in pH 5.1 acetate buffer solution, (B) 0.0020 M solution of H_2O_2, and

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(C) GOx solution (40 units mL^{-1}). The assay was performed at 35 °C by using a platinum working electrode that was held at E = 0.60 V. The solution A was spiked with three 20 μL aliquots of solution B and one 10 μL aliquot of solution C. Figure 5 shows the representative ICECEA amperometric traces for the GOx assay.

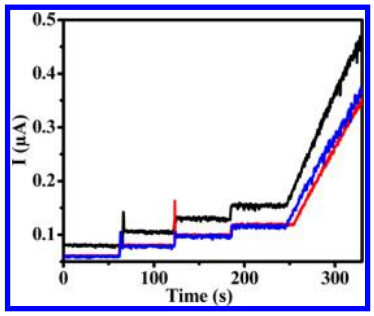

Figure 5. ICECEA amperometric traces (E = 0.60 V) recorded at a platinum electrode in a stirred solution of 0.10 M glucose in pH 5.10 acetate buffer (35 °C). The calibration $I-t$ steps are due to the additions of 2.0 μM aliquots of hydrogen peroxide. The angled $I-t$ segment was recorded after the addition of 19.9 U L^{-1} glucose oxidase.

The current steps in Figure 5 are due to the electrooxidation of H_2O_2 (solution B) that was added to a stirred solution A in the preassay calibration phase. The subsequent assay phase features an ascending $I-t$ segment that is due to the oxidation of H_2O_2, which is generated by the enzymatic reaction 6 that was triggered by the addition of GOx to the assay solution. The analysis of $I-t$ traces (Figure 5) is presented in Table 4, which

Table 4. ICECEA of 19.9 U L^{-1} Glucose Oxidase (pH 5.10, 35 °C)

run #	CS (μA μM^{-1})	AS (μA s^{-1})	units measured (U L^{-1})
1	1.22×10^{-02}	3.91×10^{-03}	19.2
2	9.60×10^{-03}	3.15×10^{-03}	19.7
3	9.20×10^{-03}	3.11×10^{-03}	20.3
avg	1.03×10^{-02}	3.39×10^{-03}	19.7
RSD	16%	13%	2.7%

shows a good precision (RSD, 2.7%, N = 3) and good accuracy (relative error, −1.0%) of the determination of GOx activity unit by the ICECEA. Again, as in the case of XOx, a good accuracy and precision was achieved regardless of the much larger RSD for the CS and AS (16% and 13%, respectively). The optical assay of glucose oxidase (Table S4, Supporting Information) was more time-consuming than the ICECEA and required a toxic dye *o*-dianisidine, auxiliary enzyme peroxidase, and four additional solutions.

Interference Studies. The interference studies were performed with the enzyme GOx and ascorbic acid. The latter is notorious for interfering with electrochemical assays because it can be easily oxidized at working electrodes made of carbon nanotubes, glassy carbon, or platinum. The addition of physiological concentration (0.10 mM) of ascorbic acid to the enzyme solution C did not change the ICECEA traces (not shown). This indicated that the ascorbic acid did not interfere with the electrochemical assaying of GOx under such conditions. Apparently, the high dilution factor (2000, =20 mL/10 μL) of the solution C during the assay lowered the concentration of ascorbic acid to a level (50 nM) that was not detectable at a platinum working electrode.

The situation changed when the dilution factor was decreased from 2000 to 20. In the presence of ascorbic acid in a solution (Figure 6A, trace 1), the angled $I-t$ segment of

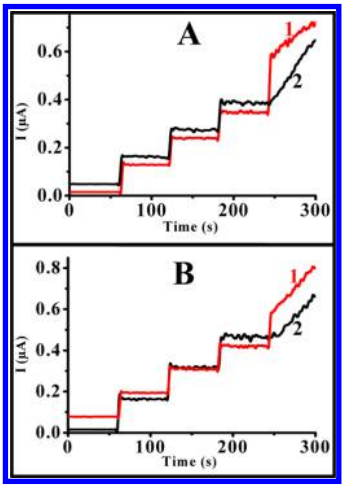

Figure 6. ICECEA amperometric traces (E = 0.60 V) recorded at a platinum electrode in in a stirred solution of 0.10 M glucose in pH 5.10 acetate buffer (35 °C). Calibration current steps are due to the additions of 10 μM aliquots of hydrogen peroxide. The angled $I-t$ segments "1" at >250 s were recorded after the addition of an aliquot of 25 U L^{-1} glucose oxidase that contained (A) 0.30 mM ascorbic acid and (B) 1.0 mM acetaminophen. Segments "2" at >250 s were recorded after the addition of the enzyme aliquots that contained (A) no ascorbic acid and (B) no acetaminophen.

ICECEA trace at >250 s shifted upward when compared to that recorded without the ascorbic acid (Figure 6A, trace 2). The shift was due to the extra current that was generated by the oxidation of ascorbic acid at the surface of platinum electrode. The second significant change was a 50% decrease in the slope of angled $I-t$ segment in the presence of ascorbic acid in the solution (Figure 6A, trace 1 vs trace 2). This indicated that the ascorbic acid at higher concentration interfered with the measurement of enzyme activity. The interference could be ascribed to the reaction of ascorbic acid, which is a relatively strong reducing agent, with hydrogen peroxide[36,37] that was generated in the enzymatic reaction 6. As hydrogen peroxide was consumed by ascorbic acid, less hydrogen peroxide was available for the oxidation at the platinum electrode. Therefore, less current was generated at the electrode leading to a decrease in the $I-t$ slope.

The additional interference studies were conducted with the acetaminophen, which is also known for interfering with electrochemical assays. In the presence of acetaminophen, the angled $I-t$ segment of ICECEA trace shifted upward (Figure 6B, trace 1) as in the case of ascorbic acid. This increase in current was due to the oxidation of acetaminophen at a platinum electrode. However, the $I-t$ segment remained practically parallel to that recorded in the absence of acetaminophen (Figure 6B, trace 2). The difference between the slopes was below 3%. This is significant because it indicates that the acetaminophen, which is a milder reducing agent than ascorbic acid, did not interfere with the determination of enzyme activity via the monitoring of hydrogen peroxide. Apparently, the unique shape of the ICECEA $I-t$ trace, which is composed of rapid and more gradual changes in current,

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allows for the selective determination of enzyme activity even in the presence of redox active species such as acetaminophen as long as their concentration stays constant during the recording of the angled $I-t$ segment. The sensitivity of $I-t$ slope to the composition of solution (Figure 6A) indicates that the ICECEA, in addition to quantifying the enzyme activity, can also quickly screen for potential interfering species, enzyme inhibitors, and enzyme substrates.

Detection Limits for Enzyme Activity Units. The capacity of ICECEA to reliably quantify enzyme activity depends on the limit of detection (LOD) of molecules that are monitored during the assay (NADH, uric acid, H_2O_2) and the linear range of their calibration plots. For the purpose of this analysis, the LOD was defined as the concentration that generated the current I_{LOD} that was three times larger than the peak-to-peak noise of step 3 in Figures 2−5. The low limit of enzyme detection (LLED) and high limit of enzyme detection (HLED) were determined (±5−10%) under the condition that (i) the enzyme was always a limiting reagent and (ii) the current measured during the assay was within the linear range of calibration plot for the monitored species. The LLED values were calculated by substituting $I_{LOD}/2$ min for the AS in eq 2. This yielded 0.18, 0.14, 0.0031, and 0.11 U L^{-1} (or 4.2, 0.72, 89, and 6.0 pM) for the ADH, LDH, XOx, and GOx, respectively. These LLED values are competitive and, in some cases, better by up to 3 orders of magnitude than those previously reported for ADH,[18] LDH,[13] XOx,[6,38] and GOx.[16,17]

As the enzyme concentration in a solution increased, the $I-t$ segments I and II became progressively less linear. Therefore, the HLED was determined based on the assay slope AS that was calculated using shorter (<30 s) linear portions of segments I and II with the correlation coefficients $R^2 > 0.99$ and $R^2 > 0.97$, respectively. Following this approach, the HLED values of 47, 55, 54, and 105 U L^{-1} (or 1.1 nM, 0.27 nM, 1.5 μM, and 5.6 nM) were obtained for the ADH, LDH, XOx, and GOx, respectively.

CONCLUSIONS

The internally calibrated electrochemical continuous enzyme assay (ICECEA) is a simple, reliable, and universal method for the rapid determination of catalytic activity of redox enzymes. In principle, the ICECEA can also be used for the determination of nonredox enzymes via the coupled assays as long as there is a species associated with their enzymatic reaction that can generate a current at a working electrode. The ICECEA is quantified via the enzyme-free calibration directly in the assay solution. This eliminates the common source of error, which is the change in the activity of working electrode surface due to its transfer between the calibration and assay solutions. The ICECEA avoids most of the problems that plague the existing optical enzyme assays, which are frequently cumbersome and involve toxic chromogenic agents and auxiliary enzymes and have a limited utility in turbid solutions. The new assay has a potential to be useful in the quantification of enzyme biomarkers for various diseases, analysis of commercial batches of enzymes, fast optimization of conditions for assaying of new enzymes, and quick corroboration of high-throughput enzyme assays. In addition, the unique integration of time-independent calibration signal with a time-dependent assay signal in one amperometric trace greatly improves the selectivity of enzyme determination and provides the means for the rapid screening for enzyme inhibitors and substrates.

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.

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Notes

The authors declare no competing financial interest.

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