Electrochemiluminescence-Based Quantitation of Classical Clinical Chemistry Analytes

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Electrochemiluminescence-Based Quantitation of Classical Clinical Chemistry Analytes

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Electrochemiluminescence (ECL)-based assays are described for the quantitation of potentially any clinical analyte that can be linked to a β-nicotinamide adenine cofactor-requiring or hydrogen peroxide-forming enzyme. Light was emitted when an appropriate voltage was applied to an electrode immersed in a solution containing the inorganic luminescent complex, ruthenium(II) tris(bipyridyl), and either NAD(P)H or H_2O_2. The detection of H_2O_2 required oxalate as a coreactant. The amount of emitted light directly related to the concentration of NAD(P)H or H_2O_2. Five classical clinical analytes were quantitated using different formats: glucose (coupled to both NADH- and H_2O_2-producing enzymes), ethanol (two NADH-producing enzymes in series), carbon dioxide (NADH-depleting enzyme), cholesterol (H_2O_2-forming enzyme), and glucose-6-phosphate dehydrogenase (temporal measurement of catalytic NADPH formation). Satisfactory correlations were found between ECL and conventional spectrophotometric analyses. The wide assortment of formats used to quantitate clinical analytes indicates that many other similarly coupled analytes may also be quantitated by ECL.

Many classical chemistry analytes are commonly quantitated by coupling them to enzyme systems that either utilize β-nicotinamide adenine cofactors or form hydrogen peroxide.[1,2] Such coupling permits indirect spectrophotometric detection of the analytes: at ultraviolet wavelengths for nicotinamide adenine cofactors (typically 340 nm) or at visible wavelengths for peroxide (often at 460, 510, or 540 nm).

As an alternative to spectrophotometry, both nicotinamide adenine cofactors[3,4] and peroxide (this work) can be detected by electrochemiluminescence (ECL). ECL, in contrast to chemiluminescence, is a luminescent chemical reaction in which light is emitted only when an appropriate voltage is applied to an electrode in contact with a solution containing an appropriate luminescent compound. One such compound is the inorganic complex, ruthenium(II) tris(bipyridyl), $Ru(bpy)_3^{2+}$.[5] In an ECL instrument, either reduced nicotinamide cofactors or peroxide can directly or indirectly promote light emission by $Ru(bpy)_3^{2+}$ [3,4] (Figure 1). There are at least three advantages of using an ECL instrument instead of a spectrophotometer to detect clinical analytes normally coupled to enzymatic transformations involving nicotinamide adenine cofactors or peroxide. First, in contrast to spectrophotometric methods where different wavelengths are used for nicotinamide adenine cofactors and peroxide, a single set of ECL instrument parameters can be used for both systems. Second, owing to the exquisite sensitivity of ECL, the same instrument can be used for immunoassays and DNA probe assays of extremely dilute analytes.[6] Finally, because NAD^+ is electrochemically regenerated from NADH in the ECL mechanism shown in Figure 1, there is the possibility that light could be catalytically emitted in the presence of an appropriate enzyme−substrate analyte mixture.[3,4]

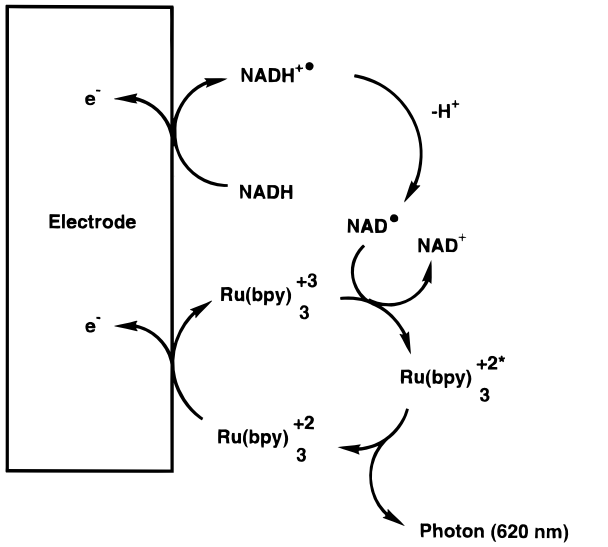

Figure 1. Proposed ECL reaction involving NADH and $Ru(bpy)_3^{2+}$. NADH and $Ru(bpy)_3^{2+}$ are both oxidized at the surface of an electrode. The one-electron-oxidized cation radical $NADH^{\bullet+}$ loses a proton to become a strongly reducing radical, $NAD^\bullet$. $NAD^\bullet$ then transfers an electron to $Ru(bpy)_3^{3+}$ to form NAD^+ and the unstable excited-state species, $Ru(bpy)_3^{2+*}$, which emits a photon when it decays to the ground state, $Ru(bpy)_3^{2+}$. Notably, the ECL reaction results in reformation of the functional reagents NAD^+ and $Ru(bpy)_3^{2+}$.

In this paper, ECL is shown to be a versatile means of analyzing a wide range of clinical analytes that are conventionally coupled to nicotinamide adenine cofactor- and peroxide-linked enzyme systems. The data demonstrate that ECL-based assays can indeed be broadly used as an alternative to spectrophotometric methods for the analysis of nicotinamide adenine cofactor- or H_2O_2-linked analytes.

EXPERIMENTAL SECTION

Apparatus. ECL measurements were made using an Origen Analyzer (IGEN, Inc., Gaithersburg, MD). The instrument

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integrates a luminometer, a potentiostat, fluid-handling components, an electrochemical flow-cell, and a 50 tube carousel and is controlled by a personal computer via operator manipulation of on-screen menus. The surfactant, Triton X-100, was routinely added to samples as an inocuous fluidics wetting agent.

Reagents. All reagents were reagent grade or better. All spectrophotometric-based diagnostic kits were purchased from Sigma as was the cholesterol calibrator solution (200 mg/dL, certified).

Bicarbonate (CO_2) Determination. For both spectrophotometric and ECL assays, solutions were degassed by sonication prior to use to remove any endogenous dissolved CO_2. Because the assays were endpoint determinations, it was sufficient to carry out incubations at room temperature (~22 °C) rather than in a thermostated incubator. Spectrophotometric assays of CO_2 standards (from dissolved sodium bicarbonate) were performed using a commercial CO_2 diagnostic kit according to manufacturer's instructions (procedure no. 131-UV, Sigma Diagnostics). For ECL analysis of CO_2, portions of the kit reagent solution (275 μL) were mixed with 5.0 μL of 0−50 mM bicarbonate. Following 10 min incubations, 25 μL of a solution of 12 μM $Ru(bpy)_3^{2+}$ and 0.6% Triton X-100 was added, the solution was mixed, and ECL was measured.

Ethanol Determination. The spectrophotometric assay for ethanol was a modification of the method recommended by the manufacturer of the ethanol diagnostic kit (procedure no. 333-UV, Sigma Diagnostics). The dry contents of commercial kit vials were reconstituted with a 3.0 mL mixture of 5.0 mM cysteine, 0.67 mM NAD^+, 1.0 mg/mL aldehyde dehydrogenase, and 270 mM Tris, pH 9.0. Aliquots (250 μL) of the reconstituted mixture were incubated for 10 min with 3.0 μL of 0.03−0.35% ethanol (w/v) in deionized water. After dilution, total absorbance changes at 340 nm were measured. For ECl-based ethanol analysis, 250 μL aliquots of the completed reaction mixtures described above were mixed with $Ru(bpy)_3^{2+}$ (25 μL of 12 μM) and Triton X-100 (25 μL of a 0.6% solution), and the ECL was measured.

For ECL detection of ethanol in human serum, ethanol was added to serum to give a final concentration of 0.19%. This ethanol-spiked serum was treated and assayed as described above for ethanol in water.

Glucose-6-phosphate Dehydrogenase Determination. For spectrophotometric enzyme analysis, reactions were initiated by the addition of glucose-6-phosphate dehydrogenase to a solution of 360 μM $NADP^+$, 350 μM glucose 6-phosphate, 3.3 mM $MgCl_2$, and 55 mM Tris-HCl, pH 7.8. Enzyme concentrations varied from 0 to 0.42 nM. The absorbance change was measured after a 30 min incubation at 30.0 ± 0.1 °C. In the ECL-based enzyme assay, an aliquot (297 μL) of the above reaction mixture was removed (after the 30 min incubation had elapsed) and mixed with 3.0 μL of 100 μM $Ru(bpy)_3^{2+}$, and ECL was measured.

ECL Quantitation of Hydrogen Peroxide. Mixtures of hydrogen peroxide (20 μL of 0−100 mM stock), oxalate (30 μL of 5.0 mM), sodium phosphate (120 μL of 0.5 M), deionized water (105 μL), and 25 μL of a solution containing both 12 μM ($Ru(bpy)_3^{2+}$ and 0.6% Triton X-100 were analyzed in an ECL instrument.

Cholesterol Determination. For spectrophotometric analysis, cholesterol calibrator (5−40 μL, Sigma Chemical Co.) was added to a solution containing cholesterol oxidase (8.0 μL of 48 units/mL) and 972 μL of a mixture of sodium phosphate (100 mM, pH 7.5) and Triton X-100 (0.05%). The rate of absorbance change was monitored at 240 nm for 30 min at 25.0 ± 0.1 °C. The molar extinction coefficient used for the cholesterol oxidase-catalyzed conversion of cholesterol to cholest-4-en-3-one was 15 500 M^{-1} cm^{-1}. ECL analysis of cholesterol required a lower pH than that used in the spectrophotometric assay. Thus, spectrophotometric assays similar to those described above were performed at pH 5.0 and 6.0 to determine whether cholesterol oxidase could function at a lower pH. The results showed that the enzyme operated efficiently at these pH values.

Conventional cholesterol oxidase assays sometimes include the enzyme catalase to generate molecular oxygen required in the chemical mechanism of cholesterol oxidase. It was found that a satisfactory level of soluble oxygen could be introduced from the air by vigorously stirring the buffer with a magnetic stir bar and stirring plate, obviating the need for catalase.

For ECL-based cholesterol analysis, a solution of cholesterol oxidase (10 μL of 48 units/mL), oxalate (60 μL of 10 mM), $Ru(bpy)_3^{2+}$ (25 μL of 12 μM, containing 0.6% Triton X-100), and cholesterol (30 μL of 2−50 μM), and buffer (120 μL of 100 mM sodium phosphate, pH 5.0, containing 0.05% Triton X-100) were mixed and incubated for 30 min, and the ECL was measured. Because of ECL inconsistencies in the results obtained using the commercial cholesterol calibrator solution (sodium azide is known to interfere with ECL), a stock solution of cholesterol was prepared for these experiments by dissolving cholesterol in a mixture of Triton X-100 and ethanol (3:2 v/v).

Glucose Determination. Spectrophotometric-based glucose assays were performed using a diagnostic kit according to manufacturer's instructions (Sigma). Two types of ECL-based glucose assays were carried out, based on either NADH formation or H_2O_2 formation. Inconsistent results were obtained quantitating glucose by NADH-based ECL using the commercial kit. These inconsistencies resulted from noncritical substances in the commercial kit (presumably azide). Because of this, individual kit components were purchased and appropriately mixed. In the NADH-based ECL assay, glucose (5.0−35.0 μM) was added to a mixture of 16.8 μg/mL hexokinase, 20 μg/mL glucose-6-phosphate dehydrogenase, 1.0 mM ATP, 1.0 mM NAD^+, 2.0 mM $MgSO_4$, and 25 mM Tris-HCl, pH 7.5. After 10 min incubations, 270 μL aliquots were mixed with 30 μL of 10 mM $Ru(bpy)_3^{2+}$, and the ECL was measured. For H_2O_2-based ECL assays, glucose oxidase (25 μL of 3.0 mg/mL) and freshly prepared β-D-(+)-glucose standards (25 μL of 1.0 to 50.0 mM) were incubated for 15 min in buffer (75 μL of 10 mM phosphate, pH 6.0). The solution was then mixed with $Ru(bpy)_3^{2+}$ (25 μL of 12 μM), oxalate (30 μL of 10 μM), and sodium acetate (120 μL of 0.1 M, pH 5.0). The ECL of the mixture was then measured.

RESULTS AND DISCUSSION

Glucose Analysis. Glucose was quantitated by coupling two enzyme-catalyzed reactions which resulted in stoichiometric NADH formation (Scheme 1). Quantitation by both the ultraviolet absorbance of NADH and by the $Ru(bpy)_3^{2+}$ ECl-promoting properties of generated NADH were satisfactory over the range of glucose concentrations tested and an excellent correlation was found between the two methods ($R^2 = 0.988$) (Figure 2).

Carbon Dioxide Analysis. Carbon dioxide was measured as bicarbonate (Scheme 2). Because NADH was consumed during this reaction, the presence of carbon dioxide was expected to result in a decrease in absorbance in the spectrophotometric assay,

Scheme 1. Enzymatic Coupling of Glucose to NADH Formation[a]

$$\text{glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{glucose-6-phosphate} + \text{NAD}^+ \xrightarrow{\text{G6PDH}} \text{gluconolactone-6-phosphate} + \text{NADH} + \text{H}^+$$

[a] HK, hexokinase; G6PDH, glucose-6-phosphate dehydrogenase.

Scheme 2. Enzymatic Coupling of Bicarbonate to NADH Formation[a]

$$\text{phosphoenolpyruvate} + \text{HCO}_3^- \xrightarrow{\text{PEPC}} \text{oxalacetate} + \text{H}_2\text{PO}_4^-$$

$$\text{oxalacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{malate} + \text{NAD}^+$$

[a] PEPC, phosphoenolpyruvate carboxylase; MDH, malate dehydrogenase.

Scheme 3. Enzymatic Coupling of Ethanol to NADH Formation[a]

$$\text{ethanol} + \text{NAD}^+ \xrightarrow{\text{AlcDH}} \text{acetaldehyde} + \text{NADH} + \text{H}^+$$

$$\text{acetaldehyde} + \text{NAD}^+ \xrightarrow{\text{AldDH}} \text{acetic acid} + \text{NADH} + \text{H}^+$$

[a] AlcDH, alcohol dehydrogenase; AldDH, aldehyde dehydrogenase.

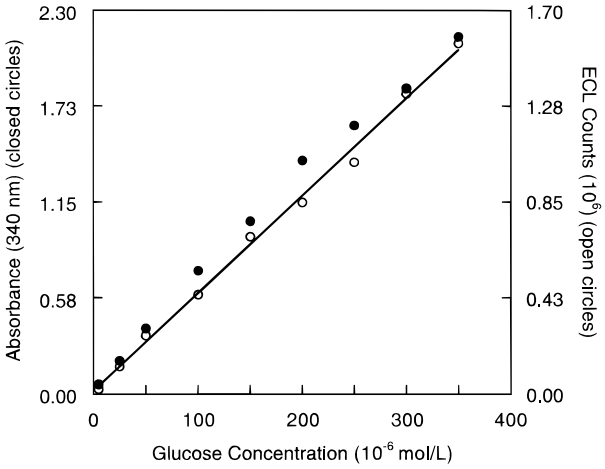

Figure 2. Comparison of spectrophotometric (closed circles) and ECL (open circles) determination of glucose based on enzyme-coupled NADH formation (Scheme 1). Glucose concentrations shown are prior to dilution for analysis.

and a decrease in light emission in the ECL assay. As shown in Figure 3, this was found to be true in both cases. A good correlation was found between the two methods ($R^2 = 0.973$) within the clinically relevant range of bicarbonate concentrations (19−29 mM) (Figure 3).

Ethanol Analysis. Ethanol was quantitated using alcohol dehydrogenase to generate NADH as shown in Scheme 3. As shown in Figure 4, the ECL results were satisfactorily linear, and comparison with a commercially available spectrophotometric method showed a good correlation ($R^2 = 0.970$). The ECL assay was enhanced by using a second enzyme, aldehyde dehydrogenase, which not only generated a second molecule of NADH to provide a stronger ECL signal, but also acted as an acetaldehyde trapping agent.

The ECL assay for ethanol was used as a test of the effects of human serum on analyte analysis. Ethanol was chosen as the

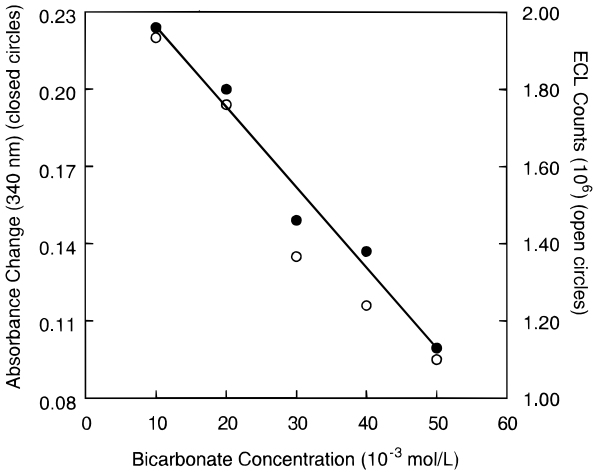

Figure 3. Comparison of spectrophotometric (closed circles) and ECL (open circles) determination of carbon dioxide coupled to NADH depletion (Scheme 2). Bicarbonate concentrations shown are prior to assay dilution.

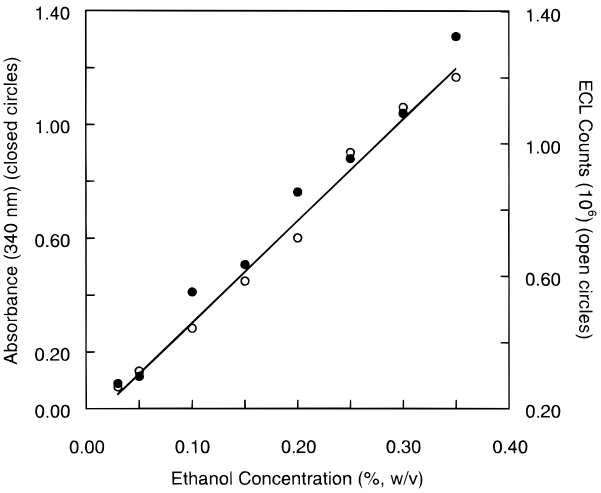

Figure 4. Comparison of spectrophotometric (closed circles) and ECL (open circles) determination of ethanol based on enzymatic coupling to NADH formation. Each molecule of ethanol was coupled to the formation two molecules of NADH as shown in Scheme 3. Ethanol concentrations shown are prior to dilution for the assays.

analyte because serum was essentially ethanol-free before we added a known concentration (0.19%). Because of the sensitivity of the ECL assay, a 100-fold dilution of the ethanol-spiked serum sample was tested. The serum present (~1% of the sample) had no effect on the analysis of ethanol. Duplicate assays gave ECL results that varied 0.6% and 4.7% from the expected value from a standard curve of ethanol in pure water (Figure 4). This shows that 100-fold diluted human serum does not affect ECL analysis of NADH. A 100-fold dilution of serum is not unreasonable for the detection of many classical clinical analytes since they are normally present in serum in concentrations well above the ECL detection limits.

Glucose-6-phosphate Dehydrogenase Analysis. In addition to NADH, NADPH is also detectable by ECL. The formation of NADPH during the glucose-6-phosphate dehydrogenase-catalyzed conversion of D-glucose 6-phosphate to D-glucono-δ-lactone 6-phosphate is shown in Scheme 4.[767] Both spectrophotometric and ECL detection of NADPH formation gave good

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Scheme 4. Glucose-6-Phosphate Dehydrogenase-Catalyzed NADPH Formation[a]

$$\text{glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{gluconolactone-6-phosphate} + \text{NADPH} + \text{H}^+$$

[a] G6PDH, glucose-6-phosphate dehydrogenase.

Scheme 5. Enzyme-Coupled Determination of Cholesterol[a]

$$\text{cholesterol} + \text{O}_2 \xrightarrow[\text{H}_2\text{O}]{\text{CO}} \text{cholest-4-en-3-one} + \text{H}_2\text{O}_2$$

[a] CO, cholesterol oxidase.

Scheme 6. Reaction between Oxalate and Hydrogen Peroxide

$$2\text{H}^+ + \text{H}_2\text{O}_2 + \text{C}_2\text{O}_4^{2-} \rightarrow 2\text{CO}_2 + \text{H}_2\text{O}$$

Scheme 7. Electrochemiluminescent Reaction of $Ru(bpy)_3^{2+}$ and Oxalate

$$\text{Ru(bpy)}_3^{2+} \rightarrow \text{Ru(bpy)}_3^{3+} + \text{e}^-$$

$$\text{Ru(bpy)}_3^{3+} + \text{C}_2\text{O}_4^{2-} \rightarrow \text{Ru(bpy)}_3^{2+} + \text{C}_2\text{O}_4^{-\bullet}$$

$$\text{C}_2\text{O}_4^{-\bullet} \rightarrow \text{CO}_2 + \text{CO}_2^{-\bullet}$$

$$\text{Ru(bpy)}_3^{3+} + \text{CO}_2^{-\bullet} \rightarrow \text{Ru(bpy)}_3^{2+*} + \text{CO}_2$$

$$\text{Ru(bpy)}_3^{2+*} \rightarrow \text{Ru(bpy)}_3^{2+} + h\nu$$

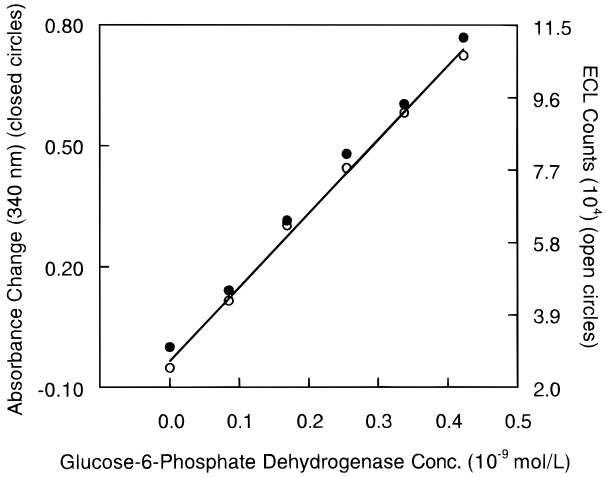

Figure 5. Comparison of spectrophotometric (closed circles) and ECL (open circles) determination of glucose-6-phosphate dehydrogenase concentration (Scheme 4). Enzymatic formation of NADPH was measured after 30 min incubations. Indicated enzyme concentrations are prior to dilution for analysis.

results with excellent correlation ($R^2 = 0.996$) (Figure 5). The data demonstrated that NADH and NADPH are essentially equivalent in promoting $Ru(bpy)_3^{2+}$ ECL. The lower detection limit of glucose-6-phosphate dehydrogenase using ECL under the conditions described here was approximately 0.10 nM.

Cholesterol Analysis. Both spectrophotometric and ECL assays for cholesterol employed cholesterol oxidase, an enzyme that produces hydrogen peroxide during the oxidation of cholesterol to cholest-4-en-3-one (Scheme 5).[8,9] As described in the Experimental Section, cholesterol oxidation is accompanied by an absorbance change at 240 nm, which can be used to quantitate cholesterol.[8,9] In the ECL assay of cholesterol described here, hydrogen peroxide formed by the action of cholesterol oxidase on cholesterol reacts with added oxalate to form water and carbon dioxide (Scheme 6). The reaction with peroxide destroys oxalate, thus preventing oxalate from participating in an ECL reaction with $Ru(bpy)_3^{2+}$. In the oxalate/$Ru(bpy)_3^{2+}$ ECL reaction, oxalate is oxidized by the ECL instrument electrode to form a strongly reducing carbon dioxide anion radical which promotes $Ru(bpy)_3^{2+}$ ECL as shown in Scheme 7.[10−12] Thus, hydrogen peroxide can

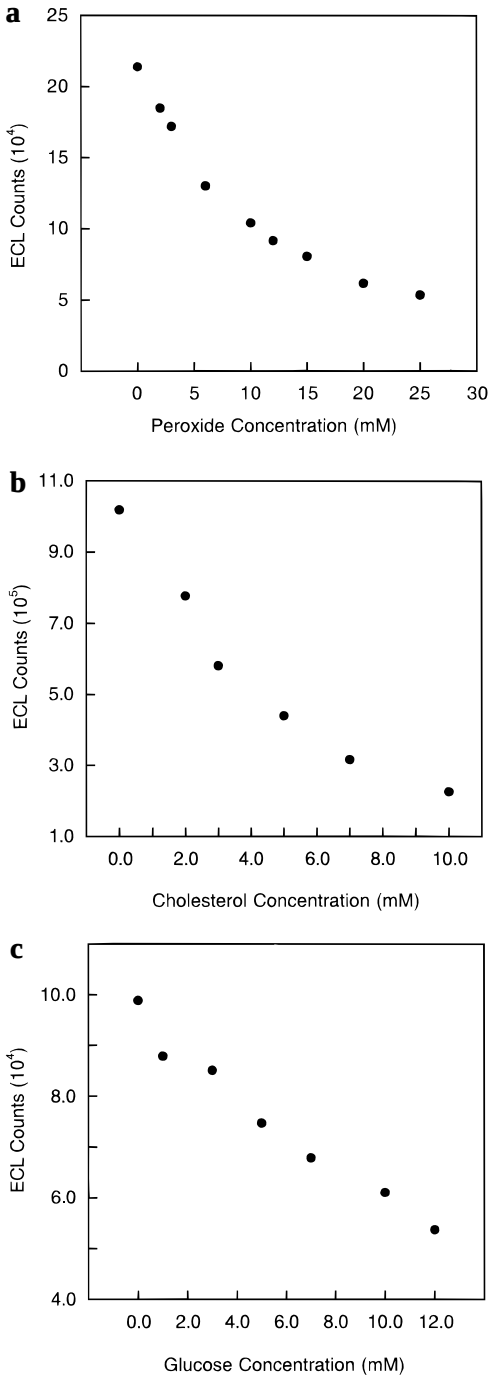

Figure 6. (a) ECL determination of hydrogen peroxide. Measurement is based on the ECL detection of remaining oxalate (Scheme 7) following the reaction with peroxide (Scheme 6). (b) ECL-based determination of cholesterol using peroxide-forming cholesterol oxidase (Scheme 5). (c) ECL-based determination of glucose using peroxide-forming glucose oxidase (Scheme 8).

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Scheme 8. Enzyme-Coupled Determination of Glucose[a]

$$\text{glucose} + O_2 \xrightarrow[H_2O]{GO} \text{gluconic acid} + H_2O_2$$

[a] GO, glucose oxidase.

be detected on the basis of its ability to diminish oxalate-promoted ECL. As shown in Scheme 7, hydrogen peroxide depletes oxalate in an equimolar stoichiometry.

The ECL intensity directly relates to the concentration of reductant, in this case, oxalate, which, in turn, is inversely proportional to the concentration of cholesterol. A technical concern was the efficiency of cholesterol oxidase at the slightly acidic pH values required for maximum ECL light generation using oxalate. The enzyme was a pH optimum of 7.5, while oxalate gives the strongest ECL signal at around pH 5 (see refs 10 and 11 and unpublished work of F. Jameison). Spectrophotometric assays performed at pH 5.0 and 6.0 showed that cholesterol oxidase was active at these pH values (data not shown).

The effect of 2−100 mM peroxide concentration on oxalate-promoted ECL was examined using a mixture of 500 μM oxalate and 1.0 μM $Ru(bpy)_3^{2+}$. As hypothesized (Schemes 6 and 7), a decrease in ECL intensity was observed with increasing peroxide concentration (Figure 6a). The plot presumably shows the effect of oxalate depletion on ECL. The ECL signal asymptotically approached zero at around 0.1 M peroxide.

Cholesterol was detected by ECL using cholesterol oxidase as shown in Schemes 5−7. Solution concentrations of cholesterol were examined within the clinical range (2−50 mM). The results showed that cholesterol was detectable over this range. The best results were obtained by diluting cholesterol samples to be within the range of 0−10 mM (Figure 6b). As with peroxide, an inverse somewhat curving relationship was observed between cholesterol concentration and ECL.

Glucose Assay. Glucose was quantitated in a similar fashion to cholesterol. Hydrogen peroxide produced from the action of glucose oxidase on β-D-(+)-glucose was coupled to a $Ru(bpy)_3^{2+}$/oxalate system (Scheme 8). In accord with the results described above with peroxide and cholesterol, an inverse relationship was observed between glucose concentration and ECL intensity (Figure 6c). Because oxalate is a poor promoter of ECL at the optimum pH of glucose oxidase, pH 6.0, the assay was performed in two steps. The enzymatic reaction was first allowed to go to completion. The enzyme reaction mixture was then mixed with a solution strongly buffered at pH 5.0, containing oxalate and $Ru(bpy)_3^{2+}$. This two-step assay permitted both rapid enzyme action and high ECL intensity.

CONCLUSION

Electrochemiluminescence is an extremely sensitive and versatile analytical tool. Subpicomolar concentrations of analytes are detectable in immunoassays using $Ru(bpy)_3^{2+}$-labeled antibodies and in DNA probe assays.[6] In the present report, the clinical utility of ECL has been extended to the area of classical clinical chemistry, where detection of extremely low concentrations is not as important as is versatility for a wide range of analytes. We show that it is probable that a single ECL analytical system could be used to quantitate classical clinical analytes, perform immunoassays, and detect DNA probes. Moreover, the same or similar instrument parameters could be used for all of these analyses.

Five classical clinical analytes were quantitated using different formats: glucose (coupled to both NADH- and H_2O_2-producing enzymes), ethanol (two NADH-producing enzymes in series), carbon dioxide (NADH-depleting enzyme), cholesterol (H_2O_2-forming enzyme), and glucose-6-phosphate dehydrogenase (enzyme rate measurement). The successful demonstration of a diverse set of assay formats opens the door to ECL assays for a wide assortment of classical clinical analytes.[1,2] The analytes shown here are only a small subset of the clinically relevant compounds conventionally or potentially detectable by the ECL effects of NAD(P)H and peroxide.[1,2] It is noteworthy that the analytes described here can also be satisfactorily detected by ECL in serum. Diluted serum had no effect on ECL analysis of ethanol. Given the relatively high concentrations of classical clinical analytes and the sensitivity of the ECL method, dilution of serum samples is an acceptable practice for ECL quantitation of clinical chemistry analytes. Despite the fact that the ECL analyses described here were carried out with solutions spiked with analyte, we have no evidence that ECL determinations could not be made on naturally occurring analytes in serum samples. These data demonstrate that ECL analysis can be used in conjunction with or as a substitute for the spectrophotometric determination of any analyte involving NAD^+/NADH, $NADP^+$/NADPH, or hydrogen peroxide-transforming systems. Furthermore, this method may prove to be more convenient than spectrophotometric nicotinamide cofactor- and H_2O_2-coupled assays as it employs a single instrument operating under similar conditions, obviating the need to assay such analytes at different wavelengths.

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