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Solid-Phase Electrochemical Enzyme Immunoassay with Attomole Detection Limit by Flow Injection Analysis

Yan Xu
Cleveland State University, y.xu@csuohio.edu

H. Brian Halsall
University of Cincinnati

William R. Heineman
University of Cincinnati

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YAN XU, H. BRIAN HALSALL and WILLIAM R. HEINEMAN

Abstract: A sandwich electrochemical enzyme immunoassay with flow injection analysis for the model antigen mouse IgG has been developed with alkaline phosphatase as the enzyme label. The enzyme substrate, 4-aminophenyl phosphate and its enzymatic reaction product, 4-aminophenol have been studied by cyclic and hydrodynamic voltammetry. The determination of 4-aminophenol by flow injection analysis with electrochemical detection (FIAEC) has a linear range of 5.0×10^{-8} to 1.0×10^{-5} M, a detection limit of 2.4×10^{-8} M, and a sample throughput of 72 samples/h. The detection limit is set by a background capacitance response, which depends on the ionic strength difference between the sample and the mobile phase. The sandwich immunoassay has been characterized with respect to substrate concentration for the enzymatic reaction, detection limit, dynamic range and sources of error. Mouse IgG can be determined with a detection limit of 0.81 pg ml^{-1} by a 30-min substrate incubation time and a six orders of magnitude linear dynamic range.

Keywords: *Flow injection analysis; electrochemical immunoassay; sandwich enzyme immunoassay; ELISA; 4-aminophenyl phosphate.*

Introduction

The rapid development of the medical and life sciences has placed more emphasis and greater demand on the methodology of fast, accurate and precise quantitation of extremely low amounts of analytes ($<10^{-18}$ mol) in small sample volumes (μl). The analytes can be drugs and their metabolites, antibodies, regulatory proteins, cancer markers and AIDS virus in blood serum or plasma, cell culture supernatants, amniotic, lymphatic, and other physiological fluids with the restriction of very low analyte content and small sample size. The combination of the analytical techniques of liquid chromatography electrochemistry (LCEC) or flow injection analysis electrochemistry (FIAEC) with enzyme immunoassay is frequently able to meet the needs of the clinical laboratory and the research community.

Electrochemical enzyme immunoassay methodology has been developed to utilize the specificity and sensitivity of an antibody (Ab)–antigen (Ag) reaction, the fast turnover

rate of substrate to an electroactive product under enzyme catalysis, and the capability of detecting a small sample volume by LCEC and FIAEC. Several heterogenous enzyme immunoassays with electrochemical detection [1–5] have been developed in this research group with detection limits of 50 pg ml^{-1} for digoxin by competitive assay [3] and 7.5 pg ml^{-1} for mouse IgG by sandwich assay [5].

Since its introduction in the 1970s [6, 7], the use of flow injection analysis (FIA) has grown enormously. This is directly attributable to the desirable features of fast analysis time, simple hardware, low cost, ease of use and versatility [8–10]. Additionally, FIA can easily function as an interface to convert existing batch methods into automated forms. Although FIA itself is unable to improve the selectivity of the analysis, it can be coupled with an assay that has high selectivity for the analyte to improve precision and sample throughput.

Several approaches [11–15] have been used to combine immunoassay with FIA. These include a homogeneous fluorescence energy-transfer immunoassay for serum albumin using stopped-flow injection analysis with merging zones [11], a homogeneous enzymatic fluorescence immunoassay for serum IgG by continuous FIA [12], FIA with chemiluminescence detection in the determination of fluorescence- and fluorescamine-labelled species [13], an "immunoprecipitin" reaction between concanavalin A (the model antibody) and yeast mannan (the model antigen) using stopped-flow merging zones analysis with turbidimetric detection [14], and liposome-enhanced flow injection immunoanalysis [15].

The feasibility and limitations of FIAEC as applied to immunoassay were first demonstrated in a competitive heterogeneous enzyme immunoassay for digoxin [3]. The detection limit of 50 pg ml^{-1} for digoxin was limited by the assay format and the blank signal of the substrate solution. This paper is concerned with the application of FIAEC to a sandwich-type enzyme immunoassay that is based on a new enzyme substrate, 4-aminophenyl phosphate [16].

Experimental

Apparatus

The FIAEC system used for this work is shown in Fig. 1. The amperometric controller was a Bioanalytical Systems LC-4A amperometric detector (BAS, West Lafayette, IN, USA). The amperometric transducer consisted of a glassy carbon working electrode, a

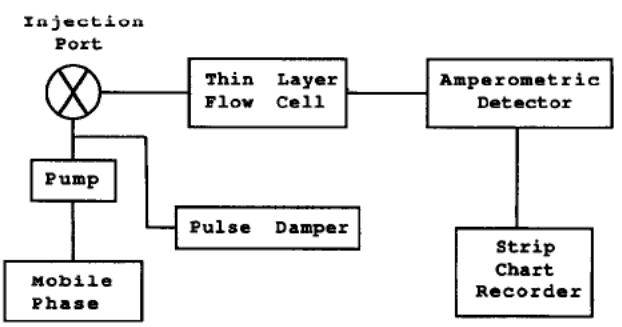


Figure 1
Schematic diagram of FIAEC apparatus.

Ag/AgCl (3 M NaCl) reference electrode, and a stainless-steel auxiliary electrode. The applied potential was 300 mV versus Ag/AgCl reference electrode unless specified otherwise. The injection valve had a 20-μl sample loop. The mobile phase was pumped at 1.0 ml min^{-1}.

The cyclic voltammogram was obtained with a BAS 100 electrochemical analyser, a Houston Instrument Hiplot™ digital plotter, and an electrochemical cell consisting of a glassy carbon electrode, a Ag/AgCl reference electrode, and a platinum wire auxiliary electrode.

Materials

Affinipure rat anti-mouse IgG (H+L) (415-005-100), Chrompure mouse IgG whole molecule (015-000-003), and alkaline phosphatase conjugated Affinipure rat anti-mouse IgG (H+L) (415-055-100) were obtained from Jackson Immunoresearch Laboratory (West Grove, PA, USA). Bovine albumin fraction V powder (the pH of a 1% w/v aqueous solution is approx. 5.2) (A-6918) and 4-aminophenol hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tris(hydroxymethyl) aminomethane (99.9+%) was obtained from Aldrich (Milwaukee, WI, USA) and the sodium azide was obtained from Eastman Organic Chemical (Rochester, NY, USA). The following chemicals were obtained from Fisher Scientific (Cincinnati, OH, USA): magnesium chloride, Tween® 20, sodium acetate (HPLC grade), sodium chloride, glacial acetic acid, ammonium chloride, and sodium bicarbonate. 4-Aminophenyl phosphate was synthesized as previously reported [16]. Nunc-immuno plate (6106-L10) was purchased from Thomas Scientific (Swedesbord, NJ, USA).

Buffers

The following aqueous buffer solutions were employed in the course of this study. Buffer A: 1.0×10^{-4} M sodium acetate–acetic acid, 0.05% (v/v) Tween® 20, and 0.02% (w/v) sodium azide at pH 5.0. Buffer B: 1.0×10^{-4} M sodium acetate–acetic acid, 0.05% (v/v) Tween® 20, 0.02% (w/v) sodium azide, and 2% (w/v) bovine albumin at pH 5.0. Buffer C: 0.10 M tris(hydroxymethyl)aminomethane, 1 mM magnesium chloride, and 0.02% (w/v) sodium azide. pH adjusted to 9.0 with hydrochloric acid.

Solutions

Primary rat anti-mouse IgG solution was prepared by a 1:2500 dilution of a stock solution (1.35 mg ml^{-1}) with buffer A. Mouse IgG standard solutions were diluted from a stock solution (5.7 mg ml^{-1}) with buffer B. The rat anti-mouse IgG alkaline phosphatase conjugate solution was prepared as a 1:2500 dilution of the stock solution with buffer B. The substrate solution was 4 mM of 4-aminophenyl phosphate in buffer C and was prepared prior to use in order to minimize its non-enzymatic hydrolysis. Buffer C was the mobile phase in the FIAEC system.

Assay procedures

In the sandwich enzyme immunoassay, the primary rat anti-mouse IgG solution (400 μl) was added to immuno-plate wells for overnight (12–14 h) adsorption after which the wells were rinsed three times with buffer B (380 μl). Each of the first two rinses was 5 min, and the third rinse was 20 min. Then, mouse IgG standard solutions (360 μl) were incubated in the wells at room temperature for 2 h, and the wells were rinsed with buffer B (380 μl) three times for 5 min each. After that, 360 μl of rat anti-

mouse IgG alkaline phosphatase conjugate solution were added to the wells and incubated for another 3 h. The wells were rinsed again consecutively with buffer C (380 μl) three times for 5 min each. Following this step, 360 μl of substrate solution (4-aminophenyl phosphate) were added to the wells and the enzymatic reaction was allowed to proceed for 30 min. At the end of the reaction, 20 μl were injected into the FIAEC system. The resulting peak currents from the oxidation of 4-aminophenol were used to prepare the standard calibration curves.

Results and Discussion

Electrochemical characteristics of enzyme substrate and its product

In previous electrochemical immunoassays [1–5], alkaline phosphatase was used as the enzyme label to convert the enzyme substrate, phenyl phosphate, to electroactive product, phenol. Phenol generated from enzymatic reaction was then detected by oxidation in a flow cell under an applied potential greater than +850 mV versus Ag/AgCl. Because of the high oxidation potential of phenol, the oxidation of electroactive impurities and the antibacterial reagent, NaN_3, in the buffer solution gave rise to a relatively large background current. Also, the oxidation product of phenol fouled the working electrode when the concentration of phenol exceeded 40 μM [16], which caused poor precision. These problems led to a search for a better enzyme substrate for alkaline phosphatase [16]. 4-Aminophenyl phosphate was chosen to take advantage of the low oxidation potential of 4-aminophenol, which is the product of the enzymatic reaction. The utility of this substrate–product system for immunoassay had been demonstrated previously by a competitive heterogenous enzyme immunoassay with LCEC for digoxin [16].

In the present study, 4-aminophenyl phosphate has shown advantages for detection by FIAEC. A cyclic voltammogram for 4-aminophenyl phosphate at a glassy carbon electrode is given in Fig. 2. The oxidation waves of 4-aminophenol and 4-aminophenyl phosphate exhibited peak potentials at +100 and +580 mV versus Ag/AgCl in buffer C, respectively. Therefore, the product, 4-aminophenol, could be easily detected by electrochemical oxidation without interference from the substrate, 4-aminophenyl phosphate. Additionally, the oxidation product of 4-aminophenol fouled the electrode less easily, which improved the precision of measurements at higher concentrations. The hydrodynamic voltammogram for 4-aminophenol in buffer C by FIAEC (Fig. 3) indicated that the limiting oxidation current starts at potentials greater than +250 mV versus Ag/AgCl, which is about 600 mV lower than that of phenol. This feature greatly reduced the background noise from the oxidation of the mobile phase by enabling a lower detection potential to be used. A potential of +300 mV was chosen for detection of 4-aminophenol in subsequent studies.

Electrochemical detection in FIA

Sample throughput in FIAEC is dependent on the time (t_a) from the sample injection to the initial appearance of peak-shaped signal from a baseline, and the baseline to baseline returning time (Δt_b) after the sample zone has passed the detector. Both of these timing factors are governed by various parameters, including the internal radius of the tubing, the length of tubing between the injector and the detector, the flow rate, and the diffusion coefficient of the analyte [8–10]. A system output may be improved by minimizing and controlling the sample dispersion process, such as by precisely

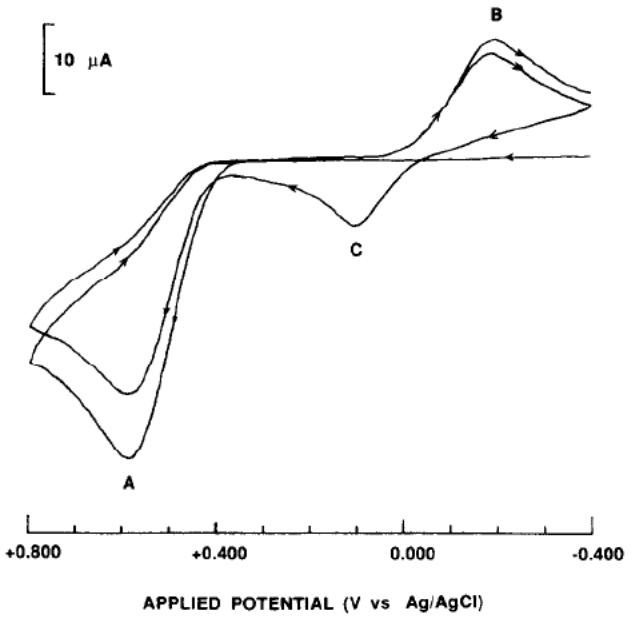
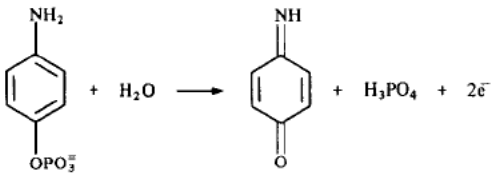
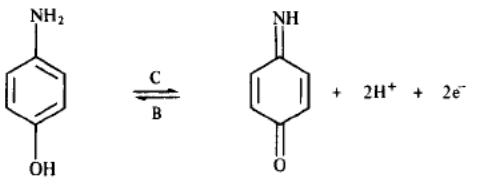


Figure 2
Cyclic voltammogram of 4-aminophenyl phosphate at a concentration of 4 mM in buffer C. Scan rate, 100 mV s^{-1}.

Peak A:



Peak B and peak C:



controlling the flow rate, using small-bore tubing and reducing the system mixing volume. A typical FIAEC readout from this study is shown in Fig. 4. Typical values of t_a and Δt_b were 4 and 46 s, respectively. The system could routinely perform at a rate of 72 samples/h by manual operation. There is room for further improvement in sample throughput by optimizing some of the parameters mentioned above and by automating the system.

A standard FIAEC calibration curve for 4-aminophenol in buffer C is given in Fig. 5. The linear range for the detection of 4-aminophenol was from 5×10^{-8} to 1×10^{-5} M [slope = 0.992 log(nA)/log(M), intercept = 7.552 log(nA)], which is compatible with the concentration of 4-aminophenol generated in a typical enzyme immunoassay. 4-Aminophenol could be determined with a precision of better than 1% over this range

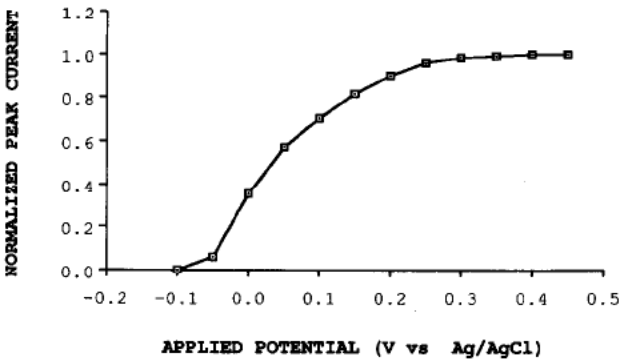


Figure 3
Hydrodynamic voltammogram of 4-aminophenol at a concentration of 1 μM in buffer C.

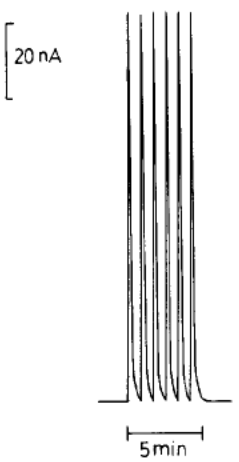


Figure 4
An illustration of sample throughput in FIAEC.
2.5 μM 4-aminophenol in buffer C.

(Table 1). At a concentration of $>1 \times 10^{-4}$ M, 4-aminophenol oxidation by exposure to air (O_2) and light (UV) occurred very rapidly. This could be minimized by deoxygenating the solution with N_2 and wrapping the container with aluminium foil. However, in an immunoassay, this problem is most easily circumvented by adjusting the substrate incubation time so that the concentration of enzyme-generated 4-aminophenol does not exceed 1×10^{-4} M.

An important phenomenon in FIAEC is a blank current response that occurs when sample is injected into the mobile phase. Since this blank response is obtained in the absence of electroactive material in the sample, it is attributed to a mixture of a capacitance response, i.e. non-faradaic charging current, caused by ionic changes in the electrical double layer associated with the electrode and reactions of organic surface functional groups such as protonation–deprotonation, and a faradaic response caused by reduction–oxidation of these surface functional groups on the carbon electrode. The magnitude of this blank response, referred to hereafter as capacitance current, is dependent on the differences in ionic strength, pH and dielectric constant between the sample and the mobile phase in the FIAEC system. Since it defines the detection limit,

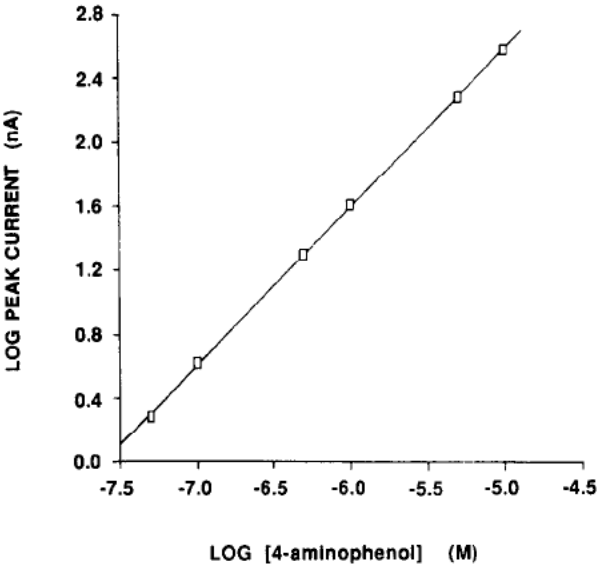


Figure 5
The standard calibration curve for the determination of 4-aminophenol in buffer C by FIAEC.

Table 1
The oxidation currents of 4-aminophenol in FIAEC

4-Aminophenol standard (M)	i_{ave}* (nA)	SD (nA)	RSD (%)
1×10^{-5}	384	2.2	0.57
5×10^{-6}	196	1.4	0.71
1×10^{-6}	41.0	0.00	0.00
5×10^{-7}	20.5	0.20	0.98
1×10^{-7}	4.21	0.033	0.78
5×10^{-8}	1.95	0.000	0.00

*The average peak current was based on four measurements.

the capacitance current is important in optimizing an analysis by FIAEC. The capacitance current was minimized in the study by matching the sample matrix with the mobile phase as shown in Fig. 6a. Figure 6b–d illustrates how the capacitance current increased due to differences in ionic strength and pH between sample and mobile phase. The response was typically a complicated pattern of both anodic and cathodic current. Of practical importance was the good reproducibility of the capacitance current for a particular sample–mobile phase system, which enabled it to be subtracted from the analytical signal due to the electroactive analyte, 4-aminophenol. If the analyte current signal is sufficiently large with respect to the capacitance current, the latter becomes insignificant and need not be corrected for. This can be achieved in enzyme immunoassays by enzymatically generating a sufficiently high concentration of product if the blank response is small enough. In this work, the mobile phase and sample matrix were matched as closely as possible to minimize the magnitude of the capacitance current, which was therefore not subtracted in the immunoassay.

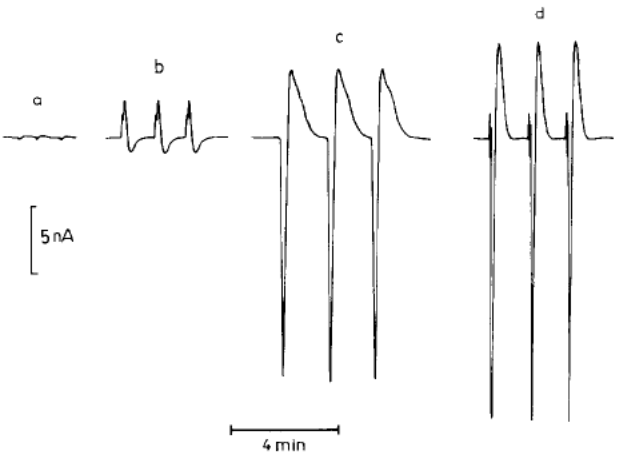


Figure 6
The capacitance current from a variety of injected solutions. (a) Buffer C; (b) deionized water; (c) $(NH_4)_2CO_3$–NH_4Cl, (0.1 M, pH 9); (d) buffer C, pH adjusted to 7 with hydrochloric acid.

Sandwich enzyme immunoassay

Mouse IgG (M_w ~150,000) was chosen as the model antigen in this study with the intent that the immunoassay procedure thus developed could be more widely applied directly or with slight modification.

Although details of optimization of the enzyme immunoassay procedure will be discussed in a separate paper devoted to a comprehensive study of antibody–solid phase interactions, a few salient points are mentioned here. The major step of the enzyme immunoassay procedure was to immobilize active primary antibody (rat anti-mouse IgG) on the surface of polystyrene immunoplate wells. In the assay, sodium acetate buffer (pH = 5) was used in all of the steps prior to the enzymatic reaction to serve this purpose. In the enzymatic reaction, buffer C was used because alkaline phosphatase displayed a maximum activity in 0.1 M tris(hydroxymethyl) aminomethane–hydrochloric acid at pH 9. The use of 0.05% Tween® 20 and 2% bovine serum albumin was to cover up the "bare" surface at the solid phase [3] and to interact with the residual negative electric potential of the solid matrix. The result was a reduction of the non-specific adsorption of antibody–enzyme conjugate and a reduction of the blank signal.

To maintain zero-order kinetics, the enzyme substrate concentration must be at least 20 times larger than the apparent Michaelis constant, $K_{m(app)}$. It has been reported that the $K_{m(app)}$ of alkaline phosphatase for 4-aminophenyl phosphate was $7.2 \pm 0.8 \times 10^{-5}$ M [17]. In this study, the concentration of 4-aminophenyl phosphate was 4 mM which was about 50 times $K_{m(app)}$.

Representative FIAEC immunoassay readouts for mouse IgG standards are shown in Fig. 7. The peak current of 4-aminophenol that was generated by enzymatic reaction was proportional to the amount of mouse IgG in the standard. A log–log plot of peak current versus mouse IgG concentration is given in Fig. 8. The detection limit of 0.81 pg ml^{-1} or 5.4 attomol ml^{-1} was calculated as the average blank signal (zero antigen concentration) plus four times the standard deviation. The linear range extended from the detection limit to 1×10^5 pg ml^{-1} [slope = 0.145 log(nA)/log(pg ml^{-1}), intercept = 1.493 log(nA)]. For the detection of mouse IgG, this work had a 10 times lower detection limit

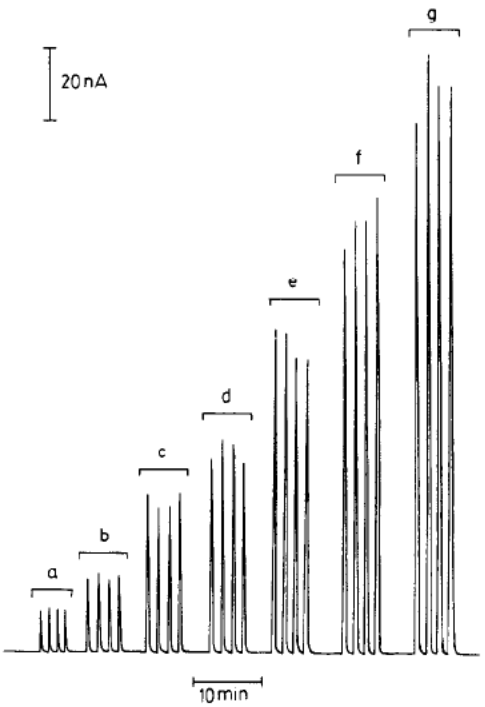


Figure 7
The readout of heterogeneous sandwich enzyme immunoassay with FIAEC detection for mouse IgG. (a) 4 mM of 4-aminophenyl phosphate in buffer C and the concentrations of mouse IgG in buffer B were: (b) 0; (c) 10; (d) 100; (e) 1000; (f) 10,000; and (g) 100,000 pg ml^{-1}.

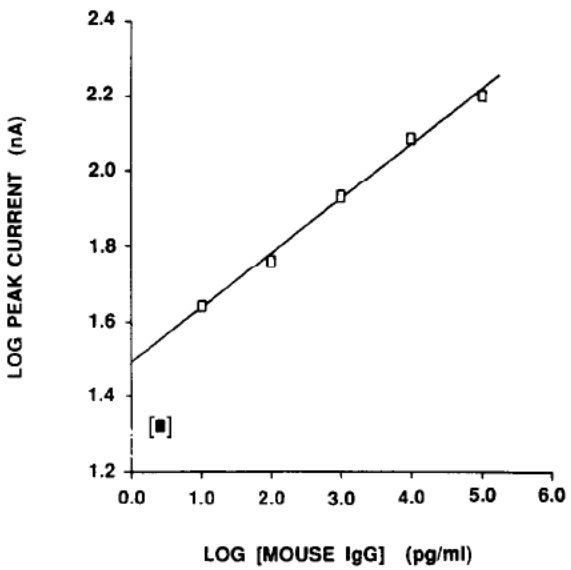


Figure 8
Log–log plot of FIAEC peak current versus mouse IgG concentration ($n = 4$) in buffer B. [■] denotes the 0.0 pg ml^{-1} mouse IgG concentration.

Table 2
The oxidation currents of 4-aminophenol generated by enzymatic reaction for a series of standards

Mouse IgG standard (pg ml^{-1})	i_{ave}* (nA)	SD (nA)	RSD (%)
0	21	2.3	11
10	44	5.0	11
100	58	7.8	13
1000	86	11.3	13
10000	123	16.1	13
100000	160	21.2	13

*The average peak current was based on four measurements.

and three orders of magnitude wider linear range than the previously reported sandwich assay for mouse IgG by LCEC detection [5]. These improvements were mainly due to improvements in the assay procedure, and the new substrate–product system. The main benefits of the FIAEC detection system are derived from the greater sample throughput and considerably simpler instrumentation compared with LCEC.

Evaluation of the error sources

Table 2 shows that the largest relative standard deviation (RSD) obtained in the construction of the standard curve for mouse IgG was 13%. Since the precision of determination of 4-aminophenol by FIAEC was <1% RSD (Table 1), the major source of error was in the immunological part of the assay, probably from inhomogeneity in the immuno-plate wells (Nunc-immuno plate product certificate). Another error source was attributed to temperature fluctuation during the enzymatic reaction, which might have affected enzyme activity. Since all injections were taken at a fixed time by manual operation, precise timing was critical.

Conclusions

It has been demonstrated that FIAEC is easily coupled to the sandwich enzyme immunoassay. With the model antigen, mouse IgG, this method has shown a wide linear dynamic range and excellent detection limits. Since a separation column was not used, FIAEC had simpler instrumentation and a shorter analysis time than LCEC. The new substrate, 4-aminophenyl phosphate has been adapted for the sandwich enzyme immunoassay with FIAEC and showed satisfactory features.

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