3,3′,5,5′-Tetramethylbenzidine as electrochemical substrate for horseradish peroxidase based enzyme immunoassays. A comparative study

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The use of 3,3′,5,5′-tetramethylbenzidine (TMB) as an electrochemical substrate for horseradish peroxidase (HRP) was investigated. HRP activity has been detected using flow injection analysis at a glassy carbon working electrode polarised at +100 mV versus Ag/AgCl in 0.1 mol l\textsuperscript{-1} citrate–phosphate buffer (pH 5.0). The optimum concentrations were 2 \times 10\textsuperscript{-6} mol l\textsuperscript{-1} TMB and 10\textsuperscript{-3} mol l\textsuperscript{-1} H\textsubscript{2}O\textsubscript{2}. The detection limit obtained after 15 min of incubation was 8.5 \times 10\textsuperscript{-11} mol l\textsuperscript{-1} HRP with the amperometric method. This limit was lower than that obtained using hydroquinone as HRP substrate and comparable to that with the p-aminophenol phosphate–alkaline phosphatase system. Better performance was achieved with amperometric than spectrophotometric detection using TMB in a competitive ELISA for rabbit immunoglobulin G as a model analyte.

Keywords: 3,3′,5,5′-Tetramethylbenzidine; horseradish peroxidase; amperometry; enzyme-linked immunosorbent assay; flow injection analysis

Enzyme immunoassays are based on selective antigen–antibody binding and a label enzyme. Depending on the assay format, the antigen or antibody is labelled and an enzyme activity measurement is performed as a final step of the assay. Fluorimetric,\textsuperscript{1–6} luminometric\textsuperscript{7–11} and colorimetric\textsuperscript{12–14} detection are widely used. The excellent sensitivity and wide linear range typical of electrochemical (particularly amperometric) detection have attracted attention in recent years, with the development of electrochemical enzyme immunoassays.\textsuperscript{15} This technique can be coupled with flow injection analysis (FIA), giving high reproducibility, partial automation and high sample throughput.\textsuperscript{16–20} Moreover, electrochemical detection is the first step for the future development of immunosensors in which the antigen–antibody reaction takes place on the surface of the transducer.\textsuperscript{21–27}

Alkaline phosphatase (AP) is the most commonly used enzyme label for enzyme linked immunosorbent assay (ELISA). This enzyme catalyses the hydrolysis of phosphate esters to give inorganic phosphate and a phenolic group. The formation of this phenolic moiety is generally followed by spectrophotometry using 4-nitrophenyl phosphate,\textsuperscript{28} by fluorescence with fluorescein phosphate,\textsuperscript{29} by chemiluminescence using dioxetane phosphate\textsuperscript{30} and by amperometry with 1-naphthyl phosphate\textsuperscript{31–33} or p-aminophenyl phosphate (PAPP). So far, PAPP has been defined as the best substrate for ELISA with amperometric detection\textsuperscript{34,35} and electrochemical immunoassays using this substrate have been reported.\textsuperscript{17,18,20,36–38} Horseradish peroxidase (HRP) is another enzyme label widely used in immunoassays. The enzyme activity can be determined by measuring the absorbance in the visible region,\textsuperscript{13,14} by fluorescence\textsuperscript{2–6,39} or by electrochemistry.\textsuperscript{19,24,25}

This paper reports on the use and analytical optimisation of 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate for the determination of HRP activity using FIA coupled with amperometric detection. This substrate has been reported to be suitable for use in ELISA with spectrophotometric detection\textsuperscript{40} and has already been used as an electrochemical mediator for cholesterol and H\textsubscript{2}O\textsubscript{2} detection.\textsuperscript{41,42} Recently, He and co-workers\textsuperscript{43,44} reported the use of TMB in a horseradish peroxidase based immunoassay with differential-pulse voltammetric detection. The electrochemical behaviours of TMB, hydroquinone (for HRP) and PAPP (for AP) were compared. TMB was found to be a good substrate for electrochemical detection of low levels of HRP. An ELISA competition assay using rabbit IgG as a model system and TMB as HRP substrate was also developed and the results are discussed.

Experimental

Reagents and materials

3,3′,5,5′-Tetramethylbenzidine dihydrochloride, hydroquinone, benzoquinone, p-aminophenol, horseradish peroxidase (EC 1.11.1.7) type VI-A, 1310 U mg\textsuperscript{-1}, alkaline phosphatase from bovine intestinal mucosa (EC 3.1.3.1) type VII-L, 1100 U mg\textsuperscript{-1}, rabbit immunoglobulin G (IgG), monoclonal anti-rabbit IgG (\(\gamma\)-chain specific) peroxidase conjugate and all other reagents of analytical-reagent grade were obtained from Sigma (St. Louis, MO, USA). A stock standard solution of TMB (0.01 mol l\textsuperscript{-1}) was prepared in distilled water and kept in a dark bottle. p-Aminophenyl phosphate was synthesised in the laboratory using a published procedure.\textsuperscript{45} Polystyrene microplates were obtained from Iwaki Glass (Iwaki City, Japan).

Apparatus

For electrochemical detection we used a thin layer cell for LCEC (liquid chromatography–electrochemistry) from Bio-Analytical Systems, West Lafayette IN, USA. This cell included a working electrode (glassy carbon disk, 3 mm diameter), a reference electrode (Ag/AgCl) and an auxiliary electrode (stainless steel). The current output was measured with a Metrohm (Herisau, Switzerland) 641 VA detector and recorded with a Model 868 recorder (Amel, Milan, Italy). For FIA, a Model 7125 HPLC valve (Rheodyne, Cotati, CA, USA) with a closed loop of 5 or 20 \(\mu l\) (Supelco, Bellefonte, PA, USA) was used. The working buffer was pumped with a Minipuls 3 peristaltic pump Gilson, (Villiers le Bel, France).

A Model 550 microplate reader (Bio-Rad, Hercules, CA, USA) was used for ELISA with spectrophotometric detection. Cyclic voltammetric studies were carried out with an Amel Model 433 polarographic analyser. The voltammetric cell consisted of a glassy carbon working electrode (3 mm diameter)
from Metrohm, a reference electrode (SCE) and a Pt counter electrode.

**Procedures**

**FIA**
The working buffer was passed through the electrochemical cell by a peristaltic pump until a constant baseline current was reached. A transient current variation was recorded after the injection of the analyte solution into the flow stream by means of the Rhodyne valve loop.

**ELISA**
A 1:10 000 dilution of rabbit IgG (solution A) was prepared from a stock standard solution (10 mg ml⁻¹) in 0.1 mol l⁻¹ phosphate-buffered saline (PBS) (pH 7.0). Anti-rabbit IgG–HRP conjugate solution (solution B) was prepared by 1:20 000 dilution of the Sigma stock standard solution (titre 1:40 000) in PBS. The substrate solution (solution C) consisted of 2 x 10⁻⁴ mol l⁻¹ TMB and 10⁻³ mol l⁻¹ H₂O₂; in 0.1 mol l⁻¹ citrate–phosphate buffer (pH 5.0). H₂O₂ was added to the TMB solution just before the measurement.

In the competitive enzyme immunoassay format, solution A (250 μl) was added to immuno-plate wells for 1 h at room temperature and the coated plates were blocked with 5% bovine serum albumine (BSA) in PBS at 4°C overnight. A 125 μl volume of solution B plus 125 μl of rabbit IgG standard solutions were then incubated in the wells at room temperature for 1 h. Between all the mentioned steps a three-cycle washing procedure with PBS was adopted. A 250 μl volume of solution C was added to the wells and the enzymatic reaction was allowed to proceed for 15 min. A 50 μl volume of 2.4 x 10⁻² mol l⁻¹ NaNO₃ were used to stop the reaction and 20 μl were injected into the FIA system.

**Results and discussion**

**Voltammetric studies**
HRP catalyses the following redox general reaction:

\[
\text{SubH}_2 \text{(red) + H}_2\text{O}_2 \rightarrow \text{Sub(ox)} + 2 \text{H}_2\text{O}
\]

The enzymatic activity can be measured by amperometric detection of the reduction current generated by Sub(ox) at an appropriate working electrode. Carbon electrodes and potassium iodide, o-phenylenediamine, hydroquinone, ferrocene and ferrocene derivatives have been already used for this purpose and for enzyme immunoassays.

Cyclic voltammetric investigation of these substrates and others used for spectrophotometric detection, such as o-dianisidine and tetramethylbenzidine, were carried out at a glassy carbon electrode. The most interesting results in terms of the generated electrocatalytic current (difference between the cathodic waves in buffer and in the presence of HRP) were observed with hydroquinone and TMB. The structure of TMB is shown in Fig. 1. Fig. 2 shows the voltammograms of TMB and hydroquinone in the potential range −500 to +800 mV; voltammograms 1A and 1B were obtained in the presence of the couples TMB and H₂O₂ and hydroquinone and H₂O₂ respectively. The buffers used were 0.1 mol l⁻¹ citrate–phosphate⁴⁰ plus 0.1 mol l⁻¹ KCl for TMB and 0.1 mol l⁻¹ phosphate⁴⁴ plus 0.1 mol l⁻¹ KCl for hydroquinone. Two-electron redox behaviour was observed for TMB with oxidation peaks at +250 and +400 mV versus SCE. The formation of radical intermediates during the electrochemical oxidation of molecules such as benzidine and dianisidine has been evidenced in earlier polarographic studies.⁴⁷ This supported the hypothesis of two subsequent one-electrode step processes for the oxidation of benzenes by HRP in the presence of H₂O₂. The formation of a radical cation and the two-step mechanism were demonstrated for TMB using spectrophotometric and ESR data.⁴⁸⁴⁹ In solution the radical cation is a semiquinone–imine in equilibrium with a charge-transfer complex (blue) of the diamine (electron donor) and the diimine (electron acceptor).⁴⁹

Our electrochemical data for TMB confirmed the previous polarographic data for the oxidation of benzidines.

The electrochemistry of hydroquinone was as reported previously.²⁴ The addition of HRP (0.1 U ml⁻¹) to the two substrate solutions resulted in the consumption of TMB and hydroquinone and the formation of reaction products. Consequently, a decrease in the oxidation and an increase in the reduction currents (Fig. 2, voltammograms 2A and 2B) were observed.

Amperometric FIA hydrodynamic voltammetry for TMB was performed in the range +350 to 0 mV to assess the best working potential. As shown in Fig. 3, substrate oxidation was observed for potentials ranging between +350 and +150 mV (curve a); curve b represents the background currents in citrate–phosphate buffer.

A working potential of +100 mV versus Ag/AgCl was selected for the measurement of HRP enzymatic activity. In fact, at this potential, the current background was near to zero and no substrate oxidation occurred. These conditions are the optimum for enzymatic activity determination in which a small amount of product [TMB (ox) in this case] needs to be measured in the presence of high concentrations of substrate. The applied

![Fig. 1 TMB structure.](image-url)
potential selected for measurement with hydroquinone was 
\(-250 \text{ mV versus Ag/AgCl}\).

**FIA measurement of reaction products and HRP activity**

The response of the two HRP reaction products [TMB (ox) and benzoquinone] in the FIA system at the selected potentials was tested. As the oxidised form of TMB was not commercially available, it was produced enzymatically using \(\text{H}_2\text{O}_2\) five times more concentrated than TMB. The production of the oxidised TMB was monitored by the measurement of the absorbance at 450 nm until the value remained constant. The concentration of TMB (ox) was calculated using a constant absorbance value and the molar absorptivity. The concentration obtained was in agreement with that expected.

Using a 5 µl loop and a flow rate of 200 µl min\(^{-1}\), an RSD of 10% was observed for consecutive injections of TMB (ox), and 2–3% when a 1 min wash at a high flow rate was carried out between the injections. This was confirmed by the flow rate study shown in Fig. 4; the sensitivity and RSD decreased in the range 200–800 µl min\(^{-1}\). A current signal about four times higher was obtained on replacing the 5 with a 20 µl sample loop. This loop and a flow rate of 800 µl min\(^{-1}\) were selected for the measurement of TMB (ox). Benzoquinone, the HRP reaction product of the hydroquinone, was detected using a 20 µl loop and a 200 µl min\(^{-1}\) flow rate. As shown in Fig. 5, the linearity range was \(5 \times 10^{-8} \text{–} 1 \times 10^{-3} \text{ mol l}^{-1}\) with a sensitivity of 36 nA µmol l\(^{-1}\) and \(5 \times 10^{-8} \text{–} 5 \times 10^{-5} \text{ mol l}^{-1}\) with a sensitivity of 16 nA µmol l\(^{-1}\) for benzoquinone and TMB, respectively (higher concentrations of benzoquinone were not soluble in the working buffer). The response (oxidation) of \(p\)-aminophenol (product of the reaction of PAPP in the presence of AP) at the applied potential of +100 mV versus Ag/AgCl is reported in Fig. 5(B) for comparison. The sensitivity was similar to that obtained with TMB (ox) and the linearity range was \(10^{-7} \text{–} 10^{-4} \text{ mol l}^{-1}\) in 0.1 mol l\(^{-1}\) diethanolamine (DEA) buffer (pH 8.0). The optimum concentration of substrates for the measurement of HRP activity was then studied. The current change was recorded after 1 min of incubation with \(10^{-3} \text{ U ml}^{-1}\) of HRP and plotted for different substrate to \(\text{H}_2\text{O}_2\) ratios. The results for TMB, reported in Fig. 6, indicated that the highest current output was achieved using \(2 \times 10^{-4} \text{ mol l}^{-1}\) TMB and \(10^{-3} \text{ mol l}^{-1}\) \(\text{H}_2\text{O}_2\). The apparent \(K_m\) for \(\text{H}_2\text{O}_2\) under these experimental conditions was \(5.4 \times 10^{-4} \text{ mol l}^{-1}\) (\(V_{\text{max}} = 355 \text{ nA min}^{-1}\)). These parameters were not calculated for TMB because of the limited solubility in the working buffer (\(3 \times 10^{-4} \text{ mol l}^{-1}\)). It should be noted that when working at the selected concentration of \(\text{H}_2\text{O}_2\) (\(10^{-3} \text{ mol l}^{-1}\)) a twofold increase in current was obtained on switching from \(1 \times 10^{-4}\) to \(2 \times 10^{-4} \text{ mol l}^{-1}\) TMB (Fig. 6). This indicates that \(2 \times 10^{-4} \text{ mol l}^{-1}\) TMB is not the ideal substrate concentration. The sensitivity of the system could be improved by increasing the solubility of TMB provided that no electroactive compounds are present.
used. The optimum concentration for hydroquinone was found to be $10^{-3}\text{ mol l}^{-1}$ with a 1 : 1 molar ratio of $\text{H}_2\text{O}_2$ owing to the spontaneous reaction of the substrates.

Calibration curves for HRP were constructed for a 15 min incubation of the enzyme with the substrate mixture at room temperature. Under the optimum conditions it was possible to measure as low as $1.7 \times 10^{-12}\text{ mol l}^{-1}$ HRP ($10^{-5}\text{ U ml}^{-1}$) with hydroquinone [Fig. 7(A)] and $8.5 \times 10^{-14}\text{ mol l}^{-1}$ HRP ($5 \times 10^{-6}\text{ U ml}^{-1}$) with TMB [Fig. 7(B)]. Using TMB as the enzymatic substrate, concentrations of HRP higher than $8.5 \times 10^{-12}\text{ mol l}^{-1}$ gave currents outside the linearity range obtained for TMB (ox). Although the electrochemical detection of benzoquinone was found to be more sensitive than that of TMB (ox) (Fig. 5), TMB appears to be more suitable for use as a substrate for HRP determination because of its limited spontaneous reaction with $\text{H}_2\text{O}_2$ (blanks after 15 min: TMB and $\text{H}_2\text{O}_2 = 4\text{ nA}$, hydroquinone and $\text{H}_2\text{O}_2 = 300\text{ nA}$; Fig. 7).

Spontaneous oxidation of HRP substrates by $\text{H}_2\text{O}_2$ has already been observed in other studies.$^{19,44}$ Using $10^{-3}\text{ mol l}^{-1}$ PAPP at $+100\text{ mV versus Ag/AgCl}$ in 0.1 mol l$^{-1}$ DEA buffer (pH 9.0), we were able to detect $4.5 \times 10^{-15}\text{ mol l}^{-1}$ of AP ($2 \times 10^{-2}\text{ mol l}^{-1}$) after 15 min of incubation (data not shown).

The enzyme-substrate incubation time in ELISA ranges from a few minutes to 1 h or more depending on the analysis time and sensitivity required. Hence the stability of the reagents under the working conditions is one of the major issues to be addressed when developing new ELISA methods. It has been already reported that $p$-aminophenol is not stable in alkaline media.$^{31}$ We observed a decrease of 35% in the electrochemical signal after 30 min in 0.1 mol l$^{-1}$ DEA buffer (pH 9.5) and of 23% in DEA (pH 9.0), whereas the signal was stable in the pH range 8-8.5. The highest sensitivity for the measurement of AP activity was attained by performing the enzymatic reaction (with $10^{-3}\text{ mol l}^{-1}$ PAPP) at pH 9.0 and then measuring at pH 8.0 (by addition of $\text{HPO}_4^{2-}$). TMB was stable in the working buffer (0.1 mol l$^{-1}$ citrate-phosphate (pH 5.0)) for at least 2 h; TMB (ox) was stable for 30 min; a 15% decrease in the signal was observed after 45 min.

**Electrochemical ELISA**

The reaction of TMB with $\text{H}_2\text{O}_2$ in the presence of HRP gives rise to a coloured product that is measured at 655 or 450 nm after $\text{H}_2\text{SO}_3$ blockage.$^{40}$ A comparison of chromogenic substrates for HRP as the label in enzyme immunoassays demonstrated that TMB gave a high sensitivity, comparable to that of a fluorimetric method using 3-$(p$-hydroxyphenyl)pro- 

Fig. 6 Current response after addition of HRP ($10^{-3}\text{ U ml}^{-1}$; incubation time, 1 min) versus $\text{H}_2\text{O}_2$ concentration. Concentration of TMB: (●) $5 \times 10^{-4}$; (△) $1 \times 10^{-4}$; and (□) $2 \times 10^{-4}\text{ mol l}^{-1}$.

Fig. 7 Calibration curves for HRP obtained after 15 min of incubation of the enzyme (A) with $10^{-3}\text{ mol l}^{-1}$ hydroquinone + $10^{-3}\text{ mol l}^{-1}\text{H}_2\text{O}_2$, and (B) with $2 \times 10^{-3}\text{ mol l}^{-1}$ TMB + $10^{-3}\text{ mol l}^{-1}\text{H}_2\text{O}_2$. Insets, expanded scales.

Conclusions

The use of TMB as an HRP substrate for ELISA with electrochemical detection has been investigated. This substrate exhibited better performance in terms of sensitivity than other substrates already used for the amperometric detection of HRP activity. The detection limit for HRP activity was of the same order of magnitude as that with the PAPP-AP electrochemical system at the same applied potential. This is very important for the future development of immunosensors for measurements in the field; in fact, using a carbon working electrode at an applied potential of $+100\text{ mV versus Ag/AgCl}$, both AP- and HRP-based ELISAs can be carried out.
Fig. 8 Calibration curves of rabbit IgG in the range $5 \times 10^{-3}$–$10 \mu g ml^{-1}$ obtained with (●) amperometric and (▲) spectrophotometric detection. Each point is the mean of four determinations.

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