Determination of horseradish peroxidase and a peroxidase-like iron porphyrin at a Nafion-modified electrode

Chantal Degrand,[a] Benoît Limoges,[b] Anne-Marie Martre[a] and Bernd Schöllhorn*[c]

[a] Equipe Electrosynthèse et Electroanalyse Bioorganique, UMR CNRS 6504, Université Blaise Pascal de Clermont-Ferrand, 24 Avenue des Landais, 63177 Aubière Cedex, France

[b] Laboratoire d'Electrochimie Moléculaire, UMR CNRS 7591, Université Paris 7 - Denis-Diderot, 2 Place Jussieu, 75251 Paris, France

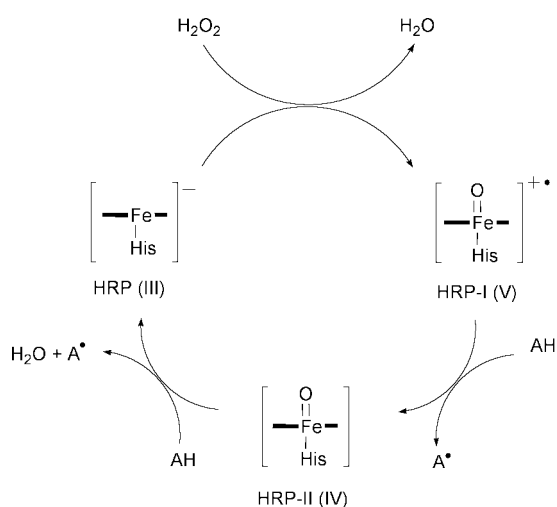[c] Ecole Normale Supérieure, Département de Chimie, UMR CNRS 8640 - PASTEUR, 24 Rue Lohmond, 75231 Paris, France

A catalytic coupling reaction between 4-amino antipyrine and a *N,N*-disubstituted aniline derivative has been exploited in the indirect electrochemical detection of horseradish peroxidase (HRP) and of a biomimetic catalyst, the iron(III) sulfonated tetraphenyl porphyrin. In the presence of hydrogen peroxide and one of the two catalysts a cationic electroactive quinone–iminium dye P^+ was formed and detected by linear scan voltammetry using a screen-printed electrode coated with a Nafion film. Detection limits of 10^{-12} M for HRP and 4×10^{-10} M for the iron porphyrin have been achieved. In conclusion the iron porphyrin is considered to be a promising alternative to the HRP label in enzyme immunoassays with electrochemical detection.

Introduction

The peroxidase-catalysed reaction is one of the most widely used reactions in enzyme immunoassays.[1] Peroxidases are a class of iron(III) porphyrins containing proteins that catalyse the oxidation of substrates by hydrogen peroxide.[2] The peroxidase reaction cycle is exemplified in Scheme 1 for horseradish peroxidase (HRP) in the presence of an electron donor AH. A two-electron oxidation of the ferrihaem prosthetic group of the native peroxidase HRP by hydrogen peroxide is involved in the first reaction step, resulting in the formation of an intermediate compound HRP-I (formal oxidation state of the iron: +5) consisting of oxyferryl iron ($Fe^{4+}=O$) and a porphyrin cation radical. In the next reaction, compound HRP-II is formed (oxidation state +4) upon one-electron reduction of compound HRP-I by an electron donor AH. In the last step, an additional electron is accepted from AH, and consequently the enzyme returns to its native state.

Chromogenic electron donors AH such as aromatic amines and phenolic compounds in the presence of H_2O_2 have been widely used for the indirect determination of the HRP label in enzyme immunoassays.[3,4] Some of these compounds are carcinogenic or hazardous, and have been replaced with a safer chromogen system consisting of 4-aminoantipyrine (AAP) and a *N,N*-disubstituted aniline derivative.[5–7] A quinone–iminium dye P^+ is thus generated through an oxidative coupling reaction [eqn. (1)]. Owing to the presence of a quinone–iminium function, the dye P^+ is intrinsically electroactive. However, the electrochemical detection of P^+ has not been exploited so far.

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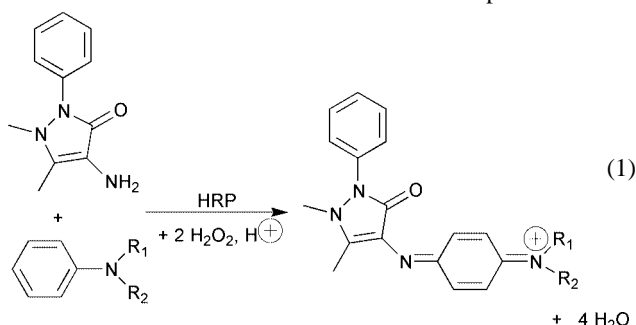
In this report, the 4-aminoantipyrine coupling reaction is shown to be adapted to the indirect picomolar electrochemical detection of HRP by linear scan voltammetry (LSV) using a screen-printed electrode (SPE) modified by a Nafion film.[8] The cation-exchange properties of the Nafion film coating are beneficially exploited to retain and preconcentrate the cation P^+ formed during the coupling reaction. Moreover, it is shown that the electrochemical procedure allows the subnanomolar detection of a peroxidase-like iron porphyrin to be achieved.

Experimental

Material and reagents

A 5 wt% Nafion solution (EW1100), 4-aminoantipyrine (AAP), 2-(*N*-ethyl-*m*-toluidino)ethanol (MEHA), citric acid disodium

Scheme 1 The peroxidase reaction cycle in the case of HRP. (His corresponds to the histidine amino acid coordinated at the proximal side of the heme.)

salt sesquihydrate and magnesium chloride hexahydrate were purchased from Aldrich (France). HRP (Type II, 150–200 units mg^{-1}, No. P8250), hydrogen peroxide (ACS reagent, 30 wt% in water), tris(hydroxymethyl)aminomethane (TRIZMA base and TRIZMA hydrochloride) were obtained from Sigma (France). Iron(III)-sulfonated tetraphenyl porphyrin (Fe-TPPS$_4$) was purchased from Midcentury (USA).

Citrate buffer (50 mM citric acid, 50 mM NaCl, pH 5.0) and TRIS buffer (50 mM TRIZMA base, 50 mM TRIZMA hydrochloride, 1 mM MgCl$_2$·6H$_2$O, and 50 mM NaCl, pH 9.2) were prepared using deionised and doubly distilled water. Stock solutions of H$_2$O$_2$ (10^{-3} M), HRP (5 × 10^{-8} M), Fe-TPPS$_4$ (2.5 × 10^{-5} M) were prepared in distilled water or buffers and stored at 4 °C as well as the substrate solution (AAP + MEHA) containing 5 × 10^{-4}M AAP and 5 × 10^{-3}M MEHA. Other chemicals used were of reagent grade.

Apparatus and electrodes

Cyclic voltammetry (CV) and linear scan voltammetry (LSV) were performed on a Princeton Applied Research Model 273 potentiostat (EG&G Instruments) controlled by an IBM personal computer with EG&G PARC Model 270 electrochemical software for parameter setup and data acquisition. All of the experiments were carried out in an electrochemical cell containing 10 mL of solution, and equipped with a platinum wire as the counter-electrode and a SCE as the reference electrode. The electrochemical cell and the stock solutions were protected from ambient light because reagents were observed to be light sensitive. The electrodes (SPEs and Nafion-SPEs) of 3.5 mm diameter were prepared as previously described.[8]
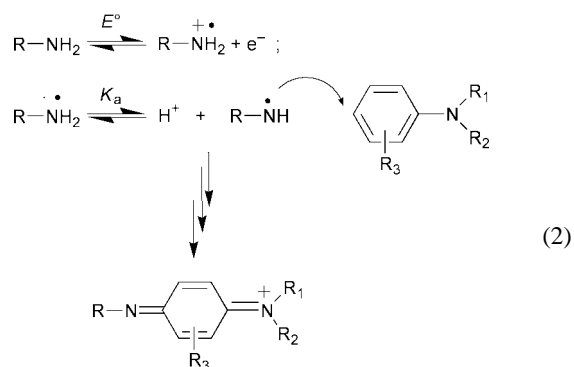
Results and discussion

Detection of HRP

The first enzymatic reaction examined was the HRP-catalysed oxidative coupling of 4-aminoantipyrine and 3-methyl-*N*-ethyl-*N*-(β-hydroxyethyl)aniline (MEHA) in the presence of hydrogen peroxide. This coupling reaction has already been thoroughly studied in order to develop a flow-through optical biosensor based on the permanent immobilization of HRP and transient retention of the cation P$^+$ on a Sephadex resin.[9] A citrate buffer (pH 5) was selected as it provided the optimal medium for the enzymatic reaction. The same non-deaerated buffer solution was adopted in the following study.

It was previously shown that cyclic voltammetry (CV) of a millimolar AAP solution (pH 4.86) yields a series of four irreversible oxidation peaks (potential range 0.64–1.30 V *vs*. Ag/AgCl) at a stationary glassy carbon electrode.[10] At a SPE, cyclic voltammetry of AAP showed two anodic peaks at low scan rates, the peak potential value E_p of the first one being observed at 0.5V (Fig. 1). No cathodic peak was observed on the reverse cathodic sweep. The first peak was split into two peaks as the potential sweep was increased (not shown). The oxidation of MEHA proceeded irreversibly and at potentials more positive than the first oxidation step of AAP (E_p = 0.75 V) (insert in Fig. 1). This latter result is consistent with previous studies which showed that AAP underwent anodic oxidation more readily than *N*-alkylanilines, which is probably due to a contribution from the enamine structure.[11] When the CV curve corresponding to the first oxidation step of AAP was recorded in the presence of MEHA, a cathodic peak was observed at −0.08 V upon reversal of the scan direction, to which was associated an anodic peak of low amplitude (E_p = 0.23 V) in a multiple sweep (Fig. 1). This peak system remained unchanged when the citrate buffer was deaerated, indicating that oxygen removal was not required,

because oxygen reduction was not detectable in the potential range of the peak system.

The LSV cathodic waves shown in Fig. 2A were recorded at a stationary SPE after HRP-catalysed coupling of AAP and MEHA in the presence of H$_2$O$_2$ during different incubation periods. A HRP concentration of 10^{-10} M was selected. The cathodic peak observed at −0.14 V was attributed to the reduction of the cationic quinone-iminium dye to the corresponding aromatic diamine (2e$^-$ process). The peak current was observed to increase linearly with the incubation period. Since the E_p value was close to the value of the cathodic peak observed when AAP was electrochemically oxidized in the presence of MEHA (Fig. 1), this suggests that the electrochemically-induced and the enzymatically HRP-catalysed coupling of AAP and MEHA led to the same product P$^+$. A mechanism for the electrochemically induced coupling reaction is proposed below.

$$R\text{—}NH_2 \underset{}{\overset{E^{\circ}}{\rightleftharpoons}} R\text{—}\overset{+\,\bullet}{N}H_2 + e^- \;;$$

$$R\text{—}\overset{\bullet}{N}H_2 \underset{}{\overset{K_a}{\rightleftharpoons}} H^+ + R\text{—}\overset{\bullet}{N}H$$

$$R\text{—}N=\!\!\!\!\bigcirc\!\!\!\!=\overset{+}{N}\langle{{R_1}\atop{R_2}} \tag{2}$$

When a Nafion-modified SPE was immersed in the solution during the incubation step preceding the voltammetric recording, the cathodic signal was shifted to more negative values (E_p = −0.443V) and the peak intensity was increased four-fold (Fig. 2B). The cathodic shift and the current amplification were consistent with the accumulation of the cathodic dye P$^+$ within the Nafion film during the incubation step. Similar results were previously obtained for other cationic species.[12] The amplification factor was even better when the HRP concentration was 10^{-11} M (amplification factor of *ca*. 11) (Fig. 3A) and a HRP detection limit[13] of 10^{-12} M could be achieved after a 35 min incubation period. In Fig. 2B, the current responses are less peak shaped than in Fig. 2A, probably because there are some kinetics and/or mass transport complications for the 2 e$^-$, 2 H$^+$ transfer of the quinone–immine dye in the Nafion-film.

An attempt was made to replace MEHA by *N,N*-dimethylaniline in order to lower the HRP detection limit at a Nafion-SPE. But no reproducible results could be obtained for incubation
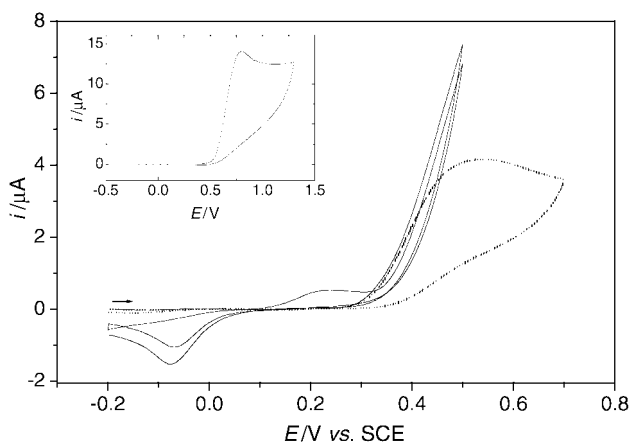

Fig. 1 CV curves recorded at a SPE in the presence of AAP (5 × 10^{-4} M) alone (dotted curve) and after addition of MEHA (10^{-3} M) (2 cycles). pH 5. Scan rate 0.02 V s^{-1}. Insert: MEHA (2 × 10^{-3} M), pH 5. Scan rate 0.1 V s^{-1}.

periods longer than 10 min. No cathodic peak appeared when MEHA was replaced by a p-substituted aniline, $e.g.$, 4′-aminoacetanilide, Fast Blue or 2,4-dimethoxyaniline, which confirms that the HRP-catalysed coupling reaction does not take place, because the dye formation involves the $para$ hydrogen.[14]

In order to compare the electrochemical and the colorimetric sensitivity of HRP, the photometric detection of HRP was monitored at 550 nm. After incubation for 35 min (same experimental conditions as in Fig. 3A), a picomolar detection limit was thus achieved by the colorimetric method; $i.e.$, the same limit as in the case of the electrochemical technique. Clearly the spectrophotometric and electrochemical detection limits of an enzyme immunoassay involving HRP as the label and a mixture of AAP + MEHA as the substrate are expected to be similar. However, the electrochemical technique incorporates advantages over the colorimetric procedure when the cationic dye P^+ is enzymatically generated in the vicinity of the Nafion film. This has been demonstrated in the case of the competitive enzyme immunoassay of the 2,4-dichlorophenox-

yacetic acid herbicide (2,4-D) using immunomagnetic beads.[15] A detection limit below 0.01 ppb ($i.e.$, 4.5×10^{-11}M of 2,4-D) was thus achieved and the electrochemical assay was shown to be $ca.$ 70-fold more sensitive than in the case of a commercial kit assay with colorimetric detection. The principle of the method is recalled in Fig. 4. The entire assay was performed in a microwell-shaped electrochemical cell. After the competitive immunoreaction between the analyte (2,4-D) and HRP-labelled 2,4-D for a limited amount of antibody-coated magnetic beads (step 1), the immunomagnetic beads were magnetically separated and washed (step 2). Then, the beads being magnetically localized on the Nafion-SPE, the HRP-catalysed coupling of AAP and MEHA proceeded in the presence of H_2O_2. The cationic dye P^+ was thus generated close to the Nafion film in which it was immediately entrapped (step 3), prior to its voltammetric detection (step 4). The beneficial effect of localizing the beads on the electrode surface was also previously demonstrated when the enzyme label was alkaline phosphatase and the enzyme product a cationic phenol derivative.[16] In this latter case, the electric signal corresponding to the anodic oxidation of the phenolic group was shown to be 40-fold increased with the magnetic accumulation.
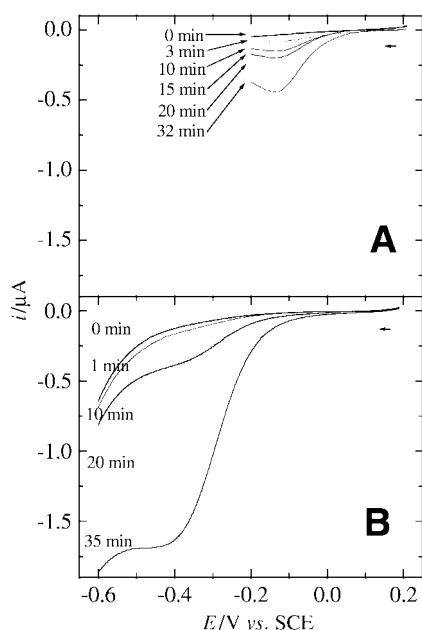
Detection of a peroxidase-like iron porphyrin

The study of potential HRP mimics such as metalloporphyrins is one of the interesting trends in enzyme immunoassays,[17–19] since HRP is expensive and its solution is not stable. The catalytic efficiency of peroxidase-like metalloporphyrins (Me-P) was previously studied for catalysing the chromogenic reaction of AAP and p-chorophenic acid with hydrogen

Fig. 2 LSV curves recorded at a SPE (A) and a Nafion-SPE (B) after oxidative coupling of AAP (5×10^{-4} M) and MEHA (5×10^{-3} M) in the presence of H_2O_2 (10^{-3} M) and HRP (10^{-10} M). pH 5. Scan rate 0.1 V s^{-1}. The incubation periods are indicated on each curve.
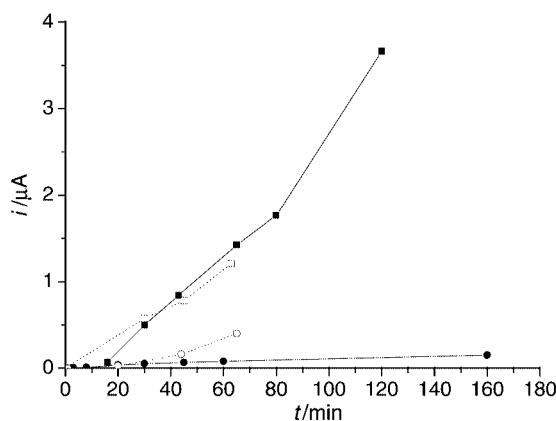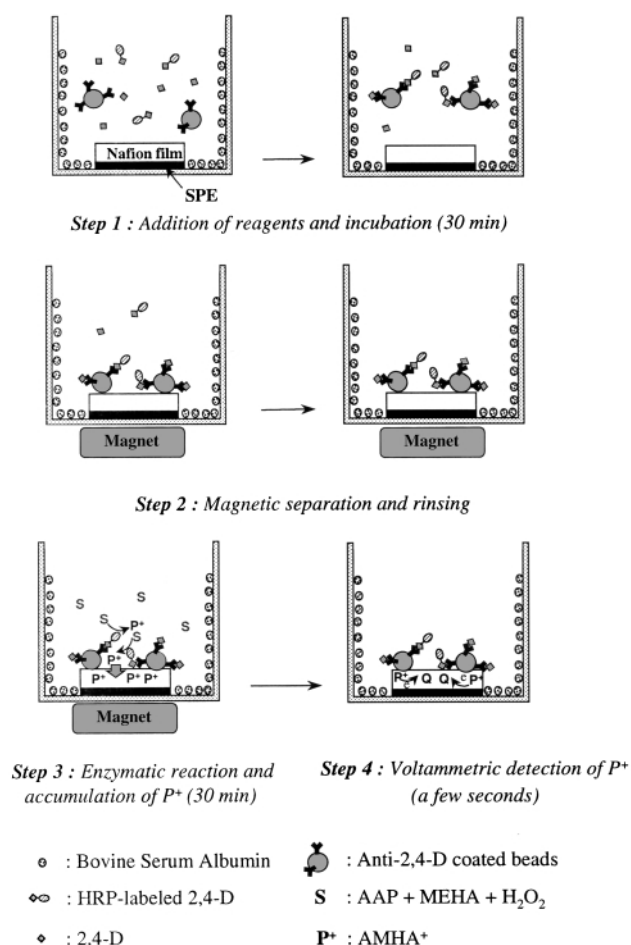
Fig. 3 Influence of the incubation period on the LSV cathodic current recorded (A) at pH 5 and measured at −0.2 V at a naked SPE (–●–●–) or at −0.4 V at a Nafion-SPE (–■–■–) in the presence of HRP (10^{-11} M), and (B) at pH 9.2 and measured at −0.4 V at a Nafion-SPE in the absence (–○–○–) and in the presence (–□–□–) of Fe-TPPS$_4$ (10^{-9} M). The concentrations of AAP, MEHA and H_2O_2 are the same as in Fig. 2.

Fig. 4 Schematic representation of the 2,4-D enzyme-linked immunomagnetic electrochemical procedure.

peroxide.[20] It was found that the catalytic activity of the water-soluble iron(III)-sulfonated tetraphenyl porphyrin (Fe-TPPS$_4$) was the highest among the Me-P systems, the optimal conditions being achieved in the 9.2–9.5 pH range. However, the catalytic activity of Fe-TPPS$_4$ was much lower than in the case of HRP (ca. 370 times). The solutions of Fe-TPPS$_4$ were shown to be stable for at least three years when stored at room temperature in the dark.[20] This iron(III) porphyrin was employed in this study.
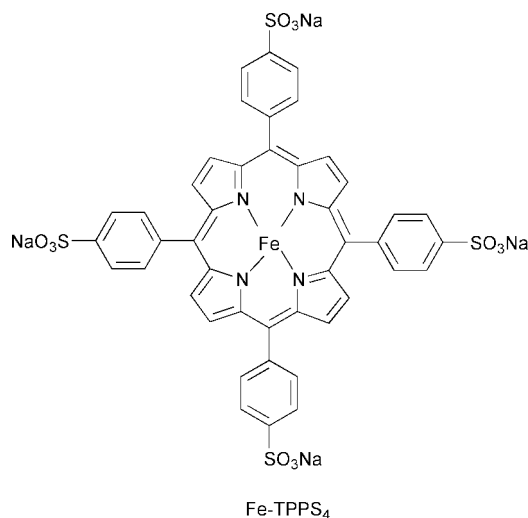
Fe-TPPS$_4$

Iron(III)-porphyrins have been extensively examined as catalysts for electrochemical reduction of molecular oxygen in non-deaerated or O$_2$ saturated solution.[21–25] The CV curves shown in Fig. 5 were recorded at a bare SPE immersed in a Fe-TPPS$_4$ solution (pH 5) saturated with N$_2$ (curve A) or O$_2$ (curve B). As expected, the redox peak system observed in deaerated solution (standard redox potential $E° = 0.25$ V) and associated with the Fe(III)-TPPS$_4$/Fe(II)-TPPS$_4$ redox couple[26] was masked by the catalytic reduction wave of dioxygen in non-deaerated solution. The quantitative detection of P$^+$ after its Fe-TPPS$_4$-catalysed generation was not possible at a bare SPE as noted in Fig. 5. Curve C shows for comparison the cathodic reduction peak of P$^+$ recorded after incubation with HRP during 32 min (see Fig. 2A). Compared to HRP a signal of much lower intensity is expected from the coupling reaction in the presence of Fe-TPPS$_4$. Conversely, a good accuracy was achieved at a Nafion-SPE, because the tetraanionic Fe-TPPS$_4$ porphyrin was repulsed by the anionic Nafion film and consequently both the
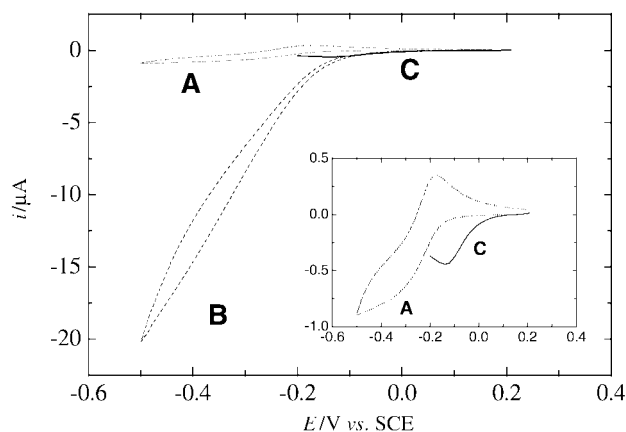
cathodic reduction of Fe-TPPS$_4$ and the catalytic process were impeded (not shown).

The cathodic waves shown in Fig. 6A were recorded at a stationary Nafion-SPE at pH 5 (non deareated solution) after Fe-TPPS$_4$ catalysed coupling of AAP and MEHA in the presence of 10^{-7} M of porphyrin (same experimental conditions as in Fig. 2B with HRP (10^{-10} M) as the catalyst). The peak currents in Fig. 6A increased linearly with the incubation time and they were of the same order of magnitude as those obtained with the 1000-fold less concentrated HRP solution (compare Fig. 2B and Fig. 6A). Assuming a linear relationship between the peak current and the Fe-TPPS$_4$ concentration, it was roughly evaluated that the catalytic activity of Fe-TPPS$_4$ was 1500 times lower that for HRP at pH 5. The electric signals were observed to be ca. 6-fold higher when the coupling reaction was carried out at pH 9.2, as shown in Fig. 6B, which corresponds to the pH optimised for the chromogenic reaction of AAP and p-chlorophenic acid with H$_2$O$_2$ catalysed by Fe-TPPS$_4$.[20] Consequently, the catalytic activity of Fe-TPPS$_4$ at pH 9.2 is expected to be ca. 250 times lower than the catalytic activity of HRP at pH 5. Fig. 3B shows the kinetic linear plots obtained at pH 9.2 in the presence of a nanomolar solution of Fe-TPPS$_4$ and in the absence of porphyrin. The non-negligible cathodic signal obtained in this latter case indicated that some spontaneous coupling of AAP and MEHA took place at pH 9.2 in the absence of enzyme. Taking into account this residual current, a detection limit of 4×10^{-10} M could be evaluated for Fe-TPPS$_4$ after 30 min of incubation.

Conclusion

The HRP-catalysed oxidative coupling of AAP and MEHA in the presence of peroxide allowed the electrochemical or colorimetric determination of HRP to be achieved with a detection limit of 10^{-12} M in each case. However, with a view to an enzyme immunoassay, the CV method involving a Nafion-SPE is definitely more sensitive than the spectrophotometric method, insofar as the cationic dye P$^+$ is generated close to the
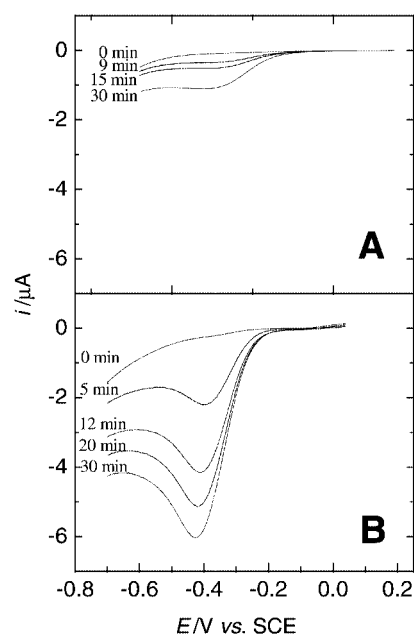

Fig. 5 CV curves (A, B) recorded at a SPE in the presence of Fe-TPPS$_4$ (1.5×10^{-4} M) in deaerated (A) and non-deaerated (B) solutions. LSV curve (C) recorded after incubation of AAP (5×10^{-4} M) + MEHA (5×10^{-3} M) in the presence of H$_2$O$_2$ (10^{-3} M) and HRP (10^{-10} M) during 20 min, pH 5. Scan rate 0.1 V s^{-1}. Inset: expanded scale of curves A and C.

Fig. 6 LSV curves recorded at a Nafion-SPE at pH 5 (A) and pH 9.2 (B) after oxidative coupling of AAP and MEHA in the presence of Fe-TPPS$_4$ (10^{-7} M). The concentrations of AAP, MEHA and H$_2$O$_2$ are the same as in Fig. 3 and the incubation periods are indicated on each curve. Scan rate 0.1 V s^{-1}.

Nafion film, which can be achieved by using immunomagnetic beads.

The stable and water-soluble anionic Fe-TPPS$_4$ porphyrin has been shown to be a promising alternative to the HRP label in the enzyme immunoassay with electrochemical detection. It has been detected at concentrations down to 4×10^{-10} M at a Nafion-SPE. The labelling of organic molecules with Fe-TPPS$_4$ (*i.e.*, covalent linking through a sulfonamide function) and the application of the tracers in an affinity assay with electrochemical detection are under investigation.

Acknowledgement

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