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Subfemtomolar Determination of Alkaline Phosphatase at a Disposable Screen-Printed Electrode Modified with a Perfluorosulfonated Ionomer Film

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A carbon-based ink composed of graphite particles and polystyrene was used in association with a manual screen-printer to prepare electrodes on a flexible polyester film. The screen-printing step was followed by a drying step which was achieved within 1 h at room temperature. The screen-printed electrode (SPE) was coated by a polyanionic Nafion film in which electroactive cationic species could be accumulated. A detection limit of 10^{-9} M was thus obtained by cyclic voltammetric (CV) determination of [[(4-hydroxyphenyl)amino]carboxyl]cobaltocenium (P^+) after accumulation for 60 min. Since this cationic phenol derivative P^+ could be generated from the corresponding anionic ester phosphate (S^-) by alkaline phosphatase (AP) hydrolysis, the new S^- substrate was synthesized and the sensitive indirect CV determination of AP was performed at a Nafion-coated SPE. The S^- substrate did not interfere on the electrochemical response of P^+ owing to the permselectivity of Nafion. An AP detection of 4×10^{-16} M was thus achieved in Tris buffer (pH 9) after hydrolysis of S^- (10^{-4} M) to P^+ (Michaelis constant $K_m = 48\ \mu M$) and simultaneous accumulation of P^+ within Nafion for 1 h. The Nafion-SPE was stuck successfully to the bottom of a microwell, making it possible to work with solution volumes ranging from 50 to 250 μL, well adapted to enzyme immunoassays.

The ultrasensitive detection of biological substances using affinity assays, such as immunoassays or nucleic acid hybridizations, is extremely important in many fields, e.g., biological and medical research, diagnostic medicine, genetics, and drug and pesticide testing. Highly sensitive immunoassays are expected to facilitate research for new diagnostic markers of diseases, better monitoring of tumor-specific products, and early detection of antigens at very low level concentrations in biological fluids. In addition, the development of highly sensitive methods allows assays to be conducted with smaller sample volumes and/or shorter analysis times. Therefore, considerable efforts have been focused on the improvement of the affinity assay sensitivity.[1−4] It depends mainly on the ability to detect the label, and so the most commonly used strategy involves an enzyme label, which provides a signal amplification through the rapid conversion of a substrate to a detectable product. The alkaline phosphatase enzyme (AP) is of particular interest because of its high turnover number and broad substrate specificity. Thus some of the most sensitive enzyme affinity assays developed to date (detection limit close to the zeptomole level) have used the AP label coupled to an ultrasensitive chemiluminescent detection method[5] or with a cascade enzyme reaction monitored by fluorescence.[6] These techniques are, however, sophisticated and costly, and so there is a continuing effort to develop new devices which are not only sensitive but also fast, reliable, low cost, and low size.

AP is able to rapidly hydrolyze a large variety of orthophosphoric monoesters into detectable alcohols. Frequently used substrates included 4-nitrophenyl phosphate (colorimetry),[7] 5-bromo-4-chloro-3-indolyl phosphate (colorimetry or chemiluminescence),[8,9] 4-methylumbelliferyl phosphate (fluorescence),[7] 1,2-dioxetane phenyl phosphate (chemiluminescence),[5,10] 4-fluorosalycilic acid phosphate (time-resolved fluorescence),[11,12] and 4-aminophenyl phosphate (amperometry).[13−16] Immunoassays with electrochemical detection can offer enhanced sensitivities and reduced instrumentation costs compared to their optical counterparts in certain circumstances, and recently an AP detection limit of 3.2×10^{-15} M has been reached with a bienzymatic biosensor coupled to a flow injection analysis system.[17] Nevertheless, it is more desirable to use nonflow instrumentation, with the electrode sensor directly immersed in the assay solution.[14,15,18,19] The advantage is to provide relatively simple and inexpensive assays,

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with a potentially higher sample throughput. However, the detection at a naked electrode is generally not very sensitive[15] and, in some case, is prone to fouling.[18] The sensitivity can be improved by selecting electrochemical conditions suitable to amplify the electrode response of the enzyme generated product,[14] whereas the fouling problem can be overcome with the use of disposable sensors.[18,19] Ideally, the best solution is to combine both of these improvements.

In order to amplify the electrode response, we have recently[20−23] developed an AP electrochemical detection method, and consequently an AP enzyme immunoassay, involving an electrode modified with Nafion, a perfluorosulfonated polyanionic ionomer, and a substrate/product couple chosen in such a way that the anionic enzyme substrate S^- is transformed catalytically into a procationic electroactive product P; i.e., the enzyme product P becomes cationic (P^+) by anodic oxidation. The modified electrode possesses the ability to amplify selectively the detection of P^+ entrapped within Nafion, whereas the anionic substrate S^-, which is electroactive at the same potential, is repelled according to the Donnan exclusion effect. Several S^-/P couples substituted by a ferrocenyl group were synthesized[20,23] and tested with two types of modified electrode, e.g., a Nafion film coated glassy carbon electrode (Nafion-GCE)[20,21,23] and a Nafion-loaded carbon paste electrode (Nafion-CPE).[22] The lowest AP detection limit (28×10^{-15} M) was obtained with the former type of modified electrode and with 6-(*N*-ferrocenoylamino)-2,4-dimethylphenyl phosphate as substrate S^-, using square wave voltammetry.[20] The corresponding ferrocene-substituted phenol generated enzymatically was entrapped within the film as a ferrocenium salt during the accumulation step. Despite its good sensitivity, this system suffered from several drawbacks:

(i) The P^+ salt was irreversibly accumulated and could not be stripped even in its neutral form P after cathodic polarization, and so the Nafion film electrode could not be used for more than one measurement.

(ii) The anodic preconcentration step took place at pH 7.4 and could not occur at the same pH as the enzyme reaction step (pH 9−10) owing to a signal inhibition. Clearly, the enzyme reaction and the accumulation could not proceed simultaneously, which lengthened the assay procedure.

(iii) The preconcentration of the P^+ salt proceeded under anodic potential, which means that the apparatus was immobilized during the accumulation step.

The use of a Nafion-CPE overcame the first drawback, since its surface could be easily refreshed between each measurement,[22] but the manual procedure was tedious. Moreover, it is difficult to conceive an automatic system for conveniently renewing the electrode surface. Another possibility to avoid the first drawback was the use of an anionic substrate leading to a less hydrophobic enzyme product which could be stripped from Nafion. A ferrocenylethyl phosphate was thus prepared, since the corresponding enzyme product, i.e., ferroceneethanol, was able to be released from the film of a Nafion-GCE as its neutral form by cathodic stripping.[23] Therefore, the Nafion-modified electrode could be reused several times, but the achieved sensitivity was lower (detection limit of 2×10^{-12} M) because of the unfavorable Michaelis constant of the selected aliphatic ester phosphate (K_m = 0.75 mM). The second drawback was also avoided with the ferrocenylethyl phosphate/ferroceneethanol couple, since ferroceneethanol allowed performance of the assay directly in the alkaline working solution (pH 9.0), which offered the possibility of performing the accumulation step during the whole enzyme incubation period.

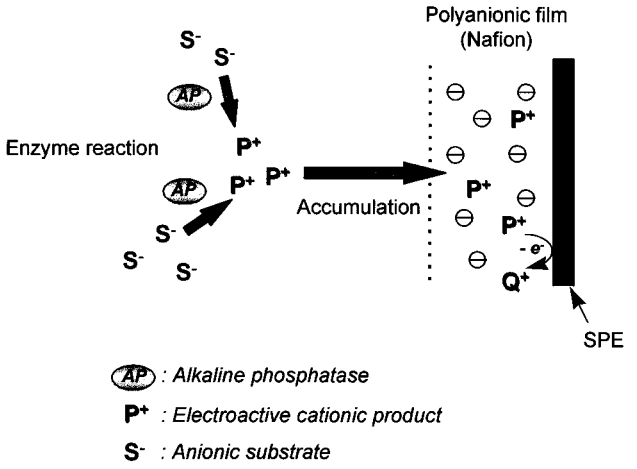
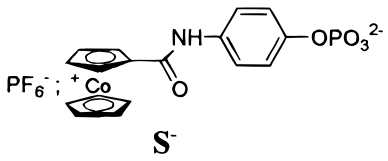
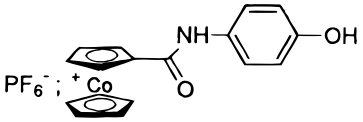


Figure 1. Schematic representation of the AP measurement.

The purpose of this study is to show that the previous drawbacks are overcome with disposable single-use screen-printed electrodes (SPE) modified with a Nafion film (Nafion-SPE), using the S^-/P^+ couple shown below. Screen-printing technology, adapted from the microelectronics industry, has the combined advantages of being inexpensive, simple, rapid, and versatile, and it allows the mass production of reproducible low-cost electrochemical sensors.[24−26] Furthermore, the SPE ink and/or surface can be easily modified in many ways.[26] The accumulation of the enzyme product P^+ proceeds under open circuit, since it is cationic (Figure 1) and the electrochemical detection is associated to the anodic oxidation of the phenolic function. Moreover, this accumulation was observed to be possible up to pH 9.4, which allowed the enzyme reaction and accumulation steps to occur simultaneously. A highly sensitive assay of AP was performed (detection limit, 0.4×10^{-15} M), which allows one to envision ultrasensitive enzyme affinity assays.



S⁻



P⁺

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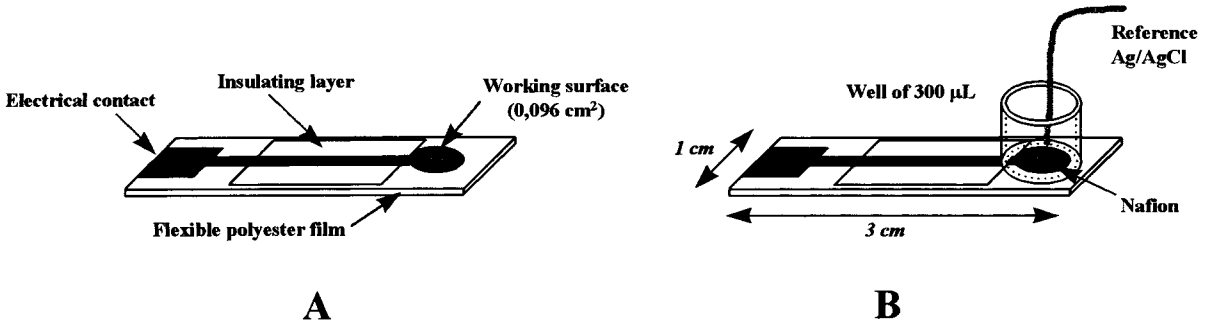

Figure 2. Scheme of the screen-printed electrodes before (A) and after (B) adaptation to a microwell.

EXPERIMENTAL SECTION

Materials and Reagents. A 5 wt % Nafion solution (EW1100) and ferrocenemethanol were purchased from Aldrich. The graphite powder was obtained from Johnson Matthey (Ultra Carbon, UCP 1M). AP from bovine intestinal mucosa (2030 units/mg of protein, 2.1 mg of protein/mL, VII-NA, P0280) and tris(hydroxymethyl)aminomethane (Tris) were supplied by Sigma. Ferroceneethanol was prepared according to a literature method.[27] A manual screen-printer from Circuit Imprimé Français (Bagneux, France) was used to build the working electrode. A Vortex mixer from Bioblock Scientific was used to shake the working solutions. The glassy carbon electrode (GCE)[28] and the carbon paste electrode (CPE)[23] were prepared as described previously.

Phosphate-buffered saline (PBS; 4.3 mM NaH_2PO_4, 15.1 mM Na_2HPO_4 and 50 mM NaCl, pH 7.4) and Tris buffer (TB; 50 mM Tris, 1 mM $MgCl_2 \cdot 6H_2O$, and 50 mM NaCl, pH 9.0) were prepared using deionized and doubly distilled water. Stock solutions of 10^{-2} M ferrocenemethanol, 10^{-2} M ferroceneethanol, and 10^{-3} M P^+ were prepared in absolute ethanol and stored at 4 °C.

Synthesis of [[(4-Hydroxyphenyl)amino]carbonyl]cobaltocenium Hexafluorophosphate (P^+) and Phosphoric Acid Ester of [[(4-Hydroxyphenyl)amino]carbonyl]cobaltocenium Hexafluorophosphate (S^-). Compound P^+ was prepared according to the procedure given by Beer et al.[29] The monoester phosphoric acid S^- was synthesized by applying the general method given by Silverberg et al.[30] Phenol P^+ (467 mg, 0.97 mmol) was dissolved in anhydrous acetonitrile (12 mL) under argon atmosphere. After cooling to −10 °C, tetrachloromethane (10 eq), *N*,*N*-diisopropylethylamine (4.2 eq), and *N*,*N*-dimethylaminopyridine (0.2 eq) were added successively under stirring. One minute later, dibenzyl phosphite (2.9 eq) was added dropwise. The internal temperature was kept below −10 °C. After the reaction was complete as determined by TLC, 0.5 M aqueous KH_2PO_4 (32 mL/100 mL of CH_3CN) was added and the mixture was allowed to warm to room temperature. The mixture was extracted three times with ethyl acetate. The combined organic phases were washed successively with water, and brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by chromatography on a Al_2O_3 column with ethyl acetate/methanol 6:1 (v/v) as eluent, and so dibenzyl phosphate was isolated in 54% yield (381 mg, 0.52 mmol): ^{1}H NMR (400 MHz, acetone-d_6) δ 9.85 (s, 1H, NH), 7.74 (d, 2H, J = 9 Hz, phenyl-H), 7.27−7.43 (m, 12H, phenyl-H), 7.22 (dd, 2H, J = 9 Hz, 1 Hz, phenyl-H), 6.49 (t, 2H, J = 2 Hz, cyclopentadienyl-H), 6.06 (t, 2H, J = 2 Hz, cyclopentadienyl-H), 6.01 (s, 5H, cyclopentadienyl-H), 5.19 (d, 4H, J = 8.4 Hz, CH_2) ppm. Dibenzyl phosphate (381 mg, 0.52 mmol) was dissolved in 40 mL of dry methanol, and then 1 g of 10% palladium on activated carbon was added. The suspension was stirred for 3 h at room temperature under hydrogen atmosphere (1 bar). The reaction was followed by TLC. When the starting material had disappeared, the hydrogenolysis was stopped and the mixture was filtered. The filtrate contained 88 mg of S^- as well as impurities. The main part of the remaining product adsorbed on the carbon catalyst and could be obtained with good purity through several washings with methanol. After the evaporation of the latter filtrates, 200 mg of S^- could be collected: ^{1}H NMR (400 MHz, methanol-d_4) δ 7.88 (d, 2H, J = 9 Hz, phenyl-H), 7.45 (d, 2H, J = 9 Hz, phenyl-H), 6.60 (t, 2H, J = 2 Hz, cyclopentadienyl-H), 6.14 (t, 2H, J = 2 Hz, cyclopentadienyl-H), 6.07 (s, 5H, cyclopentadienyl-H) ppm; MS (electrospray, H_2O) m/z (relative intensity) 324.4 ([$P^+ - PF_6^-$], 5.7), 404.5 ([$M - PF_6^-$], 44), 442.4 5 ([$M + K^+ - H^+ - PF_6^-$], 100).

In Situ Purification of the S^- Stock Solution. HPLC analysis revealed that product S^- was soiled by P^+ traces (0.1%). A S^- stock solution of initial concentration 10^{-2} M was prepared by dissolving 1.09 mg of S^- in 200 μL of TB, and then a confetti of Nafion-membrane (5.5 mm diameter, Nafion 117, Aldrich) was introduced and the solution was manually and intermittently shaken at least for 3 h before use. The P^+ traces were extracted from the stock solution under these conditions, but S^- was partially extracted, too, and its final concentration was evaluated to be ∼0.5 × 10^{-2} M by square wave voltammetry (SWV) at a CPE.

Preparation of Disposable Nafion-Coated Screen-Printed Electrodes (Nafion-SPE). A conductive carbon ink was prepared by thoroughly hand-mixing graphite powder and a polystyrene/mesitylene mixture. A commercial conductive carbon ink (Minico M3021-1RS, W. R. Grace) was also used in a few cases.

A manual screen-printer was used to produce disposable SPEs. An array of six electrodes, each of them corresponding to the pattern shown in Figure 2A, was printed on a flexible polyester film (Staedler film for overhead projection) by forcing the conductive ink to penetrate through the mesh of a screen stencil (77 threads cm^{-1}). Each SPE consisted of a disk as the working surface (3.5 mm diameter), a conductive track (20 mm × 1 mm), and a square extremity (25 mm^2) for the electrical contact. After drying for 1 h at room temperature, an insulator layer was spread manually over the conductive track, leaving the working disk area ready for Nafion modification. The resistance value of the bare

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track was determined with a two-point probe and expressed in terms of ohms per centimeter of coating ($R = 1$ kΩ cm^{-1}). The Nafion film-coated electrode surface was prepared according to the following procedure: 500 μL of Aldrich Nafion solution was combined with 250 μL of ethylene glycol. The Nafion coating was made by depositing with a syringe a microdroplet of the diluted Nafion solution on the working disk area. The solvent was allowed to evaporate overnight at 60 °C under vacuum. The film thickness was controlled by varying the concentration of the Nafion solution, and the syringed droplet volume, and it was estimated by assuming a Nafion density of 1.58 g cm^{-3}.[31]

Equipment and Electrochemical Measurements. An EG&G Par 273 potentiostat interfaced to an IBM XT 286 computer system with a PAR M270 software was used for cyclic voltammetry (CV) at a fixed potential scan rate of 50 mV s^{-1} and for SWV. The SWV parameters are given in a previous work.[28] The electrochemical experiments were carried out at room temperature in small glass vials, with a working volume of 5 mL. A few measurements were performed in a cell of small volume (50−250 μL) corresponding to the SPE-adapted microwell depicted in Figure 2B. The bottom of a commercial microwell was removed and replaced by a Nafion-SPE. Solutions were not deaerated unless otherwise stated.

For the accumulation procedure, a Nafion-SPE was immersed in the vial containing the analyte solution (P^+), shaken to enhance the mass transfer to the working electrode surface. The modified SPE was wedged in the vial to avoid its rotation during shaking. After preconcentration, the accumulated product P^+ was determined by connecting the modified SPE, the Ag/AgCl (3 M NaCl) reference electrode, and the platinum wire counter electrode to the potentiostat. A CV curve was recorded, and the resulting anodic peak current corresponding to the oxidation of the phenol function of P^+ at 0.5 V was taken as the analytical response after blank subtraction.

Determination of Alkaline Phosphatase Using S^- as the Substrate. To 4990 μL of AP in TB (AP concentrations ranging from 1.3×10^{-15} to 2.6×10^{-10} M) was added 10 μL of S^- stock solution (~0.5×10^{-2} M after in situ purification). A few experiments were carried out with 4900 μL of AP in TB (AP concentrations ranging from 4×10^{-16} to 4×10^{-15} M) and 100 μL of S^- (~0.5×10^{-2} M after in situ purification). Immediately after addition of the enzyme, a Nafion-SPE was immersed into the solution maintained under shaking during the whole enzyme incubation period (20 or 60 min) which proceeded at room temperature. The accumulated P^+ salt was then determined by CV as described above. The blank signal was obtained in the absence of AP.

Determination of the Michaelis Constant for S^-. To (4950 − x) μL of TB was added x μL of the purified S^- stock solution (~0.5×10^{-2} M in TB). A Nafion-SPE was introduced into the mixture solution under shaking for 10 min, and a blank CV curve was recorded. Then 50 μL of AP solution (1.3×10^{-10} M in TB) was added in the vial with a new Nafion-SPE, and a CV curve was recorded after shaking for 10 min. The difference between the resulting anodic peak and the preceding blank signal was taken as the analytical response. The Michaelis constant K_m of AP for S^- was deduced from the linear least-squares regression of the Lineweaver−Burk plot.[32]

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RESULTS AND DISCUSSION

Screen-Printed Electrodes. Commercial carbon- or metal-based inks are available, but their formulations are unknown and they need a heating step at elevated temperature. Therefore, we have developed our own carbon-based ink composed of graphite particles and polystyrene in mesitylene. The carbon ink formulation was optimized, and so a graphite to polystyrene ratio of 3:2 was established. It was possible to screen-print the pattern of the electrode on a flexible polyester sheet in a single step. The high carbon content provides a highly conductive thick film, without compromising its binding and adhesion properties, and it allows a sufficiently fast electron transfer rate for good electroanalytical performances ($\Delta E_p = 63 \pm 4$ mV for a 4 μM ferrocenemethanol solution). Another advantage of the formulated carbon ink is its room-temperature drying in less than 1 h. A model conductive ink system based on spherical glassy carbon microparticules and polystyrene in isophorone solution was described very recently,[33,34] which involves a drying step in a ventilated box oven at 70 °C over a period of 1 day. The polarizable potential range available with these sensors was ~3.0 V (between +1.5 and −1.5 V in TB). The observed double-layer capacitance C_d (μF cm^{-2}) was estimated from $i_c = C_d S v$, where i_c (μA) is the capacitive current, S (cm^2) is the working electrode surface, and v (V s^{-1}) is the potential scan rate. Experimental plots of i_c (at 0.05 V) versus v in PBS (pH 7.4) are linear, and the C_d value deduced from the slope was 1.6 ± 0.1 μF cm^{-2} for the unmodified SPE; i.e., it was twice as low as for the SPE built from the commercial ink (3.1 ± 0.2 μF cm^{-2}) or for the CPE (3.5 μF cm^{-2}) and it was 20 times lower than for a polished GCE (34 μF cm^{-2}). Furthermore, it did not significantly increase when it was coated by a Nafion film. Low capacitive currents are particularly attractive for CV measurements. For example, the sensitivities, i.e., the slopes of the CV calibration curves of ferroceneethanol at a GCE and at an unmodified SPE, were approximately the same (0.24 and 0.22 A M^{-1} cm^{-2}, respectively, when $v = 100$ mV s^{-1}), whereas the detection limit was ~20 times lower at the SPE (10^{-7} M) than at the GCE (2×10^{-6} M),[23] which is consistent with the C_d gain between GCE and SPE. A good reproducibility was observed for the electrochemical response of ferrocenemethanol in TB (relative standard deviation, RSD, <4% for response at 24 unmodified SPEs from 4 random batches).

Electrochemical Behavior of S^- and P^+ at an Unmodified SPE. Figure 3 shows the CV curves recorded on a SPE for S^- and P^+ in PBS (pH 7.4) deareated with argon. The ($a_1 - a_1'$) and ($a_2 - a_2'$) peak systems at −0.8 V are attributed to the cobaltocenium group (one-electron reversible reduction to cobaltocene) contained in P^+, and S^-, respectively. The corresponding standard redox potential are −0.828 and −0.838 V. The anodic oxidation (b_1') of the phenolic group of P^+ at 0.4 V was irreversible and led to peak b_1 on the reverse cathodic sweep. Fouling of the electrode surface was observed by repetitive CV scans; i.e., the anodic peak current continuously decreased. This phenomenon is characteristic of the electrooxidation of phenol derivatives, and it is associated with the electropolymerization of phenolic radicals at the electrode surface.[20] The anodic oxidation (b_2') of S^- took place beyond 1.0 V, almost merging with the solvent discharge, and it

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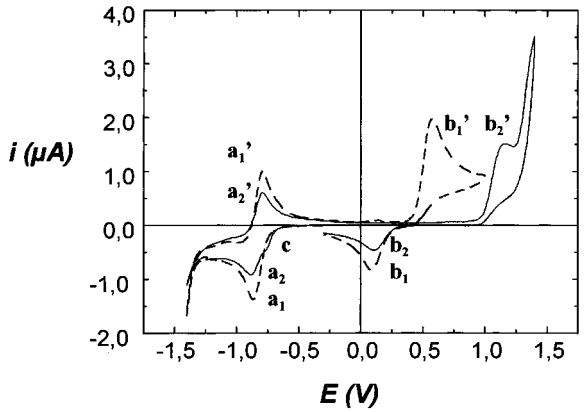

Figure 3. CV response in TB of (—) P+ (10^{-4} M) and (- - -) S^- (10^{-4} M) at a nacked SPE (scan rate, 50 mV s^{-1}).

led most probably to P^+, since peak b_2 was observed at the same potential as peak b_1 on the reverse cathodic scan. The small shoulder (c) at −0.7 V that preceded the reduction of the cobaltocenium group of S^- was related to the mediated reduction of dioxygen traces probably entrapped within the ink by redox catalysis, as shown elsewhere for cobaltocenium derivatives.[35] In nondeaerated PBS, a well-defined catalytic wave appeared (not shown) for both of the molecules S^- and P^+, which was consistent with the catalytic process. The CV curves in Figure 3 clearly indicate that at a naked SPE it is not possible to exploit the electrical signal of the cobaltocenium function of P^+ as an analytical response, since the cobaltocenium redox systems contained in P^+ and S^- are not discrete (close $E°$ values and comparable current responses).

The stability of 10^{-6} M P^+ was verified at an unmodified SPE, and no significant change of the electrode response was observed within 24 h. This is an advantage compared to unstable electroactive enzyme products such as 4-aminophenol[13] or 4-hydroquinone,[36] or to the intrinsically unstable chemiluminescent enzyme products,[5,37] when long enzyme incubation times have to be considered.

Detection of P^+ at a Nafion-SPE. It was previously shown[20−23] that a Nafion-modified electrode can act as a good electrostatic barrier against dianionic ester phosphates (RPO_3^{2-}) and that it was possible to detect preferentially the AP enzyme product even when both of the molecules possess close $E°$ values. However, the Nafion barrier was not perfectly selective, since a small residual blank signal corresponding to the ester phosphate remained.[20,23] For very sensitive AP detection, it is essential to have an excellent selectivity of the sensor for the enzyme product in the presence of a large excess of substrate. In the case of the couple S^-/P^+ involved in this study, this condition is fulfilled, since in addition to the permselectivity of the Nafion, a large difference of oxidation potentials (∼0.6V) was observed at a naked electrode between the phenolic function of P^+ and the phosphate function of S^-. In conclusion, the anodic CV peak current corresponding to the oxidation of phenol was chosen as the analytical response.

The inset in Figure 4 shows a typical anodic curve of P^+ (10^{-7} M in TB) recorded at a Nafion-SPE after 1 h of accumulation under open circuit and shaking. The anodic wave at 0.5 V corresponds to the oxidation of the phenol function of P^+ within Nafion. The anodic peak current $i_{p,a}$ (after subtraction of the base line) reflects the amount of P^+ preconcentrated into the Nafion film and therefore, the P^+ concentration in solution. Under these conditions, the Nafion film thickness was optimized, and so 1.92 μm represents a good compromise between a high sensitivity and a good reproducibility (RSD < 6% at 24 single-use Nafion-SPEs). Figure 4 shows the variation of $i_{p,a}$ with the accumulation time, and it indicates that a constant current is reached beyond 1 h. This limiting current results from the equilibrium distribution of the electroactive species between the solution and the polymer film. Figure 5 shows two calibration plots obtained for two different accumulation times, i.e., 20 (curve A) and 60 min (curve B). The electrode response of curve A was linear between 5 nM and 2 μM with a sensitivity of 21 A M^{-1} cm^{-2}, i.e., 2 orders of magnitude higher than at a naked electrode. A slight increase in

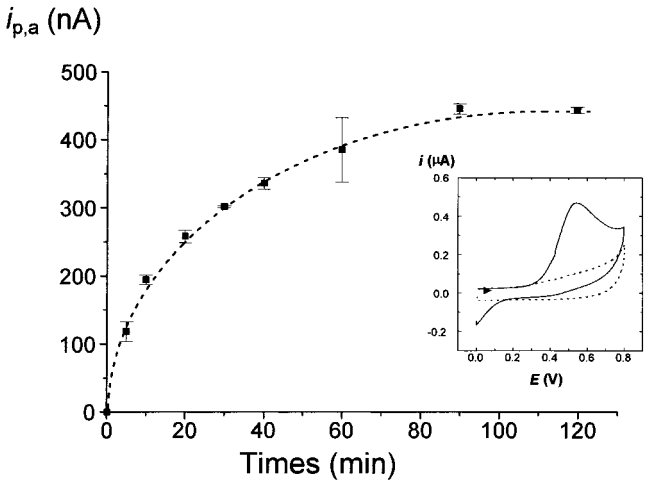

Figure 4. Dependence of the peak current (scan rate, 50 mV s^{-1}) on the accumulation time at single-use Nafion-SPEs in 10^{-7} M P^+ in TB solution (pH 9.0). Inset: CV curves at a Nafion-SPE immersed in TB containing no P^+ (- - -) and (—) 10^{-7} M P^+ (accumulation under shaking for 1 h). Error bars represent the standard deviation for peak currents at three different Nafion-SPEs.

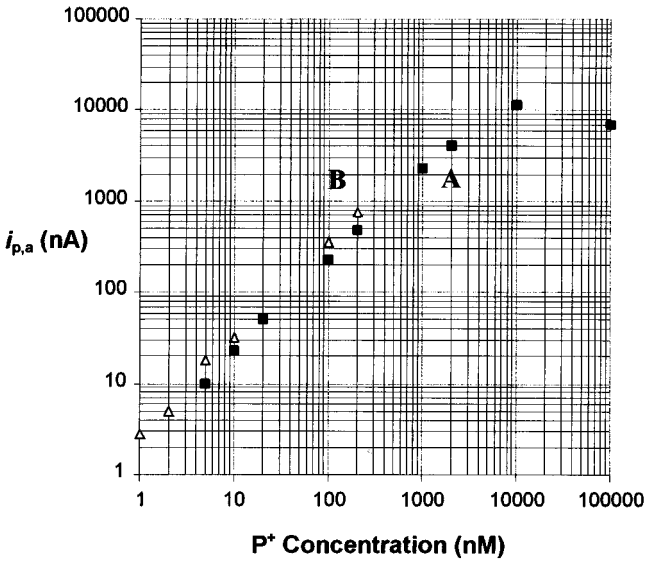

Figure 5. CV calibration plots (scan rate, 50 mV s^{-1}) of P^+ at single-use Nafion-SPEs for (A) 20 and (B) 60 min accumulation under shaking.

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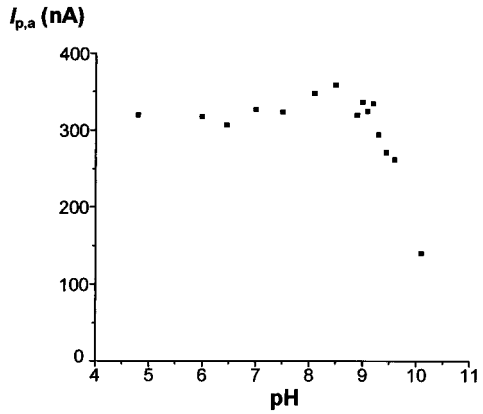

Figure 6. Influence of the pH on the accumulation of P^+ (10^{-7} M in TB) at single-use Nafion-SPEs after accumulation under shaking for 20 min.

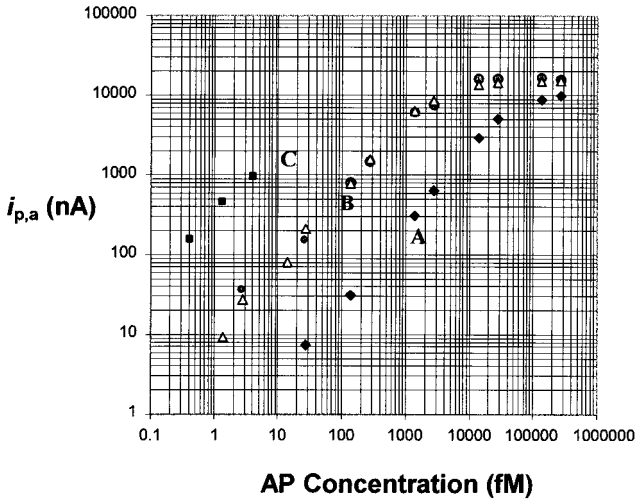

Figure 7. CV calibration plots (scan rate, 50 mV s^{-1}) of AP at single-use Nafion-SPEs immersed in TB solutions containing (A, B) 10^{-5} M S^- and (C) 10^{-4} M S^-. Accumulation under shaking for (A) 20 and (B, C) 60 min.

sensitivity (factor of 1.5) was obtained after 60 min of accumulation (Figure 5B) which is consistent with the results shown in Figure 4, and the P^+ detection limit was 10^{-9} M (S/N = 3). The peak potential (E_p) was almost independent of the P^+ concentration; i.e., a positive shift of ~10 mV per decade was observed under the conditions of Figure 5A. It confirms that the thick film is highly conductive, since the electrode resistance does not influence significantly the E_p values when the P^+ concentration is high.

It was verified that the addition of a large excess of S^- to a P^+ solution did not change the sensitivity of the P^+ calibration curve. An average electrode response of 600 ± 30 nA was obtained for 0.2 μM P^+ in TB (accumulation time of 20 min) with an excess factor of S^- ranging from 50 to 500. This result shows that there is no significant S^- influence on the P^+ detection.

Figure 6 shows the pH influence on the accumulation of P^+ (0.1 μM). Up to pH 9.3, the electrode response remains constant, whereas at higher pHs, the signal continuously decreases, which reflects the permselectivity of Nafion against the phenolate form of P^+ (pK_a ~10). The pH value 9.0 was selected for the AP assays since the optimal working pH for AP lies within the range 9−10.

Purity of the Substrate. A main and general problem encountered with AP assays (and consequently with AP enzyme immunoassays) is the substrate blank limitation. Therefore, the substrate must be of high purity to minimize the initial blank signal. The lower the blank signal, the lower the detection limit toward the enzymatic product. Thus, once the cobaltocenium phenol P^+ was prepared, the choice of the phosphorylation method involved in the synthesis of S^- was of high importance. The use of $POCl_3$ gave directly the corresponding crude phosphate S^-, which was very difficult to purify, either by column chromatography or by ion exchange resins, because of its zwiterionic character and its sensitivity to hydrolysis.[38] For these reasons we applied a general two-step method described by Silverberg et al.[30] Dibenzyl phosphate was prepared first, because it is stable[39] enough to be conveniently purified by simple-column chromatography and its benzyl groups are readily and quantitatively removed by palladium-catalyzed hydrogenolysis, a mild and clean method.[40]

HPLC analysis of the substrate S^- synthesized according to this procedure revealed an undesirable amount of ~1‰ P^+. This was confirmed by a significant CV blank signal at a Nafion-SPE, for a 10^{-4} M solution of S^-; i.e., an anodic peak was visible with $E_{p,a}$ = 0.59 V and $i_{p,a}$ = 327 nA (20 min accumulation), which corresponds to a P^+ concentration of ~10^{-7} M. After several unsuccessful attempts to purify of S^- by recrystallization, we have adopted an in situ purification method for the stock solution (10^{-2} M in 200 μL of TB), which consists in extracting residual P^+ by adding a confetti of commercial Nafion membrane. After ~3 h, the P^+ traces were almost completely extracted within the membrane, since the blank signal was nearly flat (20 min accumulation). However, some S^- was also extracted during this process, and it was evaluated that the S^- concentration dropped to ~50% of its initial value; i.e., the final concentration was ~0.5×10^{-2} M. Indeed, the SWV cathodic peak current observed at a naked CPE at −0.8 V in TB deareated with argon (reduction of the cobaltocenium group contained in S^-) dropped to half of its initial value.

AP Assay. Figure 7 shows the AP calibration plots obtained at room temperature for an initial S^- concentration of 10^{-5} M and for two different enzyme incubation times, 20 (plot A) and 60 min (plot B). For each set of data, a Nafion-SPE was introduced into the solution immediately after addition of the enzyme. Therefore, the generated product P^+ preconcentrated at the electrode surface during the whole AP incubation period. A large linearity range was observed (over 3 decades), and AP detection limits of 2.6×10^{-14} M (curve A) and 1.3×10^{-15} M (curve B) were obtained. It is worth noting that Figure 7B was plotted for two series of measurements carried out separately. The overlapping of the data illustrates the good reproducibility of the Nafion-SPEs. The plateau observed at high AP concentrations suggests that S^- was almost completely hydrolyzed into P^+ and/or that the Nafion film became saturated. A few CV curves were recorded for a 10-fold

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Table 1. Analytical Methods Used for Sensitive AP Determination[a]

substrate	AP detection limit (M)	time of analysis (temp)	recalculated detection limit (M min^{-1})	analytical method	ref
AMPPD + enhancer	1.6×10^{-17}	40 min (37 °C)	6.4×10^{-16}	chemiluminescence	5
NADP + ethanol + resorufin	6×10^{-18}	5 h (RT) + 10 min	1.9×10^{-15}	fluorescence coupled to enzyme recycling (ADH + diaphorase)	6
NADP + ethanol + 4-iodonitrotetrazolium	10^{-16}	3 h (25 °C)	1.8×10^{-14}	colorimetry coupled to enzyme recycling (ADH + diaphorase)	41
S^-	4×10^{-16}	1 h (RT)	2.4×10^{-14}	cyclic voltammetry (Nafion-SPE)	this work
FADP + proline + luminol + 4-hydroxycinnamic acid	4×10^{-15}	10 min (25 °C)	4×10^{-14}	chemiluminescence coupled to enzyme recycling (aDAAO + HRP)	42
MUP	2.5×10^{-15}	30 min (25 °C)	7.5×10^{-14}	fluorescence	7
APP	8×10^{-15}	10 min (37 °C)	8×10^{-14}	amperometry (GCE) coupled to enzyme recycling (diaphorase)	43
BCIP	2.2×10^{-14}	5 min	1.1×10^{-13}	amperometry (biosensor, HRP)	44
CSPD	8×10^{-14}	90 s	1.2×10^{-13}	chemiluminescence (CDD camera)	10
phenyl phosphate	3.2×10^{-15}	1 h (RT)	1.9×10^{-13}	amperometry with flow injection (bienzymatic biosensor, GOD + LAC)	17
cortisol-21-phosphate + lucigenin	3.55×10^{-15}	1 h (37 °C)	2.13×10^{-13}	chemiluminescence	45
4-hydroxyphenyl phosphate	2.8×10^{-14}	10 min (37 °C)	2.8×10^{-13}	amperometry (CPE)	36
FSAP + terbium:EDTA	1.2×10^{-14}	30 min (RT) + 1 min	3.6×10^{-13}	time-resolved fluorescence	11
6-(*N*-ferrocenoylamino)-2,4-dimethylphenyl phosphate	2.8×10^{-14}	15 min (37 °C) + 5 min	4.2×10^{-13}	square wave voltammetry (Nafion-GCE)	20
APP	2.5×10^{-14}	1 h (RT)	1.5×10^{-12}	amperometry with flow injection (GCE)	13
NPP	2×10^{-13}	30 min (25 °C)	6×10^{-12}	colorimetry	7
APP	10^{-11}	2 min + 45 s	2.5×10^{-11}	amperometry (SPE)	19

[a] Key: DAAO, apo-D-amino-acid oxidase; ADH, alcohol deshydrogenase; AMPPD, 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane; APP, 4-aminophenyl phosphate; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CSPD, 3-(4-methoxyspiro[1,2-dioxethane-3,2′-(5′-chloro)tricyclo[3.3.1^{3,7}]decan]-4-yl)phenyl phosphate; FADP, flavin adenine dinucleotide phosphate; FSAP, 4-fluorosalycilic acid phosphate; HRP, horseradish peroxidase; MUP, 4-methyl umbelliferryl phosphate; NADP, nicotinamide dinucleotide phosphate; NPP, 4-nitrophenyl phosphate.

S^- larger concentration (10^{-4} M), and the resulting plot (curve C) shows that a concentration as low as 4×10^{-16} M AP was measured, which corresponds to 2 amol of AP in the 5 mL solution. To our knowledge, this is the lowest AP concentration detected with an electrochemical technique, as is shown in Table 1, which compares highly sensitive measurements of AP by electrochemical as well as nonelectrochemical techniques.

The measured Michaelis constant K_m of AP for S^- was 48 μM, and this value is in the same range as for 4-nitrophenyl phosphate (82 μM),[13] 4-aminophenyl phosphate (56 μM),[13] and phenyl phosphate (36 μM).[17] It shows that the maximal velocity (V_m) is not yet reached at the substrate concentrations used in these experiments, since the velocity corresponds to $V_m/2$ when the substrate concentration is equivalent to K_m. The low detection limit of AP at a Nafion-SPE allows one to envision very sensitive enzyme immunoassays. However, they dictate working with small volumes of liquid because of the expensive reagents used (antibody, label, etc.) and of the small quantities of samples assayed (serum, urine, blood, etc.). Therefore, the Nafion-SPE was stuck successfully to the bottom of a microwell of 300 μL (Figure 2B), making it possible to work with solution volumes within the range 50−250 μL, well adapted to enzyme immunoassays. Preliminary results have shown that stronger shaking conditions are needed for the accumulation of P^+ and that the signal is decreased as the assay volume is reduced owing to a nonnegligible extraction of P^+ within the Nafion film and, therefore, its depletion in solution.[46] It was verified that P^+ did not adsorb on the microwell wall. For example, the relative electrode responses of 0.1 μM P^+ obtained after 20 min accumulation for 250, 200, 100, and 50 μL were 81, 74, 60, and 37%, respectively, compared to the response obtained in 5 mL.

CONCLUSION

The indirect electrochemical determination of AP can be achieved at subfemtomolar levels at a disposable Nafion-SPE using the enzyme substrate S^- and a 1 h incubation period. Considering the simple fabrication procedure of the Nafion-SPEs and the low cost of their mass production ($\sim$0.03 of raw material), their applications in enzyme immunoassays are very promising. An AP detection limit of 8×10^{-20} mol/200 μL ($\sim$50 000 AP units) can be envisioned insofar as the previous results can be extrapolated to small volumes (Figure 2B). Work is in progress to replace the wire Ag/AgCl reference electrode of the microwell-shaped cell shown in Figure 2B by a screen-printed Ag/AgCl electrode and to achieve ultrasensitive enzyme immunoassays.

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