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Peroxidase-Modified Carbon Fiber Microelectrodes in Flow-Through Detection of Hydrogen Peroxide and Organic Peroxides

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Mediatorless amperometric microbiosensors, prepared by immobilizing two different peroxidases [horseradish peroxidase (HRP) or peroxidase from *Arthromyces ramosus* (ARP)] on high-modulus-type carbon fibers, were applied for detection in flowing solution. The preparation and performance of the enzyme-modified carbon fibers were studied. An electrochemical flow-through cell containing the fiber electrode was evaluated and optimized. The influence of the electrode dimensions and the flow rate on the sensitivity and the sample dispersion in the cell was studied by use of bare and enzyme-modified electrodes. The peak broadening was found to be determined by the geometry of the flow cell. The response time of the enzyme-modified electrode was short (<4 s) and did not contribute significantly to the peak broadening. When operated in a flow injection (FI) system, hydrogen peroxide and organic peroxides could be detected with the enzyme-modified electrodes at an applied potential of −0.05 V vs Ag/AgCl. With the optimized cell low noise levels were obtained (<5 pA), allowing detection limits of 0.3, 6.0, and 20.0 μM for hydrogen peroxide, cumol hydroperoxide, and 2-butanone peroxide, respectively.

Measurement in extremely small sample volumes in various analytical ex- and in vivo methods requires sensitive, selective, and miniaturized detection devices.[1,2] Much work has been carried out on the development of enzyme-based microbiosensors, since they may meet the requirements.[3-15] Several advantages have been mentioned for the use of microelectrodes for detection, such as improved mass transfer with higher signal-to-noise ratios, fast response time, and the possibility of working in media with low electrical conductivity.[16] The use of enzymes to modify electrodes is well established due to the inherent selectivity with which enzymes catalyze reactions and improve analyte detection.[17]

Various materials have been tested for the construction of amperometric enzyme microsensors, mostly noble metal wires[1,7,8] and carbon fibers.[6,9,10-12,15,18-20] The latter material appears to be especially attractive, since carbon is cheap, is well studied, and has a favorable surface structure. The surface of carbonaceous materials is rich in oxygen-containing groups, which may play a key role in the electron transfer processes.[21] Several studies concerning carbon fibers as electrode material have been published recently.[15,18,22,23] Carbon fiber electrodes have been used for analysis in batch solution and for flow-through detection in flow injection analysis (FI),[10,14] column liquid chromatography (CLC),[24-26] and capillary electrophoresis (CE).[27-31]

For flow-through detection with carbon fibers, various cell designs have been proposed. Strands of fibers can be used, glued in a tub,[32,33] or embedded into epoxy resin.[20] Single

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fibers can be positioned perpendicular to the flow through a tube[34,35] or parallel with the flow into the end of a capillary.[10,30,36] Single-fiber detectors have been applied recently in combination with HPLC for the detection of nifedipine, nicardipine, and pindolol and, after modification with HRP and tyrosinase, for the detection of peroxides and phenols, respectively.[14] However, the detection required either a high working potential (+1.4 V vs SCE)[25] or the use of a mediator.[14,20]

In batch experiments[15,18] we have shown previously that mediatorless peroxidase-modified carbon fiber electrodes can be operated at –0.05 V vs Ag/AgCl, that is within the optimal potential range where the noise levels are low and interference by the reduction of dissolved oxygen or the oxidation of easily oxidizable compounds is minimal. The electrodes used were made of strands of carbon fibers, glued with a cyanoacrylate resin into glass microcapsules with the protruding part of the fibers modified with enzyme. It appeared that the carbon fibers were a material suited for the immobilization of enzyme in terms of surface coverage and activity of the immobilized enzyme. The scale of the electrodes was such that they could be prepared and handled without special equipment, while some of the advantages of microelectrodes, in particular, the possibility to work in a small volume were still present.

This paper describes the performance of these previously developed electrodes for the amperometric monitoring of peroxides in a flow-through cell. The method of preparation of the electrodes has been reevaluated for this application. High-modulus-type carbon fibers with a diameter of 7 μm (Grafil HM-S/6K 370 GPa) were used, since these showed the best performance in previous experiments. High-modulus fibers are stated to have a more graphite-like structure with a high degree of edge orientation,[16] which is the most suitable for direct electron transfer processes,[22,37] as expected with the enzyme used. The performance of enzyme electrodes in a flow-through cell depends on the electrode processes themselves as well on the efficiency of mass transport of the analytes toward the electrode surface. Therefore, the influence of the cell geometry and the flow rate on the sample dispersion (peak broadening), the response rate, and the sensitivity have been studied. Sensor characteristics are presented for both HRP- and ARP-modified electrodes.

EXPERIMENTAL SECTION

Apparatus. Amperometric detection was performed with a potentiostat Model BAS LC-4C (Bioanalytical Systems, West Lafayette, IN), the output of which was displayed on a strip chart recorder (Kipp & Zonen, Model BD 111, Amsterdam, The Netherlands). The carrier solution (0.1 M phosphate buffer at pH 6.0 or 7.0), also containing 0.01 M NaCl, was delivered by a pulse-free syringe pump (CMA/100 Microinjection Pump, CMA/Microdialysis AB, Stockholm, Sweden). Samples were injected with an injection valve (Valco, Model CI4W, Switzerland) with internal loops of 0.06 and 0.2 μL. The valve was connected to the detector cell by a 37.5 cm standard $^1/_{16}$ in. polyether ether ketone (PEEK) tube with an internal diameter of 175 μm. The homemade detector cell (Figure 4) has been described previously.[26,38] It has a T-shaped geometry with $^1/_{16}$ in. bore channels and contains, apart from the working electrode, a silver tube coated internally with silver chloride acting as a Ag/AgCl quasi-reference electrode. The coupling of the reference electrode, working electrode, and column to the cell was made with commercially available fingertights. Heat pretreatment of the electrodes was performed in a Muffle furnace oven at 700 °C for different times. Scanning electron micrographs (SEM) were taken on bare, heat pretreated and bare, and unpretreated and HRP-modified fibers with a scanning electron microscope (ISI, Santa Clara, CA, Model 100 A).

The applied potential for the detection of ferrocyanide with bare electrode was +0.6 V, while for the detection of peroxides with enzyme-modified electrodes it was –0.05 V vs Ag/AgCl. Experiments were performed without using a Faraday cage.

Chemicals. Horseradish peroxidase (HRP, EC 1.1.11.7) was purchased from Sigma Chemical Co., St. Louis, MO (Type VI, Catalog No. P 8375, 270 units mg^{-1}). Fungal peroxidase from *Arthromyces ramosus* (ARP) was received as a gift from Suntory, Japan (250 units mg^{-1}). Cumol hydroperoxide (Catalog No. 24, 750–2), 2-butanone peroxide (Catalog No. 24, 401-5), and di-*tert*-butyl peroxide (Catalog No. 16, 852–1) were received from Aldrich, Darmstadt, Germany. Potassium ferrocyanide and hydrogen peroxide were received from Merck, Darmstadt, Germany. Ferrocyanide, hydrogen peroxide, and organic peroxide solutions were prepared daily. All solutions were prepared with HPLC-grade water produced in a Milli-Q system (Millipore, Bedford, MA). The stock solutions of organic peroxides (0.1 M) were prepared with HPLC-grade acetonitrile; further dilutions were made with pure Millipore water. All chemicals were used as received.

RESULTS AND DISCUSSION

Preparation of Enzyme Electrodes. Differently prepared enzyme electrodes have been tested in a flow cell as described in the next section. A catalytic effect of the immobilized peroxidase for the electrocatalytic reduction of hydrogen peroxide or organic peroxides could be observed by recording the response current of a bare and an enzyme-modified electrode as a function of the applied potential. Figure 1 shows the variation in the response with the applied potential to injected samples containing hydrogen peroxide for (a) a bare and (b) a HRP-modified electrode both with a length of about 1.5 mm. A radical increase in the catalytic current could be observed for the enzyme-modified electrode. The electrocatalytic reduction of hydrogen peroxide at the enzyme-modified electrode started at +200 mV vs Ag/AgCl and increased when the potential was made more negative, while a very small response could be observed for bare fibers throughout the entire potential range. The obtained results indicate that HRP catalyzes the electrochemical reduction of hydrogen peroxide according to reaction cycle 1.

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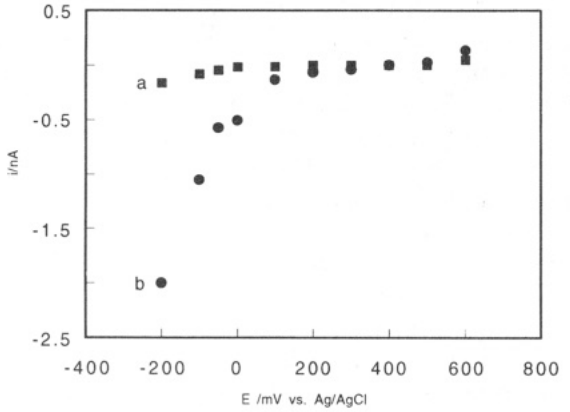
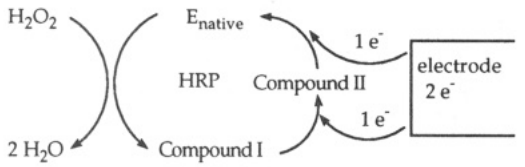


Figure 1. Hydrodynamic voltammogram registered for (a) bare and unpretreated and (b) unpretreated and HRP-modified fibers. Electrolyte 0.1 M phosphate buffer at pH 6.0; hydrogen peroxide concentration 1×10^{-3} M.

Reaction Cycle 1.



The mechanism for the electrocatalytic reaction of HRP with H_2O_2 is generally accepted according to the following. Hydrogen peroxide oxidizes the native form of HRP in a single two-electron process resulting in the oxidized form of HRP denoted compound **I** and water. In two separate one-electron transfer reactions **I** is reduced back into its native form through an intermediate state denoted compound **II**. In the case of HRP, the electrons necessary to close the enzymatic cycle can be donated by almost any reducing agent, or even by the electrode when the enzyme is immobilized on its surface. The nature of the electron transfer mechanism (direct or mediated) has been previously discussed.[21,39–42] The sensitivity of the enzyme-modified electrodes increased when the potential was made more negative, being highest at –200 mV vs Ag/AgCl. Previous experiments indicate that potentials more negative than –0.15 V caused an irreversible deactivation of the enzyme;[21] therefore, further measurements were done at –0.05 V vs Ag/AgCl.

With carbon/graphite rods,[21] graphite powder,[43] and various type of carbon fibers,[15,18] it has been observed in our laboratory that the electrode surface could be activated by heat pretreatment before immobilization of the enzyme. The heat treatment is believed to introduce quinone-type mediating functionalities on the electrode surface. Therefore, this pretreatment method was also applied for the high-modulus fibers used in this study. In contrast to the experience with graphite type fibers,[15] the heat pretreatment did not generally increase the sensitivity of HRP-modified electrodes for hydrogen peroxide. Heating the fibers longer than 30 s before enzyme immobilization drastically decreased the sensitivity. SEMs (Figure 2a) showed that unpretreated fibers possess a rough surface with a randomly distributed flakelike structure. Heat treatment slightly increased the roughness and strongly decreased the number of flakes (Figure 2b). This decrease was more pronounced for prolonged heating times. After immobilization of HRP, the difference between untreated and heated electrodes is not longer visible (Figure 2c). The presence of the flakes on the electrode surface may be beneficial for the enzyme immobilization, since it has been shown that the immobilization has to be made in a way that the active site of the immobilized enzyme is not blocked and will also

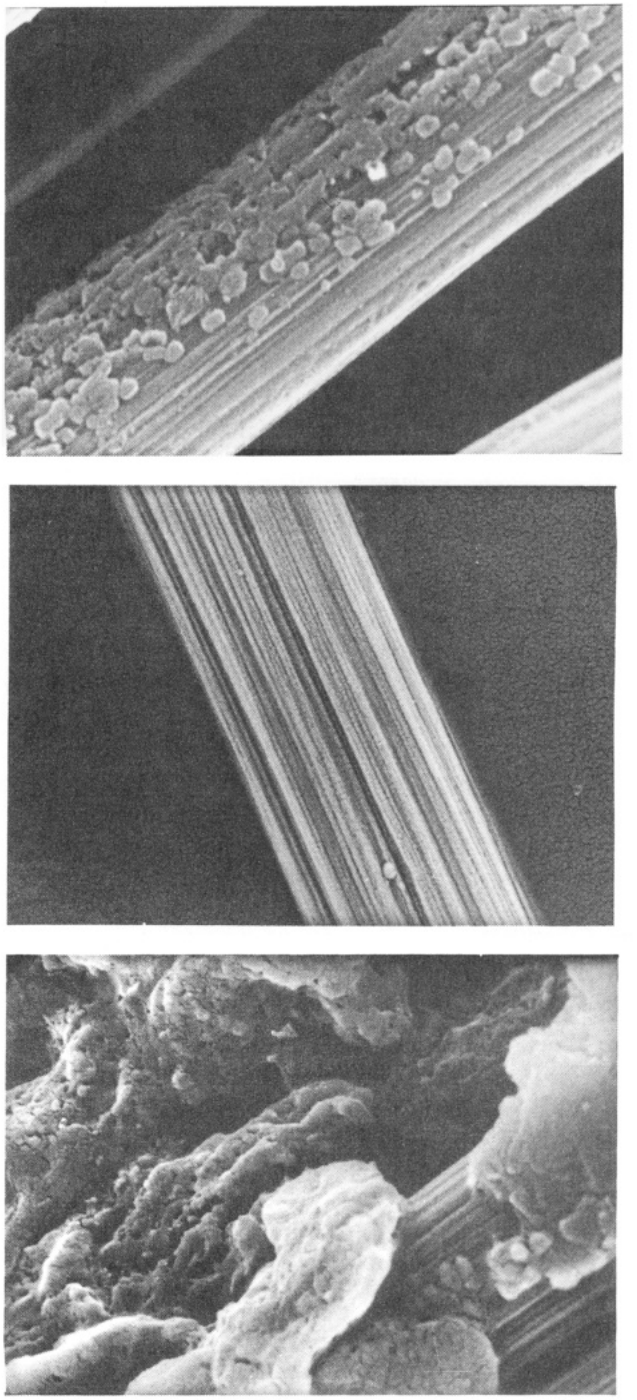


Figure 2. SEMs recorded on (a) unpretreated and bare, (b) heated (for 30 s) and bare, and (c) unpretreated and HRP-modified fibers. Magnification 10000×.

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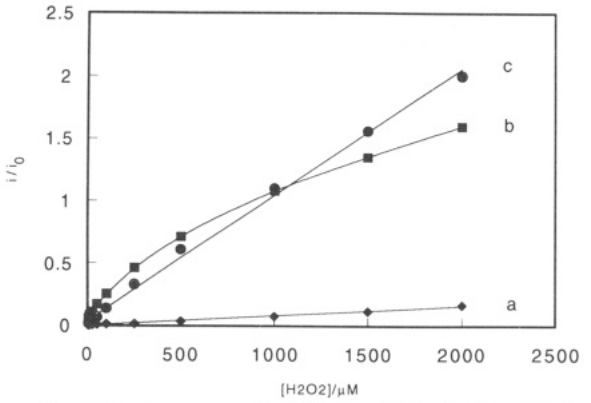

Figure 3. Calibration curves for (a) bare, (b) heated (for 30 s) and HRP-modified, and (c) unpretreated and HRP-modified fibers. Applied potential −0.05 V vs Ag/AgCl.

have a proper orientation at the electrode surface.[44] This assumption was supported by the previously made observation[15] that Polycarbon LGR fibers, with a more accentuated flakelike surface, displayed a higher activity after enzyme immobilization than smooth fiber types. Heat pretreatment caused serious nonlinearity of the calibration curves for hydrogen peroxide (Figure 3). Moreover, the pretreated electrodes showed a higher background current at −0.05 V than unpretreated ones. Therefore, in further experiments only unpretreated electrodes were used.

Optimization of the Flow-Through Cell. The electrochemical flow-through cell has been tested with the fiber electrodes in two different positions: with the carbon fiber strand parallel to the solution flow (Figure 4, top) or perpendicular to it (Figure 4, bottom). The evaluation was carried out using potassium ferrocyanide (50 μM) as the analyte and 0.1 M phosphate buffer at pH 6.0 as the carrier solution. Bare carbon fibers as working electrodes were used, and oxidative currents at an applied potential of +0.6 V vs Ag/AgCl were measured and recorded.

In the perpendicular geometry, the Ag/AgCl quasi-reference electrode had to be positioned at some distance from the working electrode to avoid short-circuiting between the silver tube and the fibers in the flowing liquid. This resulted in an effective cell volume relatively large, with a high degree of peak broadening and a low sensitivity compared with the parallel geometry. Therefore, further experiments were carried out with the parallel geometry only (Figure 4, top). Electrodes with an exposed length of either 1.5 or 3 mm were used.

To evaluate the performance of the cell geometry with the carbon fiber strand as working electrode, eliminating the effect of the enzymatic processes, the influence of experimental parameters on the sensitivity and peak broadening with bare electrodes was further studied. All further presented results are the average value of five equally prepared electrodes. Figure 5 shows the influence of the carrier flow rate (F) on the areas of the peaks obtained for injections of a 5×10^{-5} M ferrocyanide solution. The peak areas were found to be inversely proportional to the flow rate. With the 3 mm long fibers, the sensitivity was approximately 2 times higher than with the

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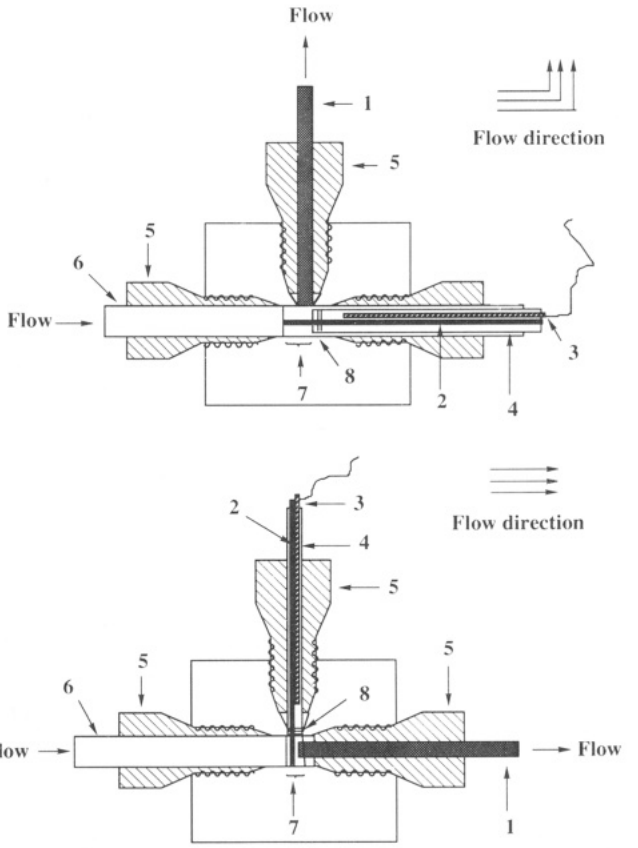

Figure 4. Scheme of the electrochemical detector cell: (1) Ag/AgCl reference electrode, (2) carbon fiber working electrode, (3) copper wire, (4) glass microcapsule, (5) fingertights, (6) PEEK tubing, (7) cell volume, and (8) glue.

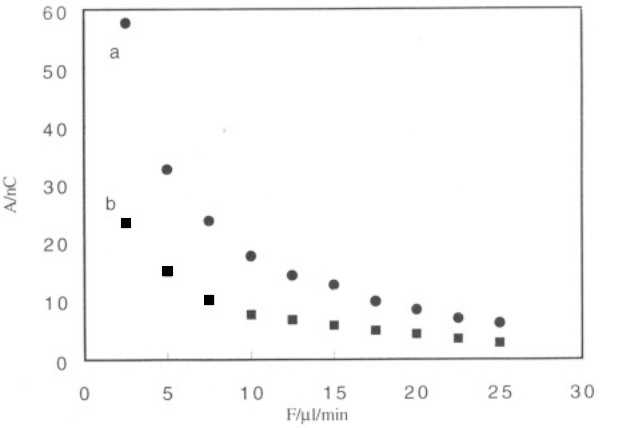

Figure 5. Dependence of the peak area with flow rate for fibers of (a) 3 and (b) of 1.5 mm length, injected solution of ferrocyanide of 50 μM, and working potential +0.6 V vs Ag/AgCl.

ones 1.5 mm long. The residence time of the analyte in the vicinity of the surface of the fibers seems to be determining the sensitivity obtained.

The observed inverse proportionality between the peak areas and the flow rate suggests that the steady-state current with a constant analyte concentration in the cell should not depend on the flow rate, since the increase of the rate of the transport of analyte to the cell would be exactly counteracted by the decrease of cell efficiency. This was confirmed by experiments in which a ferrocyanide solution was pumped continuously through the system. The current changed less than 5% when the flow rate was varied over a range from 5 to 25 μL min^{-1}.

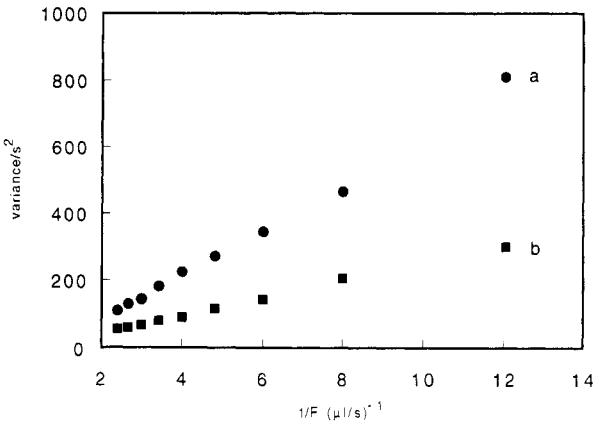

Figure 6. Influence of the flow rate on the peak variance (σ^2) with bare carbon fiber electrodes of (a) 3 and (b) 1.5 mm length. Injected volume of 60 nL of 5×10^{-5} M ferrocyanide.

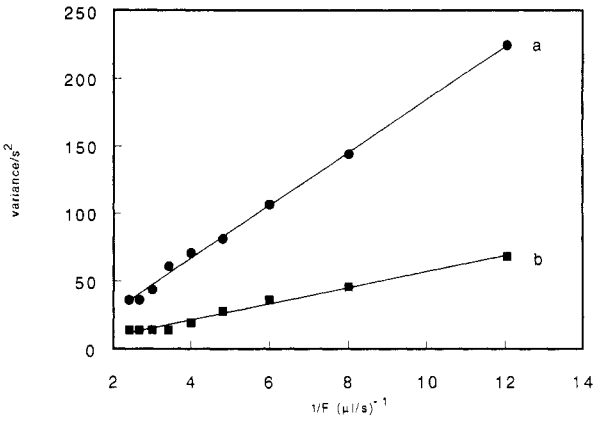

Figure 7. Influence of the flow rate on the peak variance (σ^2) with HRP-modified electrodes of (a) 3 and (b) 1.5 mm length. Injected volume of 60 nL of 1×10^{-3} M hydrogen peroxide.

In these experiments, a coulometric efficiency of 0.32% was found at a flow rate of 10 μL min^{-1}. The rather low efficiency of the cell is related to the choice of using standard construction materials with a diameter of $^1/_{16}$ in. With the presently used carbon fiber strand electrodes, this makes the cell volume relatively large (1–3 μL) for a microelectrode. To obtain higher sensitivities, tubings with a smaller diameter should be used throughout, the electrode should further be minimized, and the volume of the working electrode chamber has to be decreased.

To evaluate the effect of the enzymatic processes on the response rate of the electrodes in the flow-through setup, first the response behavior of the cell with bare electrodes had to be studied. Therefore, the dependency of the peak broadening on the flow rate was monitored. Depending on the rate-determining step in the overall process and the flow regime in the cell, different dependencies of the peak variance σ^2 (in s^2) on the flow rate F (μL min^{-1}) can be expected.[45] When the flow cell behaves as a mixing chamber, and the analyte is transported to the electrode by irregular flow, σ^2 will be proportional to $1/F^2$. When a laminar flow prevails in the cell, and the overall rate is determined by convective diffusion of the analyte to the electrode surface, σ^2 will be proportional to $1/F$. When processes other than diffusion in the solution are dominant (for instance, transport processes in an enzyme layer on the electrode), σ^2 will be independent of the flow rate.

The flow behavior of the cell was studied by injection of ferrocyanide solutions at various flow rates F. As is shown in Figure 6, peak variances σ^2_{exp} were found to be proportional to $1/F$, which is typical for laminar flow behavior.[45] With the 3 mm fibers, the peak broadening is larger than with 1.5 mm long ones. Also, some mixing-chamber behavior (σ^2_{exp} proportional to $1/F^2$) was found for the longer fibers at high flow rates; irregular flow patterns may then develop around the fibers.

From the observed relation between the contribution of peak broadening in the detector and the flow rate, it can be established whether the cell is suited for a particular separation system. If for instance a maximum peak variance of 25 s^2 is assumed, it can be seen in Figure 6 that with the 1.5 mm fibers the flow rate should be 1 μL min^{-1} or higher. This means that the present cell design is suited for liquid chromatography with conventional-scale or microbore columns, with typical flow rates of 20 and 1 μL min^{-1}, respectively, but not with packed or open capillaries.

Subsequently, HRP-modified electrodes with fiber lengths of 1.5 or 3 mm were tested in the flow cell. Peaks were recorded after injection of solutions containing hydrogen peroxide. The resulting plots of σ^2 vs $1/F$ (Figure 7) were linear, and no significant intercepts at $1/F$ equal to zero were found. This indicates that the flow rate independent processes are insignificant and that the response time of the enzyme layer is short compared with the peak broadening caused by the laminar flow in the electrochemical cell. Surprisingly, the enzyme-coated electrodes revealed more narrow peaks than those found for the bare electrodes. An explanation of this observation could be that the effective cell volume is decreased by the presence of the finite enzyme layer. Also, a more favorable flow pattern may develop in the cell by the gluing together of the fibers by the enzyme. In any case, the response time of the enzyme-covered electrodes was found to be shorter than 4 s ($\sigma^2 < 15$ s^2).

For both bare and enzyme-modified electrodes, the peak areas were approximately inversely proportional to the flow rate F with slopes for log A vs log F of 0.96 ± 0.2 (r = 0.994) and 0.96 ± 0.3 (r = 0.989), respectively. Compared with the sensitivity for ferrocyanide with bare electrodes, the sensitivities for hydrogen peroxide are lower by a factor of 30–60. Taking into account the differences in diffusion coefficients and number of electrons involved for ferrocyanide and hydrogen peroxide, this means that the yield of the electrode process for the HRP-modified electrodes is approximately 1%. It must be noticed, however, that the analyte transport in the solution may also be altered by the presence of the enzyme, as was already indicated in the peak-broadening experiments. Therefore, the above-mentioned yield of the enzymatic process on the HRP-modified electrode may be biased.

Sensor Characteristics. Relatively large differences in sensitivity were found between individual electrodes, possibly due to differences in active surface areas. However, when peak heights with different electrodes were compared relative to the background current measured at –0.05 V vs Ag/AgCl, a much better agreement was found. This is shown in Figure

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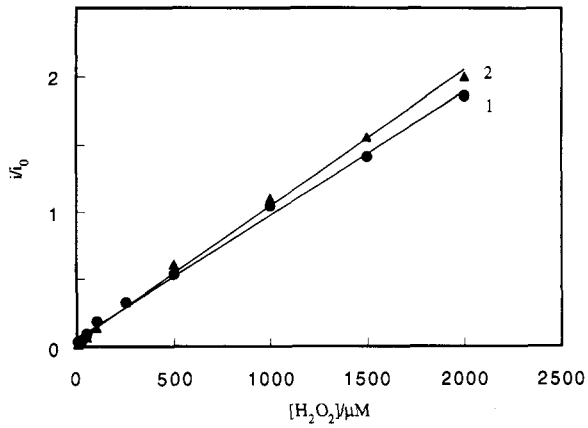

Figure 8. Calibration curves for two equally prepared, unpretreated and HRP-modified microelectrodes. Applied potential −0.05 V vs Ag/AgCl.

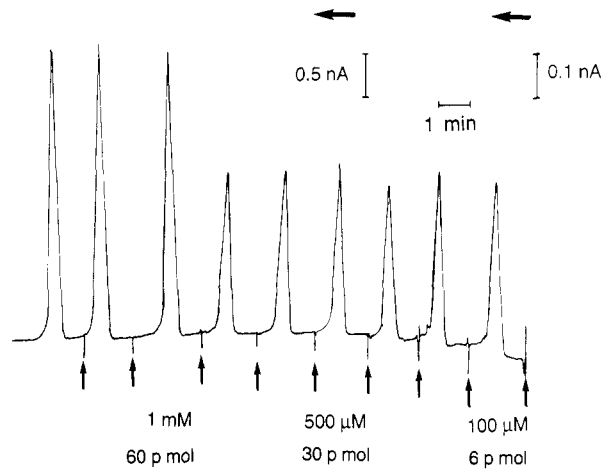

Figure 9. FI peaks recorded with ARP-modified fibers for detection of cumol hydroperoxide.

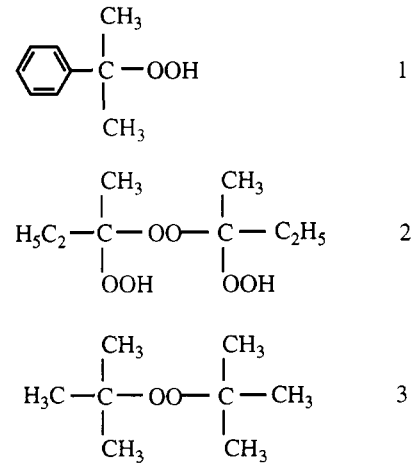

Figure 10. Formulas of the studied peroxides: cumol hydroperoxide (**1**) 2-butanone peroxide (**2**), and di-*tert*-butyl peroxide (**3**).

8 for calibration graphs for hydrogen peroxide obtained with two different HRP-modified electrodes. Very low noise levels (< 5 pA) characterized the system. The detection limit for hydrogen peroxide was 0.3 μM (signal-to-noise ratio 2). The calibration curves are linear for peroxide concentrations up to 2 mM, with a slight decrease in sensitivity at higher concentrations. From Lineweaver–Burk plots, an apparent Michaelis–Menten constant of 4–5 mM was estimated for hydrogen peroxide. At peroxide concentrations of 2.5 mM and higher, irreversible deactivation of the enzyme was noticed, possibly caused by the formation of an enzymatically inactive form of HRP (compound **III**).[46] The slope of the calibration curves (log–log plots) being close to 1.0 (0.91 ± 0.3, $r = 0.996$) indicates that the same reaction mechanism occurs for the electron transfer processes in the entire concentration range.

ARP-modified electrodes were used for the detection of not only hydrogen peroxide but also organic peroxides, i.e., cumol hydroperoxide, 2-butanone peroxide, and di-*tert*-butyl peroxide as they are of great industrial, environmental, and biological importance.[47,48] The enzyme solution was prepared at pH 7.0 according to previous experiments made in this laboratory using carbon paste as electrode material.[49] Peroxide solutions were made in acetonitrile (stock solutions) and buffer because of the attractive dynamic properties and sensitivities observed in the presence of organic media, in experiments with similar enzyme systems using carbon paste.[48,50] The enzyme electrodes responded rapidly (≤5 s) to concentration changes of hydrogen peroxide and organic peroxides whereas no signal could be detected with bare, unmodified electrodes. Figure 9 presents FI peaks recorded for detection of cumol hydroperoxide. While different sensitivities were observed for the different peroxides (cumol hydroperoxide > 2-butanone peroxide > hydrogen peroxide), all could be detected at the micromolar level. The ARP-modified electrodes displayed higher conversions for cumol hydroperoxide (3 times higher) and 2-butanone peroxide (twice as high) than for hydrogen peroxide. Detection limits of 6 μM (3.6×10^{-13} mol) and 20 μM (1.2×10^{-12} mol) were obtained for cumol hydroperoxide and 2-butanone peroxide, respectively, with corresponding slopes of the log–log calibration plots of 0.89 ± 0.2 and 1.0 ± 0.1. The sensor did not respond for di-*tert*-butyl peroxide. Cumol and 2-butanone peroxide possess polar structures, whereas di-*tert*-butyl peroxidase has a nonpolar structure (Figure 10), which may explain the insensitivity of the enzyme electrode toward this peroxide. The conversion efficiency of H_2O_2 at the ARP-modified sensor was similar to that obtained for HRP-modified ones.

The recorded signal response of individual electrodes was found to be pH dependent, the studied peroxides displaying different pH optima (see Figure 11). The highest current was obtained at pH 5.0 for cumol hydroperoxide and at pH 6.0 for 2-butanone peroxide. For the detection of hydrogen peroxide, a shift in the optimal pH value was observed when the signals obtained for HRP- and ARP-modified electrodes were compared. The HRP-modified electrode presented a decreasing signal with increasing pH (being highest at pH 5.0), while the ARP-modified electrodes displayed an optimal pH range, the signal being almost constant between pH 6 and 8. When acetaminophen or ascorbic acid was injected at a concentration corresponding to the physiological values of each (0.14 and 0.12 mM, respectively), oxidation currents of only 10 pA were registered.

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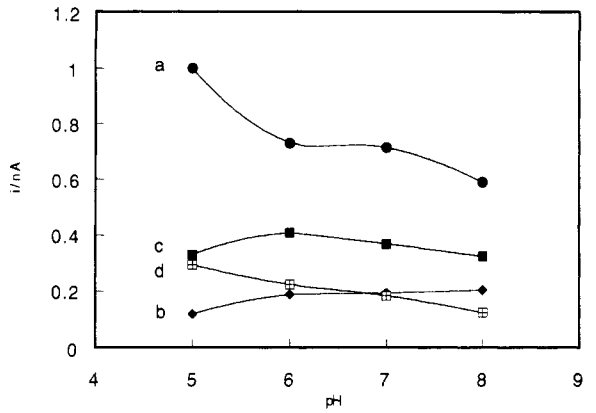

Figure 11. Variation of the current signal with pH for ARP-modified microelectrodes for (a) cumol hydroperoxide, (b) H_2O_2, (c) 2-butanone peroxide, and (d) HRP-modified fibers for hydrogen peroxide. Peroxide concentration 1×10^{-3} M.

In conclusion, this study illustrates the feasibility of using mediatorless HRP- and ARP-modified electrodes in a flow injection system for the detection of hydrogen peroxide and some organic peroxides. In addition to high sensitivity, low noise, low detection limits, and fast response times, the coupling of enzymatic reactions with the low applied sensing potential may offer a high selectivity. Future use of the described detection system is possible in various on-line measurements. Further miniaturization of the sensor should open up the possibility of its use as a detection unit even for separation systems with packed or open capillaries.

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