Anal. Chem. **1995**, *67*, 1679–1683

Determination of Organonitriles Using Enzyme-Based Selectivity Mechanisms. 2. A Nitrilase-Modified Glassy Carbon Microelectrode Sensor for Benzonitrile

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A nitrilase-modified glassy carbon microelectrode for the detection of benzonitrile in water was developed and analytically characterized. The detection scheme uses *Rhodococcus* sp. nitrilase to catalyze the hydrolysis of benzonitrile derivatives directly into carboxylic acid and ammonia. The glassy carbon microelectrode surface modification was accomplished by attaching a hydrophilic tether to the microelectrode surface and then, using the free terminal amine at its other end, biotinylating with sulfo-NHS-biotin. Afterward, avidin was attached to the spacer arm, followed by the biotinylated nitrilase through avidin–biotin coupling. Benzoic acid, an enzymatic reaction product, is electrochemically reduced at the glassy carbon microelectrode, producing a steady-state reduction current proportional to the concentration of benzonitrile in the sample. In the temperature range of 25 to −20 °C, the biotinylated nitrilase was found to be stable up to 3 times longer than the native enzyme. The activity of the biotinylated nitrilase was optimum over a pH range of 7–11, while that of the native enzyme was optimum only between pH 7 and 9. The nitrile sensor was stable for at least 7 days at 25 °C. It was demonstrated for the detection of benzonitrile at concentrations of 0.1–5 mM in both simple and benzene/chloroform/nitrobenzene containing samples.

Nitriles are widely used in the chemical and pharmaceutical industries as solvents and in the manufacture of polymer fibers, plastics, and synthetic intermediates. They are also common additives in gasoline and constitute a major fraction of several types of insecticides and herbicides. The contribution of organonitriles to environmental pollution is considered to be substantial.[1-4] Current analytical techniques for nitriles in natural samples utilize spectrophotometry or chromatography, both of which are too expensive and cumbersome for on-site or in situ monitoring and screening.[5-7] In an attempt to develop a small and fast-responding nitrile sensor which can be used for on-site monitoring and screening in environmental studies, we have selected the enzyme-based electrochemical sensing mechanism as the working scheme. Generally, in most biosensors the target substrate is selectively converted by an immobilized enzyme, and the product of this enzymatic reaction is then detected by a nonspecific transducer. Enzyme-based electrochemical sensors are widely used in different fields, and a large number of reviews have been published.[8-10]

It has been found that *Rhodococcus* sp. nitrilase enzyme can catalyze the hydrolysis of nitriles directly into the carboxylic acid derivatives and ammonia.[5] In a previous paper we reported on a biosensor for benzonitrile based on the ammonia gas-sensing electrode.[11] In that sensor, nitrilase was physically entrapped between a gas-permeable membrane and a cellulose dialysis membrane. Ammonia, one of the enzymatic reaction products, diffused through the gas-permeable membrane and was consequently detected by the ammonia gas-sensing electrode. Even though such potentiometric sensors have the advantage of being simple to use for direct measurements, they are usually very slow in terms of response time. For example, the ammonia gas-sensing electrode-based sensor for benzonitrile in aqueous solutions required ~20 min before reasonably steady readings could be obtained.[11] To improve the performance of the enzyme-based sensor, we have investigated other possible surface immobilization techniques and electrochemical detection methods. We describe here a nitrilase-modified glassy carbon microelectrode sensor for benzonitrile which utilizes an avidin–biotin coupling scheme in which benzoic acid, one of the hydrolysis products of benzonitrile, is detected amperometrically.

The coupling interaction between avidin (a glycoprotein isolated from egg white) and biotin (a naturally occurring vitamin) is one of the strongest biological recognitions in nature[12] and is widely used in protein immobilization and amino assays. The immobilization of nitrilase through the biotin–avidin–biotin molecular sandwich has been shown to be very reliable and controllable. The length of the hydrophilic arm may be adjusted by selecting an appropriate molecular link during the immobilization step. This, in turn, gives greater control over the extent and

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0003-2700/95/0367-1679$9.00/0

localization of surface coverage by the enzyme.[12-14] Thus, it is possible to prepare the surface in such a way that it provides a stable environment for the enzyme and also increases the probability of a facile electron transfer between the electrode surface and the product analyte. In addition, since other enzymes may be immobilized without changing the overall detection strategy, it makes it a general immobilization method for enzyme-based sensors.[15] Another important advantage of this scheme is that it separates the electrode surface derivatization from the enzyme modification step. This allows both processes to be optimized independently and makes it much easier to retain the activity of the enzyme after the immobilization.

The amperometric detection of enzymatic reaction products of benzonitrile hydrolysis is not a trivial task. Since the direct electrochemical reduction of benzoic acid in aqueous solutions is very difficult ($E_{red} \approx -2$ V vs SSCE),[16-18] it is necessary to accomplish the electrochemical detection by an indirect method. It has been shown that the mass-transport-limited reduction current of H^+ is independent of charge-transfer kinetics and can be used for analytical determinations.[19,20] This approach could be competitive with potentiometric measurements employing glass electrodes, especially in measuring $[H^+]$ concentrations in very small regions (e.g., near the electrode surface) or in media containing charged colloidal particles. However, steady-state voltammetric measurement of $[H^+]$ appears to be possible only with the use of microelectrodes. This is presumably due to the relatively fast removal of the H_2 formed at the electrode surface by the rapid spherical diffusion inherent with microelectrodes. Current fluctuations due to formation of H_2 bubbles, clearly observed in voltammograms taken at millimeter-sized electrodes, are drastically reduced at microelectrodes and do not appear until significantly higher $[H^+]$. This technique has been applied to both strong and weak acids for $[H^+]$ up to 0.1 M.[19] This indirect reduction of H^+ at a microelectrode has been adopted as the electrochemical transduction mechanism for the nitrile sensor described here.

EXPERIMENTAL SECTION

Instrumentation. The HPLC system used in the assay of nitrilase consisted of a Rainin solvent delivery system, Model HPXL, a Rainin detector, Model 288, and a Rainin C-18 column, Dynamax 60A. A Perkin-Elmer Lamda 6 UV–vis spectrophotometer (Perkin-Elmer Co., Norwalk, CT.) was used in the study of the stoichiometry of the biotinylated enzyme. An EG&G Model 273 potentiostat, controlled by a DEC p420-SX computer and running the M270 electrochemical research software (EG&G PAR, Princeton, NJ), was used for all the electrochemical experiments. A SLM Model SPF-500C (SLM Instruments, Inc., Urbana, IL) spectrofluorometer equipped with a customized front surface accessory was used in the microelectrode surface characterization. An Orion Model 611 pH/millivolt meter (Orion Research, Inc., MA) was also used in measurements of solution pH. A 3 mm diameter glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN), a 10 μm diameter glassy carbon microelectrode (Cypress Systems, Lawrence, KS), and a 10 μm diameter Pt microelectrode (Cypress Systems) were used as indicated. All potentials reported below are referenced against a Ag/AgCl/3 M KCl reference electrode (Bioanalytical Systems).

Chemicals. Avidin (chromatographically purified), 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide methiodide, ExtrAvidin-FITC conjugate, *o*-phenylenediamine, (Sigma); sulfo-NHS-biotin (immunopure), NHS-LC-biotin, (Pierce, IL); poly(oxyalkylenediamine) (Jeffeamine ED-600, Texaco), benzonitrile (HPLC grade), nitrobenzene, benzene, chloroform, (EM Science); potassium chloride (Aldrich); and potassium phosphate monobasic (Fisher) were all reagent grade and used without further purification. All solutions were made with 18 MΩ·cm deionized water from a Barnstead Nanopure water system (Barnstead Inc., Newton, MA).

Extraction of Nitrilase. The *Rhodococcus* sp. ATCC 39484 bacteria was chosen as the source for the production, extraction, and purification of the nitrilase enzyme, and all procedures were accomplished as previously reported.[11] *Rhodococcus* sp. nitrilase has a strict specificity for aromatic nitriles. It will hydrolyze meta- and para-substituted benzonitrile derivatives but has no effect on the ortho-substituted ones. In addition, neither mandelonitrile nor phenylglycinonitrile are hydrolyzed.

Biotinylation of Nitrilase. The biotinylation of *Rhodococcus* sp. nitrilase was accomplished according to a modified commercial protocol (Vector Laboratories, Burlingame, CA).

Biotinylation Conditions. The suitable apparent molar ratios of biotin to nitrilase were determined. Nitrilase solutions (6.9 mg/mL) were reacted with different amounts of NHS-LC-biotin (1–20 times the nitrilase concentration) at pH 7.2, and the enzyme activities were assayed during the modification. A biotin/nitrilase ratio of 4:1 was found to be the best for biotinylation of nitrilase. The nitrilase enzyme was then biotinylated directly with NHS-LC-biotin by adding 0.4 mg (0.72 μmol) of NHS-LC-biotin powder directly into 1 mL of nitrilase solution (6.9 mg, pH 7.2) and allowing the mixture to react for 60 min at 4 °C. A 40 μL aliquot of 20 mM glycine solution was then added to this solution in order to react with the excess biotin. The mixture was dialyzed against 10 mM phosphate buffer (3 × 4 L, pH 7.2) to remove low molecular weight impurities in the modified protein. The actual biotin/protein ratio was determined by the HABA dye method.

Estimation of the Biotinylation Stoichiometry. The stoichiometry of the biotinylated enzyme was determined by the spectrophotometric assay method of Green[21] based on the binding of HABA dye with avidin. When HABA is bound to avidin, it gives a new absorption band at 500 nm with an extinction coefficient (ϵ_{500nm}) of 34 000 and a change of color from yellow to red. At the same time, the 348 nm absorption band of the azo dye free anion (ϵ_{348nm} = 20 700) almost disappears. Due to the stronger affinity of biotin for avidin, these changes can be reversed by addition of 4 mol of biotin/mol of avidin. The standardized solution of avidin was then used to estimate the content of biotin and its analogues. The concentration of avidin and biotin can also be calculated from the decrease in A_{500nm} by dividing by ϵ_{500nm}. The stoichiometric displacement of the dye by biotin is specific for avidin.

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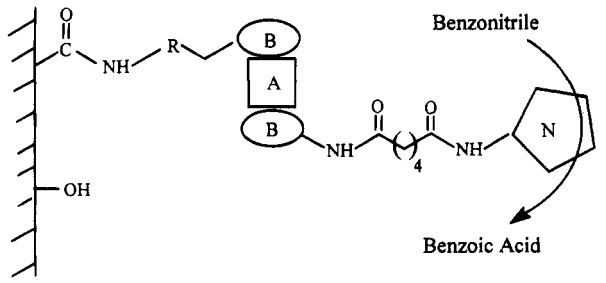

Figure 1. Schematic presentation of the nitrilase-modified glassy carbon electrode surface showing the avidin (A), biotin (B), and nitrilase (N) configuration.

Standard Curve. To prepare a standard curve, 75 μL of 10 mM HABA in 10 mM NaOH solution was added to 3 mL of 0.5 mg/mL avidin in 50 mM phosphate, 0.9% NaCl, pH 6 solution. It was well mixed, and the absorption was measured at a wavelength of 500 nm (A_{500nm}). Into the previous solution were added in 2 μL increments 0.5 mM biotin in 50 mM phosphate, 0.9% NaCl, pH 6 solution, and A_{500nm} was measured after each addition. The change in absorbance, ΔA_{500}, was plotted against the concentration of biotin to give the standard curve. According to the same procedure, a small aliquot (12 μL, 4 μg) of biotinylated enzyme solution (instead of biotin solution) was added, and the changes in A_{500nm} were measured. By comparing this value against the standard curve, the biotin content in the modified protein was determined. The standard curve obtained by this procedure was linear over a biotin concentration of 0–0.02 μmol. The actual ratio of biotin/enzyme for the biotinylated nitrilase was determined to be 1.63:1 by this method.

Electrode Derivatization. The 10 μm glassy carbon microelectrode was successively polished with 0.1 and 0.05 μm alumina on a polishing cloth (Micropolish, Mastertex, Buehler Ltd., Lake Bluff, IL) and subsequently washed and ultrasonicated in deionized water. The microelectrode was electrochemically activated in 0.1 M KH_2PO_4 + 0.15 M KCl buffer solution, pH 7, by cycling the electrode between approximately −0.2 and 1.8 V (vs Ag/AgCl) at 50 Hz for 3 s. Carboxyl groups on the electrode surface were then activated by reaction with 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide methiodide (30 mg/mL of pH 6.5 phosphate buffer) for 12 h, followed by dipping in a 0.4 mM Jeffamine ED-600 phosphate buffer solution, pH 7.4, for 2 h and 5 mg of sulfo-NHS-biotin/mL of pH 7.4 phosphate buffer solution for 2 h. In these steps, the hydrophilic tether was attached to the electrode surface at one end via an amide bond and biotinylated at the other end also through an amide linkage between the free terminal amine of Jeffamine ED-600 and the biotin. The electrode was then treated with ~25 μM avidin phosphate buffer solution at 5 °C for 6–12 h. This step attached the avidin to the electrode surface through avidin–biotin interaction. Finally, the electrode was treated with the biotinylated nitrilase at 5 °C for 12 to 24 h to attach the biotinylated nitrilase to the electrode surface, giving the desired biotin–avidin–biotin sandwich structure.[12-14] Between each of the above steps, the electrode was carefully washed with pH 7.4 phosphate buffer. A schematic presentation of the surface structure of the modified electrode is shown in Figure 1. When not in use, the modified microelectrodes were always stored in a 0.1 M phosphate buffer solution (pH 7.4) at 5 °C.

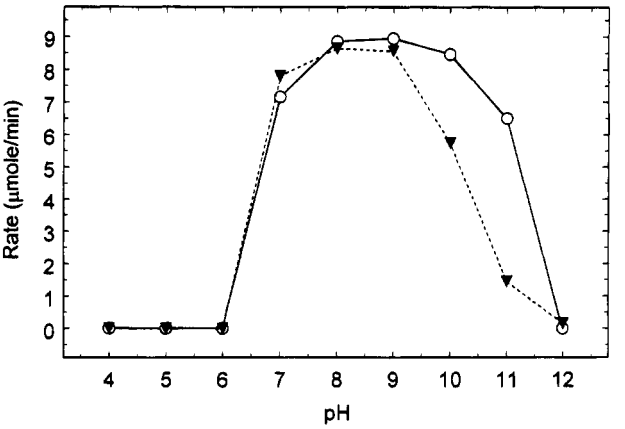

Figure 2. Effect of pH on biotinylated (○) and native nitrilase (▲).

RESULTS AND DISCUSSION

Characterization of Biotinylated Nitrilase. The activity of biotinylated enzyme was determined, as previously described,[11] by incubating the enzyme with a certain amount of benzonitrile for a specific period of time, quenching the reaction, and then determining the extent of conversion using HPLC. The effect of pH on the activity of biotinylated nitrilase was determined by measuring the activities of native nitrilase, biotinylated nitrilase, and agarose–avidin/biotinylated nitrilase complex, in a pH range of 4–12. The results, shown in Figure 2, indicate that the activity of the biotinylated nitrilase is comparable to that of the native nitrilase, with the optimum being slightly shifted to a slightly more basic range.

After immobilization via avidin cross-linked with 6% agarose beads, 1 mmol of immobilized nitrilase was able to convert 1.6 mol of benzonitrile to benzoic acid (at 25 °C after 24 h). This is a very good conversion ratio and facilitates the use of nitrilase in an analytical sensor. This high conversion ratio is generally typical for immobilized enzymes and is usually thought to be due to the formation of a more rigid three-dimensional structure after the immobilization.[22]

To demonstrate the specific binding of biotinylated nitrilase to avidin, an agarose–avidin column (1 mL) was equilibrated with phosphate buffer (pH 7.2) and loaded with 200 μL (1.4 mg) of native nitrilase. After the column was incubated for 1 h, it was washed with phosphate buffer, and then 1 mL of 8 mM benzonitrile solution was loaded on the column and assayed for 30 min. The eluent was analyzed with HPLC, which showed that no nitrilase activity was present on the column. However, when 0.2 mL (1.1 mg) of biotinylated nitrilase was loaded on the same size avidin column, washed with phosphate buffer after 1 h incubation, and assayed with benzonitrile, nitrilase activity was found to be present. This results indicates that biotin–avidin binding is specific for biotinylated nitrilase.

To determine the thermostability of biotinylated nitrilase, samples of the biotinylated nitrilase solutions were stored at 25 °C, 4 °C, and −20 °C, and enzyme activities were monitored for 1 week. At the same time, the stabilities of native nitrilase samples were monitored as the control. This procedure showed that the biotinylated nitrilase was stable for up to 3 times as long as the native enzyme.

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Electrode Modification and Characterization. Glassy carbon is a particularly attractive substrate for immobilization of enzymes because of the wide variety of surface functional groups that can be used for chemical derivatization.[1] A 10 μm diameter glassy carbon microelectrode was chosen as the working electrode due to the fact that the electrochemical transduction mechanism of the nitrile sensor is based on the reduction of the benzoic acid proton in aqueous solutions, for which the glassy carbon substrate has the widest working potential range. The Jeffamine ED-600, a poly(oxyalkylenediamine), was used as the hydrophilic tether so that it would allow the enzyme to reside a short distance away from the electrochemical double layer at the surface.[12] The extent of reaction between the surface-bound carboxylates and the primary amine of the tether and as the subsequent surface coverage by avidin have been extensively studied by Pantano and Kuhr.[14,23] They concluded that complete electrode surface coverage is possible under such modification conditions and the extent of the surface coverage can be controlled by varying the length of time of the derivatization reaction. They also demonstrated the lack of nonspecific binding of avidin to a carbon surface.[1,23] The entire electrode modification process was conducted at either 25 °C or 4 °C. There were no extreme chemical or electrochemical conditions imposed for the immobilization of nitrilase.

In order to demonstrate successful surface derivatization, a partially modified 3 mm diameter glassy carbon electrode surface was examined by spectrofluorometry using fluorescein isothiocyanate (FITC)-labeled avidin or biotin.[14,23-25] This partially modified electrode for the surface characterization was derivatized by the attachment of the hydrophilic tether of Jeffamine ED-600, biotinylation of the free terminal amine of Jeffamine ED-600, and the coupling of the FITC-avidin. The electrode was placed in a customized electrode holder in a variable angle front-surface accessory of the spectrofluorometer, and the electrode surface was aligned with the front plate of the accessory holder. The optimum incidence angle for sample excitation was experimentally determined and was carefully adjusted to minimize reflections and scattering. The excitation was set at 488 nm, and the emission spectrum was monitored by scanning from 520 to 800 nm. The background-subtracted emission spectrum of the partially modified electrode is shown in Figure 3. The background-subtracted spectrum was obtained with another clean, uncoated glassy carbon electrode of the same size. It can be seen clearly that there is an emission peak at around 615 nm corresponding to the existence of FITC-avidin on the electrode surface, which demonstrates that the modification strategy was successful in attaching the hydrophilic spacer arm and the biotin–avidin coupling to the electrode surface. It is reasonable to assume that the final step in the modification scheme results in the attachment of the biotinylated nitrilase to the already immobilized avidin on the electrode surface and the immobilization of nitrilase on the glassy carbon electrode surface.

As an alternative procedure, we also attempted to immobilize nitrilase onto a 10 μm diameter Pt disk microelectrode using one of several possible electropolymerization techniques.[22,26-28] Poly-

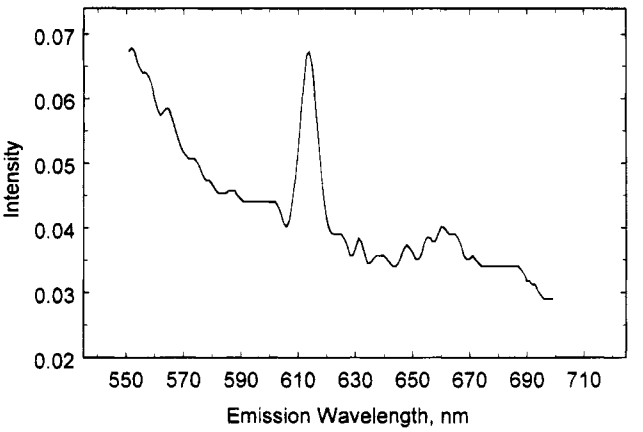

Figure 3. Background-subtracted fluorescence spectrum of a FITC-ExtrAvidin-modified glassy carbon electrode surface. Excitation wavelength, 488 nm.

(*o*-phenylenediamine) was chosen as the polymer for the immobilization because of its relatively mild polymerization conditions. However, when the potential was set at or scanned to +0.6 V, which is required for the polymerization of *o*-phenylenediamine, nitrilase lost its activity after the immobilization. The cross-linking agent glutaraldehyde is often used to increase the stability of enzymes in the immobilization.[22] However, when glutaraldehyde was added to the solution, nitrilase lost its activity even before the polymerization, presumably by the reaction of glutaraldehyde with the active sites. The failure of the electropolymerization immobilization technique for nitrilase limited our immobilization to the avidin–biotin coupling strategy.

Analytical Evaluation. The sample solutions used to characterize the analytical response of the sensor consisted of benzonitrile in deionized water with 0.1 M KCl added as supporting electrolyte. Initially, attempts were made to amperometrically monitor the stepwise current increase expected from the reduction of H^+ after addition of benzonitrile. However, at potentials sufficient for H^+ reduction, significant H_2 bubble formation occurred at the electrode surface, causing large current oscillations and making it impossible to obtain reasonably stable current values.

The situation was drastically improved, though, when the detection of the benzoic acid (produced at the electrode surface by the immobilized enzyme) was accomplished by measuring the steady-state current due to the reduction of H^+ during linear scan voltammetry (LSV) in the potential range of −0.4 to −1.2 V. Performing LSV at a microelectrode produces a sigmoidal shaped curve with the difference between the baseline and the plateau giving the steady-state current. A series of LSV experiments with scan rates between 2 and 1000 mV/s showed that satisfactory voltammograms could be obtained if scan rates were limited to <10 mV/s. Figure 4 shows representative linear scan voltammograms (5 mV/s) for samples containing 0 and 1.0 mM benzonitrile, purged and blanketed with nitrogen gas. Baseline subtraction results in an improved curve and can also be used to permit analysis of the same benzonitrile sample without N_2 purging.

The reduction current for standard additions of benzoic acid at an *unmodified* glassy carbon electrode compared to that for a *derivatized* electrode was almost 2.5 times greater, and the current

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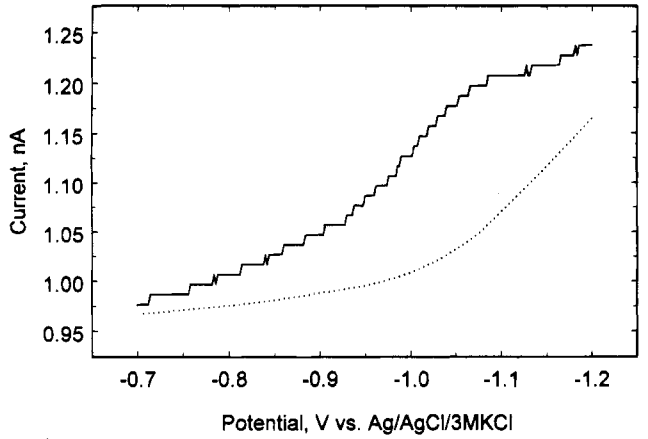

Figure 4. Voltammograms of 0 (···) and 1 mM (—) benzonitrile in 0.1 M KCl at a nitrilase-modified glassy carbon electrode. Electrode diameter, 10 μm; scan rate, 5 mV/s.

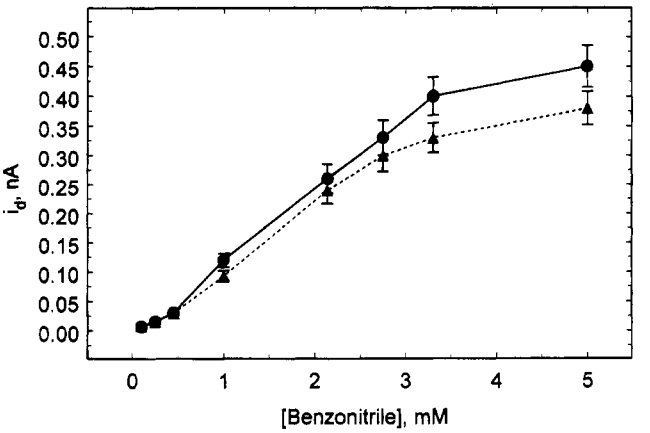

Figure 5. Calibration curve of the nitrile sensor for addition of benzonitrile in 0.1 M KCl solutions at pH 7 and 8. The steady-state reduction current, i_d, of benzoic acid is obtained from background-subtracted linear sweep voltammograms. Electrode diameter, 10 μm; scan rate, 5 mV/s.

plateau was somewhat better defined (at comparable scan rates). Both electrodes responded linearly to the addition of benzoic acid in the concentration range of 0.06–5 mM in 0.1 M KCl solutions. This shows that although the derivatization does not change the overall electrode response of the proton detection scheme to benzoic acid, it affects the electron transfer properties at the electrode surface. No attempts were made to fully optimize the derivatization conditions during the current study.

It has been reported that the reduction of H^+ can be used for analytical purposes with or without supporting electrolyte for both strong and weak acids[19,20] and that in the absence of supporting electrolyte, the reduction wave of monohydrogen weak acids exceeds that of such acids with electrolyte present by a factor of 2. However, we found that the reduction wave at the nitrilase-modified electrode was distorted without supporting electrolyte to such an extent that it was very difficult to accurately define the current plateau.

A calibration curve for standard additions of benzonitrile to deionized water with 0.1 M KCl is shown in Figure 5. The plotted points are the average values taken from five sets of data using different electrodes. The steady-state reduction current of benzoic acid increases with the concentration of benzonitrile in the solution in the range of 0.1–5 mM. The calibration curve was linear for benzonitrile concentrations of 0.1–3.3 mM, after which the response begins to taper off. It is interesting to note that the slope (sensitivity) of this linear region (0.12 nA/mM of benzonitrile) is only about 3% of the value calculated from the steady-state equation, $i_{ss} = 4nrFDC$, where the slope is $4nrFD$ (about 4 nA/mM). This could be interpreted to mean that the steady-state current is being controlled by the enzyme conversion rate and/or that the active enzyme surface area is not sufficient to support the spherical mass transport rate.

The nonlinearity of the calibration curve for concentrations of benzonitrile greater than 3.3 mM could be due to the effect of the K_m of nitrilase or a result of substrate inhibition.[29] The same nitrilase-modified electrode could be used for up to 7 days at room temperature if it was stored in a 0.1 M phosphate buffer solution (pH 7) at 5 °C at the end of each day's use. Similar results were also obtained for a series of samples which consisted of a mixture containing 5 mM benzonitrile, benzene, chloroform, and nitrobenzene in 0.1 M KCl solution. This clearly demonstrates the selectivity of the nitrilase enzyme for benzonitrile in the presence of several other aromatic compounds.

The limits of detection for the nitrilase-based sensor described here could be significantly improved in several ways. The most easily implemented would be to use an array of modified microelectrodes such as those obtained using microlithographic fabrication techniques.[30] This would allow the summing of the individual microelectrode currents while retaining the microelectrode characteristic for the reduction of the benzoic acid H^+ and should increase the detection limit of the nitrile sensor and possibly extend the linear range of the calibration curve to a wider concentration range of benzonitrile. A method for the direct deposition of avidin onto metals, such as platinum, has been recently reported.[31] This would enable the use of such microelectrode arrays for immobilization of the biotinylated nitrilase through avidin–biotin coupling while maintaining the activity of nitrilase.

ACKNOWLEDGMENT

This work was supported in part by a grant from the U.S. Environmental Protection Agency through the Tufts Center for Environmental Management. The information contained in this document does not necessarily reflect the views of the Agency or the Center, and no official endorsement should be inferred.

Received for review September 14, 1994. Accepted March 2, 1995.[⊗]

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[⊗] Abstract published in *Advance ACS Abstracts*, April 15, 1995.