Determination of Organonitriles Using Enzyme-Based Selectivity Mechanisms. 1. An Ammonia Gas Sensing Electrode-Based Sensor for Benzonitrile

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Organonitriles are considered an important class of compounds which contribute to environmental pollution. A nitrile sensor was developed to directly and selectively measure nitrile compounds in water. *Rhodococcus* sp. nitrilase catalyzes the hydrolysis of benzonitrile derivatives directly into the corresponding carboxylic acid and ammonia. The enzyme was purified and physically entrapped onto the tip of an ammonia gas sensing electrode using cellulose dialysis membranes. The resulting nitrile sensor was relatively stable for up to 4 days at 4 °C, after daily use at room temperature. It can be reliably used in the pH range of 6-9. The sensor is demonstrated for the detection of benzonitrile in neat water samples at concentration levels as low as 1×10^{-5} M.

INTRODUCTION

Nitriles are organic compounds containing the cyano carbon-nitrogen triple bond. They are widely utilized 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. Nitriles are primary irritants which can be rapidly adsorbed through the skin and are known to cause hepatic, cardiovascular, renal, gastrointestinal, and central nervous system disorders. Some nitriles in widespread use include acrylonitrile, acetonitrile, methacrylonitrile, propionitrile, and adiponitrile.[1,2] Acrylonitrile, which is an EPA priority pollutant, is among the top 50 most widely used industrial compounds in the United States.[3] It is extremely toxic to mammals with a latency period of up to 20 years.[4,5] Organonitriles are considered an important class of compounds contributing to environmental pollution.

Analysis of nitriles in natural samples is normally accomplished by spectrophotometric or chromatographic methods.[6-8] These techniques though are cumbersome and expensive for rapid on-site environmental monitoring and screening. In most cases interferences from other organics require the preliminary use of time-consuming separation techniques before qualitative or quantitative analysis can be performed. There is no method currently available for in situ or continuous monitoring of nitriles.

Enzyme-based electrochemical sensors are widely used in different fields, and a large number of reviews have been published.[9-11] The enzyme system in these sensors allows for highly selective detection of the enzyme-specific substrate without significant interferences. The product of the enzyme reaction is then detected by a nonspecific detector. Potentiometric ion selective electrodes (ISE) and gas sensing electrodes (GSE), such as the ammonia electrode, have the advantage of being simple to use for direct measurements. Several enzyme-based electrochemical sensors have been reported which utilized them as the base sensor.[11-13]

To provide the nitrile-specific function of the sensor, an enzyme is required which uses the cyano group as its sole substrate. Several microorganisms are known which contain nitrilase enzymes that utilize cyano compounds as their sole substrates.[14-23] One such bacteria, *Rhodococcus* sp., produces a nitrilase enzyme which selectively catalyzes the hydrolysis of nitriles directly into the carboxylic acid derivatives and ammonia, without the intermediacy of the amide.

In the fabrication of the electrode described here, the *Rhodococcus* sp. nitrilase is entrapped between the gas-permeable membrane of a gas-sensing ammonia electrode and a piece of cellulose dialysis membrane covering the electrode tip. The ammonia produced by the enzymatic hydrolysis reaction diffuses through the gas-permeable membrane causing a change of the ammonia GSE potential. This electrode assembly (i.e., sensor) was tested at different pHs and different concentrations of benzonitrile and was shown

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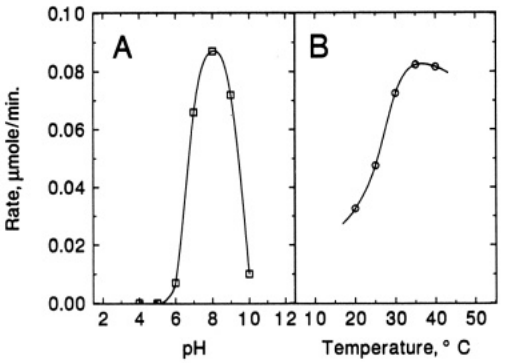

Figure 1. Dependency of *Rhodococcus* sp. nitrilase activity on (A) pH at 25 °C and (B) temperature at pH 7.

to be capable of directly detecting benzonitrile at concentration levels as low as 10 μM in aqueous solutions with no added reagents.

EXPERIMENTAL SECTION

Extraction of Nitrilase. *Rhodococcus* sp. ATCC 39484 was chosen as a suitable bacterial source for the production, extraction, and purification of the nitrilase enzyme. The freeze-dried cells were initially revived in a medium containing beef extract (0.3 g) and peptone (0.5 g) in 100 mL of tap water. Growth conditions for the organism closely followed those described for *Rhodococcus rhodochrous* J1, using 0.1–0.2% isovaleronitrile (v/v) to induce benzonitrilase activity.[7] The stock culture was maintained on 2% agar plates containing beef extract and peptone (DIFCO 0001). All purification steps were carried out at 4 °C and followed the procedure described for *R. rhodochrous* J1 nitrilase.[7] The nitrilase used in these studies was determined to be about 70% pure using SDS–PAGE electrophoresis. A prominent band corresponding to a molecular weight of 40 000 was observed, consistent with the reported molecular weight.[7]

The nitrilase was assayed by incubating an appropriate amount of the enzyme with 5 mM benzonitrile/0.1 M phosphate buffer, pH 7.2, for 10–60 min at 25 °C. The reaction was quenched by adding HCl to 0.1 M, with precipitated protein removed by centrifugation. Enzyme activity was determined by calculating the extent of conversion to benzoic acid by reversed-phase HPLC using 0.8 mM salicylic acid as internal standard.[24] The HPLC was operated using a Dynamax-60A reversed-phase C18 column, an eluent containing of 35% acetonitrile/0.1% trifluoroacetic acid and operating at a flow rate of 1 mL/min. Benzonitrile, benzoic acid, and salicylic acid had retention times of 10.1, 5.1, and 6.4 min, respectively. One unit of nitrilase activity is defined as the amount of enzyme needed to catalyze the formation of 1 μmol of carboxylic acid per minute at 25 °C. Nitrilase activity in whole cells, in crude cell-free homogenates, or in purified form (≥5 mg/mL) appears relatively stable. Less than 10% decrease was observed after prolonged storage at −70 °C.

Rhodococcus sp. nitrilase has a strict specificity for aromatic nitriles (no activity is observed with benzylcyanide), hydrolyzing benzonitrile derivatives substituted in the meta and para positions. Ortho-substituted benzonitriles are unreactive. In addition, neither mandelonitrile nor phenylglycinonitrile was hydrolyzed. Panels A and B of Figure 1show the nitrilase activity as a function of pH and temperature, respectively.

Electrode Assembly. A commercial ammonia GSE (Orion Model 95–12, Orion Research Inc., Boston, MA) was adapted as the base sensor. In order to fabricate the nitrile sensor, the ammonia GSE jacket was assembled according to the manufacturer's instructions. An o-ring was used under the top cap to create a recess where the nitrilase solution was to be entrapped, with special care taken to ensure that no air bubbles were trapped between the membranes. The recess was covered with a sheet of dialysis membrane at the electrode tip after nitrilase solution was placed into the recess and the cellulose membrane was fastened to the electrode body by a second o-ring. A diagrammatic view of the electrode structure is shown in Figure 2. Two types of cellulose dialysis membrane were tested for use with the sensor, Spectra/Por 7 with a molecular weight cutoff 25 000 and Spectra/Por 2 with a molecular weight cutoff of 12 000–14 000 (Spectrum

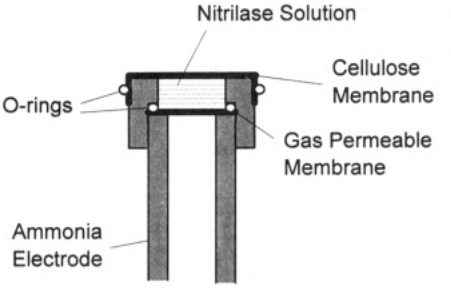

Figure 2. Diagrammatic view of the nitrile sensor showing the placement of the membranes and enzyme.

Medical Industries, Inc., Houston, Texas). Spectra/Por 7 was chosen because it provided the best overall performance in terms of both sensor response time and sensitivity.

To enhance the ammonia GSE performance at low ammonia levels, the commercial ammonia electrode internal filling solution was diluted with deionized water by 1:10. The electrode with the diluted filling solution gave a linear response with addition of ammonium chloride.

Since the response of each electrode was directly related to the amount of enzyme used to fill the intermembrane chamber, 30 μL of nitrilase solution (1.2 mg) was always used in the fabrication of the electrode, unless otherwise indicated. All data presented in this paper were obtained from the same batch of nitrilase. Just prior to each experiment, the sensor assembly was equilibrated in a phosphate buffer solution (at the same pH as the sample for 2 h at room temperature) in order to give a more stable baseline for measurement of the potential.

Instrumentation. A French pressure cell press Aminco (SLM Instruments Inc., Urbana, IL) was used in the extraction process of the nitrilase. 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.

The electrode was connected to an Orion Model 611 pH/millivolt meter (Orion Research Inc. or an EG&G Model 273 potentiostat (Princeton Applied Research, Princeton, NJ). The electrode potential was recorded with a paper strip-chart recorder ABB SE 110 (ABB Goerz AG, Vienna, Austria). A Fisher Model 120M magnetic stirrer was used during the measurements. The potential of the nitrile sensor was determined from the steady-state value after each addition of benzonitrile.

Chemicals. All chemicals used in this research were reagent grade and used without further purification. All solutions were made with 18 MΩ·cm deionized water obtained from a Barnstead Nanopure water system (Barnstead Inc., Newton, MA).

RESULTS AND DISCUSSION

The performance of the ammonia GSE-based nitrile sensor with the configuration shown in Figure 2 was initially evaluated by addition of ammonium chloride to the buffer solution. The response of this electrode with a 0.1 M KH_2PO_4 buffer solution in place of the nitrilase solution entrapped between the gas-permeable membrane and the cellulose dialysis membrane was compared to that of the original unmodified ammonia electrode. From these experiments, it was clear that this sensor configuration responded linearly to the addition of ammonium chloride. The sensitivity of the modified ammonia GSE in terms of the slope $\Delta E/\Delta[NH_4^+]$ was in most cases approximately the same or slightly less than that of the original ammonia electrode and was dependent on the solution pH. However, this modified electrode configuration had about a 50% slower response time. This appears reasonable since there are more barriers in the pathway of the ammonia diffusing into the ammonia GSE, i.e., a second sheet of dialysis membrane and the body of solution entrapped between the membranes.

The calibration curves for the nitrile sensor with additions of benzonitrile into 0.1 M phosphate buffer solution of

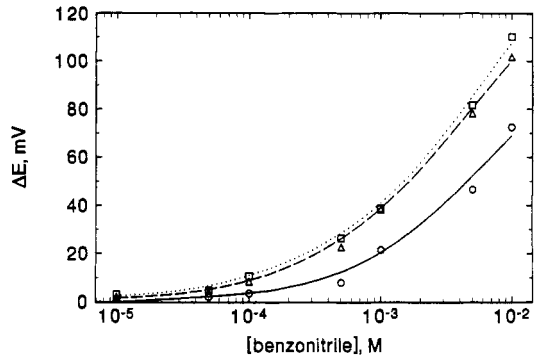

Figure 3. Calibration curves obtained with the nitrile sensor for additions of benzonitrile in solutions of pH 7 (O), pH 8 (□), and pH 9 (△).

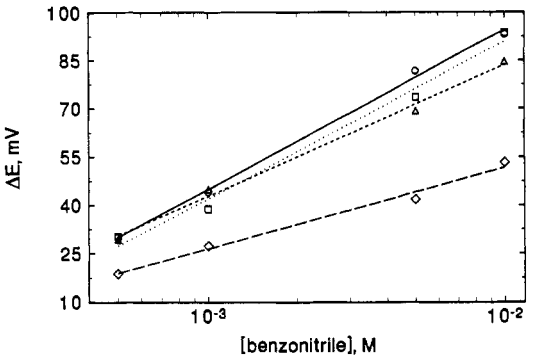

Figure 4. Performance of the sensor in a 0.1 M phosphate buffer solution of pH 8 immediately after assembly (O), after 3 days (□), after 4 days (△), and after 5 days (◇). The sensor was stored at 4 °C after each 8 h of daily use.

different pH values are shown in Figure 3. The potential difference shown, ΔE, is that of the electrode in the blank buffer solution and after the addition of the benzonitrile. The electrode response was basically linear for the concentration range of 10^{-2}–5×10^{-4} M with a slope of 59 mV/decade for the pH 8 and 9 solutions and 48 mV/decade for the pH 7 solution. For benzonitrile concentrations of approximately less than 5×10^{-5} M, the sensitivity decreased rapidly and accurate potential values were difficult to measure. This nonlinear behavior is probably due to the smaller concentration gradient formed across the membrane at low concentrations of benzonitrile. At concentrations of greater than 10^{-2} M, the curves show the effect of the decreasing solubility of the benzonitrile in water.

The amount of enzyme entrapped in the electrode had an effect on the electrode sensitivity and response time. When the volume of the nitrilase solution in the intermediate chamber was varied from 20 to 40 μL, the sensitivity of the sensor increased slightly and the potential reached a steady-state value more rapidly after each standard addition.

Effect of pH. As previously mentioned above, the activity of the nitrilase depends upon the pH of the solution it is in (Figure 1A) and the temperature (Figure 1B), with the optimum activity occurring between pH 7 and 8 and at a temperature of 34 °C. Because the optimum pH for the ammonia electrode used as the base sensor in this research is at least 11, a compromise must be made when it comes to choosing the working pH of the solution in the intermediate sensor chamber. Experimental data showed that the pH must be in the range of 6–10 for the nitrile sensor to provide a linear response with a reasonable dynamic range. The magnitude of ΔE is also proportionally related to the solution pH. The best electrode response is obtained if the pH is around 8–9. The pH also affects the detection limit. Experiments showed that if the pH was adjusted to around 7, the sensor was unable to detect benzonitrile at concentrations of less than 2.5×10^{-5} M; however, at a pH of 8 or 9 benzonitrile was detectable down to 1×10^{-5} M.

Stability of the Nitrile Sensor. The nitrile sensor was found to be relatively stable if stored in a 0.1 M KH_2PO_4 buffer solution of pH 8 at 4 °C at the end of each day's use. Under these conditions the sensor produced the same response for as long as 4 days (Figure 4). After this period the response of the sensor deteriorated gradually. During each 8 h of daily use, the nitrile sensor was used to obtain a set of data by standard addition of benzonitrile into 0.1 M KH_2PO_4 buffer solution of pH 8 at 4 °C. Experiments also showed that when the nitrile sensor performance became unacceptable, 8 h of dialysis in the 0.1 M KH_2PO_4 buffer solution of pH 8 partially restored it to the normal condition, but total recovery of sensitivity and reproducibility was never attained.

Analytical Applications of the Nitrile Sensor. The calibration curves shown in Figure 3 were obtained by the standard addition method. Each point on the graph is the arithmetic average of at least three measurements. The nitrile sensor potential decreased in a stepwise fashion corresponding to each addition of benzonitrile. There was a 2–3 min lag time before the sensor potential began decreasing after the addition, and about a 20–30-min period for the electrode potential to reach a relatively steady value, depending upon the concentration of benzonitrile. For a benzonitrile concentration of less than 2×10^{-5} M, the electrode potential change became very small and was difficult to accurately resolve. For the optimum pH range of 8–9, the change in potential with an added benzonitrile concentration of 1×10^{-5} M was approximately 2 mV. This can be considered as the detection limit of the nitrile sensor since a potential change of less than that becomes difficult to differentiate from the baseline noise.

To demonstrate the utility of this enzyme-based sensor for direct field monitoring or screening of nitrile compounds, the sensor was tested using unmodified natural spring water (Poland Spring, ME) to which various concentrations of benzonitrile had been added with a measured pH of 6.7. Additions to give concentrations of 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, and 5×10^{-5} M resulted in ΔE values of 35, 25, 10, and 4 mV, respectively.

To our knowledge this is the first demonstration of an ammonia GSE-based sensor using membrane-entrapped *Rhodococcus* sp. nitrilase for the selective detection and quantification of nitrile compounds in aqueous solution. At this point we feel that the sensitivity, dynamic range, and response time of the sensor have not been fully optimized. We are currently investigating other electrochemical techniques such as amperometry and voltammetry, and other surface immobilization techniques such as biotin/avidin attachment, to improve the desirable characteristics and further improve the analytical abilities of the sensor for field determination of organo nitriles.

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