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Amperometric Biosensor for Glutamate Using Prussian Blue-Based "Artificial Peroxidase" as a Transducer for Hydrogen Peroxide

Arkady A. Karyakin,*,† Elena E. Karyakina,† and Lo Gorton‡

Faculty of Chemistry, M. V. Lomonosov Moscow State University, 119899, Moscow, Russia, and Analytical Chemistry Department, University of Lund, POB 124, S-221 00 Lund, Sweden

The specially deposited Prussian Blue denoted as "artificial peroxidase" was used as a transducer for hydrogen peroxide. The electrocatalyst was stable, highly active, and selective to hydrogen peroxide reduction in the presence of oxygen, which allowed sensing of H_2O_2 around 0.0 V (Ag/AgCl). Glutamate oxidase was immobilized on the surface of the Prussian Blue-modified electrode in a Nafion layer using a nonaqueous enzymology approach. The calibration range for glutamate in flow injection system was 1×10^{-7}−1×10^{-4} M. The lowest concentration of glutamate detected (1×10^{-7} M) and the highest sensitivity in the linear range of 0.21 A M^{-1} cm^{-2} were achieved. The influence of reductants was practically avoided using the low potential of an indicator electrode (0.0 V Ag/AgCl). The attractive performance characteristics of the glutamate biosensor illustrate the advantages of Prussian Blue-based "artificial peroxidase" as transducer for hydrogen peroxide detection.

Accurate, rapid, cheap, and selective analysis is required nowadays for use in clinical diagnostics and food industry. The majority of known electrochemical biosensors are based on immobilized specific oxidase and amperometric detection of O_2 or H_2O_2.[1−3] Electrochemical biosensors based on detection of hydrogen peroxide at platinized electrodes were found to be the more versatile, allowing a decrease in the detection limit down to 1 μM.[4] However, all biological liquids contain a variety of electrochemically easily oxidizable reductants, e.g. ascorbate, urate, bilirubin, catecholamines, which are oxidized at similar potentials and dramatically affect the biosensor selectivity, producing parasitic anodic current.[5]

Therefore, for optimal amperometric oxidase-based biosensors a selective detection of hydrogen peroxide at low potentials is required. Unfortunately, noble metals (platinum, ruthenium, rhodium) are not selective to H_2O_2 reduction in the presence of oxygen.[6−8] The detection of H_2O_2 by its electroreduction in the presence of oxygen can be achieved using two basic approaches. The first one is bioelectrocatalytic reduction of H_2O_2 at peroxidase modified electrodes (peroxidases are responsible in nature for reduction of hydrogen peroxide).[9] However, the enzymes being biological macromolecules obviously cannot provide a complete long-term stability of the sensor due to their inherent instability, causing denaturation.

Yet an additional alternative way for a low-potential, selective detection of hydrogen peroxide was recently demonstrated.[10−12] The development of amperometric biosensors on the basis of Prussian Blue-modified electrodes was first announced by our group.[10] Through optimization of the deposition of Prussian Blue we succeed in synthesizing both stable and selective electrocatalyst to H_2O_2 reduction in the presence of oxygen.[13] The current of H_2O_2 reduction on specially deposited Prussian Blue at around 0 V vs Ag/AgCl was at least 100 times higher than the background oxygen reduction. This was even higher than on peroxidase-modified electrodes.[14] When the amount of Prussian Blue deposited was in the range of 6−10 nmol cm^{-2}, the heterogeneous rate constant for hydrogen peroxide reduction varied from 0.015 to 0.02 cm s^{-1}.[15] For comparison, the kinetic constant for H_2O_2 oxidation on platinum in neutral media was more than 1000 times lower![16] Due to both its high activity and selectivity in hydrogen peroxide reduction, we denoted our specially deposited Prussian Blue as "artificial enzyme peroxidase".

† M. V. Lomonosov Moscow State University.
‡ University of Lund.

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Glutamate is an important compound as used in food industry, being involved in neurological pathways.[17] There are several reports on glutamate biosensors based on L-glutamate oxidase (EC 1.4.1.3). Typically, glutamate oxidase was immobilized on platinum or platinized electrodes. Hydrogen peroxide generated in the enzyme-catalyzed reaction was monitored amperometrically at 0.6 V (SCE).[18] The lowest detection limit achieved was 1 μM when the sensor was used in batch mode.[19] The decrease of the detection limit down to the submicromolar region was possible due to both microfabrication and using of tubular electrodes.[20] In the presence of interferences, the selectivity for glutamate can be improved by the use of an additional Nafion membrane[21] or by coimmobilization of the specific oxidase, for instance, with ascorbate oxidase.[22]

An amperometric biosensor for glutamate made by co-immobilization of horseradish peroxidase and glutamate oxidase in carbon paste[23] or in osmium-containing hydrogel[22] was reported. Peroxidase-based biosensors had a detection limit in micromolar range of glutamate.

Here we report a glutamate biosensor made on the basis of specially deposited Prussian Blue. The attractive performance characteristics of the glutamate biosensor illustrate the advantages of Prussian Blue-based "artificial peroxidase" as transducer for hydrogen peroxide detection.

EXPERIMENTAL SECTION

Materials. All inorganic salts were obtained at highest purity. Hydrogen peroxide was purchased from Aldrich (Steinheim, Germany) as concentrated solution and was titrated before use. The solutions throughout this work were prepared using water from a Milli-Q system (Millipore, Bedford, MA). Absolute ethanol was prepared by distillation of sodium alcoholate and used immediately. Nafion (5% solution in 90% light alcohols) was obtained from Aldrich. Glutamate oxidase (EC1.4.3.11), from *Streptomyces* sp., 8 units mg^{-1}) was a gift by Dr. H. Kusakabe of Yamasa Shoyu Ltd., Japan. L-Glutamic acid (monosodium salt), D-glutamic acid, D,L-aspartic acid, and ascorbic acid (sodium salt) were obtained from Sigma. Stock solutions of these reagents were prepared in buffers immediately prior to use.

Instrumentation. Electrochemical measurements were performed using an EG&G potentiostat-galvanostat system PAR 273 (Princeton, NJ). Hydrodynamic experiments were carried out with a low-noise potentiostat (Zäta Elektronik, Lund, Sweden). Solution flow was maintained by a digital Gilson peristaltic pump (type SVA, Buchs, Switzerland).

Electrochemical Methods. A three-compartment electrochemical cell contained a platinum net auxiliary electrode and an Ag/AgCl reference electrode in 1 M KCl. The cell construction allowed deaeration of the working electrode space. Glassy carbon disk electrodes (diameter 1.5 mm) were used as working electrodes. Prior to use, the glassy carbon electrodes were mechanically polished with alumina powder (Al_2O_3, 1 m) until a mirror finish was observed.

The flow-through cell designed[24] was of the confined wall-jet type. The inlet section of the cell contained the Ag/AgCl reference electrode in a separate circular chamber filled with 0.1 M KCl from an external syringe. This chamber contacted the working electrode space by four holes (i.d. 0.3 mm) concentrically surrounding the inlet (i.d. 0.5 mm). The auxiliary electrode was a platinum wire encircling the outlet chamber. The glassy carbon disk electrodes were used as working electrodes. The distance between the nozzle and working electrode was about 2 mm. Pump pulsation damping was provided by a 3 m length Teflon tubing (i.d. 0.5 mm) and a 10 cm length cylindrical capacitor (i.d. 5 mm) placed before the cell.

The flow rates used were 0.7−0.8 mL min^{-1} for H_2O_2 analysis and 0.5 mL min^{-1} for glutamate detection. A dispersion coefficient of about 1.5 was reached by injection of 50 μL of sample.

Electrodeposition of Prussian Blue was done by applying a constant potential of 0.4 V within 60 s. The initial solution contained 2 mM $K_3[Fe(CN)_6]$ and 2 mM $FeCl_3$. The supporting electrolyte was 0.1 M KCl with various amounts of HCl. After deposition the Prussian Blue films were activated in the same supporting electrolyte solution that was used for film growth, by cycling the applied potential in a range of −0.05 0.35 V at a sweep rate of 50 mV s^{-1}. The total amount of deposited Prussian Blue (≈6 nmol cm^{-2}) was estimated from cyclic voltammograms, if a transfer of 4 electrons per unit cell is assumed.[25]

Enzyme containing Nafion membranes were prepared according to our recently reported method[26] from enzyme suspensions in 90% ethanol and 10% water. For this aim the lyophilized enzyme samples were dissolved in water to a final concentration for glutamate oxidase of 15 mg mL^{-1}. Nafion stock solution (5%) was diluted with absolute ethanol five times and neutralized by saturated NaOH in absolute ethanol. The enzyme−polyelectrolyte complex in 90% ethanol was made by further dilution of Nafion solution with absolute ethanol and subsequent mixing with the enzyme water solution. The final enzyme concentration in the complex was 1.2 − 1.3 mg mL^{-1}. The enzyme-polyelectrolyte complex contained different amounts of Nafion: 0.16%, 0.33% and 0.66%.

Enzyme-containing Nafion membranes were prepared by syringing 5 μL of the enzyme−polyelectrolyte complex onto the surface of the Prussian Blue-modified electrode and allowing the solvent to evaporate. In some experiments the casting was repeated, and membranes formed from deposition of two or three layers were prepared. After deposition the enzyme−Nafion membranes were thoroughly washed with water. Different types of glutamate electrodes were made using a slight variation in the immobilization of enzyme. Type I refers to sensing electrodes with a simple membrane made from an enzyme−polyelectrolyte complex with a Nafion content of 0.16%. Type II electrodes were

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made in similar way but from enzyme−polyelectrolyte complex containing 0.33% Nafion. To prepare type III electrodes, a more concentrated Nafion solution (0.66%) in the enzyme−polyelectrolyte complex was taken to cover electrodes prepared according to the type I procedure. The total amount of the immobilized glutamate oxidase was almost equal for type I, II, and III electrodes.

Flow injection glutamate and hydrogen peroxide analysis was carried out in 0.05 M phosphate buffer with 0.1 M KCl at −50 mV (Ag/AgCl/0.1 M KCl).

RESULTS AND DISCUSSION

Flow Injection Hydrogen Peroxide Detection Using Prussian Blue-Modified Electrodes. Prussian Blue-modified electrodes prepared according to the new deposition procedure described in Experimental Section were applied to the flow injection technique. The experiments were carried out in a solution of 0.05 M phosphate buffer (pH 6.0) containing 0.1 M KCl. Compared with our earlier data[12] the developed electrodes show an increased sensitivity toward hydrogen peroxide detection and improved stability. Prussian Blue-modified electrodes were used for flow-injection analysis of H_2O_2 without covering with an additional Nafion membrane used in previous investigations.

A linear dependence of the peak current on H_2O_2 concentration was obtained in a wide range between 0.1 μM and 0.1 mM. The response to 0.1 μM H_2O_2 was approximately 0.06 μA cm^{-2}. The sensitivity determined as the slope of the initial part of the calibration curve was 0.6 A M^{-1}cm^{-2}. The Prussian Blue-modified electrodes showed stable responses to submicromolar concentrations of H_2O_2. The reproducibility of responses of Prussian Blue-modified electrodes to 0.01 mM H_2O_2 during 50 injections exceeded 95% (standard deviation did not exceed 5%).

Flow Injection Glutamate Analysis. The enzyme glutamate oxidase was immobilized on the top of Prussian Blue-modified electrodes according to our nonaqueous enzymology approach[26] as described in the Experimental Section. The developed glutamate biosensors were tested in the flow injection mode. Stable responses with sufficiently high reproducibility were observed after continuous glutamate injections. The time for one analysis took 20−30 s, depending on the glutamate concentration. The response of the glutamate electrode was optimal at pH 6.0−7.0, which is similar for the free enzyme.[27]

Figure 1 illustrates a calibration curve for glutamate obtained with an enzyme electrode prepared from a enzyme−Nafion complex with 0.16% Nafion. The response was linear in the concentration range of 0.1−100 μM. Fitting the calibration curve to the hyperbolic Michaelis-type function, the sensitivity in the linear range obtained was 0.21 A M^{-1} cm^{-2}. This is approximately 1 order of magnitude higher than the value observed for one of the best similar glutamate biosensors in the batch mode.[19] It should be noted that in general the batch mode provides higher sensitivity than the flow injection mode because there is no dispersion of the sample.

It was possible to detect 1×10^{-7} M glutamate in the flow injection system. This is markedly lower than the detection limits (>1 μM) recently obtained for platinum- and peroxidase-based

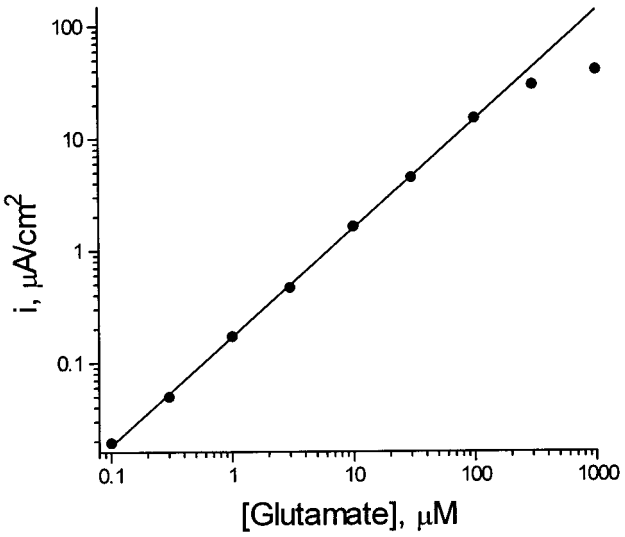

Figure 1. Calibration curve for glutamate electrode in the flow injection system: measuring potential, −50 mV (Ag/AgCl/0.1 M KCl); 0.05 M phosphate buffer, pH 6.0 with 0.1 M KCl; flow rate, 0.5 mL min^{-1}.

biosensors[18−23] and comparable with glutamate biosensors using a substrate amplification principle.[17,28]

The operational stability of the glutamate electrodes was examined for 10^{-5} M glutamate under flow injection as well as under continuous flow conditions. The electrode made from enzyme−polyelectrolyte mixture with 0.16% Nafion (type I) was rather stable in flow injection mode. After 15 injections of 10^{-5} M glutamate, the decrease of the peak did not exceed 5%. The stability of the electrode under continuous flow conditions was remarkably higher. After reaching the maximum, the current did not decrease during the first 10 min. After 30 and 60 min the remaining current was respectively 96% and 92% of its initial value.

The sensitivity and stability of glutamate-sensitive electrodes made from different enzyme−polyelectrolyte mixtures with various Nafion contents (types I−III) were compared. The lowest glutamate levels detected by type II and III electrodes were 0.3 and 1 μM, respectively, indicating that the Nafion membrane acted as a barrier for glutamate. However, the operational stability of type II and III electrodes was higher as compared with the type I electrode. The type II electrode showed less than 1% decrease of the peak after 20 injections of 10^{-5} M glutamate, where as the response of the type III electrode under similar conditions was completely stable. Under continuous flow of 10^{-5} M glutamate, the current of the type III electrode was continuously increased during first 10 min and then became stable for 1 h.

The reproducibility of the fabrication of the enzyme electrodes was tested. For this aim the enzyme immobilization was carried out using the same batch of enzyme−polyelectrolyte mixture having a Nafion content of 0.33%: freshly prepared as well as mixture kept in a refrigerator 1, 2, and 3 days. The responses of the electrodes toward injections of 10^{-5} M glutamate were compared. The experiments indicated only a minor difference in the responses of the glutamate-sensitive electrodes made using the same enzyme−polyelectrolyte mixture after storage. Thus such enzyme suspensions are completely stable and can be used for mass production of enzyme electrodes.

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Table 1. Relative Response of Glutamate Type III Biosensor to Possible Interferents

interferent	relative response
D-glutamate	<2%
D,L-aspartate	<2%
ascorbate	<30%
acetaminophen	none

[a] Ratio of response to 0.01 M interferent: response to 0.01 M L-glutamate.

The biosensors were examined for their storage and operational stability. When not in use, the electrodes were kept in a dry state in a refrigerator (4 °C). No decrease of the initial response of the enzyme electrode to 10^{-5} M of glutamate was observed after 5−7 days of storage.

D-Glutamic acid, D,L-aspartic, acetaminophen, and ascorbic acids were tested as possible interfering compounds (Table 1). The test was carried out in the flow-injection system with the Prussian Blue-based glutamate biosensor as the electrochemical detector at −50 mV (Ag/AgCl/0.1 M KCl). The biosensor was highly selective for L-glutamate. The addition of 0.01 mM D-glutamate or 0.01 mM D,L-aspartate showed minor interference (<2%) on the biosensor response to 0.01 mM L-glutamate.

Similar to our previous results,[12] acetaminophen did not affect the response of Prussian Blue-based biosensors at working potentials below 0 mV(Ag/AgCl/0.1 M KCl). Ascorbate is one of the most significant interferents in clinical analysis, because it is oxidized irreversibly in buffered neutral solutions. For type III electrodes the anodic response to ascorbic acid was less than 30% of the cathodic response to glutamate (Table 1). For comparison, in biosensors operated at higher working potentials, the response to ascorbate is usually 1−2 orders of magnitude higher than the response to analyte. A low-potential glutamate biosensor based on wired peroxidase was recently reported.[22] In the absence of any additional membranes and coimmobilized enzymes, the interference by ascorbate was quite similar to our Prussian Blue-based glutamate biosensor.

CONCLUSION

We conclude that Prussian Blue-based "artificial peroxidase" is an attractive transducer for selective low-potential detection of hydrogen peroxide. The electrocatalyst is stable, highly active, and selective to H_2O_2 reduction in the presence of oxygen, which provides the attractive performance characteristics of the corresponding biological sensors. Developing a model biosensor, we achieved in simple flow injection mode the lowest detectable glutamate concentration of 10^{-7} M, which was 1 order of magnitude lower than for similar systems in which other methods of H_2O_2 detection were used. The sensitivity of Prussian Blue-based biosensor (0.21 A M^{-1} cm^{-2}) was several times higher compared to similar bioanalytical devices.

We therefore conclude that the Prussian Blue-based electrocatalyst is a truly good choice for the development of oxidase-based biosensors. Prussian Blue-modified electrodes are (i) selective electrocatalysts for electrochemical reduction of hydrogen peroxide in the presence of oxygen, which is not a particular property of platinum; (ii) more stable and active than peroxidase modified electrodes; and (iii) much less expensive than both platinum and peroxidase electrodes.

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