



Selectivity enhancement for glutamate with a Nafion/glutamate oxidase biosensor

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Received 28 August 1995; revised 30 November 1995; accepted 7 December 1995

Abstract

Response properties and selectivity are reported for glutamate biosensors constructed with a film of Nafion between the platinum anode and a layer of immobilized glutamate oxidase. The effects of enzyme loading, sample pH and temperature are established. Operation at pH 7.8 and 37°C results in linearity up to 800 μM and a limit of detection of 0.3 μM . Nafion enhances selectivity for glutamate over test species that include ascorbic acid, uric acid and acetaminophen. Selectivity enhancement was greater over the anionic interferences because of electrostatic repulsion and the extent of this enhancement depends on the thickness of the Nafion layer. Even under ideal conditions, some interfering signal is observed when glutamate levels are ten-times less than ascorbate.

Keywords: Glutamate; Nafion/glutamate oxidase biosensor; Selectivity enhancement

1. Introduction

Numerous reports have described work to develop a functioning biosensor for glutamate [1–5]. Typically, glutamate oxidase is immobilized at the surface of an anodic electrode and the enzymatic generation of hydrogen peroxide is monitored amperometrically. The two major applications for such glutamate biosensors are as monitors for bioreactors [6] and as real-time sensors during neurochemical experiments [7]. In both cases, selectivity for glutamate over easily oxidized endogenous species is critical for acceptable accuracy.

Ascorbate represents a major potential interference, especially in neurochemical systems where high and varying levels of ascorbate can be present. Ascorbate interference is caused by direct oxidation at the electrode surface. One strategy to enhance sensor selectivity is to add a layer of Nafion which electrostatically repels anions, such as ascorbate, while freely passing hydrogen peroxide. This strategy has been used effectively with electrochemical sensors for dopamine [8,9] and blood glucose [10,11]. In both cases, Nafion coated electrodes provide sufficient selectivity enhancement for accurate measurement under conditions where the analyte concentration is greater than or equal to that of ascorbate. In some neuro-

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chemical situations, however, glutamate levels can be orders of magnitude lower than ascorbate levels, which places further demands on sensor selectivity.

In this paper, Nafion is evaluated as a means to enhance selectivity of glutamate measurements performed with the glutamate oxidase based biosensor. Selectivity for glutamate over ascorbate is characterized with and without a thin layer of Nafion positioned between the immobilized enzyme and a platinum anode. Although the response to ascorbate is attenuated significantly in the presence of the Nafion layer, this response is not completely eliminated. Even with the Nafion layer, measurement inaccuracies can be substantial depending on the relative concentrations of glutamate and ascorbate. As a result, the utility of this glutamate biosensor in neurochemical experiments will be restricted to situations where the relative amounts of ascorbate and glutamate permit accurate measurements. Besides our characterization of the Nafion membrane, the effects of enzyme loading, pH and temperature are evaluated, and conditions for the optimal glutamate response are identified.

2. Experimental

2.1. Apparatus

Amperometric measurements were made with a Model DCV-5 Voltammetry Controller from Bioanalytical Systems. (West Lafayette, IN, USA) and recorded on a Sargent-Welch Model XKR strip chart recorder. All pH measurements were made with a Ross-type combination pH electrode (Orion Model H4100-12) in conjunction with a Model ϕ 72 meter. Solution temperatures were controlled with VWR model 1140 water bath (Polyscience Corp., Niles, IL, USA).

2.2. Reagents and supplies

Platinum electrodes were disk shaped with a diameter of 1.6 mm (MF-2013). These electrodes were purchased, along with the silver–silver chloride reference electrodes (MF-2020), from

Bioanalytical Systems. Glutamate oxidase (8 U mg^{-1}) was generously donated from Hitoshi Kusakabe from Yamasa Shoyu Co. Ltd. (Choshi, Chiba 288, Japan). Nafion was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) as a 5% (w/w) suspension of the perfluorinated ion-exchange powder in a mixture of low molecular weight aliphatic alcohols and 10% water. L-Glutamic acid (monosodium salt, 99–100%), bovine serum albumin (BSA, 98–99%) and glutaraldehyde (Grade II, 25% aqueous solution) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Concentrated hydrogen peroxide (30%) was purchased from Fisher (Chicago, IL, USA) and was standardized periodically by titration with sodium thiosulfate. All other chemicals were obtained from common suppliers as reagent grade materials.

All solutions were prepared with distilled–deionized water by passing the house distilled water through a three-house Milli-Q water purification unit. The working buffer had a pH of 7.4 and contained the following salt concentrations: 50 mM NaCl, 10 mM KH_2PO_4 and 40 mM Na_2HPO_4 . Solutions of L-glutamate were prepared by dissolving L-glutamic acid (dried at 104°C for 2 h) in working buffer.

2.3. Procedures

2.3.1. Electrode preparation

Platinum electrodes were polished sequentially with 3, 1 and $0.3 \mu\text{m}$ aluminum oxide lapping films and washed in a sequence of acetone, distilled–deionized water, nitric acid (1:1) and distilled–deionized water in an ultrasonic cleaner. Polished electrodes were electrochemically pretreated in 0.5 M sulfuric acid by cycling the applied voltage between -0.4 to 1.3 V versus Ag/AgCl at a scan rate of 50 mV s^{-1} for 10 min.

Nafion layers were constructed by placing a $10 \mu\text{L}$ aliquot of a 1% Nafion suspension on the surface of a treated platinum electrode. Films were formed by allowing the solvent to evaporate at room temperature for 20 min. Different film thicknesses were generated by either using a different concentration of Nafion or applying a different volume.

Glutamate oxidase was immobilized by crosslinking with BSA and glutaraldehyde. First the platinum electrode was polished, washed and electrochemically pretreated. A Nafion film was prepared by placing one 10 μL aliquot and, subsequently, four individual 3 μL aliquots of 1% Nafion on the surface of the platinum electrode. In each case, the previous layer was allowed to dry before the next aliquot was administered. An enzyme solution was prepared by dissolving 0.5 mg of glutamate oxidase in 5 μL phosphate buffer (pH 6.86). This glutamate oxidase solution was mixed well with 2.5 μL of 10% (w/w) BSA and 1.25 μL of 2.5% glutaraldehyde. A 2 μL aliquot of this final mixture was placed on the Nafion film. Approximately 0.91 units of glutamate oxidase were immobilized on a surface area of 0.2 mm^2 . After drying at room temperature for 30 min, the electrode was washed thoroughly with phosphate buffer to remove any unretained enzyme. Different enzyme loadings were obtained by adjusting the concentration of the glutamate oxidase stock solution.

2.3.2. Sensor response measurements

Sensor responses were measured by recording anodic currents as a function of time. Unless stated otherwise, the working electrode potential was maintained at 0.65 V versus a Ag/AgCl reference electrode. Data for the response curves were collected by first immersing the sensing tip in a blank solution of working buffer to determine the background current. Microliter additions of a glutamate standard were then added sequentially and the corresponding steady-state currents were noted. In most cases, the sample solution was stirred with a magnetic stir bar. Stirring was stopped, however, to reduce noise when solutions with low glutamate concentrations were measured. Response times were measured as the time required to achieve 95% of the final steady-state response after a step change in glutamate concentration.

3. Results and discussion

Response properties for the glutamate biosen-

sor have been measured as a function of enzyme loading, solution pH and operating temperature. In addition, the effect of Nafion on electrode selectivity has been established with particular interest in the effect of Nafion layer thickness.

3.1. Operational parameters

The amount of glutamate oxidase required to saturate the sensing tip was determined by comparing the response curves obtained from a series of sensors constructed with different amounts of immobilized glutamate oxidase. The results are presented in Fig. 1. As the amount of immobilized glutamate oxidase approaches 0.91 units, the sensor response becomes independent of the amount of enzyme. Below this saturation point, however, the response is strongly affected by the amount of glutamate oxidase. The slope of the calibration curve increases with increasing glutamate oxidase concentrations, until a saturating amount is reached. Unless noted otherwise 0.91 units of glutamate oxidase were used for the construction of all subsequent sensors.

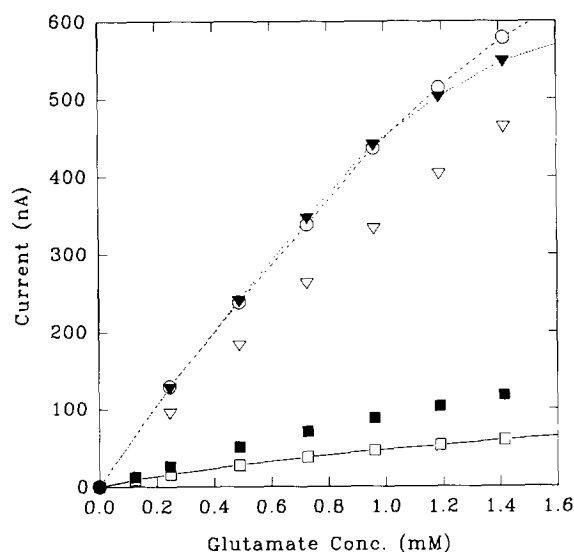


Fig. 1. Sensor response curves with enzyme loadings (U per membrane) of 0.001 (open squares); 0.01 (closed squares); 0.23 (open triangles); 0.91 (closed triangles); and 1.37 (circles).

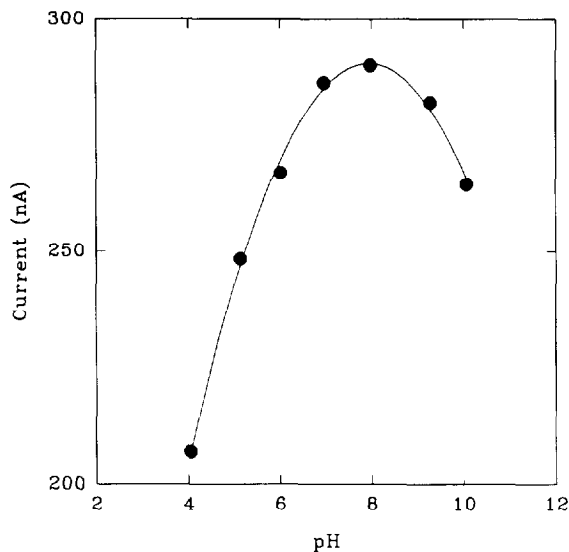


Fig. 2. pH profile for response to glutamate.

The magnitude of the sensor response depends strongly on solution pH. The sensor response was monitored at different solution pH values for a step in glutamate concentration from zero to 0.7844 mM at room temperature. Fig. 2 shows how the response depends on solution pH. The maximum response is obtained at pH 8.0 and over 95% of this maximum response is obtained over the pH range from 6.5 to 9.5. This pH profile is similar to that reported for the free enzyme [12] where the optimum pH is 7–8.

Temperature also strongly influences the sensor response as expected because of the well known effects of temperature on reaction kinetics, enzyme activity and mass transport [13]. The effect of temperature was evaluated by measuring the sensor response to 0.37 mM glutamate over a temperature range from 5.6 to 63.0°C. Enzyme loading was reduced in this experiment to 0.23 U at the electrode surface in order to amplify the temperature effects. The magnitude of the response increases dramatically as the temperature increases from 5.6 to 48°C. The temperature coefficient over this range is approximately 8.5 nA °C⁻¹. A maximum response is observed at 48°C and the response drops at a rate of 2.8 nA °C⁻¹ as the temperature increases beyond 48°C. Glutamate oxidase is a thermally stable enzyme [14]

which accounts for large responses at temperatures above 40°C. Thermal degradation of the enzyme does become a factor, however, above 48°C.

Ideal sensor response properties are obtained with at least 0.91 units of immobilized enzyme operating at pH 8.0 and 48°C. More importantly, these results demonstrate that the sensor can operate well over a wide range of pH and temperature values. Finally, the influences of pH and temperature are sufficiently strong that precautions to control these parameters are required for accurate measurements.

3.2. Nafion and selectivity

As expected, easily oxidized species, such as ascorbic acid, uric acid and acetaminophen, are positive interferences when biosensors are prepared without Nafion. The magnitude of this type of interference is illustrated by comparing the sensor responses in solutions containing only 0.369 mM glutamate and those containing 0.369 mM glutamate plus 0.369 mM of the interfering compound. Direct oxidation of the added compound increases the measured current which results in systematic errors corresponding to apparent glutamate levels of 191.9, 186.5 and 197.0%, the actual values in the presence of ascorbic acid, uric acid and acetaminophen, respectively.

Such interfering responses are reduced in the presence of Nafion and the extent of this reduction depends on the thickness of the Nafion layer.

Table 1
Ascorbate oxidation with different Nafion layer thicknesses

Thickness (μm)	Electrode response (μA) ^a	
	$E_{app} = 0.4V^b$	$E_{app} = 0.6V^b$
0	0.98 (100%)	1.08 (100%)
2.2	0.12 (12%)	0.48 (44%)
4.5	0.085 (8.7%)	0.22 (20%)
6.7	0.021 (2.1%)	0.06 (5.6%)
9.0	0.012 (1.2%)	0.03 (2.8%)
11	0.004 (0.4%)	0.014 (1.3%)

^aPer cent response with no Nafion given in parenthesis.

^bMeasured relative to Ag/AgCl.

Table 1 summarizes steady-state currents measured for 1.0 mM ascorbate solutions with different Nafion layer thicknesses for two applied potentials (0.4 and 0.6 V versus Ag/AgCl). At both potentials, the response drops significantly as the Nafion layer thickness grows from 0 to 11 μm . At 11 μm , the relative response decreased to 0.4 and 1.3% of the original values at applied potentials of 0.4 and 0.6 V, respectively.

Nafion also reduces the electrode response to hydrogen peroxide. The Nafion layer adds an additional diffusion barrier which lowers the flux of hydrogen peroxide to the electrode surface, thereby lowering the monitored current. Of course the extent of signal reduction is greater for ascorbate and the other anionic species, because both electrostatic repulsion and diffusion barrier effects are combined to reduce responses from these interferences. Relative to no Nafion, a 6.7 μm layer of Nafion results in a 35% reduction in the steady-state current for hydrogen peroxide. Under the same conditions, nearly 95% of the response to ascorbate is removed. Without Nafion, currents for ascorbate are approximately 41% of those for the same concentration of hydrogen peroxide. This percentage drops to less than 3% when the Nafion layer thickness reaches 6.7 μm . No further reduction in the relative response to ascorbate was obtained with thicker layers. It is important to stress that some response was observed for ascorbate at all Nafion layer thicknesses which suggests absolute selectivity for hydrogen peroxide is not possible with this approach.

By altering the response to hydrogen peroxide, the Nafion layer also affects the response to glutamate. Without Nafion, the mean response ($\pm 95\%$ confidence interval) for 10 consecutive measurements of 0.5 mM glutamate was 446 (± 10) nA and the corresponding response time was 7 (± 1.5) s. With a 5 μm thick Nafion film, the mean current and response time are 195 (± 10) nA and 14 (± 1) s, respectively, for seven consecutive measurements at the same glutamate concentration. Nafion causes a 2.3-fold decrease in magnitude of response and 2-fold increase in response time. Still, linear calibration curves are achieved with Nafion. Example curves are presented in Fig. 3 over wide and narrow ranges of glutamate

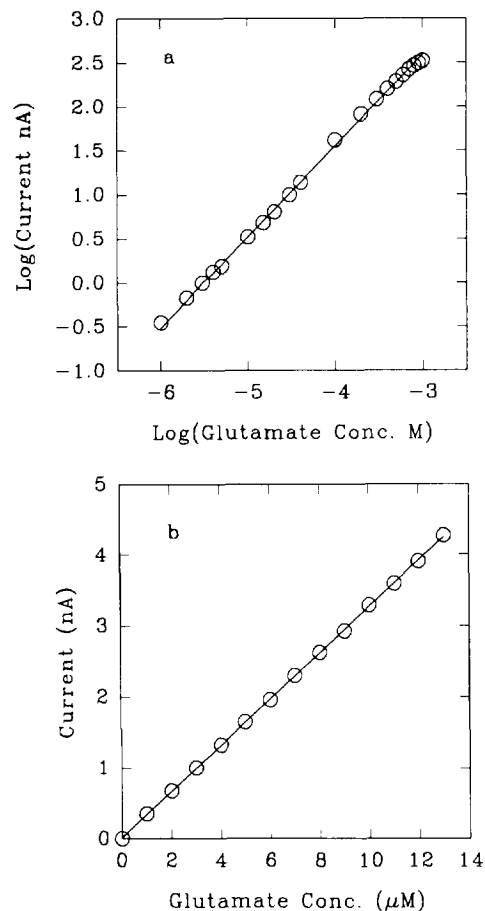


Fig. 3. Calibration lines for glutamate showing (a) log–log plot for a wide dynamic range and (b) normal plot for the micromolar concentration range.

concentrations. Fig. 3a illustrates linearity over a wide range of glutamate levels. Regression analysis of these data reveal a slope of 1.022 (± 0.008), a y -intercept of 5.64 (± 0.03) and a correlation coefficient of 99.90% for this log–log plot. Fig. 3b presents a calibration line for 1 to 13 μM glutamate. Regression analysis indicates a slope of 0.3253 (± 0.0009) nA μM^{-1} , a y -intercept of 0.018 (± 0.013) nA and a correlation coefficient of 99.99%. The calculated limit of detection ($S/N = 3$) from Fig. 3b is 0.3 μM and the response times are approximately 14 s at all concentrations. Data for both curves were collected at 37°C.

Selectivity for glutamate over anionic species is enhanced with the Nafion layer. No differences in sensor response were observed when the electrode was exposed to solutions of 0.369 mM glutamate and 0.369 mM glutamate plus 0.369 mM ascorbate or urate. This lack of response is strikingly different than that noted above in the absence of the Nafion film. Some effect of ascorbate was observed, however, when the ascorbate concentration was 10 times greater than glutamate. In this experiment, the sensor tip was immersed in an air saturated solution composed of 0.369 mM glutamate and no ascorbate. After the steady-state current was noted, solid ascorbic acid was added to give a final ascorbate concentration of 3 mM. The average relative difference in the response caused by the addition of ascorbic acid was 2.08% for six repeated trials. Ascorbate was added in this way to minimize the production of hydrogen peroxide via anaerobic oxidation [15]. The Nafion layer was much less effective in reducing interference from acetaminophen. The electrode response increased by 29.9% when comparing the responses to solutions with 0.369 mM glutamate alone and 0.369 mM glutamate plus 0.369 mM acetaminophen. Although the interference by acetaminophen is sizable with the Nafion layer, the magnitude of interference is reduced considerably compared to sensors without Nafion (see above for specific values). Enhancement in selectivity over the neutral acetaminophen is presumably caused by differential diffusion barrier effects by Nafion between acetaminophen and hydrogen peroxide.

4. Conclusion

Selectivity for glutamate can be enhanced by incorporating a thin layer of Nafion between the electrode surface and the immobilized layer of glutamate oxidase. The extent of selectivity enhancement depends on the thickness of the Nafion film. Even with the maximum thickness, however, response to interferences cannot be completely eliminated. Inaccurate measurements can result from oxidizable anionic species, such as ascorbate, when the glutamate concentration is ten-

times less than the interfering species. As a result, this electrode configuration does not provide the selectivity needed for many neurochemical experiments where ascorbate levels are known to be two orders of magnitude greater than glutamate. Under such demanding conditions, alternative selectivity enhancement strategies, such as Wilson's dual enzyme system [7] or our hydrogen peroxide gas-sensing scheme [15], are needed to ensure accuracy. Nevertheless, the Nafion/glutamate oxidase system described here offers the key advantages of being easy to construct and simple to operate, which makes it a viable alternative when the selectivity demands permit.

Acknowledgements

This work was financially supported by a grant from the National Science Foundation (BNS-8716768).

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