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Capillary Biosensor for Glutamate

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We have developed and characterized a novel glutamate biosensor which allows biological transduction of glutamate signal during transport of analyte from the sampling site to the detector. This biosensor exploits the high surface-to-volume ratio found in small-diameter fused silica capillaries. Glutamate dehydrogenase (GDH) was attached to the inner surface of a 75 μm i.d. capillary using biotin−avidin chemistry. In the presence of excess nicotinamide adenine dinucleotide (NAD^+), GDH converts glutamate to α-ketoglutarate while simultaneously reducing NAD^+ to NADH. Detection of NADH was accomplished using laser-induced fluorescence. Perfusion with 30 μM glutamate in the presence of 3 mM NAD^+ resulted in a strong increase in fluorescence, with a response time of 450 ms. This effect was abolished upon exclusion of NAD^+ from the buffer. The limit of detection is 3 μM (S/N = 3), with a linear working range from 3 to 300 μM. Efficiency of the GDH-modified capillary ranged between 20% and 92% and was positively correlated with concentration of glutamate. The effect of linear velocity was also examined and was shown to be indirectly related to efficiency, with maximum response observed at 4.5 cm/min. In summary, we have demonstrated the successful attachment of glutamate dehydrogenase to the inner wall of a small-diameter fused silica capillary while retaining enzymatic activity. The resulting biosensor exhibits characteristics amenable for in vivo applications. Future efforts will be directed toward the incorporation of this biosensor into current technologies, such as capillary electrophoresis and microdialysis.

Changes in extracellular glutamate concentration have generated considerable interest in the study of ischemia-induced excitotoxicity.[1] A substantial proportion of neuronal damage is the result of events occurring within the first 5 min following the ischemic event.[2] To develop better cerebroprotective strategies, it is therefore of interest to determine the precise temporal relationship between glutamate release and neuronal destruction. Presently available analytical tools do not possess the resolution with respect to time or the sensitivity which is necessary to make such measurements. This work is directed toward the development of a novel analytical biosensor for glutamate which has the capability to make measurements in "real-time", the selectivity necessary for in vivo analyses, and the sensitivity necessary to monitor physiologically relevant concentrations.

A primary difficulty in the analysis of glutamate arises from the simple nature of the molecule, which possesses few functional groups that can be exploited using conventional means of detection. For this reason, many approaches make use of chemical derivatization procedures to convert glutamate to a detectable product.[3,4] Difficulties arise in derivatization of small volumes, however, and current research efforts in this area involve the development of on-line derivatization schemes[5,6] which will reduce sample handling and dilution.

An alternative approach to chemical derivatization procedures is the use of an enzyme to produce a detectable analyte upon conversion of the substrate. Enzyme-based biosensors have generated considerable interest in recent years.[7,8] Glutamate biosensors have been fabricated using glutamate oxidase[8−10] and glutamate dehydrogenase.[11,12] Glutamate dehydrogenase (GDH), a 330 kDa hexamer,[13] is a particularly attractive candidate for biological transduction systems, given that it is fast acting (V_{max} = 3.3 mol of NADH s^{-1} mg of GDH) and has a high affinity for glutamate ($K_m = 2.2 \times 10^{-5}$ mol). Furthermore, GDH requires the cofactor nicotinamide adenine dinucleotide (NAD^+), which is reduced to NADH upon conversion of glutamate to α-ketoglutarate. Detection of NADH may be accomplished using a variety of techniques (electrochemistry, UV absorbance, and fluorescence detection), lending great flexibility to instrumental design.

Biosensors utilizing glutamate-specific enzymes have been reported in a variety of formats; however, a majority fall into two categories. Electrode-based designs comprise the first category and involve either the incorporation of enzyme-containing biocatalytic membranes attached to the electrode surface,[10,14] immobilization of enzyme in polymers using flow-through electrodes,[15,16] or direct derivatization of the electrode surface.[12,17,18] A second category of biosensors is based on flow injection analysis. Glutamate and lactate biosensors using a flow injection

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format have been reported that use enzyme-modified packing material[8,9] or inclusion of enzyme in perfusion buffer.[19]

To reduce dilution problems associated with many derivatization procedures, it is of interest to physically separate the enzyme from the reaction mixture. Biotin−avidin tethering systems have shown great utility in this regard by serving as a means to immobilize enzymes to surfaces. By using the *N*-hydroxysuccinimide ester of biotin (NHS-biotin), it is possible to exploit primary amines, such as those found on exposed lysine residues, for derivatization of the enzyme with a biotin molecule.[20] This scheme has been used successfully for the immobilization of GDH to carbon fiber electrodes[12,21,22] while retaining enzymatic activity. Attachment chemistries have also been developed for immobilization of biotin to silica surfaces,[23] using (aminopropyl)triethoxysilane (APTES) to form a primary amine on the surface of the capillary.[24−26] The primary amine may then undergo nucleophilic substitution with NHS-biotin in the same manner described previously for exposed lysine residues on the enzyme. This procedure has been used successfully to attach trypsin to the inner surface of a fused silica capillary while retaining enzymatic activity.[23,27,28]

The aim of this research was to develop a novel glutamate biosensor by directly attaching of GDH to the wall of a 75 μm i.d. silica capillary. This is the first report of an enzyme biosensor based on this format. The novel design exploits the high surface-to-volume ratio attainable with small-diameter fused silica capillaries, thus maximizing the interaction between glutamate and GDH. This paper describes the development and characterization of the glutamate biosensor with respect to response time, sensitivity, optimization of linear velocity, and efficiency. The demonstrated improvements over previous designs, particularly in terms of increased limits of detection and decreased response time, provide support for the application of this biosensor to the study of neurochemical questions.

EXPERIMENTAL SECTION

Materials. Fused silica capillary (150 μm o.d., 75 μm, i.d) was obtained from Polymicro Technologies (Phoenix, AZ). Dialysis fiber (6.4 mm o.d.; MW cutoff, 12 000−14 000) was purchased from Spectra/Por (Houston, TX). Bovine liver L-glutamate dehydrogenase was purchased as an ammonium sulfate suspension from Sigma Chemical Co. (St. Louis, MO). Also purchased from Sigma were biotinamidocaproic acid 3-sulfo-*N*-hydroxysuccinimide ester (NHS-LC-biotin), β-nicotinamide adenine dinucleotide, reduced form (NADH), and L-lysine. ExtrAvidin was obtained from Sigma Immuno Chemicals (St. Louis, MO). β-Nicotinamide adenine dinucleotide (NAD^+) was supplied by Fluka (Buchs, Switzerland), and 3-(aminopropyl)triethoxysilane (APTES) was from Aldrich (Milwaukee, WI). Reagent grade sodium hydroxide (NaOH), acetone, and methanol (MeOH) were used as received from Fisher (Fair Lawn, NJ). All other chemicals were obtained from Fisher.

Phosphate buffer consisted of 50 mM sodium phosphate and 150 mM sodium chloride (pH 8.5). For glutamate experiments, 3 mM NAD^+ was added to the buffer. Glutamate and NADH standards were constructed by serial dilution of a 30 mM stock solution in the appropriate run buffer. All solutions were prepared fresh daily and were passed through a 0.2 μm filter prior to use.

Methods. (i) Biotinylation of Glutamate Dehydrogenase. NHS-LC-biotin (3.6 μmol) was added to a solution of 7.7 μM GDH (corresponding to 2.3 mg of protein), 4.5 μM NAD^+, and 9 μM glutamate in 100 mM phosphate buffer (pH 8.5), and the mixture was allowed to incubate at room temperature for a period of 1 h. The biotinylation reaction was quenched through the addition of lysine in high concentration, and the resulting mixture was dialyzed in phosphate pH 8.5 buffer at 4 °C for a period of 18 h, over which time buffer was refreshed twice. Dialysate was centrifuged to remove particulate matter, and the enzymatic activity of the resulting suspension was measured using a UV−vis spectrommeter. Suspensions with initial rate measures less than 40 μmol of NADH s^{-1} mg^{-1} of protein were not used for capillary derivatization procedures. Protein determination of the resulting superfusate by biuret assay demonstrated negligible loss of protein from the biotinylation procedure (2.4 mg/mL, not significant).

(ii) Capillary Derivatization. An 80 cm length of bare fused silica capillary was cleaned by sequential perfusion with 1.0 mL volumes of 0.1 N NaOH, distilled water (dH_2O), MeOH, dH_2O, and finally acetone. The capillary was treated with APTES (2% in dry acetone) and was allowed to incubate at 45 °C for a minimum of 12 h to allow the formation of the aminopropyl derivative of glass. Further information concerning this attachment procedure has been published elsewhere.[24,25] Following return to room temperature and a rinse with 2 mL of 50 mM carbonate buffer (pH 8.3), the capillary was subsequently treated with 40 μL of NHS-LC-biotin (5 mg/mL in carbonate pH 8.3). This volume is sufficient to derivatize the proximal end of the capillary so that GDH would not be present in the detector window. Following an incubation period of 20 min at 4 °C, the capillary was returned to room temperature and rinsed in the reverse direction with 100 mM phosphate buffer (pH 8.5). The capillary was then treated with 40 μL of ExtrAvidin (0.1 mg/mL in phosphate pH 8.5 buffer) and incubated for 1 h at room temperature. It was then rinsed in the reverse direction with dH_2O and phosphate pH 8.5 buffer and treated with 40 μL of the biotinylated GDH suspension. Following overnight refrigeration, the capillary was rinsed in the reverse direction with 1 mL of phosphate pH 8.5 buffer to remove non-immobilized enzyme and was inserted into a capillary holder constructed in-house for attachment to a microscope stage (Ernst Leitz Wetzler, Germany).

(iii) Instrumental Setup. A schematic of the instrumental setup is shown in Figure 1. Briefly, a window was burned in the polyimide coating of the biosensor distal to the derivatized area through brief exposure to flame. Flow was accomplished either

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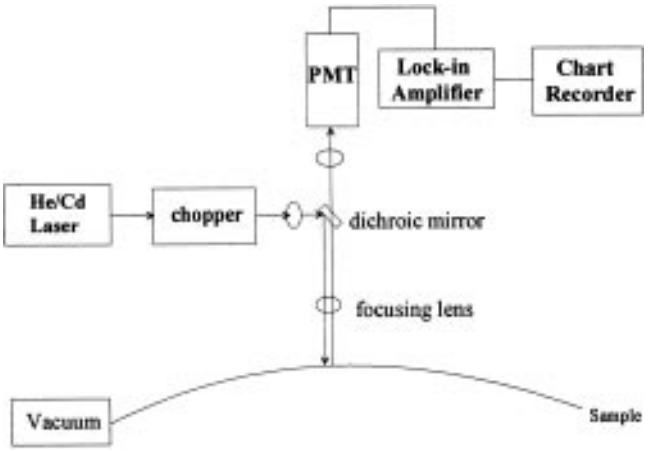

Figure 1. Instrumental schematic for glutamate biosensor capillary. Glutamate dehydrogenase is attached to the proximal end of the capillary. Detection is accomplished using the 325 nm line of a He/Cd laser, focused on the inner surface of the biosensor distal to the derivatization area. Signal is monitored using a photomultiplier tube coupled to a mechanical chopper and lock-in amplifier for signal-to-noise enhancement.

by positive pressure supplied by a perfusion syringe pump (KD Scientific, Boston, MA) attached to the derivatized end of the biosensor or by negative pressure from a vacuum (100 mmHg) applied to the distal portion of the biosensor. For glutamate experiments, the biosensor was perfused continuously with phosphate buffer containing 3 mM NAD^+ (pH 8.5). During NADH measurements, the biosensor was perfused with phosphate buffer devoid of NAD^+. Sample introduction was accomplished either by rapid switching of inlet vial (vacuum mode) or by exchanging contents of perfusion syringe (pump mode).

Detection was accomplished through excitation of the sample with the 325 nm line from a He/Cd laser (Omnichrome, Chino, CA), which was modulated at a rate of 3500 Hz using a mechanical chopper (Oriel, Stratford, CT). The excitation beam was filtered using a UG-11 coated low-pass filter (Barr Associates, Westford, MA) and dichroic mirrors (45°, reflects 325 nm, transmits 442 nm; CVI Laser Corp., Albuquerque, NM) and was focused to an ~50 μm spot on the interior surface of the fused silica capillary using a quartz microscope collimator (Carl Zeiss, Thornwood, NY). Final detection volume was ~15 pL. Fluorescence was passed through a 380 nm high-pass filter (Oriel) and was collected in an epilluminescence configuration. Signal was transduced to current using a photomultiplier tube (Hamamatsu, Hamamatsu City, Japan) and was amplified using a lock-in amplifier (Stanford Research Systems, Sunnyvale, CA). The phase angle was experimentally determined to be optimal for NADH detection using our instrumental configuration.

Experimental Procedures. (i) Frontal Analysis Experiment. Frontal analysis of glutamate was conducted by continuously perfusing the biosensor with buffer containing 3 mM NAD^+ through application of a vacuum. Change in fluorescence upon introduction of 30 μM glutamate to the biosensor was monitored using a strip chart recorder. A control experiment was conducted by repeating the above experiment, with the exception that the buffer used was devoid of NAD^+.

(ii) Standard Curve/Efficiency Measurements. A standard curve was constructed to determine the limits of detection and the linear working range of the biosensor. The biosensor was perfused continuously at a rate of 4.5 cm/min using a precision syringe pump with phosphate buffer containing 15 mM NAD^+. Following the establishment of a stable baseline, the perfusion fluid was switched to one containing either 3 μM, 30 μM, 300 μM, 3 mM, or 30 mM glutamate, and the increase in fluorescence was measured using a strip chart recorder. The process was repeated until all standards had been perfused through the biosensor.

In the same manner, a standard curve was constructed for NADH in phosphate buffer (no NAD^+) using concentrations identical to those used in the glutamate experiment. Efficiency of the enzymatic conversion process was estimated as the ratio between the signal observed for glutamate and that observed for NADH at equivalent concentrations.

(iii) Linear Velocity Experiment. The effect of changing the linear velocity of the perfusion buffer on the efficiency of glutamate conversion was measured in pump mode by perfusing the biosensor at either 4.5, 18.1, 90, 188, or 379 cm/min with phosphate buffer containing 3 mM NAD^+. The response of the biosensor to injection of 300 μM glutamate was monitored using a strip chart recorder.

(iv) Characterization of Derivatized Area. To characterize the length of capillary to which GDH was attached, efficiency measurements were taken in the manner described above for several different lengths of the derivatized capillary. Following an initial measurement of enzymatic efficiency upon perfusion with 30 μM glutamate by application of a vacuum, a 1 cm length of capillary was removed from the derivatized end. This procedure was repeated until no signal was obtained upon perfusion of the biosensor with glutamate.

RESULTS AND DISCUSSION

Frontal Analysis. Upon perfusion with 30 μM glutamate, fluorescence intensity increased to a maximum (S/N > 20), with a response time of 450 ms (Figure 2A). Perfusion of the biosensor with glutamate in a buffer devoid of NAD^+ resulted in no change in fluorescence intensity above baseline, indicating that the observed response was due to enzymatically generated NADH (Figure 2B). The response time for this biosensor to glutamate is well within the acceptable range for in vivo neurochemical analyses. Previous reports on the development of flow injection analysis-based glutamate biosensors have demonstrated continual improvement in response time from 7.5 min[9] to less than 2.5 min.[8] Electrode-based designs have had considerably more success with achievement of subsecond response times;[12] however, sensitivity issues arise, presumably stemming from enzyme modification procedures.[22]

The response time of this biosensor is sufficient for application to the study of ischemia; however, further improvements are likely. Comparison of response time between glutamate injection and NADH injection demonstrated no difference between rise time of enzymatically generated NADH from exogenously applied NADH (450 ms vs 500 ms, not shown). This indicates that the main limitation to faster response times may be the use of a laminar flow profile imposed by application of positive or negative pressure for sample introduction. Use of smaller diameter capillaries, better injection techniques, or electroosmotic flow, which exhibits a planar flow profile, may provide significant improvements in the response time of the capillary biosensor.

Efficiency. Efficiency of conversion was measured as the ratio between enzymatically generated NADH and an equivalent

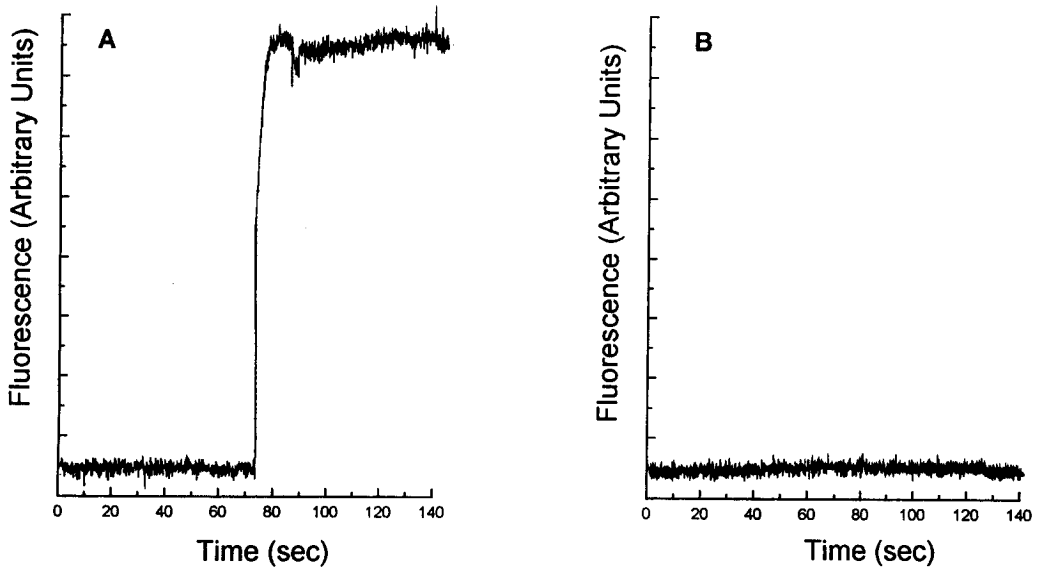

Figure 2. (A) Response time of glutamate dehydrogenase-modified capillary (75 μm i.d., 150 μm o.d., 80 cm total length) to 30 μM glutamate in phosphate buffer containing 3 mM NAD^+. The distal end of the biosensor was attached to a vacuum operating at 100 mmHg, and sample was introduced by rapidly switching the proximal end from a vial containing buffer to one containing glutamate. Time of response was ~450 ms (bar = 5 s). (B) Perfusion of the biosensor with 30 μM glutamate in phosphate buffer devoid of NAD^+ resulted in no change in fluorescence intensity.

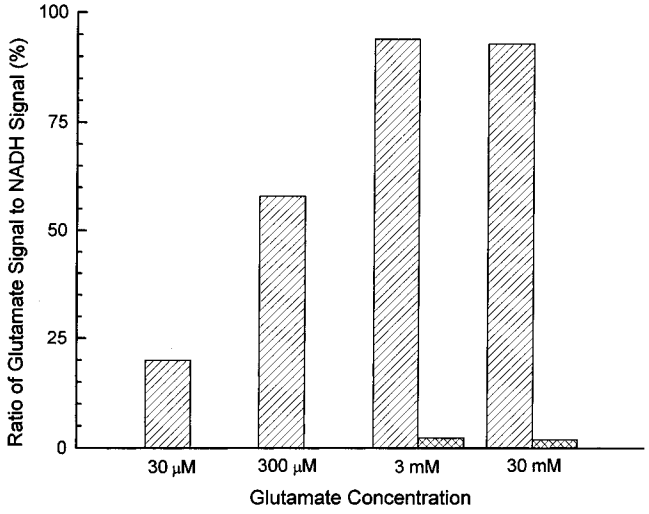

Figure 3. Efficiency of glutamate dehydrogenase in GDH-modified capillary on the first and second day of operation (75 μm i.d., 150 μm o.d., 80 cm length). The derivatized capillary was perfused continuously with phosphate buffer containing 3 mM NAD^+ at a rate of 4.5 cm/min, and the fluorescence signal obtained from perfusion with either glutamate or NADH was monitored. The ratio of fluorescence signal obtained from enzymatically generated NADH to that of native NADH is plotted for four tested concentrations. Efficiency of glutamate conversion on day 1 was 92% at 30 mM glutamate and was 93.5% at 3 mM glutamate. Efficiency decreased to 58% at 300 μM and to 20% at 30 μM. On day 2, efficiency of glutamate conversion dropped to 2% for 3 and 30 mM concentrations. At concentrations under 3 mM, signal was below the limits of detection.

concentration of native NADH injected into the biosensor. Efficiency ranged between 20% and 92% according to concentration of glutamate (Figure 3). Michaelis−Menton kinetics predict that enzyme velocity will decrease at substrate concentrations below the K_m of the enzyme. While we have not measured this parameter on the immobilized GDH, solution measurements of the biotinylated form gave a value for K_m of 2.2×10^{-5} M. Any inactivation of the enzyme as a result of the attachment procedure would serve to increase this value. Thus, the observed profile of efficiency is consistent with our expectations based on enzyme kinetics.

It is likely that enzymatic efficiency in the lower concentration range may be increased through optimization of experimental parameters. The volume of a 1 cm segment of a 75 μm i.d. capillary is 44 nL. When perfusing the capillary at 4.5 cm/min (0.2 μL/min), this allows ~2.2 min for interaction between enzyme and substrate to occur. Increases in the length of derivatized area or decreases in the diameter of the capillary should further optimize this interaction.

Sensitivity. Limits of detection and working range were determined by constructing a standard curve for glutamate ranging in concentration from 3 μM to 30 mM (Figure 4). The limits of detection, based on a signal-to-noise ratio of 3, were determined to be 3 μM. Extracellular glutamate concentration in the rat hippocampus has been estimated from microdialysis experiments to be ~20 μM.[29] Thus, our detection limits are comfortably within the appropriate range for in vivo applications. However, optimization of laser optics and focusing is likely to result in further improvements with respect to sensitivity.

Response of the capillary microreactor to glutamate in the presence of 15 mM NAD^+ was linear from 3 to 300 μM ($r = 0.998$, $p < 0.005$). However, above 300 μM, a roll-off in fluorescence intensity was observed. Michaelis−Menten kinetics measurements of our enzymatic suspension yielded values for V_{max} of 3.3 mol of NADH s^{-1} mg^{-1} of GDH prior to biotinylation and 5.1×10^{-3} mol of NADH s^{-1} mg^{-1} of GDH subsequent to biotinylation. Decreases in V_{max} upon chemical derivatization of glutamate dehydrogenase have been reported previously[11] and may be indicative of "overderivatization" of the enzyme. Should this be the case, derivatization of GDH with more dilute biotin solutions should alleviate the effect and increase the dynamic range of this sensor. Nonetheless, the roll-off

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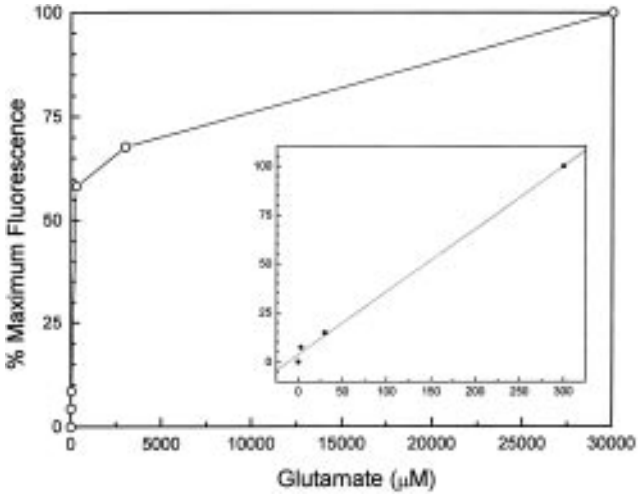

Figure 4. Standard curve of glutamate. The GDH-modified capillary (75 μm i.d., 150 μm o.d., 80 cm length) was perfused continuously at a rate of 4.5 cm/min with phosphate buffer containing 3 mM NAD^+, and the fluorescence response to injection of various concentrations of glutamate was monitored. The linear working range of the biosensor is from 3 to 300 μM glutamate ($y = 7.7x + 0.6$; $r = 0.998$, $p < 0.005$). The limit of detection for glutamate is 3 μM (S/N = 3).

observed at higher glutamate concentrations is consistent with our knowledge of the kinetics of the enzyme.

The linear working range of the biosensor may also be manipulated through control of the length of derivatized area. However, given the mild fluorescence of the enzyme, it is necessary to limit the area of derivatization such that the detector window remains clear of GDH. For this reason, derivatization procedures were designed to constrain enzyme attachment to the initial segment of the capillary. The actual length of derivatization in the capillary was experimentally characterized by monitoring the change in efficiency during removal of 1 cm lengths from the capillary entrance. Removal of 1 cm lengths yielded a linear decrease in efficiency until 11 cm had been removed ($r = 0.996$, $p < 0.0001$). Beyond this point, enzymatic activity was slight (efficiency < 4%). Further truncation of the biosensor resulted in gradual decreases of activity until 30 cm had been removed. At this point, production of enzymatically generated NADH was completely abolished. The profile of change in fluorescence intensity in this region was gradual and was consistent with sporadic attachment of enzyme. Improvements in detection limits may be possible by increasing the length of capillary derivatization to 40 or 50 cm while still avoiding enzyme attachment in the detector window ($l = 65$ cm). Further improvements may also be obtained through the use of smaller diameter capillaries while controlling for linear velocity. Both of these modifications should result in increased interaction between glutamate and GDH so that efficiency, and thus detection limits, are optimized.

Linear Velocity. The efficiency of enzymatic conversion was also measured as a function of linear velocity. The effect of linear velocity on fluorescence intensity is shown in Figure 5. Fluorescence intensity was virtually constant at perfusion linear velocities above 90 cm/min. However, below 90 cm/min, it was inversely proportional to linear velocity, with a maximum signal observed at 4.5 cm/min. This may be explained as due to the increased time for interaction allowed at lower linear velocities. At the maximum linear velocity tested (379 cm/min), analyte occupied the derivatized region of the biosensor for ~1.6 s, while at 4.5 cm/min, the analyte spent about 2.2 min in this region.

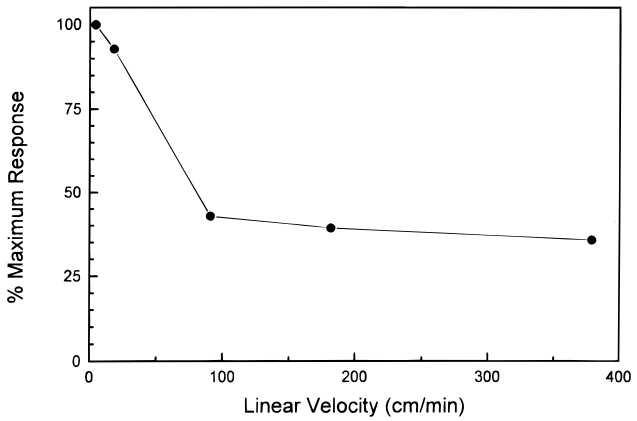

Figure 5. Effect of linear velocity of perfusion buffer on fluorescence intensity. A GDH-modified capillary (75 μm i.d., 150 μm o.d., 80 cm total length) was perfused continuously with phosphate buffer containing 3 mM NAD^+, and the response to injection of 300 μM glutamate was monitored at linear velocities ranging between 4.5 (0.2 μL/min) and 375 cm/min (16.5 μL/min).

The optimal use of lower linear velocities supports the incorporation of this biosensor into existing technologies, such as microdialysis and capillary electrophoresis. Use of low flow rates has been reported to provide optimal recovery of analyte in microdialysis experiments.[30] However, rates less than 0.2 μL/min are not commonly employed due to the increased length of time required to attain a sample of sufficient volume for analysis.[31] Microdialysis probes may easily be constructed by inserting two lengths of fused silica into a small length of dialysis fiber.[32] Incorporation of this biosensor as an outlet line for a microdialysis probe would thus be facile and would allow "real-time" monitoring of changes in extracellular glutamate as changes in fluorescence. By incorporating an on-line analysis scheme to the microdialysis experiment, constraints of long sample collection periods could be avoided. This would allow a further reduction in microdialysis perfusion flow rates and concomitant increases in sample recovery.

To assess the applicability of this sensor for in vivo analyses, we recently tested its performance in physiological saline (pH 7.3). No alteration in biosensor characteristics in terms of enzyme efficiency or detection limits were observed under these conditions. Prior to use in vivo, it will be necessary also to test the effect of NAD^+ perfusion on the dynamics of glutamate neurotransmission. However, given results by Mitani et al.,[33] we expect negligible effects in this regard.

Low flow rates associated with capillary electrophoresis separations make this separation technique another attractive candidate for coupling with the capillary-based biosensor. Use of the glutamate biosensor in such a format would allow the conversion of glutamate to NADH in the initial segment of the capillary, with subsequent separation and detection. This could be employed either as an on-line analysis (with limited area of derivatization) or through fluid junction coupling of a fully derivatized biosensor to a separation capillary.[27] Coupling of the biosensor to such a separation technique could thus provide increased information concerning the extracellular space without much loss in time resolution.

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Stability. The stability of the biosensor was assessed by comparing efficiency of glutamate conversion on the second day of use (Figure 3). The efficiency of glutamate conversion at 3 and 30 mM dropped to ~2% on the second day of testing. A likely cause of this dramatic decrease in performance is the storage procedure for the biosensor. In these experiments, the biosensor was stored in phosphate buffer at 4 °C. Performance of GDH-modified electrodes has been reported to be seriously degraded following storage in solution as opposed to air.[22] Under air storage at room temperature, these biosensors have retained activity for more than 3 weeks.[34] Trypsin-modified capillaries[35] and GDH-modified fiber optics[34] have shown similar characteristics. Further investigation into the appropriate storage methods for the GDH-modified capillary is presently under way.

CONCLUSION

A novel capillary biosensor was developed for the analysis of glutamate which allows the transduction of the analyte to a detectable signal on-line and without dilution of sample. The high sensitivity of this biosensor, along with its fast time of response demonstrate its suitability for in vivo application to neurochemical questions. Optimization of the sensor may be achieved through decreases in capillary diameter and increases in the length derivatized. Extracellular glutamate analysis may be accomplished in vitro through operation of the sensor in the current form. However, future efforts will involve the incorporation of this enzyme-modified capillary into existing technologies, such as microdialysis and capillary zone electrophoresis. The development of such interfaces should extend the use of the biosensor to application in the more complex matrices found in vivo.

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