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Letter

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Glutaraldehyde Cross-Linked Glutamate Oxidase Coated Microelectrode Arrays: Selectivity and Resting Levels of Glutamate in the CNS

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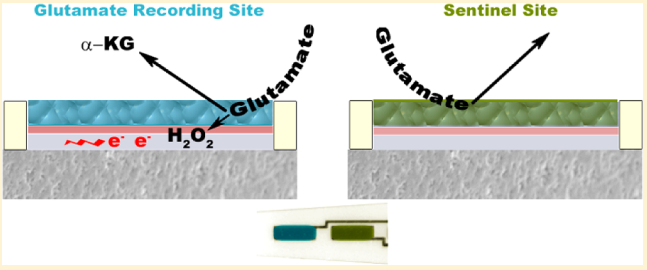
ABSTRACT: Glutaraldehyde is widely used as a cross-linking agent for enzyme immobilization onto microelectrodes. Recent studies and prior reports indicate changes in enzyme activity and selectivity with certain glutaraldehyde cross-linking procedures that may jeopardize the performance of microelectrode recordings and lead to falsely elevated responses in biological systems. In this study, the sensitivity of glutaraldehyde cross-linked glutamate oxidase-based microelectrode arrays to 22 amino acids was tested and compared to glutamate. As expected, responses to electroactive amino acids (Cys, Tyr, Trp) were detected at both nonenzyme-coated and enzyme-coated microelectrodes sites, while the remaining amino acids yielded no detectable responses. Electroactive amino acids were effectively blocked with a *m*-phenylene diamine (mPD) layer and, subsequently, no responses were detected. Preliminary results on the use of poly(ethylene glycol) diglycidyl ether (PEGDE) as a potentially more reliable cross-linking agent for the immobilization of glutamate oxidase onto ceramic-based microelectrode arrays are reported and show no significant advantages over glutaraldehyde as we observe comparable selectivities and responses. These results support that glutaraldehyde-cross-linked glutamate oxidase retains sufficient enzyme specificity for accurate in vivo brain measures of tonic and phasic glutamate levels when immobilized using specific "wet" coating procedures.



KEYWORDS: *Glutamate, electrode, amino acid, selectivity, microelectrode, brain, neurotransmitter, glutaraldehyde, PEGDE*

Many enzyme immobilization methods have been reported including sol gel, entrapment in overoxidized polypyrole or *o*-phenylenediamine, hydrogels, and more recently cross-linking with poly(ethylene glycol) diglycidyl ether (PEGDE).[1−7] The most common method of enzyme immobilization is cross-linking with glutaraldehyde.[8−10] Although glutaraldehyde is a fairly simple molecule, it can take many forms when dissolved in water. Thus, the reaction of glutaraldehyde with reactive groups on proteins is likely complex.[11] Most methods involve mixing glutaraldehyde with water and proteins; however, some methods involve immobilizing the proteins to the microelectrode surface first, then exposing this dried protein mixture to glutaraldehyde fumes.[7,11] Alternately, our group and others combine the glutaraldehyde with bovine serum albumin (BSA) followed by addition of glutamate oxidase in water or phosphate buffered saline (PBS). BSA is often included in the cross-linked matrix to protect enzyme activity by creating a 3D network for enzyme entrapment.[9,10] Although different successful methodologies have been reported, we apply the protein and glutaraldehyde mixture to the microelectrode surface then allow it to dry. We allow the glutaraldehyde to react with dissolved BSA before addition of enzymes. This "wet" method may allow enzyme molecules to be immobilized in a conformation that is more similar to the "free" enzyme conformation than methods involving vapor cross-linking of dried enzymes or direct cross-linking of dissolved enzymes.

Selectivity is of key importance to chemical sensors, especially for direct in vivo sensing in the CNS where there are a host of neurochemicals present and in situ calibrations are impractical or impossible. Free glutamate oxidase is extremely selective for glutamate over other amino acids, and this enzyme has been used extensively to quantify glutamate in vivo in the CNS by glutaraldehyde cross-linked immobilization onto microelectrode surfaces including ceramic-based microelectrode arrays (MEA).[10−15] Recently, the selectivity of this broad class of sensors has been questioned because of suspected enzyme conformation changes that occur during immobilization with glutaraldehyde.[16] Here we test the selectivity of glutamate oxidase-coated ceramic-based platinum coated microelectrodes versus other amino acids using a "wet" method of glutaraldehyde-based immobilization that has been used since our first report on glutamate sensing.[15] We describe in detail the immobilization method and report preliminary data showing how to adapt this

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method to use PEGDE-based immobilization. We also compare the enzyme responses for PEGDE-based immobilized glutamate oxidase with those for glutaraldehyde-based glutamate oxidase immobilization.

RESULTS AND DISCUSSION

A ceramic substrate forms a rigid, nonconducting platform for microelectrode arrays (Figure 1). The recording sites are formed by sputter-coating ultrapure platinum (Pt; ~0.25 μm thick) using photolithography. Patterned polyimide with openings at the recording sites insulates the connecting lines. Cross-linked glutamate oxidase and BSA immobilized onto the recording sites converts glutamate and O_2 to α-ketoglutarate, NH_3, and H_2O_2. H_2O_2 is oxidized at a potential of +0.7 V vs Ag/Ag^+ at the Pt recording sites. The size-exclusion mPD layer permits H_2O_2 to reach the Pt recording sites while effectively preventing larger molecules such as ascorbic acid, dopamine, and amino acids from accessing the platinum sites. The manufacturing process yields Pt recording sites with highly similar recording properties, which cannot be obtained with handmade wire electrodes. Furthermore, the differential coatings between the glutamate and sentinel sites and the close proximity of sites allows channel subtraction that effectively removes interferents from signals.[10]

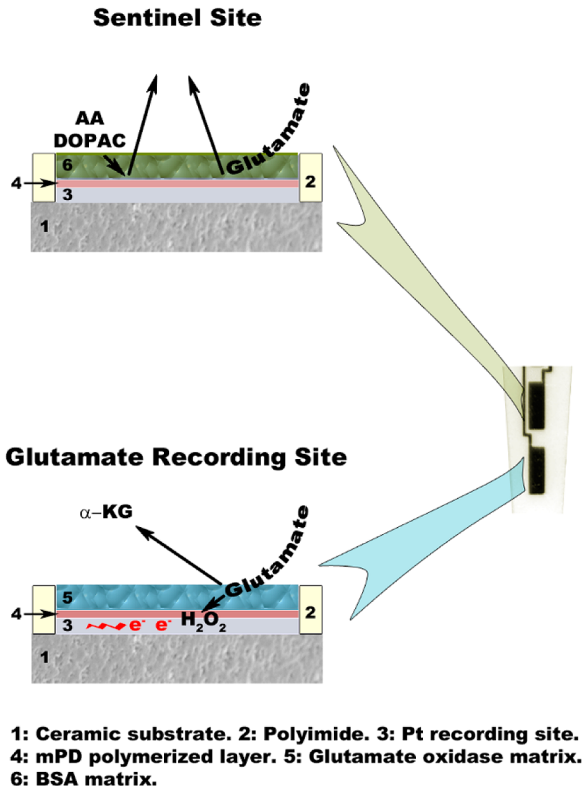

Figure 1. Schematic drawing of glutamate recording site and sentinel site layers and their functions. The image on the right is a magnification of a microelectrode array tip with 50 × 150 μm^2 sites.

Figure 2 shows optical images of an uncoated MEA and a cross-linked glutamate oxidase-coated MEA (see Methods section for a description of the coating procedure). To determine the thickness of the enzyme coating, we used atomic force microscopy (AFM) as described in ref 17 and found that the average coating thickness of the glutaraldehyde-cross-linked enzyme was 2.3 ± 0.1 μm. This coating is much thinner than protein layers on wire-type microelectrodes. [7]

Figure 3 shows the average glutamate MEA responses relative to glutamate for each of 22 amino acids (means ± SEMs). All amino acids were undetectable except for glutamate, Cys, Tyr, and Trp. In every case when an amino acid other than glutamate showed a response at the glutamate oxidase-coated sites, a comparable response was also observed at the control (sentinel) sites, that is, the sites without glutamate oxidase. Table 1 lists the selectivity of glutaraldehyde cross-linked glutamate MEAs without mPD for each amino acid tested to assess the responses of each amino acid so those amino acids that potentially interfere could be identified. Cys yielded the poorest selectivity because it is electroactive; however, when channel subtraction (self-referencing) between glutamate oxidase-coated sites and sentinel sites was applied, the selectivity improved to 250 ± 80. Others have previously

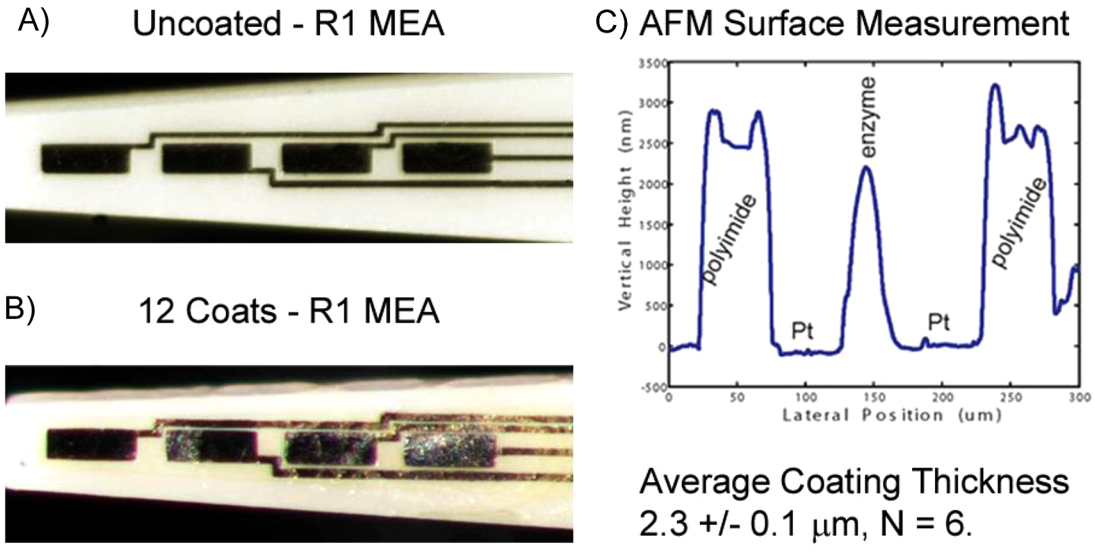

Figure 2. Images of (A) uncoated and (B) glutaraldehyde-cross-linked-enzyme coated MEAs. Panel (C) is a representative AFM scan of one recording site on the MEA surface showing the enzyme thickness scanning from top to bottom of the image across a recording site as shown in the photographs.

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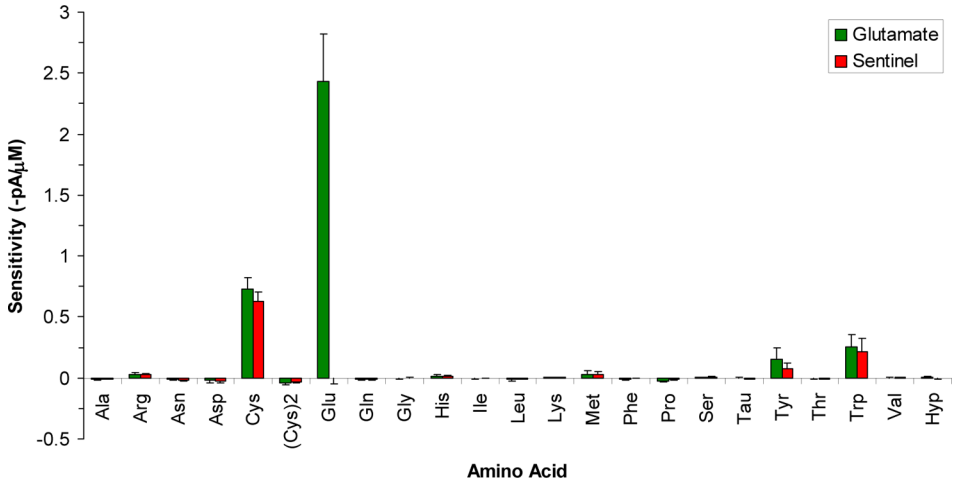

Figure 3. Averages of 10 working (glutamate) and 10 control (sentinel) site responses from the MEAs for each of the 23 amino acids tested. Glutamate sites were coated with glutamate oxidase and BSA cross-linked with glutaraldehyde. Sentinel sites were coated with glutaraldehyde cross-linked BSA only. Five MEAs without the mPD layer were tested each with two glutamate and two sentinel sites. Amino acids with measurable responses at glutamate sites showed similar responses at the sentinel sites. These amino acids are electroactive and detectable on MEAs without a size-exclusion layer such as mPD. Values are means ± SEMs.

Table 1. Glutamate Oxidase-Coated Microelectrode Amino Acid Selectivity Ratios (glutamate vs amino acid) without the Use of Self-Referencing Subtraction[a]

amino acid	selectivity	mPD selectivity
Ala	ND	–[b]
Arg	520 ± 300	–[b]
Asn	ND	ND
Asp	ND	ND
Cys[c]	6.2 ± 3	18 ± 60[d]
(Cys)2	ND	–[b]
Gln	ND	ND
Glu	1	1
Gly	ND	–[b]
His	270 ± 60	590 ± 100
Ile	ND	–[b]
Leu	ND	–[b]
Lys	370 ± 80	–[b]
Met	230 ± 80	270 ± 100
Phe	ND	–[b]
Pro	ND	–[b]
Ser	750 ± 200	–[b]
Tau	950 ± 600	–[b]
Tyr	210 ± 80	490 ± 100
Thr	ND	–[b]
Trp	260 ± 200	490 ± 100
Val	960 ± 400	–[b]
Hyp	410 ± 60	–[b]

[a]Note that the selectivity values increase with sentinel electrode subtraction. Selectivity was calculated as the glutamate sensitivity divided by the test amino acid sensitivity. Higher numbers indicate better selectivity; that is, a selectivity of 100 means that the microelectrode is 100 times more sensitive to glutamate than the tested amino acid. Only electroactive amino acids or ones that were cited as being problematic in ref 16 were tested with mPD-coated MEAs. All amino acid final concentrations were 100 μM except for 42 μM for Tyr. ND = not detectable. [b]Not tested. [c]Channel subtraction increased selectivity ratio for Cys to 250 ± 80 on non-mPD plated microelectrodes. [d]N = 8 recording sites on 4 MEAs.

reported the direct oxidation and quantification of amino acids: Arg,[18,19] Cys,[20,21] Met,[20] Trp,[22−24] and Tyr.[24] The control sites were less sensitive (83 ± 7% of glutamate response for Arg, Cys, His, Met, Trp, and Tyr), possibly because of coating differences between the control and glutamate sensitive sites. Although electroactive amino acids could be detected at microelectrode Pt surfaces without selective coatings (e.g., mPD), such exclusion coatings are used for in vivo experiments to minimize or eliminate interfering agents.[25,26] Table 1 also lists the selectivity of mPD-coated, glutaraldehyde cross-linked glutamate microelectrode arrays for glutamate over electroactive amino acids, specifically those cited by Vasylieva and colleagues as being potentially problematic in vivo.[16] The mPD effectively blocked potentially interfering electroactive species including amino acids. The published resting levels of amino acids range from 1 to 10 μM for most amino acids and 41 μM for glutamine. These are below the test concentrations used here, which yielded no responses. In addition to using exclusion layers, employing a control channel (sentinel) for channel subtraction further minimizes signals from interfering, electroactive chemicals or noise.[10,27,28] Thus, using our approach, electroactive amino acids do not appear to interfere appreciably with in vivo glutamate detection in the CNS.

Figure 4 shows a representative trace for the current response of a mPD-coated glutaraldehyde cross-linked glutamate microelectrode after addition of a 20 μM step in concentration

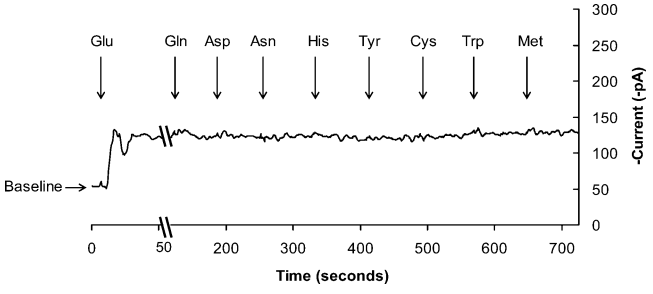

Figure 4. Representative glutaraldehyde cross-linked glutamate microelectrode response to 20 μM Glu followed by 100 μM of each of Gln, Asp, Asn, His, Cys, Trp, Met, and 42 μM Tyr. A 10 point moving average was applied for clarity. The MEA limit of detection for glutamate was 0.6 μM (signal-to-noise = 3).

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of Glu followed by 100 μM sequential additions of Gln, Asp, Asn, His, Cys, Met, and 42 μM Trp. These amino acids were chosen because they were electroactive or they were cited as being problematic in ref 16. Changes in the current responses were undetectable for each of the tested amino acids. Using extracellular amino acid concentrations in dialysates measured previously by others using HPLC[16] and the concentration-dependent microelectrode responses measured here, we calculated expected theoretical errors for the MEAs. For example, glutamine, which has a relatively high resting concentration of 41 μM measured by HPLC, would only yield a 0.03% error in the presence of a 4 μM glutamate signal. The sum of the estimated amino acid responses would be at most ~1% of the glutamate response using the MEAs described here.

Vasylieva and colleagues have compared glutamate oxidase-coated microelectrodes immobilized with different cross-linking agents including glutaraldehyde and PEGDE and found decreased amino acid selectivity for glutamate oxidase electrodes cross-linked with glutaraldehyde fumes, which they reported as similar to the "wet" coating procedures that we describe here.[7,16] They propose that decreased selectivity could yield erroneous results for basal glutamate measurements in vivo in the brain. In their glutaraldehyde immobilization method, a mixture containing glutamate oxidase and BSA was applied to a Pt-wire microelectrode then allowed to dry. Cross-linking with glutaraldehyde was achieved by exposure of the dried protein to glutaraldehyde fumes.[7,12,16] A dry method of immobilization such as this could alter the enzyme conformation, resulting in sensitivity and selectivity changes. Critically, this method differs from our "wet" method where glutaraldehyde is reacted with a BSA solution before adding glutamate oxidase. Vasylieva and co-workers tested a small sample (n = 4) of electrodes where glutaraldehyde was combined with a solution of glutamate oxidase and BSA then applied to the wire. These showed smaller but not statistically different responses to the tested amino acids. Our results demonstrate that allowing the glutaraldehyde to cross-link with the BSA before addition of glutamate oxidase yields no detectable loss of specificity. In addition, Vasylieva and colleagues' studies were carried out on Pt-wire electrodes rather than MEAs, which have faster response times as a result of the thinner enzyme layers (compare Figure 2 above to Figure 1b in ref 7). Immobilization procedures must be carefully chosen to promote intermolecular cross-linking and minimize enzyme conformation distortion.[16,29−33]

Enzyme immobilization with PEGDE has recently been proposed as a more reliable and selective alternative to glutaraldehyde.[16] Vasylieva and co-workers dip coated their wire-based microelectrodes in a more concentrated and viscous coating mixture of PEGDE, BSA, and glutamate oxidase compared to the mixture used in our glutaraldehyde-based method. Here, we adapted PEGDE for use on MEAs. Our coating procedure required a less concentrated mixture that could flow through a microsyringe for application onto recording sites. PEGDE-based glutamate MEAs had similar sensitivities to glutamate (Figure 5, $F_{(2,18)}$ = 0.813, p = 0.459) and linearities compared to glutaraldehyde cross-linking. These results indicate that PEGDE-based immobilization is compatible with our coating procedure and MEAs.

We also made a direct comparison of the selectivity of glutaraldehyde and PEGDE cross-linked glutamate microelectrodes for several key amino acids (Figure 5). Vasylieva and co-workers reported poor selectivity of Asn, Asp, Gln, and His when using glutaraldehyde fumes over PEGDE on

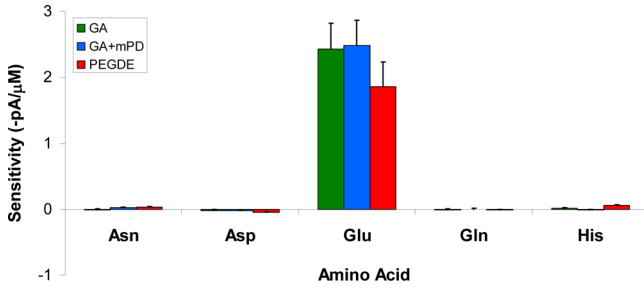

Figure 5. Comparison of glutaraldehyde (GA, n = 10 on 5 MEAs), PEGDE (n = 14 on 4 MEAs), and glutaraldehyde cross-linked glutamate electrodes with mPD (GA+mPD, n = 10 on 5 MEAs) to selected amino acids. Error bars represent SEMs. Sentinel sites showed similar responses as glutamate oxidase-coated sites for all amino acids with no response to glutamate (not shown).

Pt wire-based electrodes.[16] However, our studies support the finding that glutaraldehyde cross-linking results in no significant loss of glutamate oxidase selectivity for glutamate over other amino acids using our coating procedure. We do acknowledge that the PEGDE immobilization procedure we used is not exactly as reported by Vasylieva and co-workers. As with glutaraldehyde cross-linking, PEGDE-based glutamate microelectrodes also responded to the electroactive amino acids Cys, Met, Trp, and Tyr in the absence of an mPD exclusion layer (data not shown).

The present studies support the idea that, using specific glutaraldehyde cross-linking procedures, glutamate-coated microelectrodes can be prepared that show high sensitivity and selectivity to glutamate. Here, we observed minimal responses to interfering amino acids with glutaraldehyde-cross-linked glutamate-oxidase-coated MEAs prepared using a "wet" deposition procedure. Furthermore, glutamate MEA biosensors employed for in vivo experiments use multiple means to minimize potential interference from electroactive molecules (e.g., exclusion layers and self-referencing subtraction). At issue also are the resting levels of glutamate reported in vivo. It is suggested that the purported lack of selectivity of enzyme-coated MEAs results in higher levels of resting glutamate than with recordings employing enzyme-coated wire electrodes and some microdialysis measures.[16] We have recorded glutamate resting levels in a variety of brain regions, species, and animal strains as listed in Table 2. In addition, Table 2 lists resting levels of glutamate measured by microdialysis in numerous brain areas and species. While there appears to be a wide range of different extracellular glutamate levels reported using either method, resting levels of glutamate reported with either microdialysis or glutamate-oxidase coated MEAs are within the range of glutamate affinities for glutamate transporters.[34]

In general, microdialysis levels tend to be lower than values measured using MEAs, but with the wide range of concentrations reported, the distributions of values overlap, including those obtained using no-net-flux microdialysis (Table 2). Taken together, these data show that the "wet" method of glutaraldehyde-based enzyme immobilization described here preserves glutamate oxidase selectivity toward amino acid substrates when combined with MEA technology. In addition, these preliminary studies support the use of PEGDE as a cross-linking agent on MEAs giving similar results to glutaraldehyde. Additional studies will be needed to more thoroughly compare the performance of MEAs versus wire electrodes using the different cross-linking procedures and to investigate resting levels of glutamate in different brain regions and species.

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Table 2. Overlap of Resting Glutamate Measurements with Enzyme-Based MEAs and Microdialysis

enzyme-based MEAs				microdialysis			
concentration (μM)	species	location	ref	concentration (μM)	species	location	ref
0.8	Balb/c Glud1wt mice	striatum	35	0.275	Wistar rat	hippocampus	51
1.4	Fischer 344 rat	striatum	28	0.356	Wistar rat	medial prefrontal cortex	51
~1.4	C57BL/6-VAMP2$^{\pm}$ mice	dentate gyrus hippocampus	36	0.45	Long Evans rat	prefrontal cortex	52
1.5	DRD4$^{+/+}$ mice	striatum	37	0.54	CD-COBS rat	prefrontal cortex	53
1.6	Fischer 344 rat	frontal cortex	28	0.6	Wistar rat	striatum	54
1.9	Sprague–Dawley rat	CA3 hippocampus	38	0.85	Wistar rat	striatum	55
2.1	Sprague–Dawley rat	cuneate nucleus	39	1	Wistar rat	hippocampus	56
2.2	Sprague–Dawley rat	striatum	40	1.1	Macaque (nonhuman primate)	visual cortex	57
2.8	Fischer 344 rat	CA3 hippocampus	41	1.3	Sprague–Dawley rat	striatum	58
3	Sprague–Dawley rat	striatum	42	1.3	Wistar rat	prefrontal cortex	55
3.3	C57BL/6 mice	prefrontal cortex	43	1.4[a]	Sprague–Dawley rat	nucleus accumbens	59
3.7	Macaque (nonhuman primate)	motor cortex	44	1.5	Wistar rat	entopenduncular nucleus	60
3.8	Macaque (nonhuman primate)	premotor cortex	44	1.7	Wistar rat	hippocampus	61
~4	Fischer 344 rat	prefrontal cortex	45	2.1	Wistar rat	hippocampus	62
4.1	Sprague–Dawley rat	striatum	26	2.4[a]	Human	cortex	63
4.6	Sprague–Dawley rat	ventral posterior medial thalamus	46	2.6[a]	Human	hippocampus	63
5	C57BL/6 mice	striatum	43	2.9	Spague Dawley rat	striatum	64
6.3	Flinders Resistant Line rat	prefrontal cortex	47	3.1	Wistar rat	hippocampus	65
7.3	Long Evans rat	striatum	25	4	Wistar rat	frontal cortex	16
10.9	Macaque (nonhuman primate)	putamen	48	4.7	Wistar rat	prefrontal cortex	66
11.2	Sprague–Dawley rat	prefrontal cortex	49	8.2[a]	Mice	striatum	67
34.7	Long Evans rat	prefrontal cortex	50	14	Human	cortex	68
44.9	Long Evans rat	prefrontal cortex	25	16	Human	frontal cerebral cortex	69
				24.7	Sprague–Dawley rat	striatum	70
				28.8	Macaque (nonhuman primate)	putamen	71

[a]Zero-flow/no-net-flux.

METHODS

Chemicals. L-Amino acids including Alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine hydrochloride (Cys), cystine [(Cys)2], sodium glutamate (Glu), glutamine (Gln), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine monohydrochloride (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), taurine (Tau), tyrosine (Tyr), threonine (Thr), tryptophan (Trp), valine (Val), *trans*-4-hydroxyl-L-proline (Hyp), BSA, glutaraldehyde, poly(ethylene glycol) diglycidyl ether (PEGDE; average MW = 500), *m*-phenylene diamine (mPD), sodium chloride, sodium phosphate monobasic, and sodium phosphate dibasic were purchased from Sigma (Sigma Aldrich, St. Louis, MO).

All amino acids were dissolved in 0.05 M PBS to yield 40 mM stock solutions with the exception of the following. The pH was lowered by dropwise addition of 1 M HCl to aid in dissolution of Tyr and (Cys)2 yielding 16 mM stock solutions of each. Solutions of Asp and Glu were prepared at 20 mM.

Microelectrode Arrays. Glutamate sensitive ceramic-based platinum microelectrode arrays (MEAs) were prepared and coated as previously described.[10,13,15,27] The MEAs had 4 in-line 50 × 150 μm^2 platinum recording sites with 50 μm spacing between recording sites. The recording sites were arranged in pairs for channel subtraction using self-referencing recording methods.[10] Deionized water (0.985 μL) was added to 0.1 g BSA in a 1 mL microcentrifuge tube. After allowing the BSA to dissolve, 5 μL of glutaraldehyde (25% in water) was added then allowed to react for approximately 5 min. All mixtures are in w/v %. A color change from light to darker yellow was observed for this 1% BSA and 0.125% glutaraldehyde mixture. A volume of 4 μL of the BSA and glutaraldehyde mixture was combined with 0.5 unit of glutamate oxidase (0.5 unit/μL) and stirred to form the 0.1 units/μL glutamate oxidase, 0.8% BSA, and 0.1% glutaraldehyde mixture. Under a microscope, a drop of the enzyme coating solution was applied by hand to two of the four recording sites closest to the MEA tip using a microsyringe. The surface tension of the solution prevents spillover onto adjoining sites. Four coats were applied allowing each coat to dry (1–2 min) before the next application. The remaining two recording sites were coated with four applications of 1% BSA and 0.125% glutaraldehyde in water. The sites without glutamate oxidase served as controls (sentinels). The size and spacing of the recording sites allows manual coating without cross contamination between the glutamate and sentinel sites. Coated MEAs were cured at room temperature for 2–7 days. This is necessary to achieve stable recordings in vivo for up to 3 weeks.[25]

Several MEAs were tested using PEGDE as an alternative to glutaraldehyde for cross-linking. A procedure was adapted from Vasylieva and colleagues for coating individual sites on MEAs with PEGDE cross-linking.[7,16] A solution of 1% BSA and 0.5% PEGDE was prepared in 0.05 mM PBS. A volume of 9 μL of the BSA/PEGDE solution was combined with 1 unit glutamate oxidase and stirred to form the 0.1 units/μL glutamate oxidase, 0.9% BSA, and 0.45% PEGDE mixture. As with the previously described glutaraldehyde-based coating procedure, this mixture was coated onto recording sites using a microsyringe. Multiple coats (6–11) were applied to recording sites and allowed to dry between applications. Six enzyme applications were required for adequate sensitivity; however, no increase was observed with additional coats up to 11. Coated MEAs were cured for 2 h at 55 °C. Fourteen out of 16 sites on 4 MEAs were sensitive to glutamate after coating.

To block electroactive substances, mPD was applied after enzyme immobilization. We have found that applying mPD post enzyme immobilization yields a highly responsive and selective microelectrode for glutamate measures. The microelectrode potential was cycled between +0.25 and +0.75 V vs Ag/Ag+ in a degassed PBS solution

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containing 5 mM mPD at 0.05 V/s for 20 min. When electroplating mPD onto the Pt surface, mPD plates onto the metal surface rather than the enzyme layer. The mPD layer on the recording sites does not impede glutamate or other analyte molecules from reaching the enzyme layers. The resulting mPD layer blocked molecules from diffusing to the recording sites based on size while allowing small molecules such as H_2O_2 to pass to the recording sites for detection. Although a selective layer such as Nafion or mPD is required for in vivo testing, this layer was omitted for part of this in vitro study to enable selectivity studies of electroactive amino acids. Limits of detection of MEAs were calculated as 3 times the standard deviation of the baseline current noise divided by the sensitivity.

In Vitro Testing. Amino acid selectivity tests were performed on five glutaraldehyde cross-linked MEAs coated for glutamate detection. Each MEA had four recording sites. Amperometry at +0.7 V vs a Ag/Ag+ reference electrode was used to record the current associated with H_2O_2 oxidation; H_2O_2 is produced by oxidation of glutamate by glutamate oxidase. A total of 10 glutamate oxidase coated and 10 control recording sites were used to calculate mean responses for each amino acid. Aliquots (100 μL) of stock amino acid solutions were added to a stirred 0.05 M PBS solution (40 mL, 37 °C, pH = 7.4) to obtain final concentrations of 100 μM except for 50 μM for Glu and Asp and 40 μM for Tyr and Cys. 100 μM was chosen as it is many times higher than reported for in vivo amino acid concentrations.[16] Final concentrations of Asp, Tyr, and Cys were selected due to solubility limitations of stock solutions. The amino acid addition order was randomized across MEAs; however, amino acids yielding large responses (Cys, Met, Tyr, Trp, Glu) were added last to allow accurate current measures for all additions. The microelectrode array current was recorded after each addition. The change in baseline current was calculated and then divided by the amino acid concentration to yield the sensitivity in pA/μM for each amino acid. Selectivity was calculated as the glutamate sensitivity divided by the test amino acid sensitivity. A value of 500 was included in the selectivity average for any amino acid addition where there was a negative change in current meaning the amino acid was not detectable by the electrodes. These are due to random noise. Data were collected using a FAST16mkII instrument (Quanteon L.L.C., Nicholasville, KY). Analysis was performed in Matlab and Excel.

Atomic Force Microscopy. Briefly, the topography of MEA Pt recording sites was measured by atomic force microscopy using a Veeco MultiMode nanoscope (Veeco Instruments, Inc., Plainview, NY).[17] All MEA samples were studied at 25 °C under normal atmospheric pressure. A standard, uncoated contact-mode silicon atomic force microscopy probe with a tip radius of ~10 nm and half cone angle (contact mode, measured along the cantilever axis) ranging from 20° to 25° was used in the current study (Ted Pella, Inc., Redding, CA). The nanoscope maintained the probe in a fixed position while allowing the sample to move beneath the cantilever using a 2.4 Hz scan rate in a rastered fashion.

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Author Contributions

Participated in research design: J.J.B. and G.A.G. Conducted experiments and performed data analysis: J.J.B. and V.A.D. Wrote manuscript: J.J.B., V.A.D., J.E.Q., F.P., P.H., and G.A.G.

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Notes

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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ABBREVIATIONS

Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; BSA, bovine serum albumin; Cys, cysteine; (Cys)2, cystine; DI, deionized; Glu, glutamate; Gln, glutamine; Gly, glycine; His, histidine; Hyp, trans-4-hydroxyl-L-proline; Ile, isoleucine; Leu, leucine; Lys, lysine; MEA, microelectrode array; Met, methionine; mPD, *m*-phenylene diamine; ND, not detectable; PBS, phosphate buffered saline; PEGDE, poly(ethylene glycol) diglycidyl ether; Phe, phenylalanine; Pro, proline; SEM, standard error of mean; Ser, serine; Tau, taurine; Tyr, tyrosine; Thr, threonine; Trp, tryptophan; Val, valine

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