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Feasibility study using surface-enhanced Raman spectroscopy for the quantitative detection of excitatory amino acids

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Abstract. The release of excitatory amino acids (EAAs) from injured neurons has been associated with secondary injury following head trauma. The development of a rapid and sensitive method for the quantification of EAAs may provide a means for clinical management of patients affected by head trauma. We explore the potential application of surface-enhanced Raman spectroscopy (SERS) for rapid quantification of the concentration of EAAs in aqueous silver colloids. The EAAs glutamate (Glu) and aspartate (Asp) are released following head injury and have been observed to exhibit SERS spectra that should enable them to be distinguished in a complex aqueous media. Of the two EAAs, the concentration of Glu has been shown to be more indicative of injury to the central nervous system. Using 30-s scans and a 50-mW argon laser, aqueous Glu is quantifiable from 0.4 to 5 μmol/L and is spectrally distinguishable from Asp. In addition, initial *in vivo* microdialysis experiments suggest that this SERS system is capable of measuring chemical changes following head trauma in the rat brain. Compared with current high-performance liquid chromatography (HPLC) techniques for amino acid detection, the short scanning and processing time associated with the SERS approach enables measurement on a near-real-time basis, providing clinical information in anticipation of pharmaceutical intervention. [DOI: 10.1117/1.1528208]

Keywords: surface-enhanced Raman scattering; excitatory amino acids; glutamate; aspartate; silver colloid.

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1 Introduction

About 2 million Americans are hospitalized each year due to head injuries, which has been reported as the most common cause of injury-related death in America.[1] While 80 to 90% of head injury patients admitted to hospitals suffer mild or moderate injury, up to 5% of those patients die due to secondary mechanisms associated with the initial injury. Of the patients admitted with severe injury, as many as one-third die due to secondary mechanisms.[2]

Elevated excitatory amino acid (EAA) concentrations accompany brain trauma and ischemia and have been implicated in secondary injury to the nervous system.[3–5] An evaluation of current scientific literature regarding the two amino acids glutamate (Glu) and aspartate (Asp) suggests that the concentration of Glu is more indicative of and sensitive to injuries involving the central nervous system. While the intact and healthy brain is quite resistant to Glu, an injured one is susceptible to additional neurotoxic injury. Injured neurons release large quantities of Glu that injure more neurons, inducing a self-propagating cycle.[6] Sudden increases in Glu concentration have been observed as an antecedent to patient death as much as several days following a severe head injury.[2,7] EAAs are also presumed to play pathogenic roles in neurological disorders including ischemic stroke, multiple sclerosis, Parkinson's and Alzheimer's diseases, epilepsy, and chronic schizophrenia.[6]

EAAs act as neurotransmitters that mediate postsynaptic receptors and channels that control Ca^{2+} flux. Mechanisms proposed to account for the neurotoxic effects observed include acute neuronal swelling caused by excessive Ca^{2+} and Na^{+} and an increase to toxic levels of Ca^{2+}-mediated nitric oxide production. Recent research has further implicated Glu in neuronal death after ischemia because Glu receptor blockade has been shown to be neuroprotective in animals.[8]

EAA concentrations in cerebrospinal fluid (CSF) or brain extracellular fluid (ECF) samples can be collected using several methods. These body fluids contain many of the same amino acids and neurotransmitters but often differ in concentration levels. In a traditional method, raw CSF samples are deproteinized with a sulfosalicylic or trichloroacetic acid solution. This deproteinization step is circumvented when microdialysis techniques are employed.[9–12] In this procedure, a dialysate fluid is flushed through a hollow tube with porous walls that is inserted into the brain or spinal cord. Samples are taken of this fluid after different chemical species diffuse across the membrane from nearby extracellular fluid. Samples

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are then frozen awaiting amino acid separation by high-performance liquid chromatography[13] (HPLC). Chemical tagging of the amino acids for use with fluorescence or electrochemical detection techniques is then necessary due to the low concentration of the analytes in these fluids. The reported human extracellular concentration of Glu by HPLC is at least 0.6 μmol/L; the toxic concentration is at least 2 to 5 μmol/L. The time required to perform HPLC assays may eliminate valuable clinical information while neurotoxicity induces further brain injury over the course of minutes and hours. For this reason, a near-real-time method of monitoring EAA concentrations may prove invaluable in the development of drug therapies using specific amino acid antagonists.[6]

Surface-enhanced Raman spectroscopy (SERS) techniques have been previously applied to other neurotransmitters that are found in the central nervous system. Lee et al. used a silver electrode SERS technique to examine dopamine, epinephrine, and norepinephrine.[14] Of these compounds, dopamine was apparently the most sensitive, with quantitative measurements obtainable at 1 μmol/L concentration. However, dopamine concentrations in the brain are thought to be much lower.[15] They added potential confounders known to exist in CSF and ECF such as ascorbate, glutathione, and acetylcholine, but determined that these chemicals were not SERS active. Since these confounders were unable to generate SERS spectra, they did not affect the SERS spectra of the analytes of interest.

Kneipp et al. combined dopamine and norepinephrine with a different silver colloid than the one used in this research.[16] They showed that quantitative measurements could be made of a single analyte in the presence of other SERS-active analytes in an aqueous solution using a SERS technique. They presented spectra of dopamine at 0.5 μmol/L, with some spectral features observable at concentrations 100 times lower. In addition, they added albumin as a confounder, but determined that it was not SERS active with their colloid.

Both traditional dispersive Raman and SERS spectral attributes have been reported for the EAAs we studied in this paper. Traditional dispersive Raman detection limits fall far short of detecting typical physiological concentrations.[17,18] Chumanov et al. showed aqueous SERS spectra for 68 and 75 μmol/L Glu and Asp (respectively) along with a broad examination of other amino acids.[19] They did not report on these amino acids in physiological solutions nor did they suggest what SERS detection limits exist for any amino acids. While they did report general enhancement factors for amino acids of at least 10^6, the concentration of EAAs reported were well above expected *in vivo* values for mammals.

The study presented in this paper examined the SERS spectra of the EAAs in aqueous solution and determined the detection limit of Glu using silver colloids prepared according to the Creighton method.[20] The goal was to determine if SERS is sensitive enough to detect micromolar concentration levels of Glu reported for CSF or brain ECF. For brain ECF, preliminary research described here, along with several other reports, suggests that Glu is one of the highest concentrated and most SERS-active amino acids following brain injury.[21–24] In both extracellular brain fluid and CSF, Asp has been reported[4,10,25] at lower concentrations than Glu. The ability to detect the SERS spectral attributes of Glu in the presence of confounding biological molecules of approximately the same molecular weight was investigated. The time constraints of colloidal SERS measurements, which must be prompt enough to have the potential for rapid detection at the bedside, were also investigated.

2 Materials and Methods

Traditional dispersive Raman scattering and SERS scattering measurements were conducted with a 500M SPEX spectrometer equipped with a CCD camera operated in multichannel detection mode. The samples were excited by the 514.5-nm line of an argon laser (CR-4 supergraphite ion laser by Coherent) operating at a power of 50 ± 1 mW. Sample excitation and Raman signal collection was performed using a previously described confocal Raman system,[26] although the confocal attributes of the system were not required in this study. Thirty-second data acquisition was used throughout the study. All measurements were performed under the same conditions (i.e., laser power, spot size, etc.).

The silver colloids were prepared according to the procedure previously described by Creighton et al.[20] One part (1 mmol/L) aqueous silver nitrate ($AgNO_3$) was added dropwise to 3 parts (2 mmol/L) aqueous sodium borohydride ($NaBH_4$) at room temperature to produce 20 to 30 ml of colloid. $AgNO_3$ and $NaBH_4$ were purchased from Alfa Aesar and were used within 8 h of stock preparation due to known long-term instability and decomposition of these components in water. The $AgNO_3$ stock was handled and stored under dark-room conditions due to suspected light sensitivity. The mixture was constantly and gently stirred during preparation to homogenize the colloidal particles, but was then allowed to sit for at least 2 min before the addition of any adsorbate. A decrease in SERS enhancement due to a reduced ability to aggregate over time was noted by previous investigators, thus colloids over 30 min old were never used.[27] The color of the pH 9.3 colloidal solution ranged from light yellow to golden brown, with darker colloids producing greater enhancement of the Raman signal. Therefore, only golden brown solutions were employed for this study. All solutions were prepared using Millipore water in the pH range 5.5 to 6.0 with a resistivity of 18 mΩ. The pH was measured with a pH meter (Piccolo Plus by Hanna), which was calibrated using standard buffer solutions of pH 7.01 and 4.01. The amino acid solutions were always mixed with the silver colloidal solution in a one-to-one milliliter ratio. All of the samples from each data set were mixed with the colloid as quickly as possible and spectra were collected starting 10 min later, which took into account slower reaction times for lower concentrated samples. Amino acids and γ-amino-butyric acid (GABA) were obtained from Sigma. For the studies performed in this paper, the same colloid preparation was used in each case for the calibration and validation data sets.

This feasibility study begins with an examination of homogeneous amino acid solutions. This simplifies control of each amino acid concentration and eliminates confounding effects. This univariate approach enables the determination of Raman enhancement from each analyte ranging from below normal to extreme pathologic concentrations. Furthermore, the effects of suspected confounding analytes can then be examined individually.

The normal dispersive Raman spectra of 50 mmol/L Glu and Asp solutions were obtained initially and served as relative reference spectra for the SERS measurements. Following a qualitative comparison of the dispersive Raman and SERS spectra, a quantitative analysis of the relationship between Glu concentration and SERS spectral intensity was performed by tracking the changes in the band observed at 830 cm^{-1}. This quantitative study examined 3 samples from each Glu concentration between 0.4 and 5 μmol/L (24 samples total) and was prepared from a single silver colloid. An additional set of 8 samples of variable concentration was prepared using the same colloid. The spectra were calibrated by subtracting features produced by the aqueous colloid along with any broad baseline shifts indicative of Raleigh scatter (as influenced by the general increase of colloidal particle size due to analyte induced aggregation).[28] A supplementary qualitative study was performed to ascertain the low end of the SERS detection range of an aqueous homogeneous Glu solution.

The SERS sensitivity of a variety of amino acids relative to Glu (Gln, Phe, Tyr, Gly, Ala, Asp, and GABA) was tested. Additionally, Asp was added to Glu in similar, lower, and higher concentrations to determine the sensitivity and specificity of the SERS system to changes in Glu in the presence of the similar SERS active confounder Asp.

The time-dependent kinetics of silver colloids with Glu was examined. SERS measurements were started immediately after the samples were prepared with 30-s scanning repeated for a 15-min period.

Finally, a preliminary *in vivo* microdialysis experiment was performed on two rats. This surgical procedure is described in detail elsewhere.[29] The animals were exposed to middle cerebral artery occlusion (MCAO), a procedure that produces localized ischemia in the brain to resemble head trauma.[23,24] A microdialysis probe was then inserted into the damaged area of the brain and the concentrations of the various components of extracellular brain fluid were sampled across the dialysis membrane. The small pore size of the probe membrane allowed analytes such as amino acids to pass into our SERS detection system, while rejecting the flow of any larger confounding substances that occur naturally in the brain. An artificial cerebral spinal fluid was pumped using a 2 μL/min flow rate and 30 μL samples were collected every 15 mins.

3 Results

Representative normal dispersive Raman and SERS spectra of Glu and Asp are shown in Figures 1 and 2, respectively. The Raman spectra of 50 mmol/L Glu and Asp confirms earlier spectral observations of other investigators showing a strong COO^- band at 1416 cm^{-1}, with a similar peak in the SERS spectra.[17,18] Both the (COO^-) band at 1387 cm^{-1} and the (CH_2) band at 1048 cm^{-1} were prominent.[19] In addition, the EAAs were readily distinguishable in a mixed solution (Figure 3).

For comparison with other investigators previous work, absolute SERS enhancement factors of the prominent 5 μmol/L Glu peaks were estimated to be at least 10^7 by adding a 1 mol/L methanol standard following a procedure outlined by Kneipp et al.[30] Aqueous MeOH shows a singular feature at 1020 cm^{-1} in the 900 to 1200-cm^{-1} region. When this Glu

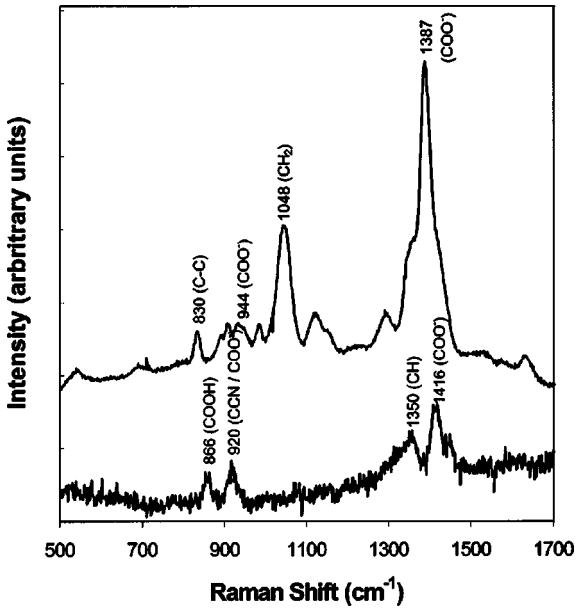

Fig. 1 Background corrected SERS and Raman spectra of Glu: (top) 3 μmol/L, SERS; (bottom) 50 mmol/L, Raman. SERS intensity is shown here reduced by a factor of 10 for comparison.

+MeOH mixture is added to a silver colloid, the resultant spectrum includes Glu SERS features plus the unenhanced features of MeOH. We further assume that the probing volumes among our different solutions are constant and that the Raman cross sections of the different molecules are close to the same size (within an order of magnitude). We then take into account the relative concentrations in solution and compare the relative peak heights of the highest MeOH features with the highest Glu SERS feature. For the concentrations employed here Glu is 5×10^6 less concentrated than MeOH

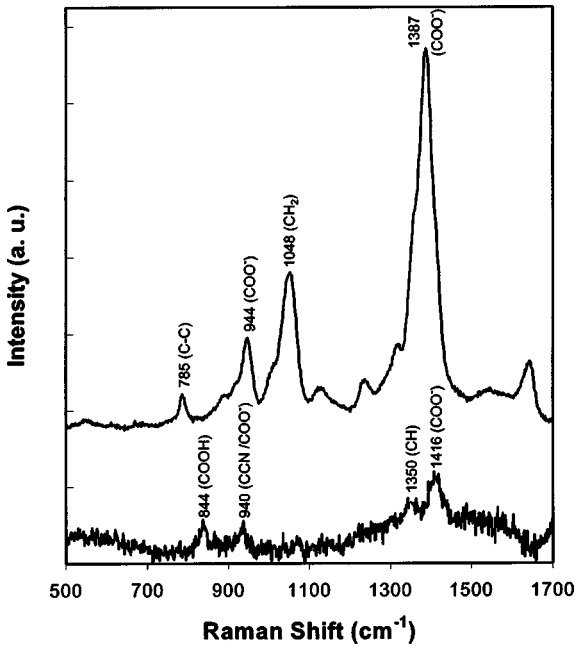

Fig. 2 Background-corrected SERS and Raman spectra of L-aspartic acid: (top) 100 μmol/L, SERS; (bottom) 50 mmol/L, Raman. SERS intensity is shown here reduced by a factor of 10 for comparison.

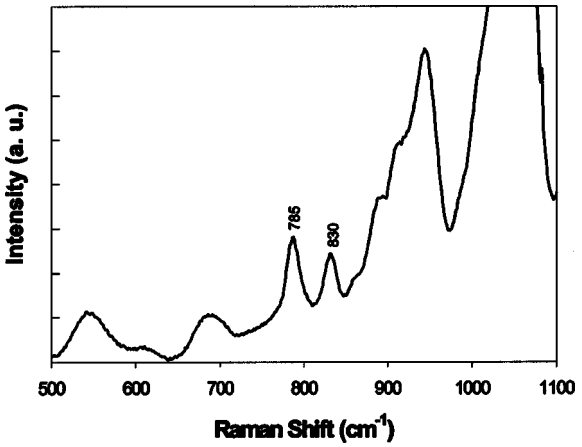

Fig. 3 Plot of 5 μmol/L Glu in the presence of 5 μmol/L Asp. The Glu peak at 830 cm^{-1} is differentiated easily in the presence of Asp, since Asp has no spectral features in the 800- to 850-cm^{-1} range. The C–C peak of Asp is shifted to 785 cm^{-1}.

and produces 12 times the photons for the highest peak. Therefore, this method estimates that the SERS enhancement of Glu is 6×10^7. Similarly, the SERS enhancement of a 100 μmol/L concentration of Asp was deduced to have an enhancement of 10^6.

The colloidal SERS qualitative detection limit of our system (SNR=3) was 0.4 μmol/L Glu using 30-s scans and 50 mW. The 1387- and 1048-cm^{-1} bands were the only features distinguishable from the background noise at this low concentration.

Figure 4 shows the correlation of the 830-cm^{-1} band intensity compared to known Glu concentrations in SERS measurements. This moderately intense band was chosen due to its uniqueness among amino acids.[19] The calibrated sample data followed a log-log linear model ($R^2=0.986$) for concentrations between 0.4 and 5 μmol/L as expected.[27,31] No shift of spectral traits was observed as the adsorbate concentration varied. The Glu concentration model was reproducible using spectral intensity at 830 cm^{-1} with an average relative error of correlation of 13.1%. The independently collected sample set yielded an average error of prediction (AREP) of 12.6% compared with the initial model.

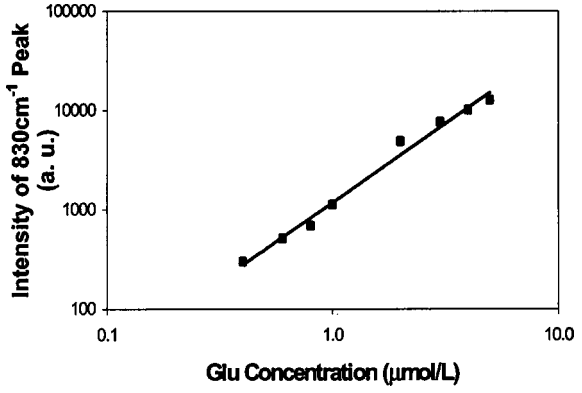

Fig. 4 SERS spectral intensity of the unique 830-cm^{-1} Glu peak versus concentration. When exhibited as a log-log plot, the colloid yields a linear model that predicts concentration between 0.4 and 5 μmol/L.

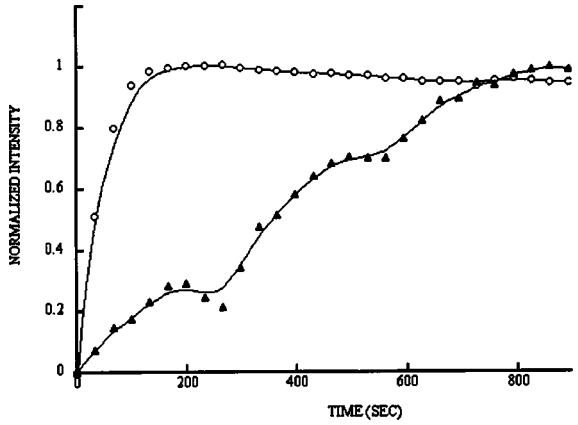

Fig. 5 Normalized SERS intensity (1387-cm^{-1} peak) of 1 μmol/L (triangle) and 5 μmol/L (circle) Glu versus time. Aggregation reactions between the colloid and lower concentrated amino acids took longer to reach steady state conditions compared to higher concentrated amino acid solutions. However, the curves plateau past 10 min. Other spectra collected for quantitative measurement in this study were taken starting at 10 min, based on these results.

The initial kinetic study is illustrated in Figure 5. Depicted is the time dependence of the 1387-cm^{-1} band intensity of the Glu/colloidal mixture at 1 and 5 μmol/L concentrations. For the 5 μmol/L sample, this band intensity quickly reached its maximum 2 min after the amino acid sample was mixed with silver colloids. For the 1 μmol/L sample, the process was slower; it took approximately 10 min for the COO^- band intensity to reach a reaction rate that approached steady state.

Figure 6 shows the extinction spectra of glutamate at a range of concentrations. The initial unaggregated colloid exhibits a predominant feature centered around 395 nm. This feature is a product of the Mie scattering observed around particles approximately the size of the wavelength of light. It decreases as these initial particles are consumed in the aggregation that produces increasing levels of SERS enhancement (compare with Figure 5).

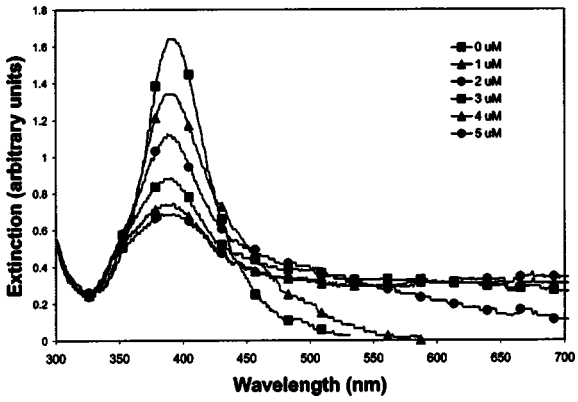

Fig. 6 Absorption spectra of the Glu-colloid complex across the dynamic concentration range of the colloid for this analyte. The height of the 390-nm absorption feature, attributed to unaggregated silver particles, is inversely proportional to SERS enhancement. We find no further change in the absorption spectra for this colloid above 10 μmol/L.

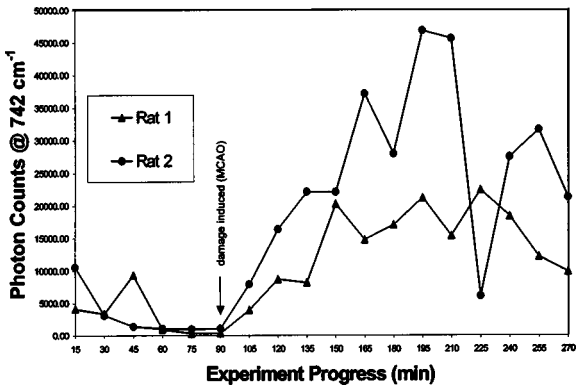

Fig. 7 Initial SERS examination of rat extracellular brain dialysate before and during ischemic conditions. This initial study of two rats shows significant SERS differences before and after MCAO. The microdialysis probe was inserted at 0 min and MCAO was induced at 90 min. The peak measured at 742 cm^{-1} was never attributed specifically to Glu but showed a compelling similarity to changes expected in EAA concentrations following head injury.

As depicted in Figure 7, the preliminary data taken from two rat models shows a trend in the SERS *in vivo* analysis that mimics the known trend of glutamate to fluctuate over time after brain injury from the MCAO procedure.[23,24] After the probe insertion at time zero, a small rise in glutamate is expected due to damage from the insertion of the probe itself. Healthy brain physiology then reasserts itself and baseline levels of Glu are restored. Glu levels are expected to rise sharply after ischemia is induced and are observed to peak around 180 min. After about 1.5 to 2 h into ischemic conditions, as most of the cells in the traumatized region are then dead, Glu levels are expected to begin a slow decline.

4 Discussion

As depicted in Figures 1, 2, and 3, the SERS spectral peak of Glu at 830 cm^{-1} is distinguishable from the 785-cm^{-1} Asp peak. Assuming this peak is attributed to (C-C) stretching, then a difference due to the different carbon-chain length of the two molecules is expected. As anticipated from an examination of Chumanov et al.[19] and confirmed in our studies, the 800 to 850-cm^{-1} area of Glu is unique among the common amino acids found in proteins.

In general for a mixed solution, quantitative colloidal SERS methods are confounded when detecting analytes due to the varying ability of different molecules to cause adsorption, induce aggregation, and influence SERS enhancement. This is due in part to the competition of different analytes to displace the negatively charged ions known to exist on the surface of the metal particles. In practice, amino acids with the greatest ability to cause adsorption should be visible in a mixed solution even to the exclusion or suppression of more concentrated but less active analytes. Glu was found to be by far the most SERS active amino acid tested. For example, the Creighton SERS colloid was shown to be many times ($\approx$30) less sensitive to Asp than to Glu. While isolated aqueous Asp is not considered SERS active below 50 μmol/L, it is known that aliphatic amino acids can sensitize a silver colloid in much the same way as certain ions (Na^+,Mg^{2+},Cl^-). This occurred when the EAAs were mixed (Figure 3) and spectral attributes were observed from Asp at concentrations far below what would be expected from an isolated solution.

Since Asp and other amino acids share the 1387- and 1048-cm^{-1} bands, only the 830-cm^{-1} peak is unique to Glu. This peak was found to be quantifiable to 0.4 μmol/L using the instrument setup and dosimetry already described. A lower detection limit of Glu can potentially be achieved using longer scanning time or higher laser power since the SNR would be expected to increase. For example, isolated Glu at a 0.1 μmol/L concentration was distinguishable from the background by increasing the scanning time to 300 s. Thus, there is a trade-off between resolution versus the time required to obtain the information, however, for this clinical application 0.4 μmol/L resolution is sufficient. Above 5 μmol/L Glu, only slight increases in SERS peak heights are expected due to a saturation of the binding sites of the silver colloid. If the concentration of an unknown Glu sample is thought to be at or above 5 μmol/L, dilutions of the unknown solution can be made until the intensity of the 830-cm^{-1} peak falls within the 0.4 to 5 μmol/L Glu calibration curve. In addition, extinction spectra of the colloid could be generated to determine the aggregation state and concomitant SERS enhancement[20] (Figure 6).

Kinetic aggregation predictions for a particular colloid preparation were found to be reproducible and predictable. In an effort to prove the reproducibility of these colloids, a calibration curve (Figure 4) was produced using known concentrations of amino acids and then unknown concentrations were determined with the use of the model produced.

The reproducibility and good quantitative results observed in this paper was due in part to the careful control of the colloid manufacturing process. Regarding colloid manufacture in general, care must be taken to ensure reproducible results given the variables inherent in colloidal systems. Glassware cleanliness, water quality, and the age of the stock solutions play important roles in determining the relative SERS activity of a given colloidal preparation. If any of these factors vary, the particle size, as noted by the color of the colloid, is affected. For example, from Mie theory it is known that the color of the solution relates directly to particle size in colloidal solutions where particles are approximately the wavelength of light.[32] The light brownish-yellow (larger particles) colloids that were used for this study were determined to produce better enhancement and reach steady state aggregation quicker than yellow (smaller particles) or brown (much larger particles) colloids in concurrence with earlier work by Torres and Winefordner.[27]

The rate of aggregation of an EAA with the silver colloid is dependent on the limited surface area of the silver particles in solution and can be described with ''active site'' analogy. This analogy predicts that the detected peak intensities used in our model take longer to reach steady state kinetic conditions under low concentrations than high or saturated adsorbate concentrations (Figure 5). The surface area, as predicted by the particle size and quantity, should limit this rate. For low concentrations of analyte, this analogy predicts that some minimum threshold value of active surface sites must be activated to produce detectable aggregation. As more of the active sites are occupied, a decrease in the reaction rate is expected. Diminishing increases in peak intensity for Glu concentrations were observed above 5 μmol/L due to this

saturation effect. The nonlinearities in the model observed at medium concentrations ($\approx$2 μmol/L) can also be attributed to this enzyme-like sigmoidal kinetics.

Reproducible timing of the reaction between the colloid and the analyte was a primary concern for quantitative measurements. Ideally, SERS spectra should be obtained at a time when the aggregation reaction between the colloid and the analyte has reached steady state conditions. In practice, unreacted colloids over 30-min old were discarded since it was found that these solutions became increasingly stable and resistant to aggregation caused by the introduction of an analyte. Similarly, once an analyte was added to a freshly prepared colloid it was noticed that the newly created colloidal complex became relatively stable after a short period of time. Steady state aggregation conditions of the EAA/colloidal solution served to produce a steady SERS signal level. Thus based on the results of Figure 5, a 10-min time estimate of steady state conditions was used in this study as the earliest possible measurement that produced a high confidence of reproducibility as confirmed by the less than 13% AREP of the independent sample set.

Regarding the *in vivo* results, as mentioned, the SERS peak differences were observed to change in a fashion that correlates directly with previous studies of brain injury induced using the MCAO procedure.[23,24] Although the spectral peak differences are indicative of head injury, further *in vivo* studies are required to determine if these changes are directly or indirectly related to changes in glutamate levels. One outlier was noted at 225 min but was verified in both of the SERS scans taken of this sample, which suggested a sample-handling error.

5 Conclusion

The combination of the strong SERS activity of Glu obtainable in a timely fashion and sensitive even in the presence other similar confounders makes a compelling argument for the use of colloidal SERS systems for monitoring brain ECF and CSF samples. SERS is an especially promising technique for use with clear biological solutions such as CSF microdialysis samples. These samples do not contain large scattering or fluorescing particles that typically confound Raman and SERS measurements of biological media. Even with a minimal 50-mW laser power and 30-s scan time, the current protocol provides detection limits that can ascertain subphysiological levels of Glu in an aqueous solution rivaling time-consuming conventional HPLC methods. The uniqueness of the SERS Glu spectrum should enable us to quantitatively distinguish it in complex solutions such as ECF as well. This initial system also showed the potential of detecting chemical changes in traumatized rat brain tissues. Future work will focus on obtaining more *in vivo* samples and the possible application of postprocessing techniques such as multivariate partial least-squares regression for analyte quantification.

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References

1. J. F. Kraus, ''Epidemiology of head injury,'' Chap. 2 in *Neurotrauma*, R. K. Narayan et al., Eds., pp. 13–14, McGraw-Hill, New York (1996).
2. A. Zauner and R. Bullock, ''The role of excitatory amino acids in severe brain trauma: opportunities for therapy: a review,'' *J. Neurotrauma* **12**, 547–554 (1995).
3. A. J. Baker, R. J. Moulton, V. H. MacMillan, and P. M. Shedden, ''Excitatory amino acids in cerebrospinal fluid following traumatic brain injury in humans,'' *J. Neurosurg.* **79**, 369–372 (1993).
4. A. M. Palmer, D. W. Marion, M. L. Botscheller, D. M. Bowen, and S. T. DeKosky, ''Increased transmitter amino acid concentration in human ventricular CSF after brain trauma,'' *NeuroReport* **6**, 153–156 (1994).
5. J. P. W. F. Lakke, A. W. Teelken, H. v. D. Voet, and B. G. Wolthers, ''Amino acid abnormalities in cerebrospinal fluid and blood serum of patients with parkinson's disease, other heredodegenerative disorders and head injuries,'' *Adv. Neurol.* **45**, 243–247 (1986).
6. R. J. Thomas, ''Excitatory amino acids in health and disease,'' *J. Am. Geriatr. Soc.* **43**, 1279–1289 (1995).
7. A. Zauner, R. Bullock, A. J. Kuta, J. Woodward, and H. F. Young, ''Glutamate release and cerebral blood flow after severe human head injury,'' *Acta Neurochir. Suppl. (Wien)* **67**(Suppl.), 40–44 (1996).
8. D. W. Choi, M. Maulucci-Gedde, and A. R. Kreigstein, ''Glutamate neurotoxicity in cortical cell culture,'' *J. Neurotrauma* **7**, 357–368 (1990).
9. N. Shimada, R. Graf, G. Rosner, and W. Heiss, ''Ischemia-induced accumulation of extracellular amino acids in cerebral cortex, white matter, and cerebrospinal fluid,'' *J. Neurochem.* **60**, 66–71 (1993).
10. H. Benveniste, J. Drejer, A. Schousboe, and N. H. Diemer, ''Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis,'' *J. Neurochem.* **43**, 1369–1374 (1984).
11. M. L. Boatell, G. Bendahan, and N. Mahy, ''Time-related cortical amino acid changes after basal forebrain lesion: a microdialysis study,'' *J. Neurochem.* **64**, 285–291 (1995).
12. L. Hillered, L. Persson, H. Carlson, U. Ungerstedt, E. Ronne-Engstrom, and P. Nilsson, ''Studies on excitatory amino acid receptor-linked brain disorders in rat and man using *in vivo* microdialysis,'' *Clin. Neuropharmacol* **15**(Suppl. 1 Pt. A), 695A–696A (1992).
13. M. H. Joseph and C. A. Marsden, ''Amino acids and small peptides,'' Chap. 2 in *HPLC of Small Molecules—A Practical Approach*, C. K. Lim, Ed., pp. 13–28, IRL, Washington, D.C. (1986).
14. N. Lee, Y. Hsieh, R. F. Paisley, and M. D. Morris, ''Surface enhanced Raman spectroscopy of the catecholamine neurotransmitters and related compounds,'' *Anal. Chem.* **60**, 442–226 (1988).
15. H. Benveniste, ''Brain microdialysis,'' *J. Neurochem.* **52**, 1667–1679 (1989).
16. K. Kneipp, Y. Wang, R. R. Dasari, and M. S. Feld, ''Near-infrared surface-enhanced Raman scattering (NIR-SERS) of neurotransmitters in colloidal silver solutions,'' *Spectrochim. Acta* **51A**, 481–487 (1995).
17. H. F. Shurvell and F. J. Bergin, ''Raman spectra of L(+)gGlutamic acid and related compounds,'' *J. Raman Spectrosc.* **20**, 163–168 (1989).
18. J. T. Lopez Navarrete, V. Hernandez, and F. J. Ramirez, ''IR and Raman spectra of L-aspartic acid and isotropic derivatives,'' *Biopolymers* **34**, 1065–1077 (1994).
19. G. D. Chumanov, R. G. Efremov, and I. R. Nabiev, ''Surface-enhanced Raman spectroscopy of biomolecules: Part I.—water-soluble proteins, dipeptides and amino acids,'' *J. Raman Spectrosc.* **21**, 43–48 (1990).
20. J. A. Creighton, C. G. Blatchford, and M. G. Albrecht, ''Plasma resonance enhancement of Raman scattering by pyridine adsorbed on silver or gold sol particles of size comparable to the excitation wavelength,'' *J. Chem. Soc., Faraday Trans. 2* **75**, 790–798 (1979).
21. H. Katoh, K. Sima, H. Nawashiro, K. Wada, and H. Chigasaki, ''The effect of MK-801 on extracellular neuroactive amino acids in hippocampus after closed head injury followed by hypoxia in rats,'' *Brain Res.* **758**, 153–162 (1997).
22. A. M. Palmer, D. W. Marion, Michelle L. Botscheller, P. E. Swedlow, S. D. Styren, and S. T. DeKosky, ''Traumatic brain injury-induced excitotoxicity assessed in a controlled cortical impact model,'' *J. Neurochem.* **61**, 2015–2020 (1993).

23. S. P. Butcher, R. Bullock, D. I. Graham, J. McCulloch, ''Correlation between amino acid release and neuropathologic outcome in rat brain following middle cerebral artery occlusion,'' *Stroke* **21**, 1727–1733 (1990).
24. C. J. Winfree, C. J. Baker, E. S. Connolly Jr., A. J. Fiore, and R. A. Solomon, ''Mild hypothermia reduces penumbral glutamate levels in the rat permanent focal cerebral ischemia model,'' *Neurosurgery* **38**, 1216–1222 (1996).
25. J. Castillo, A. Davalos, J. Naveiro, and M. Noya, ''Neuroexcitatory amino acids and their relation to infarct size and neurological deficit in ischemic stroke,'' *Stroke* **27**, 1060–1065 (1996).
26. F. H. M. Jongsma, R. J. Erckens, J. P. Wicksted, N. J. C. Bauer, F. Hendrikse, W. F. March, and M. Motamedi, ''Confocal Raman spectroscopy system for noncontact scanning of ocular tissues: an *in vitro* study,'' *Opt. Eng.* **36**, 3193–31997 (1997).
27. E. L. Torres and J. D. Winefordner, ''Trace determination of nitrogen-containing drugs by surface enhanced Raman scattering spectrometry on silver colloids,'' *Anal. Chem.* **59**, 1626–1632 (1987).
28. R. Montes, C. Contreras, A. Ruperez, and J. J. Laserna, ''Improvement in fingerprinting capability of surface-enhanced Raman spectrometry by simultaneous measurement of scattering signal and transmitted light,'' *Anal. Chem.* **64**, 2715–2719 (1992).
29. D. P. O'Neal, ''The application of surface-enhanced Raman spectroscopy for the detection of excitatory amino acids,'' MS Thesis, Texas A & M University (May 1999).
30. K. Kneipp, R. R. Dasari, and Y. Wang, ''Near-infrared surface-enhanced Raman scattering (NIR SERS) on colloidal silver and gold,'' *Appl. Spectrosc.* **48**, 951–955 (1994).
31. K. Kneipp, Y. Wang, R. R. Dasari, and M. S. Feld, ''Approach to single molecule detection using surface-enhanced resonance Raman scattering (SERRS): a study using rhodamine 6G on colloidal silver,'' *Appl. Spectrosc.* **49**, 780–784 (1995).
32. A. Henglein, ''Reactions of organic free radicals at colloidal silver in aqueous solution. Electron pool effect and water decomposition,'' *J. Phys. Chem.* **83**, 2209–2216 (1979).