Published in final edited form as:

Anal Bioanal Chem. 2011 June ; 400(7): 2013–2023. doi:10.1007/s00216-011-4956-9.

Collection, storage, and electrophoretic analysis of nanoliter microdialysis samples collected from awake animals in vivo

Meng Wang,

Department of Chemistry, University of Michigan, 930 North University Avenue, Ann Arbor, MI 48109, USA

Neil D. Hershey,

Department of Chemistry, University of Michigan, 930 North University Avenue, Ann Arbor, MI 48109, USA

Omar S. Mabrouk, and

Department of Chemistry, University of Michigan, 930 North University Avenue, Ann Arbor, MI 48109, USA

Robert T. Kennedy

Department of Chemistry, University of Michigan, 930 North University Avenue, Ann Arbor, MI 48109, USA

Department of Pharmacology, University of Michigan, Ann Arbor, MI 48109, USA

Robert T. Kennedy: rtkenn@umich.edu

Abstract

Microdialysis sampling is an important tool for chemical monitoring in living systems. Temporal resolution is an important figure of merit that is determined by sampling frequency, assay sensitivity, and dispersion of chemical zones during transport from sampling device to fraction collector or analytical system. Temporal resolution has recently been improved by segmenting flow into plugs, so that nanoliter fractions are collected at intervals of 0.1–2 s, thus eliminating temporal distortion associated with dispersion in continuous flow. Such systems, however, have yet to be used with behaving subjects. Furthermore, long-term storage of nanoliter samples created by segmented flow has not been reported. In this work, we have addressed these challenges. A microdialysis probe was integrated to a plug generator that could be stably mounted onto behaving animals. Long-term storage of dialysate plugs was achieved by collecting plugs into high-purity perfluoroalkoxy tubes, placing the tube into hexane and then freezing at -80°C . Slow warming with even temperatures prevented plug coalescence during sample thawing. As a demonstration of the system, plugs were collected from the striatum of behaving rats using a 0.5-mm-long microdialysis probe. Resulting plugs were analyzed 1–4 days later by chip-based electrophoresis. To improve throughput of plug analysis over previous work, the speed of electrophoretic separation was increased by using forced air cooling and 1-butyl-2,3-dimethylimidazolium tetrafluoroborate as a separation buffer additive, allowing resolution of six neuroactive amino acids in 30 s. Concentration changes induced by K^{+} microinjections were monitored with 10 s temporal resolution. The improvements reported in this work make it possible to apply segmented flow microdialysis to the study of behaving animals and enable experiments where the analytical system cannot be placed close to the animal.

Keywords

Behaving; Electrophoresis; Microdialysis; Offline analysis; Plug storage; Segmented flow; Spatial resolution; Temporal resolution

Introduction

Microdialysis is a widely used *in vivo* sampling technique that has become especially useful for studying brain function and pathologies. When coupled to analytical methods such as high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE), microdialysis is an effective approach to characterize dynamics of neurochemical concentration in response to stimuli [1, 2]. An important figure of merit for *in vivo* monitoring is temporal resolution. Because neurotransmitter release events related to behavior or disease states are often observed on the order of seconds [3, 4], it is necessary that monitoring methods have comparable temporal resolution in order to accurately follow these dynamics. In state-of-the-art technology, an inherent limitation to obtaining high temporal resolution with microdialysis is Taylor dispersion, i.e., broadening of concentration zones due to flow and diffusion, during transfer from the dialysis probe to the analytical system or fraction collector [5]. Although temporal resolution of 3–30 s using microdialysis has been reported [6–11], these studies were generally performed using high sampling flow rate (e.g., over 1 $\mu\text{l}/\text{min}$), anesthetized subjects, or short, small-bore connector capillaries. These conditions suppress Taylor dispersion, but they sacrifice other useful features such as higher relative recovery for better sensitivity that requires low sampling flow rate (50–300 nL/min) [12] or the possibilities of conducting behavioral experiments or clinical applications where long and larger bore connector capillaries are necessary [6, 13].

Use of segmented flow coupled with microdialysis can reduce Taylor dispersion [5]. In this approach, a dialysate stream is converted into nanoliter sample plugs carried by an immiscible oil phase to prevent mixing between adjacent plugs during transport or storage. This approach allows temporal resolution that is largely independent of flow rate and tubing inner diameter (i.d.) or length [14].

Segmented flow microdialysis has been coupled to microchip CE to monitor dynamics of neuroactive amino acids from anesthetized animals. In an online method, temporal resolution was limited by CE separation time, which was 25–50 s [15]. With offline analysis (i.e., fraction collection separate from analysis), higher temporal resolution, such as 9 s *in vivo*, could be obtained by allowing more time for analysis of collected samples [14]. Offline analysis is also desirable if the analytical instrument cannot be located near the subject as might occur in collaborative projects or clinical applications [13].

Despite the usefulness of offline segmented flow microdialysis, several practical issues have not been addressed. While dialysate plugs could be collected in tubing and analyzed shortly after, methods for storing these sample fractions over a long time (e.g., several days) has not been developed. Previous *in vivo* applications of segmented flow microdialysis have been carried out with anesthetized animals [5, 14, 15], but demonstration on awake animals, which is necessary for behavioral studies, has yet to be shown. It is also desirable to improve CE conditions so as to increase throughput of offline analysis.

In this paper, we report progress in these directions. A procedure to preserve uniform segmented flow format in high-purity perfluoroalkoxy (HPFA) tubing after freezing dialysate plugs at -80°C was found. A dialysis/plug sampler [14] was integrated into a headpiece that is robust enough to be mounted onto behaving rats. Electrophoresis times

were reduced to 30 s using higher electric field, chip cooling, and novel buffer additives. In addition, to achieve both high temporal and spatial resolution, we characterized microdialysis probes with only 0.5 mm active sampling length. By using segmented flow, low sampling flow rate (50 or 100 nl/min) compatible with small probes could be used while temporal resolution was still better than 15 s.

Experimental

Reagents and chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except the salts for artificial cerebral spinal fluid (aCSF) were from Fisher Scientific (Chicago, IL) and naphthalene-2,3-dicarboxaldehyde (NDA) was from Invitrogen (Eugene, OR). All aqueous solutions were prepared with water purified and deionized to 18 M Ω resistivity using a Series 1090 E-pure system (Barnstead Thermolyne Cooperation, Dubuque, IA). Amino acid standards were dissolved in aCSF (145 mM NaCl, 2.68 mM KCl, 1.01 mM MgSO₄, 1.22 mM CaCl₂, 1.55 mM Na₂HPO₄, 0.45 mM NaH₂PO₄, pH 7.4). Octadecyltrichlorosilane (OTCS) was stored in a desiccator and was opened in dry N₂ atmosphere in an Aldrich Atmosbag (St. Louis, MO).

Microdialysis probes

Side-by-side microdialysis probes with 18 kDa molecular weight cutoff membranes made from regenerated cellulose hollow fibers were made in-house as described elsewhere [16]. Probes had 200 μ m diameter, 0.5 mm sampling length, and 40 μ m i.d. $\times$ 100 μ m outer diameter (o.d.) fused silica capillaries for inlet and outlet tubing. Microdialysis probes were integrated with a guide cannula (Plastics One, Roanoke, VA) for later fixation onto the skull of behaving rats.

Headpiece for awake animals

Samples were segmented and reagents added as the dialysate emerged from the probe using a microfluidic chip. Details of the fabrication procedure and dimensions of the polydimethylsiloxane (PDMS)-based dialysis/plug sampler, as shown in Fig. 1 (right), can be found in previous report [14]. To make the system compatible with behaving animals, tubing on the opposite side to the microdialysis probe [including capillaries for the delivery of FC-77, NDA, and cyanide and HPFA tubing (Upchurch Scientific, Oak Harbor, OR) for plug transfer] were threaded through a hole drilled at the bottom of a 0.6 or 1.5 ml Fisher clear microcentrifuge tube (cap removed) until the PDMS chip was loosely stuck in the middle of the tube. The hole was then sealed with Loctite super glue gel (Henkel Consumer Adhesives, Avon, OH) before injecting uncured PDMS mixture into the tube with a syringe until the whole tube was filled with PDMS. The whole device was left in room temperature for two days or until PDMS was fully cured (see Fig. 1, middle).

Segmented flow CE chip

The electrophoretic chip for analysis of segmented flow was the same as described before [15]. Selective patterning of channel surface with OTCS to create an hydrophobic segmented flow channel was also the same as that described before [15, 17, 18].

Temporal resolution and relative recovery

To measure temporal resolution, dialysate was collected at 0.05 to 0.6 μ l/min sampling flow rate and mixed online with NDA and cyanide solutions [14, 15, 19]. At each flow rate, step change of the concentration of a mixture of amino acid standards [γ -aminobutyric acid (GABA), taurine (Tau), serine (Ser), glycine (Gly), glutamic acid (Glu), and aspartic acid

(Asp)] from 10 to 50 μM was made at the surface of the probe by rapidly spiking the solution with amino acids. The total fluorescence intensity of plugs was recorded downstream on HPFA tubing. Temporal resolution was defined as the time the signal ranged from 10% to 90% of the maximum signal. Relative recovery at each sampling flow rate was determined as the ratio of fluorescence intensity by microdialysis sampling 10 μM standards to the fluorescence intensity by directly infusing 10 μM standards.

Electrophoresis conditions

The operational scheme of segmented flow electrophoresis chip as well as the configuration of epi-confocal microscope for laser-induced fluorescence (LIF) detection have been reported before [15]. The chip uses a separation length of 11 cm in a channel that is $42\ \mu\text{m} \times 6\ \mu\text{m}$ deep. Fisher brand Tite-Seal caps with Pt electrodes fixed in the middle were used to cover buffer reservoirs. To prevent arcing at the high voltages used, exposed metal parts (e.g., electrodes or metal alligator clips) were wrapped by polyvinyl chloride electrical insulating tape and a $1'' \times 1''$ piece of glass was placed vertically between gating buffer reservoir and electrophoresis waste reservoir where positive and negative voltages were applied. To suppress Joule heating caused by high electric field, nitrogen gas was forced along the bottom of the chip. Initial separation buffer consisted of 10 mM sodium tetraborate at pH 10. Hydroxyl- β -cyclodextrin (HP- β -CD), HP- γ -CD, 1-butyl-2,3-dimethylimidazolium tetrafluoroborate, or tetrabutylphosphonium methanesulfonate was added to the initial buffer at different concentrations for separation optimization. Resolutions between neuroactive amino acids and possible interference from other amino acids in dialysate were evaluated at each buffer composition using 10 μM amino acids standard solution.

Storage of dialysate plugs and test of sample stability

A 30 cm length of $150\ \mu\text{m}$ i.d. $\times$ $360\ \mu\text{m}$ o.d. HPFA tubing was used to collect dialysate plugs. After collection, the ends of the tubing were sealed by capillary wax (Hampton Research, Aliso Viejo, CA). The tubing was coiled and placed in a 100 ml glass bottle filled with degassed hexane. The orientation of the coiled capillary was not found to have an effect on the performance of the device. A stir bar was placed in the bottle for use in future thawing step. The bottle was then wrapped in aluminum foil to keep out light and stored in -80°C freezer.

Offline CE was performed on selected days after sample collection. On the day of analysis, the bottle containing tube of sample plugs was taken out of the freezer and immediately immersed in a beaker filled with room temperature water. The beaker was placed on a stir plate to constantly stir hexane in the bottle using the pre-placed stir bar. After 10–15 min, the bottle was removed from the water bath. The tube with plugs was removed from the bottle and connected to another syringe filled with FC-77. The plug train was pumped onto CE chip at 100 or 200 nl/min for analysis.

To test the stability of NDA-tagged amino acid standards (NDA-AAAs), 10 μM standard solution was derivatized and was either stored at -80°C or room temperature (also immersed in hexane and kept in dark). Peak height of each amino acid on 1–3 days after sample collection was recorded and compared. With NDA labeled *in vivo* dialysate, measurements were performed through up to 8 days after sample collection. Calibration curve was made on each analysis day to quantify the concentration of amino acids over storage period.

Surgery and in vivo experiments

In vivo microdialysis experiments with awake rats were performed on male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 250–350 g. For implantation of a guide cannula, rats were anesthetized with a combination of ketamine (65 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and medetomidine (0.5 mg/kg; Pfizer Animal Health, New York, NY) intraperitoneally. Using aseptic technique, a guide cannula was implanted in the right hemisphere anterior +1.0 mm from bregma, lateral +2.0 mm from midline, ventral –5.5 mm from dura [20] and secured in place with dental cement (A-M Systems, Sequim, WA). The guide cannula was sealed by means of a solid metal stylet (Plastics One). Animals were allowed to recover for 2–3 days before experimentation, which was found sufficient for our application. Longer periods may be desirable for more physiological experiments.

On the day of experiment, the animal was weighed, briefly anesthetized with isoflurane (Baxter Healthcare, Deerfield, IL), and the dialysis/plug sampler headpiece was inserted with the probe extending 5 mm past the tip of the guide cannula. The animal was placed in a Ratturn (Bioanalytical Systems, West Lafayette, IN) to prevent the headpiece from being disturbed by normal movements of the animal (Fig. 1, left). The system was equilibrated by perfusing aCSF at 0.1 μ l/min flow rate through the probe for 1 h before beginning measurements. 200 nl of 75 mM potassium aCSF was delivered into the brain through a microinjector (4.5 cm long $\times$ 20 μ m i.d. $\times$ 90 μ m o.d. fused silica capillary) by applying 80 psi air pressure for 4 s using an electrically actuated solenoid (Picospritzer, General Valve, Fairfield, NJ). The microinjector was pre-filled with test solution and glued next to the dialysis probe as shown in Fig. 1 (middle).

Results and discussion

Dialysis/plug sampler headpiece for awake animals

The dialysis/plug sampler headpiece used in this work was similar to that used previously but engineered to be suitable for mounting on behaving animals [14]. As before, the dialysate inlet and derivatization reagent outlets were placed on opposite sides of the segmented flow channel (Fig. 1, right). In this way, when the microdialysis probe is placed downward into the brain of the animal, the other fluid access tubing is brought out from the top and therefore does not interfere with animal movement. To give strength to the device and prevent breakage during animal movement, we encased the whole chip in a microcentrifuge tube (Fig. 1, middle). The total weight of the device was 2–3 g, which is light enough to be carried by the rat on the head (Fig. 1, left). The encasing PDMS was cured under room temperature to prevent shrinkage of HPFA tubing by heat, which was found to be the major cause of leakage at the HPFA–PDMS connection and failure of the device. With these precautions, the dialysis/plug sampler headpiece proved to be robust, and we experienced no device failures with animals drinking, feeding, grooming, burrowing, and moving about the cage. It is likely that the device could be used in more active behaviors, such as mating, similar to regular microdialysis probes. In principle, the same approach could be used with commercially available microdialysis probes; however, we used probes fabricated in-house because of the flexibility of different probe lengths and lower dead volume that improves temporal resolution.

Temporal resolution and relative recovery of 0.5 mm microdialysis probes

Microdialysis sampling is generally considered to have poor spatial resolution due to its large probe size, i.e., 1–4 mm in length, which makes it impractical to accurately implant a dialysis probe in brain regions smaller than a few mm^3 . In traditional continuous flow microdialysis, shorter probe (e.g., <1 mm) would result in lower relative recovery and lower sensitivity. Although this can be partially compensated by using lower sampling flow rate

(e.g., 50–300 nL/min), temporal resolution is generally worsened due to excessive Taylor dispersion at low flow rate and after long transport time. On the contrary, segmented flow microdialysis could maintain good temporal resolution even at low flow rate, which makes it suitable to be coupled to miniaturized microdialysis probes. In this work, we used microdialysis probes that have only 0.5 mm sampling length. As shown in Fig. 2a, at 0.05–0.6 $\mu\text{L}/\text{min}$ sampling flow rates, temporal resolution of 3–15 s and relative recovery of 10–60% could be obtained. Notably, at 0.05 $\mu\text{L}/\text{min}$, relative recovery was reasonably high at ~60%, which generated sufficient analyte concentration in the dialysate, and temporal resolution was still better than 15 s. When we compared temporal resolutions with 0.5, 2, and 3 mm microdialysis probes (Fig. 2b), they all fell between 2 and 25 s, although within different flow rate ranges. Smaller probes had better temporal resolution than large ones under low flow rates, because temporal resolution depended only upon dispersion within the probe itself [5]. These results demonstrate that with segmented flow, miniaturized microdialysis probes can be used to improve spatial resolution without sacrificing temporal resolution or relative recovery.

CE resolution of NDA-AAs

NDA is an attractive fluorogenic reagent to label amino acids and primary amines in dialysate for CE–LIF detection, because its derivatives exhibit good stability, high quantum yields, and fast reaction kinetics (120–240 s) [10, 19, 21–23]. Compared to another widely used fluorogenic reagent *o*-phthalaldehyde (OPA), NDA offers 10- to 50-fold improvement in detection sensitivity [10, 19, 24, 25]. This is especially useful in LIF detection on microchip, where detection limits are generally higher than capillary-based systems [25–27]. However, in contrast to a free zone CE method to successfully resolve neuroactive OPA-AAs such as GABA, glutamate, aspartate, taurine, and glycine in vivo [25], reports of rapid and satisfactory CE separation of NDA-AAs have been scarce. A micellar electrokinetic chromatography (MEKC) method was proven effective in simultaneously monitoring those neurotransmitters in vivo by capillary-based system [22]. However, from our experience, it was not very suitable for microchip CE, which only holds a small volume of buffer supplies in its reservoirs, as analyte migration order in MEKC can be sensitive to slight changes in buffer composition or pH during operation. Other reported methods based on free zone CE of NDA-AAs in vivo have only been able to resolve a subset of these compounds [10, 15, 28, 29].

We searched for a CE buffer condition that was capable of simultaneously resolving all the NDA-AAs mentioned above in vivo. Initially, we found that 10 mM sodium tetraborate buffer at pH 10 without any additives roughly met our needs, although resolution in GABA/serine and taurine/glycine pairs were less than 1.5 [14] (Fig. 3a, bottom). In order to improve resolution, cyclodextrins (HP- β -CD or HP- γ -CD) and ionic liquids (IL) [30, 31] (1-butyl-2,3-dimethylimidazolium tetrafluoroborate or tetrabutylphosphonium methanesulfonate) were added at different concentrations to the initial borate buffer. When cyclodextrins were used as buffer additive, baseline separation of neuroactive NDA-AAs could be achieved in vitro under several conditions (e.g., 0.2 mM HP- β -CD, 0.2 or 0.7 mM HP- γ -CD). However, in vivo detection was still impossible due to interference from other amino acids contained in the dialysate (data not shown). On the other hand, 10 mM 1-butyl-2,3-dimethylimidazolium tetrafluoroborate (abbreviated as IL in Fig. 3) could improve resolutions of GABA/serine and taurine/glycine pairs (Fig. 3a, top) without significantly altering the migration order of amino acids compared to plain borate buffer. Test with the separation of 21 NDA-AAs in vitro using this buffer condition also suggested that no interference from other amino acids would be expected in vivo (Fig. 3b). Therefore, 10 mM sodium tetraborate with 10 mM 1-butyl-2,3-dimethylimidazolium tetrafluoroborate at pH 10

was chosen as the electrophoresis buffer. Future improvement in separation condition or detector might allow other amino acids and amines to be resolved and detected.

To accelerate separation and increase analysis throughput, electric field up to 1.1 kV/cm (−14 kV separation voltage and +10 kV gating voltage) was applied after taking precautions to avoid arcing (Fig. 4a). The effect of Joule heating observed above 650 kV/cm was eliminated with forced gas cooling as shown in Fig. 4b. Under these conditions, separations could be achieved in 30 s (Fig. 4c). At even higher electric field, however, electrolysis at the surface of Pt electrodes became more significant and rendered continuous operation unstable.

Conditions for freezing segmented samples

The dialysis/plug sampler used in our experiment could generate 2 nl plugs with 5 nl oil gaps. Therefore, an array of ~760 dialysate plugs could be stored in a 30 cm length of 150 μm i.d. HPFA tubing, which corresponded to ~15 min sampling at 0.1 $\mu\text{l}/\text{min}$ sampling flow rate (or 3 min at 0.5 $\mu\text{l}/\text{min}$). Such sample collection time would be enough for the study of fast neurotransmitter dynamics in response to short infusions of chemical agents into the brain.

In order to preserve neurotransmitters in these dialysate fractions for later offline analysis, they should be stored at low temperature, preferably frozen at -80°C [32]. However, freezing and thawing nanoliter plugs segmented by oil poses challenges and special procedures were necessary to keep uniform plugs during or after freezing. Placing the tube of plugs directly in the freezer often resulted in air bubbles in either aqueous or oil phase (FC-77) after thawing (Fig. 5a). We hypothesized that this was due to air permeable HPFA allowing gas exchange during freeze–thaw. To avoid this effect, we stored the tube in a liquid filled glass container to prevent such exchange. Hexane was chosen as the liquid because it does not freeze at -80°C and is immiscible with either aqueous plugs or the carrier phase. This property eliminates the possibility of sample concentration change by water evaporation through HPFA walls to the storage media during long-term storage. It was found that after storing plug tubes in degassed hexane, no air bubbles were observed after thawing (Fig. 5b and c).

Another problem was plug coalescence due to plugs moving at different velocities during thaw. This phenomenon was believed to be due to uneven heating rate in different sections of the tube that caused temperature-induced flow. Therefore, ensuring even and steady temperature increase throughout the whole tube during the thawing process was essential in reducing or eliminating coalescence problem. To achieve this, the hexane filled bottle was placed in a water bath and stirred to prevent rapid and uneven temperature changes. With these procedures, uniform segmented flow format could be observed throughout most, if not all, parts of the tube (Fig. 5b).

FC-77 has a freezing point of -95°C making it suitable for storage at -80°C . We also tested other commonly used carrier phases such as FC-40, FC-70, FC-3283, and perfluorodecalin, which all have a freezing point higher than -80°C and would therefore freeze together with aqueous plugs. We found that a nonfreezing oil like FC-77 had a higher success rate of keeping uniform plugs throughout the tube than oils that freeze at -80°C . This observation can probably be explained by the abrupt volume change during solid–liquid phase conversion. FC-77 would not undergo such phase conversion, thus reducing the possibility of plug movement or coalescence.

The viscosity of the carrier phase also has an impact on plug uniformity. When plugs were stored at -20°C , FC-70 (f.p. -25°C), FC-40 (f.p. -50°C), and FC-3283 (f.p. -50°C) could

also be used as nonfreezing carrier phases. Under such condition, we observed that more viscous oils like FC-70 resulted in higher success rate of keeping uniform plug array. This is probably because temperature-induced flow is more difficult with viscous oils, making plug coalescence less likely. However, due to large viscosity of FC-70 (24 cp) and the high back pressure it will induce in plug collection, it is unsuitable to be used with segmented flow microdialysis sampling. However, results from this study can be useful for freezing plug array in other applications where more viscous oils and higher storage temperatures (e.g., > -20°C) are feasible.

Stability of NDA-AA adducts in vitro and in vivo during long-term storage

In our experiment, we derivatized samples prior to storage. No difference in the electropherograms was observed before or after storage. We first examined the stability of NDA adducts of amino acid standards. 10 μ M amino acids including glutamate, aspartate, GABA, taurine, and glycine were derivatized by NDA/cyanide and stored in the dark either at -80°C or room temperature for 1–3 days. As shown in Fig. 6a, samples stored at -80°C remained intact throughout the period. The percentage of loss calculated from a two-sample *t*-test ($\alpha = 0.05$) was less than 7% for all the analytes. On the contrary, analysis of samples stored at room temperature presented a drastic plunge in peak height only after 1 day (Fig. 6b).

We then examined the stability of NDA-AAs from in vivo dialysate frozen at -80°C. Figure 6c shows measured concentrations of the same set of neuroactive amino acids from the striatum of an awake rat stored over 8 days ($n = 20$ sample plugs). On the 4th day, no obvious decrease was observed in the measured concentration of GABA, taurine, or glycine, while 10% of glutamate and aspartate was degraded. After 4 days, however, degradation in low concentration analytes, such as glutamate, aspartate and GABA, became statistically significant ($p < 0.05$).

These results suggest that in order to accurately quantify the absolute concentration of amino acids in the dialysate, it is desirable to analyze fractions soon after collection. On the other hand, however, freezing NDA-derivatized amino acids could be an useful approach to extend the window of sample analysis when percent change of analyte with respect to its basal concentration is of more interest (i.e., analyte's basal concentration acts as the internal standard), which is often the case in many in vivo applications. This is based on the assumption that the decomposition rate of an NDA-AA adduct is independent of the concentration of the amino acid. Under such situation, dialysate could be stored for up to 4 days, without severe degradation in amino acids that might harm their detectability.

Offline measurements of K⁺ stimulated neurotransmitter efflux in the striatum of behaving rats

To demonstrate the suitability of the offline method for monitoring rapid changes, we microinjected potassium ions into the striatum of awake rats and analyzed the responses using offline CE method after sample storage. 75 mM K⁺ was microinjected over 4 s into the brain while sampling with a 0.5 mm probe at 0.1 μ l/min. Under these conditions, temporal resolution was 9.5 s and relative recovery was ~30%. Dialysate fractions were stored for 1–4 days and then pumped into the electrophoresis chip at 0.1 μ l/min. As the total flow rate of segmented flow during sample collection was 0.7 μ l/min, the analysis time was six times greater than collection time. Figure 7 shows in vivo electropherograms of dialysate collected at basal (A) and levels obtained during K⁺ stimulation (B). Basal dialysate concentrations of glutamate, aspartate, GABA, taurine, and glycine in awake rats were found to be 0.3 ± 0.17 , 0.19 ± 0.07 , 0.20 ± 0.05 , 1.7 ± 1.1 , and 1.3 ± 0.4 μ M (mean $\pm$ SEM,

$n = 4$), respectively, which were in good agreement with previous reports [33, 34] when accounting for the differences in relative recovery in different works.

Figure 8 shows responses of glutamate (A), aspartate (B), GABA (C), taurine (D), and glycine (E) upon K^+ microinjection. On average, extracellular level was elevated by $1,570 \pm 90\%$ in glutamate, $530 \pm 80\%$ in aspartate, $7,000 \pm 1,400\%$ in GABA, $180 \pm 40\%$ in taurine, and $180 \pm 20\%$ in glycine (mean $\pm$ SEM, $n = 4$). For glutamate, aspartate, GABA and glycine, it took ~ 10 s to reach peak concentrations, which was the same as in our previous report [14] using similar experiment procedures with anesthetized rats. The increase induced in taurine, however, was slower both in the time to show the increase after injection and in the time it took to reach peak concentration. The results reveal differences in the dynamics of neurochemicals in response to the same stimulation.

Interestingly, the smaller probes used here resulted in significant differences from experiments performed following microinjection at larger probes. For example, in our previous report using 2 mm probes, no increase in taurine was observed, while glutamate and GABA were elevated to a few hundred percentage relative to basal level [14]. Using 0.5 mm probes in this work, however, not only $\sim 180\%$ increase in taurine became obvious, measured release of glutamate and GABA also increased to a few thousand percentage of basal level. Since the former tests were done with anesthetized rats, we repeated the procedure on awake rats using a slightly different CE system and obtained the same results (data not shown), which suggests that anesthetization was not the main reason leading to such differences. We speculate that this is because smaller probes can focus the sampling region to the center of the liquid stimulus sphere resulted from microinjection, where the strongest response was likely to be induced, while larger probes average the responses from both the center and the peripheral regions of the stimulus sphere. This further suggests that using miniaturized probes with improved spatial resolution is useful in revealing true dynamics induced by locally applied stimuli.

Conclusions

In this report, we have discussed improvements made in some practical aspects of offline segmented flow microdialysis. Demonstration of the technique with awake animals extended its application to behavioral studies. By using miniaturized probes, spatial resolution was improved without sacrificing temporal resolution or sensitivity. Combining offline fraction collection and long-term storage of samples allows animal experiments to be separated from the analytical measurements in both time and space. Thus, conditions for both types of experiments can be optimized. Work flow efficiency may be improved because both aspects of the experiment (animal and analytical) do not need to be working at the same time. Finally, collaborations are facilitated by allowing both parts of the experiments to be performed in different locations.

Acknowledgments

Meng Wang was supported by the American Chemical Society, Division of Analytical Chemistry Fellowship, sponsored by the Society for Analytical Chemists of Pittsburgh. This work was supported by National Institutes of Health grant R37 EB003320 (RTK).

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Fig. 1. Illustration of the headpiece for segmented flow microdialysis on behaving animals. *Left:* Picture of a behaving rat with the headpiece mounted on his head during experiment. *Middle:* Picture of finished headpiece after encasing dialysis/plug sampler with guide cannula in a microcentrifuge tube (with enlarged picture of the sampling area of a 0.5 mm probe with a microinjector). *Right:* Schematic drawing of channel network and connection tubing for the dialysis/plug sampler

Fig. 2.

a Dependence of temporal resolution and relative recovery on sampling flow rate using 0.5 mm microdialysis probes. **b** Comparison of temporal resolution of 0.5, 2, and 3 mm microdialysis probes at different flow rate. In both **a** and **b**, water fraction was 0.29. $n = 3$ and *error bars* indicate standard deviation

Fig. 3. Optimization of CE separation buffer. **a** Comparison of separation of NDA-AA standards in 10 mM sodium borate buffer at pH 10 with different concentrations of 1-butyl-2,3-dimethylimidazolium tetrafluoroborate (abbreviated as IL) as additive. 10 mM IL (underlined) was determined to be the optimum concentration. Peak identities were labeled in 10 mM IL electropherogram as an example. **b** Electropherogram of 21 NDA-AA standards using optimized separation buffer. Electric field was 720 V/cm. Separation distance was 11 cm. Injection time was 0.08 s

Fig. 4.

Optimization of CE electric field. **a** Serial electropherograms of seven amino acid standards at different electric field. **b** Dependence of current on electric field with or without air cooling. **c** Electropherogram of seven amino acid standards at 1.1 kV/cm electric field. Other conditions were the same as in Fig. 3

Fig. 5.

Brightfield pictures of plugs in HPFA tubing after freezing at -80°C overnight. **a** Air bubbles were formed in carrier phase when tube of plugs were directly placed in the freezer. **b** Segmented flow remained uniform after storage with optimal procedures described in text. Carrier phase was FC-77. **c** Picture of HPFA tubing filled with plugs immersed in hexane in a glass container with pre-placed stir bar

Fig. 6. Stability of NDA-AAs after storage under different conditions. **a** Peak height of 10 μ M NDA-AA standards stored at -80°C for 3 days. **b** Peak height of the same standards stored at room temperature. **c** Measured concentration of amino acids in in vivo dialysate after derivatized by NDA/cyanide and stored at 80°C for 8 days. Measurements were made on the 2nd, 4th, 6th, and 8th days after sample collection. Dialysate was collected from the striatum of a awake rat using 0.5 mm microdialysis probe at 0.1 $\mu\text{l}/\text{min}$ sampling flow rate. Data was the average of at least 20 electropherograms, while sample plugs were pumped at 0.2 $\mu\text{l}/\text{min}$ into the CE chip. Error bar = ± 1 standard deviation

Fig. 7.

In vivo electropherograms collected from the striatum of behaving rats at A basal level; B elevated level during K^+ stimulation. *Insets* show enlarged electropherograms in the *dotted box*. Sampling flow rate was 0.1 μ l/min with 0.5 mm microdialysis probe. Separation conditions were the same as in Fig. 4c

Fig. 8. The effect of microinjecting 200 nl 75 mM potassium on glutamate (a), aspartate (b), GABA (c), taurine (d), and glycine (e). Data are expressed as percentage of basal level (mean $\pm$ SEM, $n = 4$). Black arrows indicate the start of microinjection