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Complete List of Authors:	Gunawardhana, Shamal; University of Kansas, Adams Institute for Bioanalytical Chemistry; University of Kansas, Department of Chemistry Lunte, Susan; University of Kansas, Adams Institute for Bioanalytical Chemistry; University of Kansas, Department of Chemistry; University of Kansas, Department of Pharmaceutical Chemistry

Continuous monitoring of adenosine and its metabolites using microdialysis coupled to microchip electrophoresis with amperometric detection

Shamal M. Gunawardhana[a,b] and Susan M. Lunte[a,b,c]*

[a] Ralph N. Adams Institute for Bioanalytical Chemistry, University of Kansas, Lawrence, KS, USA
[b] Department of Chemistry, University of Kansas, Lawrence, KS, USA
[c] Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS, USA

* Corresponding author

Abstract

Rapid monitoring of concentration changes of neurotransmitters and energy metabolites is important for understanding the biochemistry of neurological disease as well as for developing therapeutic options. This paper describes the development of a separation-based sensor using microchip electrophoresis (ME) with electrochemical (EC) detection coupled to microdialysis (MD) sampling for continuous on-line monitoring of adenosine and its downstream metabolites. The device was fabricated completely in PDMS. End-channel electrochemical detection was accomplished using a carbon fiber working electrode embedded in the PDMS. The separation conditions for adenosine, inosine, hypoxanthine, and guanosine were investigated using a ME-EC chip with a 5-cm long separation channel. The best resolution was achieved using a background electrolyte consisting of 35 mM sodium borate at pH 10, 15% dimethyl sulfoxide (DMSO), and 2 mM sodium dodecyl sulphate (SDS), and a field strength of 222 V/cm. Under these conditions, all four purines were separated in less than 85 s. Using a working electrode detection potential of 1.4 vs Ag/AgCl, the limits of detection were 25, 33, 10, and 25 μM for adenosine, inosine, hypoxanthine, and guanosine, respectively. The ME-EC chip was then coupled to microdialysis sampling using a novel all-PDMS microdialysis-microchip interface that was reversibly sealed. This made alignment of the working electrode with the end of the separation channel much easier and more reproducible than could be obtained with previous MD-ME-EC systems. The integrated device was then used to monitor the enzymatic conversion of adenosine to inosine *in vitro*.

Introduction

The adenosine family of compounds is comprised of many important biologically active substances, including DNA, RNA, and adenosine triphosphate (ATP), that are critical for many key biological pathways and are involved in several disease states.[1,2] Due to their important role in cellular energy metabolism, neuronal signaling and neuromodulation, adenosine and its up- and downstream metabolites such as ATP, inosine, and hypoxanthine have been considered as biomarkers for cerebral ischemia, a condition commonly developed during traumatic brain injury and stroke.[3,4] As such, significant attention has been directed toward monitoring their extracellular concentrations in the brain.[5,6] Fast scan cyclic voltammetry and enzyme-modified amperometric sensors have been explored to monitor the extracellular concentration of adenosine,[7-10] ATP,[11] and hypoxanthine.[12] However, these methods are generally capable of monitoring only one compound at a time. To better understand the biochemistry of brain injury and stroke, as well as to develop better therapeutic options, a method that is capable of simultaneous monitoring of extracellular concentration changes of multiple biomarkers is necessary. For adenosine and its major downstream metabolites, this has generally been achieved using microdialysis sampling followed by off-line analysis by liquid chromatography (LC) with UV detection,[13,14] fluorescence detection,[15] or mass spectrometry.[16]

Microdialysis is a continuous sampling technique that has been widely used both *in vivo* and *in vitro*.[17,18] Because sampling is based on analyte diffusion across a membrane, the dialysate sample contains only small molecules and excludes proteins. To maximize analyte recovery across the membrane, sampling is performed at low flow rates, typically from 0.1 to 2 μL/min.[18] However, since most conventional separation-based methods of analysis require several microliters (5–20) of sample, the temporal resolution that can be obtained using off-line analysis is generally between 5 and 30 min. In contrast, the sample requirements for capillary and microchip electrophoresis are in the low nanoliter to picoliter range, making it possible to analyze smaller sample volumes and, therefore, obtain better temporal resoluton.[17] In addition, ME separations are significantly faster than similar separations performed using conventional liquid chromatography or capillary electrophoresis. For this reason, microchip electrophoresis is considered an excellent candidate for on-line near real-time analysis of microdialysis samples.

ME has improved significantly since its introduction in the early 1990s, and is now a well-established analytical tool in the field of separation science.[19] To date, several excellent reviews have been published that discuss various aspects and recent developments in microchip electrophoresis separations.[20-23] In particular, ME has been widely employed for biomedical applications.[22,23] The planar format of ME enabled the development of microchip electrophoresis-based micro-total-analysis systems, where all the necessary components required for chemical analysis are integrated into a single device.[24] The planar separation platform of ME also facilitates coupling of the microchip with a variety of detection techniques, including laser-induced fluorescence (LIF),[22] mass spectrometry (MS),[25] and electrochemical detection,[26] as well as sampling techniques such as microdialysis and push-pull perfusion.[17,27] LIF is the most common detection technique employed for ME; however, analytes must be naturally fluorescent or be derivatized with a fluorophore in order to be determined by this method. Mass spectrometric detection is hampered by the high salt content of the electrophoresis buffer, and the instrumentation is fairly large and expensive. LIF and MS detection also require sophisticated optics and interfaces, respectively, for integration with ME separation platforms.

Electrochemical detection, particularly amperometric detection, has some distinct advantages over other methods of detection for ME. The instrumentation required for amperometric detection is relatively simple and inexpensive. Direct integration of the electrodes and associated electronics with ME separation systems can be accomplished without the use of sophisticated interfaces or optics. This feature makes it possible to miniaturize the ME-EC system for use in point-of-care applications or on-animal sensors.[28-30] Microdialysis coupled to ME-EC has previously been employed for continuous monitoring of many biologically important analytes including catecholamines and reactive oxygen and nitrogen species such as hydrogen peroxide and nitrite.[31-33]

In this paper, we report the development of a separation-based sensor for the continuous monitoring of adenosine and its metabolites using microdialysis sampling coupled to microchip electrophoresis with electrochemical detection. The ultimate goal of this study is to employ the sensor to continuously monitor concentration changes of adenosine and its metabolites in brain extracellular fluid after severe traumatic brain injury.

Materials and methods

Chemicals

The following chemicals were purchased from the designated sources and used as received: adenosine, inosine, hypoxanthine, guanosine, boric acid, monosodium phosphate, disodium phosphate and dimethylsulfoxide (Sigma Aldrich, St. Louis, MO, USA), NaOH, H_2SO_4, HCl, acetonitrile, and 2-propanol (IPA) (Fisher Scientific, Fairlawn, NJ, USA); sodium dodecyl sulfate (SDS) (Thermo Scientific, Waltham, MA, USA); SU-8 10 and SU-8 developer (Micro-Chem, Newton, MA, USA); and poly(dimethyl siloxane) and curing agent (Sylgard 184 silicon elastomer base and curing agent, Dow Corning Corp., Midland, MI, USA). The 33-μm diameter carbon fibers (Avco Specialty Materials, Lowell, MA, USA), copper wire (22 gage. Westlake Hardware, Lawrence, KS, USA); epoxy (J-B Weld, Sulphur Springs, TX, USA), and colloidal silver liquid (Ted Pella, Inc., Redding, CA, USA) were used for electrode preparation. Solutions were prepared in 18.2 (MΩcm) water (Millipore, Kansas City, MO, USA). Adenosine deaminase was purchased from Calzyme Laboratories, Inc. (San Luis Obispo, CA, USA) and stored in a –20 °C freezer until used.

PDMS microchips fabrication

All PDMS microchips and electrode substrates were fabricated at the Ralph N. Adams Institute Microfabrication Facility at the University of Kansas. A detailed description of the microchip fabrication procedure can be found elsewhere.[34] Briefly, a 15 μm thick layer of SU-8 10 negative photoresist was spin coated using a Cee 100 spin coater (Brewer Science, Rolla, MO, USA) onto a 4-inch diameter silicon wafer. The wafer was then soft baked for 2 min at 65 °C followed by 5 min at 95 °C. To copy the microchannel design onto the photoresist layer, the silicon wafer with the negative photoresist film was covered with a transparency mask (Infinite Graphics, Minneapolis, MN, USA) with the desired microchannel design and then exposed to UV (344 mJ/cm^2) using a UV flood source (ABM Inc., Scotts Valley, CA, USA). After the photoresist was exposed to UV, the silicon master was baked at 65 °C for 1 min and at 95 °C for 2 min on a programmable hotplate (Thermo Scientific, Waltham, MA, USA). SU-8 10 developer was used to develop the silicon master, and it was rinsed with isopropyl alcohol and dried with N_2 gas prior to hard bake at 200 °C for 2 h. To fabricate PDMS microchips, a mixture of PDMS and curing agent (10:1) was poured onto the silicon master and allowed to cure overnight at 70 °C. Once

hardened, the PDMS microchips can be easily peeled from the silicon master. Simple 'T' microchips fabricated using this procedure consisted of a 5 cm long separation channel and 0.75 cm long side arms with 15 μm channel depth and 40 μm width. Double T microchips have exactly the same channel dimensions as simple T microchips, except that they also contain a 600 μm wide cross channel connected to the top of the separation channel for microdialysis interface. Sample, buffer, and waste reservoirs were cut at the ends of the microchannels using a 4 mm diameter biopsy puncher (Harris Uni-Core, Ted Pella) in the PDMS chip.

Construction of reversibly sealed MD-ME interface

The procedure used for the construction of the device is shown in Figure 1. To connect the microdialysis probe outlet to the wider microdialysis flow channel in the double T microchip, a 1.5 cm long 400 μm i.d. stainless steel tube was used. To direct the MD flow to the separation channel, a hole (500 μm inner diameter) was made at the end of the top wide flow channel of the PDMS microchip. The stainless steel tube was then inserted through this hole to direct the microdialysis flow to the microchannel. To minimize the backpressure at the chip/substrate interface, the outlet of the stainless-steel tube was kept about 2 mm above the substrate. To secure the stainless-steel tube above the microchip surface, the tube was first inserted through a thick (~1 cm) PDMS block and then the PDMS block with the tube was permanently sealed to the chip surface using plasma oxidation (Model: BD-20, Electro-technic Products, Inc., Chicago, IL, USA).

Integration of carbon fiber electrode on to MD-ME-EC PDMS platform

Carbon fiber microelectrodes (CF, 33 μm diameter) were integrated into PDMS substrates according to the procedure previously described.[35] Briefly, a trench of 33 μm depth and 4 cm length in a PDMS substrate was fabricated using the procedure described in microchips fabrication section. The PDMS substrate containing the trench was sealed onto a glass plate to add structural rigidity. The CF was then carefully rolled into the trench. One end of the CF was connected to a copper wire using silver colloidal and epoxy resin to make the electrical contact between the CF electrode and the potentiostat. Electrode platforms fabricated using this procedure last for several months and can be reused with new CF as necessary.

Microchip electrophoresis

All the ME-EC analyses were carried out using devices with either a simple T or double T configuration. Spellman CZE 1000R (Hauppauge, NY, USA) high voltage power supplies were used for all separations, and LabView (National Instruments, Austin, TX, USA) software written in-house was used to control the voltage output.

Simple T microchips

For the separation optimization experiments, a simple T microchip was used. Microchannels were flushed sequentially with isopropyl alcohol, 0.1 M NaOH, and run buffer for 5 min using negative pressure (aspirator) prior to electrophoresis separations. SDS was added to the run buffer to maintain a stable electroosmotic flow (EOF). Analytes were separated using normal polarity at 222 V/cm electric field strength. Gated injection was used for sample introduction. This was performed by floating the high voltage at the buffer reservoir for 1 s.

Double T microchips

A microchip conditioning protocol similar to that described above was used to condition the separation channel of the double T microchip. The top wider channel (MD interface channel) was conditioned with only run buffer by pumping it through the top channel using polyimide

tubing (0.127 mm ID, Index Health & Science, Tampa, FL, USA) connected to a syringe pump. A single high voltage power supply was used for the electrophoretic separation. A flow-gated sample injection method with 1900 V and injection time of 1.5 s was used for introduction of microdialysate to the separation channel.

Electrochemical detection

For both method optimization studies and on-line microdialysis sample analysis, a 3-electrode detection system was utilized for amperometric detection. Unless otherwise mentioned, BAS 4C-LC Epsilon potentiostat (Bioanalytical Systems, West Lafayette, IN, USA) was used for the electrochemical measurements. To minimize noise, end-channel detection was employed. In end-channel electrochemical detection, the CF microelectrode was aligned 5 μm downstream of the end of the microchannel. Ag/AgCl (Bioanalytical Systems) and Pt wire (1 mm diameter) were used as reference and counter electrodes.

Sample preparation and on-line analysis

Stock solutions of adenosine (2.5 mM) and inosine (2.5 mM) were prepared in ultrapure water. The hypoxanthine (2.5 mM) and guanosine (2.5 mM) stock solutions were prepared in 0.06 M NaOH. All solutions were stored at 4 °C in the dark. Working standards of adenosine (100 μM), guanosine (100 μM), hypoxanthine (50 μM), and inosine (150 μM) were prepared by diluting stock solutions with the appropriate run buffer at the time of analysis. All MD-ME-EC experiments were performed using homemade 3 cm linear microdialysis probes with 20 kDa molecular weight cut-off. The complete procedure for fabrication of linear MD probes can be found in reference [36].[36] Briefly, two polyimide tubes (163 μm O.D and 122 μm I.D and lengths of 30 cm and 15 cm, Cole-Parmer, Vernon Hills, IL USA) were glued to a 3 cm long polyacrylonitrile (PAN) membrane. The two free ends of the polyimide tubes were glued to 0.5 cm long polypropylene tubes (0.5 mm O.D) to set up the connections to syringe pump and stainless-steel guide tube. A flow rate of 1 μL/min was employed in all on-line experiments conducted to monitor the conversion of adenosine to inosine. Adenosine deaminase (ADA) enzyme was dissolved in 15 mM phosphate at pH 7.4, and the enzyme reaction mixture was kept in a water bath at 37 °C throughout the duration of the experiment. 2.5 mM guanosine was used as the internal standard.

Results and discussion

Amperometric detection

The goal of this research was to develop a method for the continuous monitoring of adenosine, inosine, hypoxanthine, and guanosine based on microdialysis sampling coupled to microchip electrophoresis with amperometric detection. Because of their role in many important biological processes, the direct electrochemical detection of purine-based compounds has gained significant attention.[37-40] However, many purine-based compounds, including the compounds targeted in this study, are oxidized at high electrode potentials.[37,38,41] It has also been shown that the electrochemical oxidation of these compounds proceeds via adsorption onto an active electrode surface.[37,42] Due to these considerations, carbon-based materials, such as glassy carbon and carbon fibers, have been used for the investigation of the electrochemical properties of adenosine, inosine, hypoxanthine, and guanosine.[38,43-45] Compared to metal-based electrodes, carbon-based electrodes are inert to surface oxide formation and exhibit more stable background currents at high oxidation potentials. Based on the considerations of the

sensitivity, stability, and ease of integration with microchip electrophoresis, a carbon fiber was chosen as the working electrode material for the present study. Figure 2 shows the hydrodynamic voltammograms (HDV) obtained for the three adenosine family compounds by ME-EC using a carbon fiber electrode with a sodium borate background electrolyte at pH 10. It can be seen from the HDV that the onset potential for the oxidation of inosine is at least 200 mV higher than that of the hypoxanthine. Therefore, to obtain the best sensitivity for all the analytes of interest, a potential of +1.4 V vs Ag/AgCl was selected as the detection potential in these studies.

Separation optimization

Separation optimization of the four purine compounds was performed using a simple T PDMS microchip with 5 cm long separation channel. Because of the high pKas of the analytes of interest (except for adenosine),[46,47] sodium borate at pH 10.0 was selected as the background electrolyte (BGE) for initial electrophoresis separation. Under these conditions, it was expected that inosine, guanosine, and hypoxanthine would be negatively charged.

Unfortunately, the use of borate alone as the BGE did not produce acceptable resolution of the four analytes (Figure 3A). Surprisingly, the peaks for the structurally very different compounds hypoxanthine and guanosine significantly overlapped at all the borate concentrations tested. Modification of the run buffer with common EOF modifiers, such as methanol and acetonitrile, did not improve resolution. However, as can be seen in Figure 3B, the addition of a small amount of dimethyl sulfoxide (DMSO) to the BGE improved the resolution significantly. This resolution enhancement can be explained by considering the ability of DMSO to slow the EOF as well as to disrupt interactions between water and anionic analytes.[48] The reduction in EOF is not surprising, since DMSO increases the viscosity and decreases the dielectric constant of the BGE. This reduces the zeta potential of the channel wall.[48] In addition, DMSO has a specific ability to reduce the solvation of anionic species by disrupting the hydrogen bonding between water and analytes, causing changes to the hydration radii of the analytes.[48]

The use of borate as the background electrolyte provided several additional important advantages for the separation of the four biomarkers of interest. Its relatively low conductivity permitted the use of high concentrations without generating excessive Joule heating in the PDMS microchannels. Increasing the borate concentration decreases the EOF, which was ultimately helpful for improving the resolution (Figure 3C). In addition to the low conductivity and the high buffer capacity of borate at pH 10.0, it also has a unique ability to form complexes with compounds containing cis diol groups.[49] All the purine nucleosides used in this study, except hypoxanthine, possess cis diol groups and can form diol-boronate complexes that possess an additional negative charge over the native compound. In particular, this approach aided in the separation of adenosine, which is originally neutral but forms a negatively charged adenosine-boronate complex. Similarly, the borate complexes of guanosine and inosine become (–2) charged.

Using the PDMS microchip with end-channel amperometric detection, near-baseline resolution was achieved for the four biomarkers using a BGE consisting of 35 mM borate with 2 mM SDS and 15% DMSO (v/v). SDS was included in the run buffer to maintain the stability of the EOF in the PDMS chips. Under these conditions, the four purine compounds were separated in under 85 s (Figure 3D). The separation efficiencies obtained for the analytes were between 360,000 and 440,000 theoretical plates per meter. The limits of detection were 10 μM, 25 μM, 25 μM,

and 33 μM for hypoxanthine, adenosine, guanosine, and inosine, respectively (Table 1). These values are higher than the detection limits that have been reported using CE-UV,[50] HPLC-UV, [51] and mass spectrometry.[52] However, electrochemical detection should provide greater selectivity than UV detection for biological samples and is easier to implement in the microchip format for on-line monitoring. Mass spectrometric detection is very selective but requires expensive and relatively large instrumentation. This makes it more difficult to implement for near- or on-animal on-line monitoring applications.

On-line MD-ME-EC analysis

On-line coupling of microdialysis sampling with microchip electrophoresis was first reported in 2004 by Huynh *et al.* and has since gained significant attention in bioanalytical applications.[53] A stable MD-ME interface that can reproducibly introduce the sample into the separation channel is important for successful on-line monitoring. To date, three different MD-ME interfaces have been described in the literature. These are the flow-gated sample injection interface,[31] on-chip flow splitter (segmented flow),[54] and integrated pneumatic valve-based MD-ME interface.[55,56] In the present study, a flow-gated sample injection interface is employed. Since flow-gated interfaces work by manipulating voltages, they are more amenable to remote control than the other methods and, therefore, are better suited to on-animal sensing applications.[28]

Most early applications of on-line MD-ME employed LIF detection. However, more recently, several electrochemical detection-based on-line MD-ME analysis platforms have been described.[32,57] In particular, the Martin group developed a novel multilayer pneumatic valve-based ME platform for on-line analysis of microdialysis samples that has been successfully employed for on-line MD-ME analysis with electrochemical detection.[55] Our group reported the use of an all-glass microchip device for MD-ME-EC that employed a flow-gated interface and integrated Pt electrodes for on-line analysis of microdialysis samples. This device was demonstrated for monitoring peroxide generated from enzyme reactions and for monitoring nitroglycerin metabolism in freely roaming sheep.[28,31] More recently, Saylor *et al*. reported the use of a pyrolyzed photoresist carbon film working electrode (PPF) for detection of catecholamines in anesthetized rats using a PDMS/glass hybrid MD-ME-EC platform.[33] In both of these applications, either the whole microchip or part of the chip was irreversibly bonded to the substrate to prevent delamination of the chip due to the hydrodynamic pressure generated at the chip/substrate interface by the microdialysis flow.

While irreversibly sealed microchips have the advantage of providing a robust MD-ME interface, when it comes to implementing electrochemical detection, irreversibly bonding of the microchip to the substrate has several disadvantages. If the channels are damaged due to clogging or Joule heating, the entire device has to be discarded. In addition, when the microchip is permanently bonded to the substrate, it is difficult to reproducibly align the microchannel with the electrode. Reproducible electrode-channel alignment is critical to obtain the best signal-to-noise ratio, applied working electrode potential, and reproducible detector response. To address the drawbacks associated with the use of irreversibly sealed devices with electrochemical detection for MD-ME-EC, a simple reversibly sealed all-PDMS microchip device was developed for on-line analysis.

The procedure for the fabrication of the reversibly sealed device is described in detail in the Experimental Section. Since the microchip is reversibly sealed with the substrate containing the electrode, aligning the microchannel with the CF fiber was much less difficult. More importantly,

the PDMS-CF substrate could be reused for multiple experiments. The MD-ME-EC devices fabricated in this manner were tested at several microdialysis flow rates, and it was found that the device could withstand flow rates up to 5 µL/min. Typical flow rates used for microdialysis sampling are in the range of 0.1-2 µL/min.[18] Therefore, the device described can be used for other MD-ME-EC applications.

Once the stability of the new reversibly sealed MD-ME-EC platform was demonstrated, the device was evaluated for the continuous on-line monitoring of the enzymatic conversion of adenosine to inosine by adenosine deaminase. A linear microdialysis probe was used to sample the products generated in a 2 mL polypropylene vial containing adenosine (2.5 mM), adenosine deaminase (22 mg/mL), and the internal standard guanosine (2.5 mM). In these studies, it was possible to continuously monitor the reaction for more than 3 h without any chip failure. Figure 4 shows representative electropherograms recorded over the 1.5 -h period with the developed MD-ME-EC system. Five stacked curves—a,b,c,d, and e—corresponding to different sampling time points of the continuous online monitoring of adenosine metabolites are shown in Figure 4. Figure 4a shows baseline prior to addition of ADA and guanosine. The electropherograms obtained following the addition of ADA and guanosine are shown in Figure 4b. After 20 min, the appearance of guanosine (used as the internal standard) is detected as shown in Figure 4c. This provides an idea of the "lag time" of the system due to tubing. Adenosine was then added to the vial and its appearance is shown Figure 4d at approximately 30 min. In the three consecutive injections shown in Figure 4e (starting at 40.1 min), the appearance and growth of a peak for inosine due to the enzymatic conversion of adenosine by adenosine deaminase is observed.

In these electropherograms, an unidentified matrix (peak 3) present in the ADA sample was found to co-migrate with adenosine (peak 4); thus it was not possible to quantitate adenosine in these studies. The first noticeable appearance of inosine (peak 2) was observed within 512 s from the initial mixing of adenosine with the enzyme. As expected, the inosine signal rapidly increased within the first few minutes of the reaction and then levelled off over time (Figure 5). The experimental lag time, or the time from the changing concentration in the reaction mixture until the change was measured by the device, was about 506 s. The identification of the analytes was based on the migration times recorded with standards at the beginning of the experiment. They were further verified after the experiment using standard addition.

The time required to observe the first appearance of guanosine, the internal standard, was used to estimate the response time of the device. The response time for the device used in these studies was approximately 90 s or equal to a run time of a single sample injection. Although having the stainless-steel connector tube 2 mm above the substrate helped to alleviate the back-pressure at the chip/substrate interface, it is obvious that this introduces additional dead volume into the system. This volume was estimated to be about 0.4 µL. The total length of the tubing from the probe to the device used for this experiment was approximately 16.5 cm (3.6 µL volume). However, it is worth noting that the lag time and the dead volume of the device are very dependent on the length and inner diameter of the tubing and connections as well as the flow rate. Therefore, by varying these parameters, lag time and the dead volume of the device can be further optimized based on the experimental requirements. Furthermore, using a higher PDMS:curing agent ratio (Ex: 20:1), one could fabricate PDMS microchips with stronger adhesion to the substrate. This would make it possible to use a smaller gap between the stainless steel tube and the electrode platform.

Conclusions

A microchip electrophoresis separation-based system coupled to electrochemical detection has been developed for simultaneous monitoring of adenosine and its major downstream metabolites, namely, inosine and hypoxanthine. An innovative approach to integrate microdialysis sampling with an all-PDMS microchip device consisting of a CF working electrode for amperometric detection was developed. The availability of the developed reversibly sealed MD-ME-EC device for long-time continuous on-line monitoring was successfully demonstrated using an *in vitro* assessment of enzymatic conversion of adenosine to inosine. Current research is focused on achieving lower limits of detection for the four purine analytes so that the system can be employed *in vivo* to investigate their role in neurodegenerative processes.

Conflicts of interest

There are no conflicts to declare.

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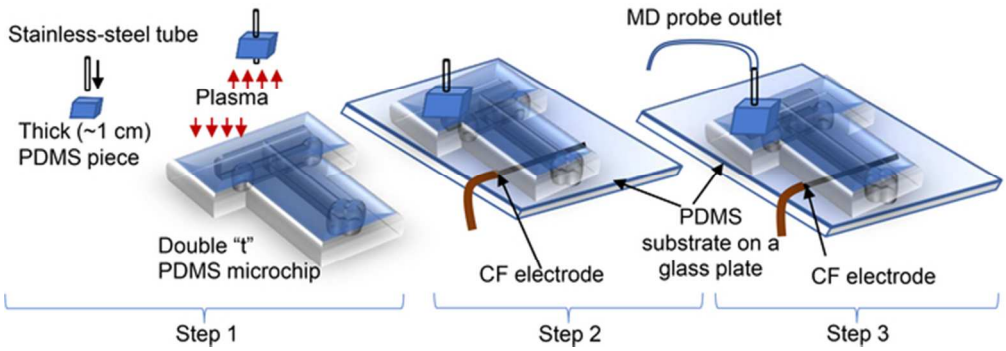


Figure 1: Procedure for the construction of reversibly sealed MD/ME platform; (1) insert stainless steel tube into the PDMS block and permanently bond the block to the microchip; (2) reversibly align the separation channel with CF electrode; (3) attach microdialysis sampling outlet to the microchip device.

58x19mm (300 x 300 DPI)

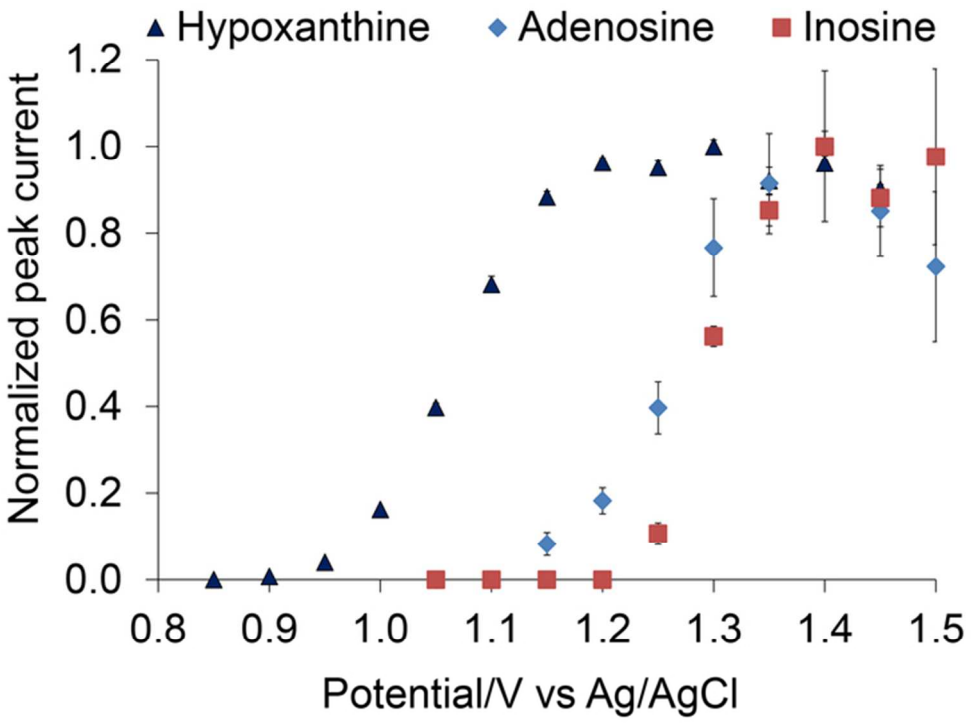


Figure 2: Hydrodynamic voltammograms of hypoxanthine, adenosine and inosine with end-channel detection at CF working electrode. BGE, 35 mM sodium borate at pH 10 with 15% DMSO and 2 mM SDS.

63x48mm (300 x 300 DPI)

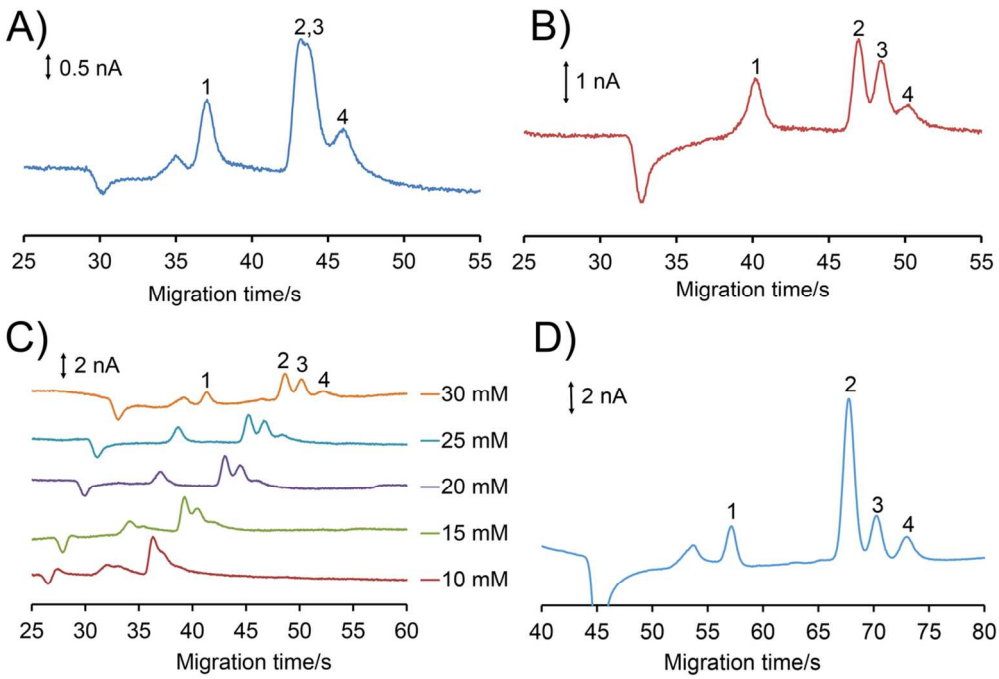


Figure 3: Optimization of the separation of 1) adenosine, 2) hypoxanthine, 3) guanosine and 4) inosine by ME-EC. A) Separation of the four compounds in 25 mM sodium borate at pH 10.0 with no DMSO in the BGE B) Effect of DMSO on separation; BGE contains 25 mM sodium borate at pH 10.0 with 2 mM SDS. C) Effect of borate concentration on separation; BGE contains 15% DMSO (v/v) and 2 mM SDS. D) Separation of the four biomarkers under optimal separation conditions: 35 mM borate at pH 10 with 15 % DMSO (v/v) and 2 mM SDS. All separations were studied using 5 cm separation channel at 222 cm/V field strength at end-channel detection.

114x76mm (300 x 300 DPI)

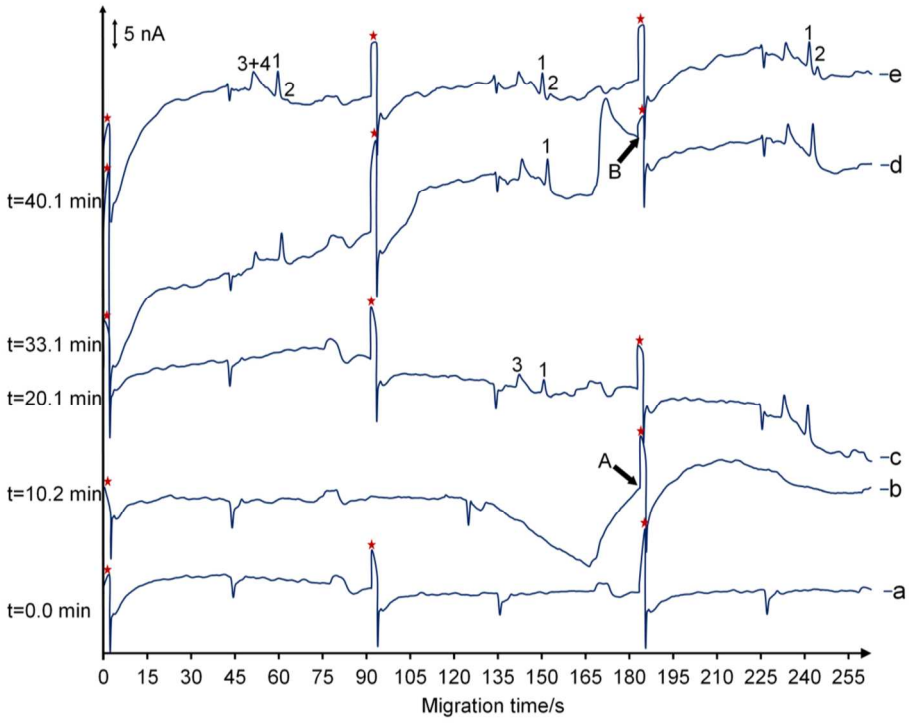


Figure 4: Continuous on-line monitoring of enzymatic conversion of adenosine to inosine using reversibly sealed MD-ME-EC device; '*' represents the time of sample injections. (a) Baseline, (b) ADA and guanosine addition, (c) appearance of guanosine peak, (d) adenosine addition, (e) appearance and growth of inosine peak The peak identities are (1) guanosine (internal standard) (2) inosine, (3) unknown in sample matrix, and (4) adenosine. A and B represent the times for the addition of guanosine and adenosine to the reaction mixture, respectively.

127x94mm (300 x 300 DPI)

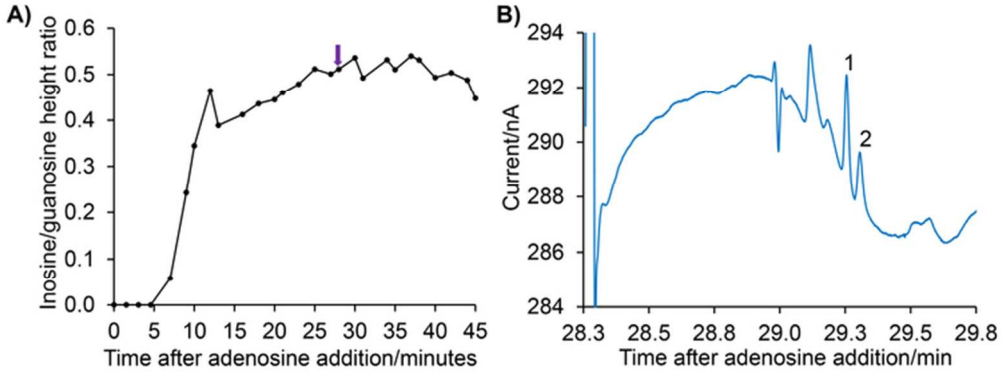


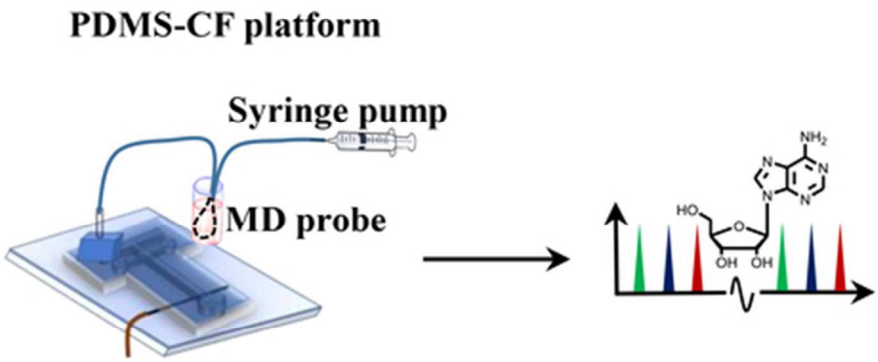
Figure 5: A) Time plot of the enzymatic conversion of adenosine to inosine by adenosine deaminase enzyme followed by MD-ME-EC. B) An electropherogram of a single sample injection extracted from the continuous electropherogram recorded for over one and half hours. The arrow on the time plot indicates the start of the sample injection of the electropherohram. The peak identities are (1) guanosine and (2) inosine.

63x23mm (300 x 300 DPI)

Analyte	R/from previous peak	N/per meter	LOD/ μM	LDR/μM
Adenosine	N/A	375,584 (± 7,345)	25	75-400
Hypoxanthine	5.97 (± 0.07)	388,787 (± 7,087)	10	20-100
Guanosine	1.30 (± 0.01)	441,652 (± 12,551)	25	75-400
Inosine	1.39 (± 0.04)	364,353 (± 12,565)	33	75-150

Table 1. Figures of merit for end-channel detection; BGE: 35 mM borate at pH 10 with 15% DMSO and 2 mM SDS (n = 3 sample injections). Where R- Resolution, N- Number of theoretical plates, LOD- Limits of detection, LDR- Linear dynamic range

45x12mm (300 x 300 DPI)



Graphical abstract: Reversibly sealed all-PDMS device for continuous monitoring of adenosine and its metabolites using microdialysis and ME-EC

44x22mm (300 x 300 DPI)