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Analysis of Raw Biofluids by Mass Spectrometry Using Microfluidic Diffusion-Based Separation

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Abstract

Elucidation and monitoring of biomarkers continues to expand because of their medical value and potential to reduce healthcare costs. For example, biomarkers are used extensively to track physiology associated with drug addiction, disease progression, aging, and industrial processes. While longitudinal analyses are of great value from a biological or healthcare perspective, the cost associated with replicate analyses is preventing the expansion of frequent routine testing. Frequent testing could deepen our understanding of disease emergence and aid adoption of personalized healthcare. To address this need, we have developed a system for measuring metabolite abundance from raw biofluids. Using a metabolite extraction chip (MEC), based upon diffusive extraction of small molecules and metabolites from biofluids using microfluidics, we show that biologically relevant markers can be measured in blood and urine. Previously it was shown that the MEC could be used to track metabolic changes in real-time. We now demonstrate that the device can be adapted to high-throughput screening using standard liquid chromatography mass spectrometry instrumentation (LCMS). The results provide insight into the sensitivity of the system and its application for the analysis of human biofluids. Quantitative analysis of clinical predictors including nicotine, caffeine, and glutathione are described

Graphical Abstract

Introduction

Longitudinal monitoring of physiology has gained popularity through the use of personal tracking devices. A wave of products has hit the personalized health/fitness market place

such as the iWatch and FitBit which track movement, heart rate, even sleep quality. Another group of products such as the Scout™ by Scanadu Inc. is aimed at the citizen health and medical markets. It collects basic physiological data such as heart rate, blood pressure, temperature, and blood oxygenation through the skin. These products are based on the premise that long-term tracking can improve health and facilitate lifestyle changes with beneficial outcomes. This trend is an extension of real-time monitoring of human biomarkers for disease control such as in the case of diabetes and glucose monitors^{1,2}. Considering this movement, it is somewhat surprising that biomedical and biochemical researchers have yet to adopt experimental protocols that collect samples with time resolution sufficient to facilitate accurate tracking and trending. This is despite clear evidence for the value of such data^{1,2}. A reason for this is that researchers have generally been forced to choose between collecting data targeted at a few specific markers with good time resolution, or collecting data with significant physiological depth but with poor time resolution. To date, the success of omics methods and subsequent biomarker discovery has largely been predicated on depth of coverage. Transcriptomics and metabolomics studies look at 1000s of molecular features, but often use only a single time point for each person or animal^{5–11}. The ability to quantify multiple biomarkers in a sample with high temporal resolution has the potential to dramatically improve biomarker accuracy through pattern recognition, tracking and trending while aiding in new biomarker discovery by finding responsive biomarkers with short half-life^{3–9}.

Ambient ionization methods have been shown to remove matrix effects and are capable of handling raw samples for MS, however these methods are not capable of real-time analysis, as they require offline sample deposition onto a surface before analysis^{10,11}. We have developed a RT technology that allows for the measurement of physiologically important biomarkers directly from biofluids such as cell culture media and blood¹². This technology facilitates digital evaluation of extra cellular metabolite, drug, and chemical levels by automating metabolite extraction from complex fluids using a microfluidic metabolite extraction chip (MEC). The device takes advantage of the inertial lift and viscous drag forces acting on particles of various sizes to achieve differential migration and separation of metabolites from cellular debris and proteins^{13,14}. The first application of the MEC was for untargeted monitoring of extra cellular metabolic changes in real-time associated with microbial growth and response to stress¹. Batch cultures of *Escherichia coli* were monitored during exponential growth using the MEC directly coupled to a mass spectrometer. Metabolic changes associated with growth, mild oxidative stress and cellular death. This technology, while still in its infancy, has the potential to revolutionize metabolic studies and improve human health by providing researchers with the means to improve biomarker discovery and by automating detection of disease via quantification of human biomarkers directly from biofluids.

Despite the recent wave of new analytical tools being developed for personalized medicine, from a research standpoint, mass spectrometry (MS) remains the leading technology for quantification of biomarkers because of the diversity and sensitivity for biological molecule detection^{12,15–17}. When coupled with liquid chromatography, MS (LCMS) can be used to track thousands of different proteins and metabolites making it a very versatile detector for biomarker quantification^{15,16}. MS based metabolite biomarker analysis is already an integral

component in the battle against addiction^{3,4}, cancer⁵⁻⁷, Parkinson's⁸, and Crohn's disease⁹. This is because dysregulated metabolic pathways produce distinct metabolite abundance profiles enabling biomarker identification via MS based profiling¹⁶⁻¹⁸. For example, in kidney diseases creatinine levels provide a reliable biomarker for monitoring kidney function¹⁸⁻²⁰. In aging and disease, estimates of redox balance based upon the ratios of reduced to oxidized glutathione (GSH/GSSG) can provide metrics for progression and accumulated tissue damage¹⁹⁻²⁸. Our research using the MEC showed metabolite abundance can change rapidly in response to even minor perturbations, emphasizing that important metabolic changes occur that current experimental and equipment set ups cannot capture adequately due to low time resolution²⁹⁻³⁹. In metabolic profiling, the rate-limiting step for long term metabolite monitoring is the extraction of metabolites and their subsequent computational interpretation⁴⁰. This bottleneck could be avoided if sample processing and data analysis were automated.

Here we establish the ability of our automated MEC system to reproducibly quantify metabolites, while realizing a new application of the technology for low-cost high-throughput sample analysis. To do so, we implement new versions of the MEC that include two, three and four loop logarithmic spirals, which facilitate the extraction. The new chip designs enable us to selectively control the amount of diffusion dependent on sample complexity and concentration.

Results and Discussion

A system capable of rapid and automated extraction of metabolites from human bodily fluids that can operate in a high throughput manner could revolutionize metabolic studies by allowing direct analysis of blood and urine without sample preparation. Biofouling is a major issue with blood and urine even when injected after substantial dilution. Tests conducted on our LCMS system in which blood was analysed without processing resulted in contamination or clogging of the ESI needle after as few as one sample. Similar problems were encountered when unprocessed cellular lysate was analysed directly from a syringe¹ with a standard direct-infusion protocol. Such problems have precluded real-time and prep less analysis of biofluids not only by mass spectrometry, but by nearly all analytical methods.

To overcome such obstacles, MECs that consisted of two (MECX2), three (MECX3) or four (MECX4) loop logarithmic spiral micro-channels were fabricated (Figure 1). During operation, an isocratic flow of 40 $\mu\text{l}/\text{min}$ (100% H_2O , 0.1% formic acid) was maintained through the MEC and into the MS. Periodic injections of sample into channel two were made using a robotic arm auto-sampler (Figure 2). The MEC was designed to use the unique properties of a microfluidic environment at liquid chromatography flow rates to enable the system to diffusively extract small molecules from mixtures of metabolite standards, urine and blood without turbulent mixing^{1, 26}. As the injected samples traversed the MEC, diffusion of solute molecules from channel two to channel one occurred with different efficiencies depending on which chip was used. The MECX2 has the shortest diffusion path and consequently the lowest diffusion efficiency for a given flow rate, which allows metabolites to diffuse into the analysis channel, while proteins and larger biomolecules for

the most part remain in the waste channel similar to Bhagat et al.¹³. To test this, signal intensities for a set of common metabolites of interest were measured using each of the three MECs. Ion intensity increased as expected with the longer path MECX3 and MECX4 (Figure 1B). This is because the length of the flow path in the MEC controls the amount of analyte that diffuses across the micro-channels and into the mass spectrometer. In this way, different MEC designs can be used to tune the amount of diffusion based on the desired MS signal intensity.

For blood and urine analysis the channel length, flow rate, and injection volume were varied. The goal was to identify parameters that optimized metabolite diffusion for mass analysis, while minimizing build-up of background signal during long periods of continuous instrument operation (>8 hrs). Automated injection of sample into the MEC was facilitated by a robotic auto-sampler placed in line with a 2-way six port valve (Figure 2). In valve position 1, the auto-sampler syringe loaded the sample of interest (metabolite standards/urine/blood) into the sample loop. In position 2, the sample loop was placed inline causing the sample to be injected into channel two of the MEC (Figure 2B). The periodicity of injections resulted in pulses of metabolites that were easily detected as ion pulses using ESI-MS.

To evaluate the sensitivity and quantitative potential of the MEC system, standard curves for caffeine, creatinine, dopamine, glutathione (oxidized), lysine, arginine, and nicotine were completed. Injections were made in five minute cycles using 100 picograms to one nanogram per injection. The observed standard curves were linear and displayed good reproducibility (<10% Standard Error) using both the MECX3 and MECX4 (Figure 3). This demonstrates that the system has relevant sensitivity and can be used as a quantitative analytical device

The next step was to evaluate general MEC performance when biofluids were analysed directly, without processing. Preliminary analyses showed that MECX2 was the optimal design for analysing small molecules in blood and urine, because of their relatively high concentration and correspondingly high signal intensity. Ion signals smoothly and reproducibly appeared as transients on the mass spectrometer as shown by the chromatographic profile of the total ion current (Figure 4). Averaging across a pulse of metabolites extracted from a 10 μ L injection of urine revealed roughly 50 molecular features with a signal to noise (s/n) level > 10 (Figure 4B). The most intense ion was creatinine, m/z 114 ($M+H^+$). This is consistent with known urine content and the low molecular weight/high diffusional coefficient of the metabolite. The averaged signal from an injection of 1 μ L of whole blood was more complex, having roughly 100 features with > 10 s/n ratio (Figure 4C). Avoiding overly intense signals from highly abundant metabolites is important to prevent carryover and degradation of system performance over time. Injection of larger volumes caused a slow increase in baseline ion intensity. The background was mostly associated with blood protein rather than metabolites. Blood was the most concentrated sample and required use of the MECX2 to avoid background signals. Urine could be analysed with any of the MECX chips, but the MEC2 provided sufficient signal intensity with no carryover.

Glutathione is a molecular marker for assessing the balance between oxidation and reduction in human blood. The ratio of oxidized (GSSG) to reduced (GSH) forms changes in response to stress, disease and aging^{21–29}, making these compounds of significant biological interest. GSSG has a molecular weight of 612 Daltons (613.159 m/z, M+H⁺) and GSH 307 Daltons (308.083 m/z, M+H⁺). We expected to observe GSH in the blood samples, as it is reported to be present at mM concentrations in human blood. GSSG however, was at the limit of detection for the system. This indicated that very low concentrations of oxidized GSSG were present in the fresh samples used here. This is consistent with other reports that suggest relatively high ratios of GSSG:GSH are an artefact of handling related oxidation of GSH from the metabolite extraction process³⁰. The GSSG:GSH ratio detected in our samples was ~0.5. We hypothesized this was because there is no extraction step in our method, as GSSG/GSH levels are measured directly from blood. To test this interpretation, samples were exposure to air for 12 hours in closed vials and reanalysed. The ratio of GSSG:GSH increased in these samples by relative abundance. Table 1 lists other putative metabolites identified either by comparison to a standard or by accurate mass in blood and/or urine. Table 2 shows the reproducibility between samples and the abundance of measured metabolites in raw biofluid and standard. The % recovery was determined by taking the abundance of recovered signal in urine and blood over the abundance in H₂O, after subtracting endogenous levels. Table 2 shows the reproducibility between samples and the abundance of measured metabolites in raw biofluid and standard. The % recovery was determined from the ratio of the intensity of the observed signal in urine and blood over the intensity in H₂O, after correcting for endogenous signal. For the majority of metabolites, matrix effects from the sample were as expected and can easily become quantitative using standard addition methods. Two metabolites, glutathione and nicotine, had lower signals than expected while lysine was higher. We attribute this to matrix effects during analysis and endogenous levels. Because the signal was reproducible, this too can be corrected using standard curves.

Conclusions

Routine screening of biofluids has the potential to enhance preventative medicine and improve diagnostics. However, a major hurdle is the high cost of analysis due to the analytical challenge of detection and quantification of metabolites in complex biological fluids. The data presented here demonstrates that a diffusion-based microfluidics approach can be quantitative and robust. Importantly, the MEC described here has the potential to dramatically reduce the time and cost of mass spectrometry analysis by reducing sample handling and analysis time.

Optimization of the MEC for use with an HPLC and autosampler required a range of conditions to be tested. For example, the flow rate determines the contact time of the solvent streams as well as the laminar behaviour in the channels. A second consideration is the efficiency of the electrospray source. While micro-flow sources such as the unit that we used are tolerant to a range of flows, ionization efficiency and spray stability must be taken into account when adjusting flow rates. With the current MEC channel width and depth, we settled upon 40 µl as the best option for diffusion efficiency, time per sample, and robust operation. Another factor to be considered was diameter of the tubing. In our experience

nano-flow rates (nl/min) are challenging to sustain when dealing with raw biofluids, especially blood. We had difficulty transporting blood in PEEK tubing of less than 0.008 inch internal diameter, without exceeding the pressure tolerance of our set up. Therefore, in our effort to develop a system that is straightforward to operate, a modular design in which the MEC itself can be quickly changed to increase or decrease signal based on diffusion efficiency was adopted. This was implemented by introducing new chips designated MECX2, MECX3, and MECX4 that had longer channels to increase signal.

Future versions of the MEC will see improved performance through increasing the pressure tolerance. This will allow the velocity of the flow to be increased, decreasing analysis time per sample and lowering flow rates. This will also reduce problems associated with challenging samples such as blood and will facilitate moving toward nanospray which should improve overall sensitivity. Even without improvement, the LC-MEC-MS system can be used to quantify a variety of potential biomarkers directly from human biofluids, reducing sample handling and instrument time.

Experimental

Metabolite Extraction Chip

The Metabolite Extraction Chip (MEC) was designed to be a low cost and robust device for molecular separations. This is described in detail in Heinemann et al.¹². Changes to the MEC are as noted below. Briefly, guides for MEC fluidic channels were made using high resolution printing on standard office transparencies. Thermoplastic molds for polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning) chips were fabricated using a 3D printer from PETG (Ultimaker 2). The PDMS layer for the MEC was mixed at a ratio of 10:1, and was cured in a vacuum oven for 8 hours at 65°C. MEC were then sealed with oxygen plasma (MARCH etcher, Nordson) to a glass slide. To interface the microfluidics chip with a high pressure liquid chromatography (LC) system, PEEK Tubing with 1/32" outer diameter 0.005 mm ID (Upchurch) was inserted and sealed into a blunted 18 Ga syringe needle using PDMS. The design of the chips was optimized for diffusional separation of small molecules from blood, urine and cellular media. To do so, MECs of varying length were evaluated by changing the length of the logarithmic spiral microchannels (2–4 loops) with two inlets and two outlets (Figure 1). The specific model of MEC is indicated by X followed by the number of loops in the spiral (eg MECX2). The inlet and outlet channels were 250 µm wide, with the central microchannel 500 µm in width. All port and channels heights were 10–12 µm.

Metabolite Samples

Standards of arginine, caffeine, creatinine, dopamine, glutathione (reduced and oxidized), lysine, and nicotine (Sigma-Aldrich) were dissolved in H₂O at a concentration of 1 mg/ml. The Nicotine 99% standard was already in solution and only required dilution. Stock solutions were stored at –20 °C.

Blood and urine samples were collected after informed consent was obtained from all human subjects. All experiments involving human biofluids were performed in compliance with the

relevant laws and institutional guidelines of Montana State University Institutional Biosafety Committee and Federal assurances (FWA00000165, protocol 43-2014). This project was approved by the Montana State University Institutional Biosafety Committee.

Urine samples were stored as collected in sterile vials at 4 °C until analysis. Whole blood was drawn at the Montana State University student health center in 5 ml vials with sodium citrate as an anticoagulant. Samples were transferred to 250 µl snap cap HPLC auto sampler vials (Agilent Technologies) immediately prior to analysis. Any handling or pre-processing of the samples was avoided, as the purpose of this study using MEC technology was to fully automate the metabolite extraction process directly from raw biofluid. Auto sampler temperature was kept at 4 °C.

Mass Spectrometry

ESI-TOF analyses were performed using an isocratic flow (1290 UPLC, Agilent Technologies) of 40 µl/min (100% H₂O with 0.1% formic acid) coupled to a Q-TOF (6520, Agilent Technologies). The system was operated in positive electrospray ionization mode, using extended dynamic range (50–1700 m/z), 1 scan per second. Drying gas was used at 6 L/min, and a temperature of 350 °C. The Agilent 1290 auto sampler was configured to allow its use directly with MEC chip (Figure 2).

Interfacing the Metabolite Extraction Chip with Mass Spectrometer

Standard mass spectrometry-based metabolite analysis uses the LC in tandem with the mass spectrometer (LCMS) to allow chromatographic clean-up and separation of the samples. In reverse-phase chromatography (RP-LC), salts and other low molecular weight polar species present at high concentration in biofluids pass through the column while metabolites are retained. Following a wash period, gradient-based separation of the molecules of interest takes place. RP-LC serves two critical purposes; One, it helps keep the ionization source of the mass spectrometer clean and functioning properly. Two, it spreads the elution of molecules of interest across the gradient, reducing ion suppression. In the MEC setup, the LC is used only to establish continuous solvent flow through the chip using a single solvent. Periodic direct injections of sample into the solvent stream occur through the MEC inlet (Figure 1). For this project, the LC auto-sampler was configured to allow direct injection of raw samples in the mass spec (Figure 2). To achieve this the 1290 pump flow was split into two equal streams before the auto-sampler using a P-450 flow splitter (Rheodyne). The flow from splitter outlet 1 goes directly to inlet 1 on the MEC. The flow from outlet 2 passes through the 6 port valve configured with a sample loop that is filled from the auto sampler in position 1 and injected into the MEC in position 2. Standard solutions were injected into the MEC in volumes between 0.1 to 1 µl dependent on the concentration. For urine samples, direct injection of 0.1 µl on the MECX2 generated satisfactory signal, without saturating the chip or the MS. Injection volumes of 0.01 µl were sufficient on MECX2 for whole blood samples. A sample pulse was collected every five minutes, with each injection spanning two minutes for the standards and the urine samples. Whole blood took slightly longer to run through the chip, so total time was seven minutes per sample. Blank injections between whole blood runs were used to avoid buildup of background signals during extended analyses.

Data Analysis

Data is recorded as ionization pulses on the MS, where pulse intensity is controlled by the volume of injections. The pulsed nature of the signal is due to switching of the 6-port valve which directed a sample to the MEC. Data was analysed using MassHunter Quantitative software for QTOF (Agilent Technologies). Ion intensity of each m/z was summed for the duration of the pulse and placed into a list. All samples were manually validated with Masshunter Qualitative analysis (Agilent Technologies). The m/z intensities were exported as a .csv file.

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Figure 1.

Diffusion of small molecules on different metabolite extraction chips (MEC). Three different chips were used (x-axis) from left to right MECX2, MECX3, MECX4 (a) Images of the variable loop MEC, from left to right MECX2, MECX3, MECX4 coincide with x-axis (b) Relative spectral intensity from 1 ng of Caffeine, Creatinine, Dopamine, Lysine, Arginine, and Nicotine standards on each chip were conducted in triplicate with error bars representing standard deviation. In most cases error bars are too small to be visualized.

Figure 2.

Setup of integrated auto-sampler, valve, pumps, MEC, and mass spectrometer. The isocratic flow from the LC is split in two solvent streams. Stream 1 goes directly to channel 1 of the MEC. Stream 2 goes through the valve before entering the MEC via channel 2. (a) valve is in position 1, syringe pump is set to load sample loop from samples in auto-sampler tray; (b) valve is in position 2, LC pump is directed to flow through sample loop effectively injecting sample into the MEC.

Figure 3. Quantitative measurements using the MEC. Standard curves for (A) Caffeine, (B) Creatinine, (C) Lysine, (D) Arginine, (E) Nicotine, and (F) GSSG, in the MECX3 (dark line) and MECX4 (light line), Injections of 1, 250, 500 and 1000 pg were made. All analyses were conducted in triplicate. Error bars represent standard deviation (95% confidence interval) are hidden by the markers in most cases.

Figure 4.

Mass spectrometry data from the MEC. A) Total ion chromatogram showing 3 separate injections of urine on the MECX2 chip. B) Mass spectrum of urine from the MECX2. Spectrum averaged from the region indicated in A. C) Total ion chromatogram showing 3 separate injections of whole blood on the MECX2 chip. D) Mass spectrum of blood. Spectrum shows an average of the region indicated in C. E) Close up of lower intensity ions from panel D.

Table 1

Compounds detected in biofluids

m/z	Formula	Compound	Fluid
137.0595	C ₈ H ₁₀ O ₂	4-Hydroxyphenylacetaldehyde	Urine
163.1240	C ₁₀ H ₁₄ N ₂	L-Nicotine *	Urine
170.0900	C ₇ H ₁₁ N ₃ O ₂	3-Methyl-L-Histidine	Urine
172.9220	C ₃ H ₃ BrO ₂	3-bromo-2E-acrylic acid	Urine
188.8939	C ₂ H ₄ S ₅	Lenthionine	Urine
204.1251	C ₉ H ₁₇ NO ₄	Acetylcarnitine	Urine
209.1227	C ₉ H ₁₈ N ₂ O ₂	3[(Methylbutyl)nitrosamino]-2-butanone	Urine
229.1549	C ₁₁ H ₂₀ N ₂ O ₃	L-isoleucyl-L-proline	Urine
249.1058	C ₉ H ₁₆ N ₂ O ₆	Glutamate Threonine dipeptide	Urine
114.0654	C ₄ H ₇ N ₃ O	Creatinine *	Blood/Urine
132.0060	C ₂ H ₇ NO ₂ S	Hypotaurine	Blood
132.0754	C ₄ H ₉ N ₃ O ₂	Creatine	Blood
203.0549	C ₆ H ₁₂ O ₆	Glucose	Blood
215.0161	C ₆ H ₈ O ₇	Citric acid	Blood
308.0933	C ₁₀ H ₁₇ N ₃ O ₆ S	Glutathione (reduced) *	Blood
346.0144	C ₁₀ H ₁₂ BrN ₅ O ₄	8-Bromoadenosine	Blood
548.2933	C ₂₇ H ₄₁ N ₅ O ₅ S	Ile-Met-Val-Trp	Blood
555.2753	C ₂₅ H ₃₄ N ₁₀ O ₅	Gly-His-Arg-Trp	Blood
561.0937	C ₁₇ H ₂₆ N ₂ O ₁₅ P ₂	dTDO-4-oxo-5-C-methyl-L-rhamose	Blood
568.3354	C ₂₃ H ₄₁ N ₁₁ O ₆	His-Lys-Gln-Arg	Blood

* identification confirmed with authentic standard

Table 2
Recovery of 100ng compound dissolved in 1µl H₂O mixed into biofluids, compared to control

ID	H ₂ O Control		Urine		Whole Blood	
	m/z	Avg. +/- %SE	Avg. +/- %SE	% Recovery	Avg. +/- %SE	% Recovery
Caffeine	195	1176556+/-6.31%	330412+/-3.12%	28.08	485170+/-12.02%	41.16
Creatinine	114	2996612+/-0.15%	4570140+/-1.92%	135.50	1887180+/-2.76%	59.42
Lysine	147	389712+/-7.49%	740752+/-26.42%	180.02	408351+/-3.86%	104.78
Arginine	175	2122521+/-2.95%	1464663+/-12.38%	69.00	1025972+/-7.61%	48.33
Nicotine	164	1290831+/-4.03%	313373+/-6.88%	18.85	137572+/-8.48%	10.65
GSSG	613	409163+/-17.08%	113021+/-2.12%	26.81	42829+/-3.33%	7.02
GSH	308	1261270+/-2.95%	NA	ND	1370440+/-7.85%	106.36