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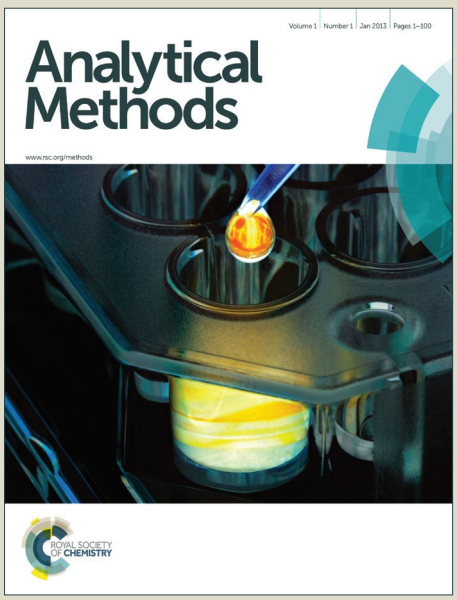

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Integrated Electrodes and Electrospray Emitter for Polymer Microfluidic Nanospray-MS Interface

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Interfacing of microfluidic devices to mass spectrometry has challenges including dilution from sheath liquid junctions, fragile electrodes, and excessive dead volumes which prevent optimum performance and common use. The goal of this work is to develop a stable nanospray chip-MS interface that contains easily integrated electrodes and an embedded capillary emitter to mitigate current chip-MS problems. This system uses a hybrid polystyrene-poly(dimethylsiloxane) (PS-PDMS) microfluidic platform with an embedded electrode and integrated capillary emitter used as the nanospray interface. Two chip designs were used to evaluate the performance, illustrate on-chip reaction capabilities. By direct infusion, this system showed good performance with LODs of GSH and caffeine of 9 nM and 1 nM, R^2 of 0.996 and 0.992 and sensitivity of 12 counts/nM and 332 counts/nM over a linear dynamic range of 40 nM to 50 µM and 1 to 50 µM respectively. A reaction was performed on the chip with syringe pumps showing the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) using H_2O_2. The on-chip reaction of GSH oxidation to GSSG, with online-MS detection, successfully demonstrate the stability and robustness of the nanospray interface.

Introduction

Coupling of microfluidic devices to mass spectrometry (MS) holds the potential to yield high sensitivity multi-analyte data for low sample volume analyses[1,2,3] but

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common use is limited due to a lack of robust electrospray (ES) interfaces. The combination of chips to MS is highly desirable because the miniaturized flow rates used in chips increases the electrospray efficiency and sensitivity of MS as well as improved identification of structurally diverse analytes. Such systems can aid in the development of biochemical assays, integrated separation techniques, and simplified sample preparation. Though the inherent low flow rates of microfluidic platforms lend themselves to increased ES performance, the interface of these two systems is not trivial. Inherent problems with interfacing microfluidic devices to MS include ES instability, dilution from sheath liquid, and a lack of a robust method to apply ES voltage. An ideal interface would have zero dead-volume, straightforward voltage interfacing and utilize a small internal diameter (i.d.) ES emitter.

Current methods to interface chip to MS vary depending on the microfluidic materials used (glass or polymer based), composition of the emitter and method to apply the ES voltage.[4,5,6] Glass chips have been interfaced with MS[7] by spraying from the corner of the chip with the spray voltage applied at a side reservoir.[2] While all glass chips benefit from high electroosmotic flow (EOF) and reproducible fabrication of electrodes, their fabrication is not trivial. Currently polymer based chips are more common microfluidic devices, but not easily adapted to MS analysis.

Polymer based chips are a popular material for microfluidic devices because of their low cost, ease of fabrication, and ability to integrate capillaries and electrodes. Currently, the common method of interfacing a polymer microfluidic device to MS is using an integrated polydimethylsiloxane (PDMS) emitter which reduces dead volume.[8,9,10,11,12,13] Since PDMS is hydrophobic, it encourages the aqueous droplets to be sprayed into the MS. This discourages droplet congregation and encourages small droplets to be formed, increasing ionization efficiency.[14,15] Nevertheless, creating a polymer emitter with dimensions on the scale of a capillary emitter (30 μm) and diminishing the background noise from PDMS is difficult.

Capillary based emitter tips are common in microscale LC as they can be pulled down to a tapered 1 μm tip, which offers increased ES efficiency. To date, capillary emitters have only been used with glass devices with respect to MS.[16,17] Polymer microfluidic devices have been known to have integrated capillaries, but used electrochemical detection.[18]

There are several ways to apply ES voltage to polymer-based microfluidic device for MS. Liquid junction is the most common,[8] but the spray voltage can also be applied at the syringe tip[9] or at an electrode inserted in the PDMS chip. If a more rigid polymer like polystyrene (PS) is used as the microfluidic device base, an electrode integrated directly under the channels allowing for the voltage to be applied closer to the emitter. This eliminates the connection that is required for liquid junction. There have been reports of interfacing chip-MS through nanoESI, and all with integrated emitters.[9,19,20] Oleshuk et al. report an embedded electrode, but had problems with blocking the channel and the thin electrode breaking.[19]

The microfluidic device proposed here takes advantage of the ease of fabrication of polymer based devices while integrating a capillary with ES electrodes. The microfludic device is a PS base with integrated capillary and electrodes with a PDMS microfluidic chip. The polystyrene base allows zero dead-volume integration of both the electrode and the capillary emitter tip and voltage to be applied directly to the chip which simplifies chip-MS interfacing. The voltage is applied and controlled directly from the MS controlled ES power supply. The integrated capillary yields a near zero dead volume and integrated nanospray emitter. The epoxy-based sealing PS to PDMS approach[21] can withstand the back pressure requirements of generating electrospray with the integrated emitter (11-30 μm). This system offers a robust,

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easily fabricated, high sensitivity interfaced between PDMS-PS based chips and ES-MS.

Method and Materials:

Reagents and Chemicals

High purity water was purchased from Honeywell and used to make all samples. Ammonium formate, glutathione and caffeine were purchased from Alfa Aesar (Wardhill, MA, USA). Ornithine and lysine were purchased from Acros (Morris Plains, NJ, USA). Formic acid was purchased from Fischer (MA, USA). 35% hydrogen peroxide was purchased from Sigma Aldrich (MO, USA) and used for making hydrogen peroxide solution with 40 mM ammonium formate/ammonium hydroxide (pH=10). All capillary was purchased from Polymicro Technologies (Lisle, IL, USA). PDMS kit was purchased from Ellsworth Adhesives (Germantown, WI, USA). PS was purchased in powder form (mean particle size 250 µm) from Goodfellow (Coraopolis, PA, USA).

Microfluidic Device Fabrication

Lithographic fabrication of PDMS and silicon master were manufactured as reported previously.[22] The two designs used were a straight channel (1 cm x 700 µm x 50 µm) and a Y shaped channel (1 cm x 700 µm x 50 µm straight, 0.5 cm x 400 µm branches). The method followed for PS has been previously established and involved heating of polystyrene powder around electrode materials and fused silica capillary.[18]

The aligning for integration of palladium electrodes in PS base fabrication was completed using an aluminum weigh boat.[18] When the straight channel design is desired, a straight channel cured PDMS chip is coated with marker and stamped on the bottom of the weigh boat. Holes in the weigh boat are punched with a 20 gauge syringe needle: a hole for capillaries at the entrance and exit of the channel and a hole for the electrode towards the exit of the channel. There are two different PS designs that are used in this paper. Both systems are illustrated in Figure 1a-b.

Once the PS is polished and the PDMS is cured, a selective stamp bonding is utilized to seal the PDMS on top of the integrated capillaries and electrode. To create the integrated emitter, the exit capillary was pulled to a tapered tip using a Sutter laser puller (model P2000, Novato, CA, USA). The program used was Heat: 350, Filament: 0, Velocity: 50, Delay: 135 and Pull: 35. The device is taped to the lid of the laser puller, and all of the capillaries are contained within the lid, while the tip is being pulled on the capillary. A 30 µm nanospray tip was formed after etching in 51% HF for 4 minutes (Figure 1d). For the oxidation of glutathione, the tip was pulled to create an 11 µm tip.

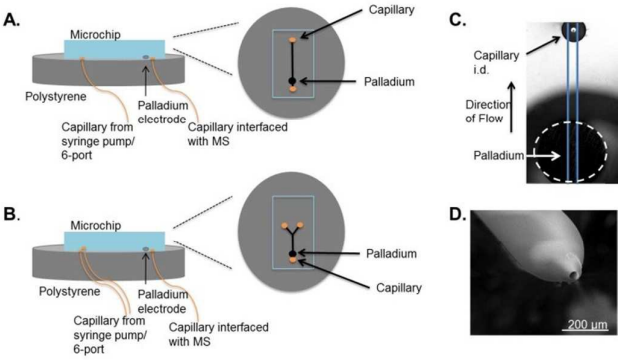

Figure 1. PS-PDMS Hybrid Interface Configurations. In all configurations (A-C), the spray voltage is applied at the embedded electrode. **(A)** Chip configuration used for injections of 1 µM Ornithine. There is one integrated capillary and electrode. **(B)** Configuration used for oxidation of 50 µM glutathione. There are three integrated capillaries and an electrode. **(C)** Image of the microchannel showing the 75 µm i.d. capillary and 1 mm palladium electrode. Blue lines are used to outline channel, with white lines being used to outline the palladium electrode. **(D)** SEM of 100 µm i.d. capillary pulled tip 30 mm that has been etched with 51% HF for 4 minutes.

Syringe Pump Experiments

Two syringe pumps were connected to the microfluidic device as shown in Figure 1b. 50 µM GSH (in 0.1% formic acid) in syringe pump A and 0.5% hydrogen peroxide (pH=10, 40 mM Ammonium Formate) in syringe pump B. Syringe pump B was set to 20% the flow rate of syringe pump A, 100 and 500 nL/min, respectively. When the oxidation was

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desired, the flow rate of syringe pump B was raised to 500 nL/min for 10 seconds. In other words, the flow rate ratio between syringe pump A and B is originally 4:1. For an injection, the ratio is adjusted to be 1:1 for 10 seconds and then changed back to 4:1.

Mass Spectrometry

A Thermo LTQ XL ion trap mass spectrometer was used for all experiments (Walthram, MA, USA). Syringe pumps (KD Scientific, MA) were used for directly infusing solutions. Evaluation of electrospray voltage showed reproducible and stable signal for voltages between 2-2.5kV (Supplemental Information S1). The nanospray source was used to apply +2.5 kV to the chip. A Rheodyne (WA, USA) six port injector was used for injection of ornithine and hydrogen peroxide. SGE 500 μL glass syringes (AUS) were used for all experiments.

Microscopy

Fluorescence imaging was performed with an upright fluorescence microscope (Olympus EX 60, Center Valley, PA, USA) equipped with a 100 W Hg Arc lamp and a cooled 12-bit monochrome Qicam Fast digital CCD camera (Qimaging, Montreal, Canada), utilizing a 4x objective. Streampix was used to record videos and detect the average grey level. The intensity of fluorescein was recorded through the average grey level and the data exported into excel. The data is originally in frames/second (x) and intensity of fluorescein (y). frames are recorded at 10.48 Hz, frames/second and converted to minutes time scale. The intensity of fluorescein was plotted vs. time to produce.

The pulled capillary tip orifice was imaged using an Inspect F50 scanning electron microscope (SEM, FEI). Two type of tips were measured, a tip etched in 51% HF for 4 minutes, and on rare occasions when the tip clogged, a tip that was pulled a broken with a ceramic scorer (Figure 1d).[23] SEM is driven by is xTm (4.1.4.2010, FEI). The scan rate was 1 μs for Figure 1d. The capillary was placed perpendicular to the stage for the imaging.

Data Analysis

All MS data was collected using Xcalibur (Thermo Scientific, Walthram, MA, USA). All data was boxcar averaged over 7 points. For GSH, 308 m/z was plotted in Xcalibur over time and that was exported for excel. For GSSG, 307 m/z and 613 m/z were plotted over time and exported to excel. The intensity of GSSG was divided by GSH to determine the ratio. This ratio was plotted over time to illustrate the change in oxidation. For the calibration curves of GSH and caffeine, all concentrations were infused for 1 minute, the intensities were averaged, and then plotted versus concentration. The best fit line was plotted and the sensitivity was taken from the slope. The LOD was found by taking the standard deviation of 3 blanks, multiplying it by 3 and dividing it by the sensitivity. The LOQ was found using the standard deviation of the blank (n=3), multiplying it by 10 and dividing it by the sensitivity.

Results and Discussion:

Experiments were performed using two variations of a microfluidic device coupled to MS with embedded electrodes and an embedded ES emitter found in Figure 1a-b. The design of the chip was adjusted to a Figure 1a for direct infusion or b configuration for use with dual syringe pumps. The devices contain embedded exit and entrance capillaries if syringe pumps were used. All of the devices contain an embedded electrode. Since the channel is directly in line with the entrance/exit capillaries, a zero dead volume connection is achieved. The exit capillary is an integrated emitter tip interfacing to the MS with a pulled tip to enhance ES efficiency. The pulled emitter tip shows a smooth orifice and an average size of 30 μm i.d. (Figure 1e). The emitter is pulled on the exit capillary that was 15 cm × 100 μm i.d. In the event that the emitter tip clogged, the tip could be cut off with a scribe and a new tip pulled. The difference in length of the exit capillary changed the dead time by < 20 seconds.

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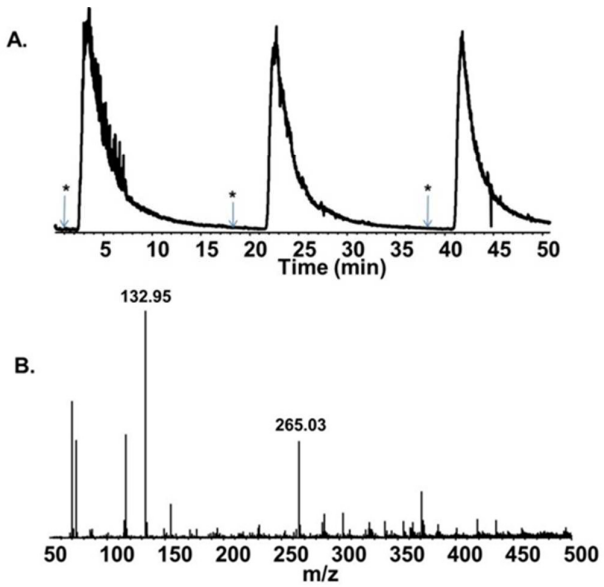

Figure 2. **Injections of Ornithine. (A) Reconstructed ion Flow Injection-gram.** Injections of 1 µM ornithine (M+H=133) with a six-port injector and a 27 nL sample loop. The flow rate was 1 µL/min. The MS was in positive mode and had a spray voltage of +2.50 kV. The RSD for these injections is 5.7% The mass spectra can be found in figure. Y axis is given as relative signal intensity. **(B). Mass Spectrum of ornithine injection.** 265 m/z is most likely two ornithine molecules with a positive charge. This aggregation is due to high flow rate and concentration. * indicate where injections were made. Y axis is given as relative signal intensity.

By embedding the palladium electrode in rigid PS, the durability of the device is enhanced. Previous work has shown that carbon or platinum based materials can be embedded as electrodes for PS as well.[22] Electrodes were stable throughout the course of the experiments with the limiting factor in experiments was clogging of the emitter tip.

The straight channel device (Figure 1a) was tested for compatibility with ES-MS. The integrated electrode was used to apply the spray voltage. The entrance capillary was connected to a syringe containing 5mM ammonium formate buffer (pH=6.5) as the carrier stream. 1 µM ornithine (M+H=133) was injected onto the chip using a 27 nL loop with a 6-port injection valve. Figure 2a and b shows the flow injection plot and mass spectrum corresponding to the injection. Within the spectrum, 265 m/z can be seen in Figure 2b which corresponds to a dimer of two ornithine molecules with one positive charge $[2M+H]^+$. The RSD was found to be 5.7% based on signal intensity. The purpose of the injections was to demonstrate that the microfluidic device could be properly coupled to the MS. The ease of fabrication and stability of the electrode gave us confidence to examine other analytes and on-chip reaction schemes.

Calibration curves were generated for both GSH and caffeine using the chip in Figure 1a with both the entrance and exit capillaries being embedded. GSH was directly infused at six different concentrations. The LOD calculated for GSH is 32 nM and the R^2 value for the curve is 0.996. The S/N of 40 nM GSH is 6.5 and can be found in Table 1. The LOD for caffeine lower than that for GSH. The differences in sensitivity between GSH and caffeine are attributed to their differing polarities and proton affinities.[15]

The purpose of this study was not to develop a device that details some new mixing approach; rather, we wanted to simulate an on-chip process (as opposed to the off-chip injection process we used in other experiments) so that the ability of the device to monitor on-chip processes could be assessed.

Table 1. Figures of Merit. Calibration curves for GSH and Caffeine were created using a straight channel chip (Figure 1a) with two embedded capillaries (entrance and exit). The distal capillary was pulled to a tapered tip to act as the emitter. Concentrations of 40 nM, 1,5,10,25,50 µM were directly infused at 500 nL/min. Caffeine was infused at 1, 25, and 50 µM at 500 nL/min.

	GSH	Caffeine
Sensitivity	12 nM^{-1}	3 nM^{-1}
R^2	0.996	0.992
LOD	9 nM	<1 nM
LOQ	32 nM	1 nM

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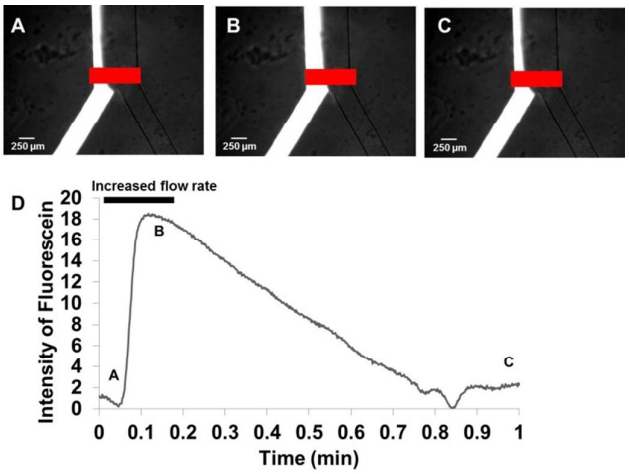

Figure 3 Microscope Imagine of Y channel Device. Microscope imagine of Y channel microfluidic chip filled with water and fluorescein. **(A)** is before the fluorescein syringe pump is increased. Red bar indicates poiont of detection. **(B)** is after the fluorescein flow rate has been increased to 500 nL/min for 10 seconds.**(C)** is after the fluorescein flow rate was reduced back to 100 nL/min. **(D)** is the measured intensity of 100 µM fluorescein. The flow rate of the syringe pump containing fluorescein increased for 10 seconds (t=0.00 min to t=0.16 min) and then reduced back to the original flow rate (100 nL/min). Images A-C are marked on graph of fluorescein with the corresponding letters. Arrows denote direction of flow

A Y channel chip was fabricated (Figure 1b) to examine the feasibility of running on chip reactions. Syringes are attached to each of the entrance capillaries. Flow studies were initially performed with a microscope to visualize how the dual flow rates interact in the channel. Fluorescein and water were chosen as the two fluids to demonstrate the ratio of mixing in the straight channel. This chip configuration used a pulled 30 µm emitter tip to imitate the conditions of the microfluidic device -MS used in the reaction conditions described below. Two syringe pumps were used: water in syringe pump A and 100 µM fluorescein in syringe pump B. Initially, Syringe pump A and B flowed at 500 and 100 nL/min, respectively, a 4:1 ratio. The flow rate of syringe pump B was increased to 500 nL/min for 10 seconds, making the flow rates a 1:1 ratio which should lead to fluorescein filling 50% of the channel. After the pulse of increased flow from fluorescein, it is predicted that the mixing ratio will return to the initial 20% ratio. The intensity of fluorescein was imaged and plotted in Figure 3d. When syringe pump B is pulsed there is an increase of fluorescein intensity that lingers after the syringe pump is turned off. Figure 3a-c show the channel initially at 4:1 ratio, then syringe pump B is increased to 500 nL/min (1:1 ratio), once it has been increased, and after it has been reduced back to 100 nL/min (4:1 ratio). Before syringe pump B has been increased, fluorescence covers 20% of the channel, as expected. After the flow rate increase, fluorescein fills approximately 50% of the channel. Depicted in Figures 3a-c, fluorescein and water flowed side by side right after the channel branches. However, at the exit the two solutions were fully mixed at the end of the channel, 1cm from the point of mixing. Figure 3d indicates that the system took approximately 30 seconds to fully recover back to baseline after the change in flow ratios. At higher flow rates there is less time for complete diffusion. Therefore the two solutions have diminished mixing at higher flow rates. This information gave light to using lower flow rates, which is compatible with nano-spray-MS.

Following the evaluation of the mixing streams, the system was used to show the feasibility of performing on-chip reactions with MS detection. As the Y based chip showed sufficient mixing, this device was implemented for the GSH oxidation (Figure 1b). Due to rapid reaction kinetics,

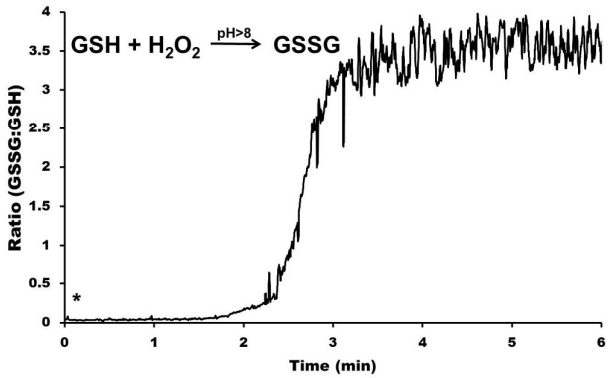

Figure 4 Oxidation of Glutathione. The oxidation of GSH to GSSG using two syringe pumps. Syringe pump A and B contains 50 µM GSH in 0.1% formic acid (pH=3) and 0.5% H_2O_2 in 40 mM ammonium formate (pH=10). The flow rate of syringe pump B was increased to equal that of syringe pump A for 10 seconds. The intensity of GSSG (M+H= 613, M+2H= 307) increases and the intensity of GSH (M+H=308) decreases, due to the oxidation. * Increase of flow rate of H_2O_2 for 10 seconds.

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the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) using H_2O_2 was examined.[24] The pH of the reaction is important to ensure that GSH has the deprotonated thiolate (GS-) so they may act as strong nucleophiles and rapidly self-oxidize in the presence of an oxidizing agent. Hydrogen peroxide was placed in 40 mM ammonium formate (pH 10) buffer to ensure thiolate formation. To initiate the reaction, a pulse of hydrogen peroxide is sent into the Y channel allowing it to mix with GSH (see methods and materials). The M+H peak being detected for GSH and GSSG are 308 m/z and 307 + 613 m/z respectively. Figure 4 shows the oxidation of GSH to GSSG by plotting the ratios of their respective intensities. The introduction of the H_2O_2 began at t=0. The dead time of the chip is around 3 minutes, giving the H_2O_2 sufficient time to oxidize the GSH. The GSSG concentration changes from near zero to almost 4, indicating that this reaction is substantial and levels off after about 1 minute of reaction (t=4 minutes). The signal ratio plateau is likely due to residual peroxide and high pH buffer carrying over in the system.

Conclusions

A PDMS-PS nanospray interface with an embedded electrode and capillaries was fabricated. The robust embedded electrode allowed for a simple and direct application of the spray voltage. The integrated capillary emitter created a virtually zero dead volume microfluidic device. The oxidation of glutathione was able to demonstrate the stability of this microfluidic device coupled to MS. Because of the ease of applying different voltages throughout the chip via our integrated electrodes and generating a sheathless microfluidic device to MS interface, implementation in a capillary electrophoresis or monolith LC is a likely next step for our system.[25] We anticipate future work to involve coupling this technique with on-chip cell culture.[26,27] The monitoring of cell release could also be possible, having compounds diffusion across a membrane. This interface allows for lab on a chip technique to be stably coupled with MS.

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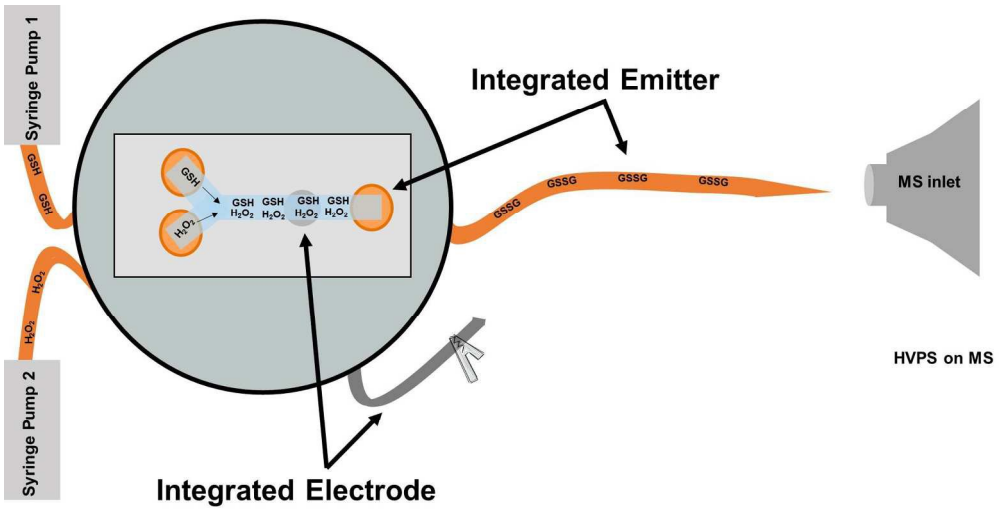

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