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Integrated Microfluidic Enzyme Reactor Mass Spectrometry Platform for Detection of Anthrax Lethal Factor

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***Abstract*—In this work, we have developed a coupled microfluidic enzyme reactor mass spectrometry platform for the detection of protein toxins such as anthrax lethal factor. The lethal toxin produced during *Bacillus anthracis* infection is a complex protective antigen, which localizes the toxin to the cell receptor and lethal factor (LF). We have demonstrated, in this work, the applicability of a microfluidic reactor for the capture and concentration of enzyme reaction solid-phase. The reaction solid-phase consists of anti-LF monoclonal antibodies immobilized on magnetic protein G beads for the capture of LF. The captured LF, on exposure to optimized peptide substrate, hydrolyzes into two smaller peptide products. These cleavage products were then analyzed by mass spectrometer coupled to the microfluidic reactor. This resulted in efficient sample preparation, high sensitivity, larger reaction sites, less reagents consumption and shorter analysis time. We have showed here reproducible detection of anthrax lethal factor in concentration range of 40 to 0.5 ng/mL with a detection limit of 1 ng/mL. The enzymatic reaction and the analysis were performed in less than 15 minutes, indicating a rapid diagnostic tool for early anthrax prognosis.**

I. Introduction

Anthrax lethal factor (LF) caused by infection with *Bacillus anthracis* (a spore forming Gram-positive bacterium) and protein toxins such as botulinum neurotoxin and ricin are likely agents of bioterrorism. In the US bioterrorism attacks of 2001, pulmonary anthrax had 45% fatality rate despite aggressive treatment and supportive care. Therefore developing a rapid, specific, and sensitive screening assay, which can be used in case of national emergency, is a high priority for Centers for Disease Control and Prevention. The major drawbacks of current standardized methods such as ELISA, mouse bio-assays or fluorescence-based assays are lack of sensitivity, speed (analysis takes 1-3 days) and functionality.

The objective of this work is to develop a microfluidic system coupled to mass spectrometer to enable enzymatic assays with (a) enhanced reaction efficiency, (b) reduced analysis time, (c) better sensitivity and (d) less reagents than current assays. We herein report a prototype integrated microfluidic enzyme reactor-mass spectrometry (MS) platform for the detection of functional lethal factor (LF). The two exotoxins of B. Anthracis are the binary combinations of protective antigen (PA) and either edema factor (EF) or lethal factor (LF). The complex of PA and EF forms edema toxin (ETx) and PA complexed with LF forms lethal toxin (LTx). LF is a zinc-dependent-endoproteinase, which is known to target the amino terminus of five members of the nitrogen-activated protein kinase kinase (MAPKK) family of response regulators [1]. The exploitation of LF zinc–dependence for endoproteinase activity with strands and subtype-specific peptide substrates forms the foundation of our detection method.

II. Method of Detection

As stated earlier, the method is based on detection of bacterial protein toxin endopeptidase activities (Endopep MS) [2]. Endopep MS incorporates three levels of specificity into the detection scheme: (a) LF-specific antibody capture, (b) LF-specific peptide cleavage and (c) MS peptide detection. The detection of mass-specific peak intensities gives certainty of the cleaved products and LF concentration, which is not possible with fluorescence resonance energy transfer (FRET)-based LF cleavage assays. Additionally, microfluidics provides for effective reaction control and reduces assay time, because of the size effects in liquid micro-space.

Figure 1 illustrates the typical work-flow of capturing protein toxins and analyzing their products using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The monoclonal antibodies (mAb) are prepared in CDC and reported elsewhere [1]. Dynal magnetic protein-G beads used to bind and cross-link mAbs are prepared according to manufacture's instructions (Invitrogen Corp, Carlsbad, CA). The magnetic protein-G beads immobilized with monoclonal anti-LF form the reaction solid-phase. Then, the protein toxins are captured and concentrated on the mAb-cross linked magnetic beads. The captured protein toxins=beads complex are then exposed to an optimized MAPKK peptide

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substrate [1], which hydrolyzes them into smaller products (Product 1 and Product 2). The LF cleavage products are then analyzed by MALDI-TOF MS. It is important to note here that only a small volume (0.05% of actual specimen) is spotted on MALDI from each sample. In other words, the MS technique is a micro-scale technology following a macro-scale sample preparation. Therefore, in this work, we have incorporated a micro-environment for sample preparation, in the way of microfluidic enzyme reactor.

The microfluidic format allows for miniaturization of sample preparation unit as a result (a) much less of highly infectious specimens and chemicals are used, (b) expensive MALDI-MS detection is avoided, (c) enzymatic reactions rates are enhanced due to microfluidic size effects, (d) specific interface area is increased, and (e) diffusion time and distance are reduced. Furthermore, the microfluidic enzyme reactor is coupled to a standard high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI/MS/MS) detection platform for protein toxin analysis. In the next section, we will discuss the development and integration of microfluidic enzyme reactor to the MS system.

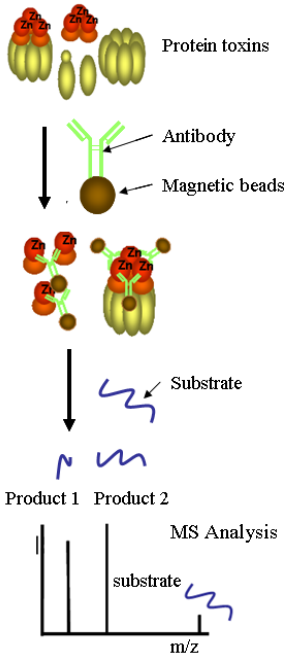

Fig. 1. Illustration of a typical work-flow for the detection of protein toxins using antibody cross-linked magnetic beads by mass spectrometry (MS).

III. Design and Fabrication of Microfluidic Enzyme Reactor

The sample preparation unit, namely the enzyme reactor is designed as long microchannel with inlet, outlet ports and a reaction chamber, where the solid-phase is formed. Figure 2 illustrates the design of the microfluidic enzyme reactor. The reaction solid-phase consists of antibody coated magnetic beads cross-linked to the protein toxins. To capture the solid-phase in the chamber, a two-level strategy is followed: (a) a dam structure (as shown in figure 2a) is constructed to pack the magnetic beads (diameter: 2.8 µm) on one side [3], (b) Ni-Fe patterns (lines) are fabricated on the same side of the dam to act as magnetic traps for bead distribution [4]. The two ends of the microchannel are connected to inlet/outlet connections in the form of generic Nanoport™ assembly (Upchurch Scientific). The microchannels are designed with a width of 250 µm, depth 100 µm and 7.5 mm in length. The design for the enzyme reactor are created in AutoCAD and then transferred onto glass masks coated with chrome.

Next, the microfluidic enzyme reactor is fabricated in three steps: (a) bottom microchannel is created by PDMS molding (after development of SU-8 structures by photolithography). This step creates the long microchannel and the dam structure (100 µm in height and 2 mm width), (b) PDMS top cover (200 µm thick) is then created with shallow 1 µm groove (just over the dam structure), and (c) Ni-Fe patterns are fabricated by photolithography on plasma cleaned Corning 7740 Glass substrate. The Ni-Fe patterns are 2 µm thick with a pitch of 4 µm (Figure 2b). As stated earlier, these patterns act as magnetic bead traps, thus eliminating magnetic bead agglomeration to enhance reaction surface area, reaction efficiency and reduce inlet pressure. The three layers fabricated are then bonded together by plasma activation and heating to 100 °C for 2 minutes.

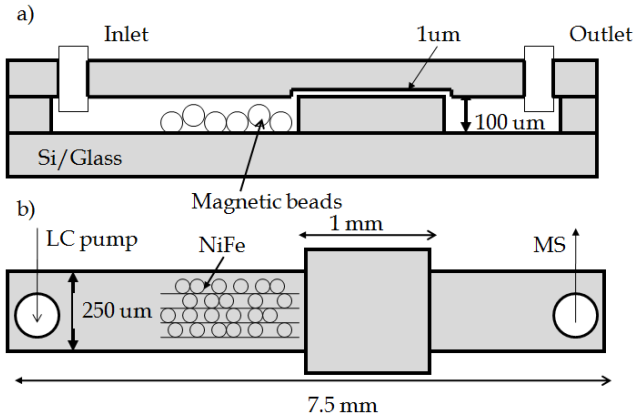

Fig 2. Schematic illustration of the microfluidic enzyme reactor (a) cross-sectional view, (b) overview with magnetic beads trapped on to Ni-Fe patterns in the microchannel.

Next, the ends of the microchannel (Figure 2b) are connected to the inlet/outlet connectors using Nanoport™ assembly. Figure 3 shows the image of the completed two-channel microfluidic enzyme reactor with inlet/outlet connections. An important advantage of this method is that the number of channels (sample preparation unit) can be easily multiplexed, resulting in high throughput analysis. In fact for some analysis in this work, we fabricated between 5 to 10 microchannels, which in turn were connected to a 10-port switching valve for automated analysis.

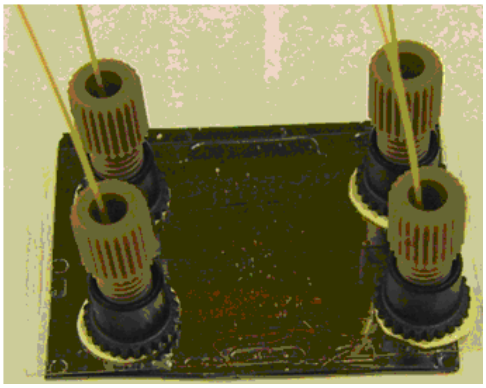

Fig. 3. Image of a two channel microfluidic enzyme reactor with inlet and outlet ports, with Ni-Fe magnetic traps fabricated underneath.

IV. Experimental Testing of Coupled Microfluidic Enzyme Reactor Mass Spectroemtry Platform

Figure 4 illustrates the experimental test setup showing the coupling the 10-channel microfluidic enzyme reactor to a 10-port switching valve, sample injection and detection unit. On the inlet end (sample injection), the microchannels through the valve assembly is connected to a autosampler and Agilent 1200 series LC pump for injection of internal standard and peptide substrate. On the outlet (detection) end, the microchannels are connected through a HPLC column to MS/MS detection system (Applied Biosystems 4000Q-TRAP triple quadrupole mass spectrometer).

The analysis of LF by MS/MS detection technique used the following detection sequence. Dynal 2.8 µm magnetic beads coated with antigen-antibody complex were loaded into the microchannel. Then, the peptide substrate and the CDC internal standard were injected into the microfluidic cell by the LC pump. Following the enzymatic reaction, the reaction products (product 1 and product 2) along with the excess substrate and internal standard were continuously collected on the HPLC column. The products were automatically eluted from the HPLC pump by a binary pump and detected by the MS/MS method. The entire operation was automated by using Analyst® software.

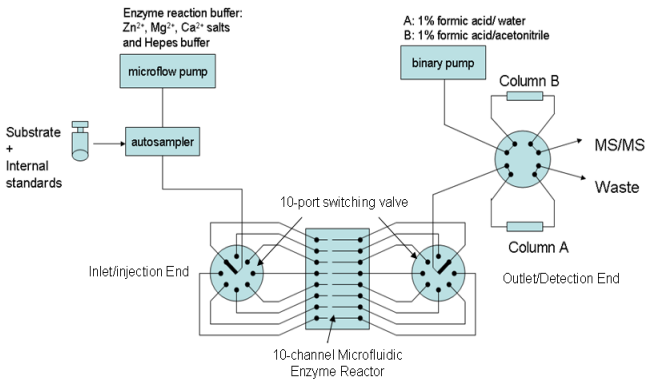

Fig. 4. Illustration of the experimental test setup for the coupled microfluidic enzyme reactor mass spectrometry platform to detect quantitatively anthrax lethal factor.

V. Results and Discussion

Figure 5 shows the image of magnetic beads packed microchannel near the dam structure. The inset shows the distribution of magnetic beads along the Ni-Fe patterns. One obvious observation was that because of the distribution of magnetic beads, the inlet line pressure drastically reduced from 60-100 bar range to about 10 bar. This pressure reduction had a huge impact on the reliability of inlet/outlet port connectors. Then the flow rate for injection and concentration of peptide substrate were optimized at 6 nM and 2 µL/min respectively, after a series of test runs.

Next, to validate the detection concept and specificity of the reaction solid-phase for LF activity, MS/MS detection was conducted after enzymatic reaction with substrate in either absence or presence of 5 ng/mL LF. Isotopically labeled internal standards (ISTD) were used for accurate quantification. Figure 6 shows the MS spectra of products, along with ISTD in the absence and presence of LF. In absence of LF, only ISTD peak (799.4/833.5 amu) was visible after 15-minutes of run time. No product peaks were seen, with only a very small background signal. However, with 5 ng/mL LF in addition to ISTD, doubly charged product peak was visible with masses 795.9/826.5 amu and 795.9/729.4 amu.

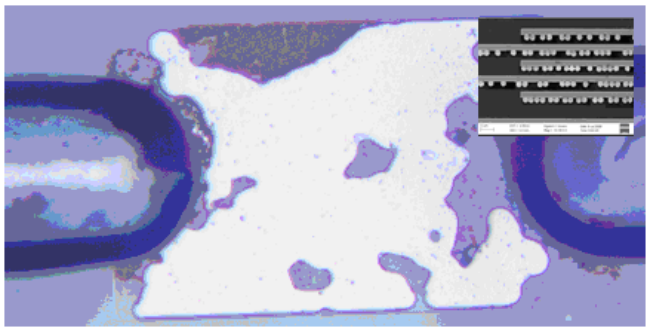

Fig. 5. Magnified view of the dam region and channels on both sides. (Inset) SEM image of magnetic beads trapped on Ni-Fe patterns

Before, LF activity could be analyzed for range of LF concentrations (calibration curve); the purge rate for removing functionalized beads was optimized at 80-100 µL of TW/PBS buffer. Next, the LF activity was monitored for LF concentration from 40 to 0.1 ng/mL. In this work, two volumes of reaction solid-phase (0.5µL and 1µL of magnetic beads) were analyzed after substrate cleavage.

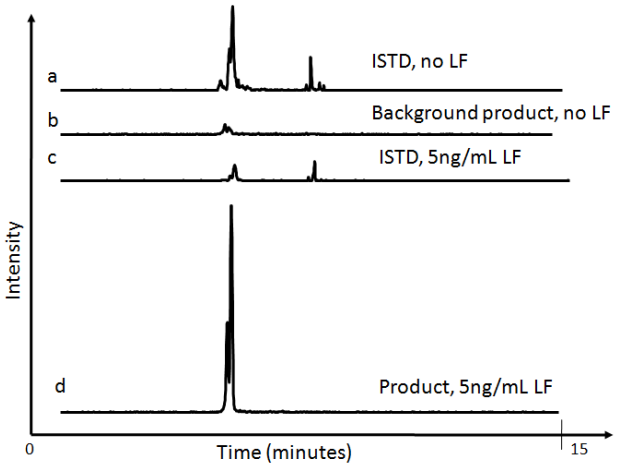

Fig 6. HPLC-ESI/MS/MS spectra after product cleavage with 6 nM peptide substrate and reaction buffer with no LF (a and b), with 5 ng/mL LF (c and d) for a 15-min run time.

Figure 7 shows the response ratio (product/ISTD intensity) from triplicate analyses versus LF concentration range. Both reaction volumes showed a linear response to concentration up to 0.5 ng/mL with reproducibility of ±5%. At less than 0.5 ng/mL of LF, the enzyme reaction became diffusion-limited showing a quadratic signature. An enzyme reaction kinetics study and optimization of substrate availability will help us understand this phenomenon better. The limit of detection was calculated to be 1 ng/mL for these reaction volumes. This limit of detection is significantly lower than 20 ng/mL limit for LF ELISA [5]. Even lower detection limits can be achieved by optimizing the sample availability and incubation times.

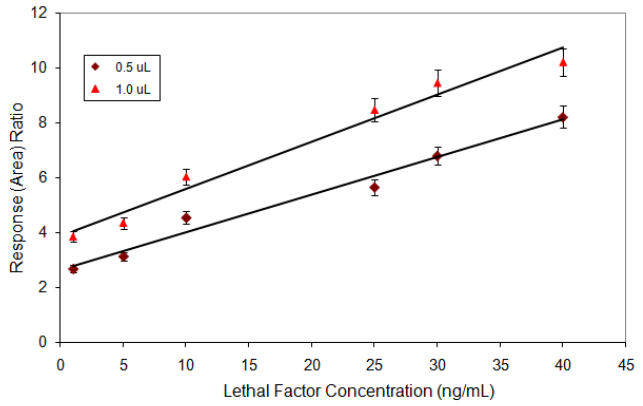

Fig. 7. Calibration curve of LF activity showing response ratio (ratio of product intensity to ISTD intensity) for LF concentration (40 to 0.5 ng/mL). Two volumes of reaction solid-phase of 0.5 and 1.0µL are used in these analyses.

Lastly, the reproducibility and stability of the reaction solid-phase (LF activity) were studied in time. The MS spectra showed no significant variation in product response ratio on repeated substrate injections and cleavage product collection even after 16 hours [6]. Method validation at even lower LF (less than 0.1 ng/mL) by optimization of peptide design to LF activity and quantification with other established methods at CDC (such as ELISA, MALDI) is currently in progress. Future studies include correlation of anthrax toxemia with clinical status as infection progresses and effect of protective antigen (PA83 and PA63) on LF assay.

VI. Conclusion

In conclusion, this method represents a novel, highly specific, rapid and sensitive diagnostic tool for protein toxins with potential for advancing our understanding of toxemia and *Bacillus anthracis* infection. The combination of LF-specific antibody capture, LF-specific peptide cleavage and MS peptide detection provides three levels of specificity for an unambiguous diagnostic method for anthrax lethal factor. Furthermore, the integration of an efficient sample preparation unit namely, the microfluidic enzyme reactor enabled shorter reaction and analysis times (less than 15 minutes in total). We also showed reproducible LF detection in the concentration range of 40 to 0.5 ng/mL, with detection limit of 1 ng/mL. We believe that microfluidics will play an important role in development of robust, high throughput and efficient detection systems and ultimately envision a combined system that could enable all analytical processes on-chip.

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