INTEGRATED TOP-DOWN/BOTTOM-UP MASS SPECTROMETRY OF PROTEINS USING A DROPLET MICROFLUIDIC PLATFORM

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ABSTRACT

We have used droplet microfluidic (DMF) platform as front-end sample preparation technique prior to ESI mass-spectrometric analysis of protein samples. DMF is a micro-electromechanical system (MEMS) technology capable of moving, splitting, merging and dispensing of sub-microlitre droplets. We report on integration of DMF with ESI-MS using a liquid handling robot and demonstrate sample enrichment using functionalized magnetic beads to perform on-chip enrichment of a HIS-tagged protein from a clarified cell lysate droplet, followed by alternatively a bottom-up or top-down proteomics mass-spectrometric workflow. This demonstrates the utility of DMF for low-volume automated sample handling for mass spectrometric analysis.

KEYWORDS: Droplet Microfluidics, Mass Spectrometry, On-Chip Enrichment

INTRODUCTION

The use of mass spectrometry in the biosciences has undergone huge growth in recent years due to sustained effort in the development of new ionisation techniques, more powerful mass analyzers and better bioinformatics tools. This makes it now possible to introduce increasingly complex crude biological-mixtures into mass spectrometers. The amount of sample required for mass spectrometric analysis is frequently determined not by instrument sensitivity but by the ability to isolate, prepare and deliver trace analytes to the instrument. Losses during conventional sample preparation and the challenges in working with small volumes on the benchtop compound the problem. As such, there is a growing interest in the manipulation and characterization of small sample volumes, driven by demand from end-users. Droplet microfluidic (DMF) technology is an attractive option, because in contrast to bulk fluid flow in microfluidic channels it takes advantage of droplet microfluidics. In this technique, droplets of fluid are manipulated across a surface of patterned electrodes by means of electromechanical forces. Droplets on these devices are isolated from their surroundings, rather than being embedded in a stream of fluid and in theory only the surface of the chip offers an opportunity to lose low abundance analytes. In the case of sample-limited analyses, the entire droplet can be manipulated with minimal analyte dilution. Furthermore, DMF separation techniques have been developed to concentrate samples by reducing the container volume through splitting or evaporation. This is particularly relevant for low concentration analytes since sample concentration increases inversely with decreasing droplet volume.

In this paper the use of Droplet Microfluidics (DMF) for front-end sample preparation prior to ESI mass spectrometric analysis of protein samples has been investigated. DMF is a micro-electromechanical system (MEMS) technology capable of moving, splitting, merging and dispensing of sub-microlitre droplets [1-4]. Here, such devices have been directly integrated with ESI-MS using a liquid handling robot. We demonstrate on-chip sample enrichment using functionalized magnetic beads to perform enrichment of HIS-tagged *E. coli* Bacterioferritin Comigratory Protein (BCP) protein from a clarified cell lysate, followed by either a bottom-up and top-down proteomics mass-spectrometric workflow. These experiments demonstrate the utility of DMF platforms for low-volume automated sample handling in combination with mass spectrometric analysis.

EXPERIMENTAL

BCP was expressed with a His-Tag to facilitate enrichment as described previously [5, 6]. Cells were harvested by centrifugation and lysed using BugBuster protein extraction reagent (Novagen) and the clarified cell lysate was diluted 1 in 10 before use with 50 mM Tris-HCl, pH 7.5. The DMF devices are based on a co-planar low-voltage design [7]. A thermally oxidized silicon wafer was sputtered with tantalum and electrodes patterned using a dry-etch process. The electrodes were anodized to form a 38nm thick pin-hole free Ta_2O_5 dielectric. Finally, Teflon-C was spincoated onto the chip surface to form a 16nm thick hydrophobic layer on top of the final device. The devices were computer-controlled (LabView, National Instruments) using a digital I/O expander (Agilent Technologies) for application of the control voltages. The liquid handling robot was based on a modified 2700 MALDI spotter (Waters, UK), also under computer control. One of the two arms of the robot was utilized to pickup and dispense sample/solvents from a 96 well microtiter plate, the other transferred samples into the injection valve of a HPLC. An electromagnet (Magnet Schultz, Germany) was placed below the DMF chip to pull the magnetic beads down to the surface of the chip.

The on-chip enrichment of BCP from the crude cell lysate was carried out as follows: Functionalized Ni-affinity magnetic beads (BcMag: His-Tag, BioClone) were used to selectively bind proteins containing 6AA histidine motif. A 1µl lysate drop-

let (total protein concentration 100 µg/ml) and 1µl aliquot of bead solution (diluted 1:10) were dispensed on the chip and combined and mixed using DMF actuation. After 10min, the magnetic field was switched on, pulling the beads down to the chip surface within ~5s (Figure 1), allowing the removal of supernatant using the liquid handling robot. The beads were re-suspended by addition of 1ul droplet of wash buffer (50mM Tris-HCl, pH 7.0) using DMF. For efficient re-suspension, the applied voltage to the DMF electrodes was pulsed several times (DMF-assisted mixing). Typically, this wash procedure was repeated three times.

For top-down analysis, BCP was eluted from the beads by using DMF to perform an addition of 1 µl of elution buffer (25mM Tris-HCl, 250mM imidazole, pH 7.0). The beads were then pulled down using the electromagnet. The robot was then

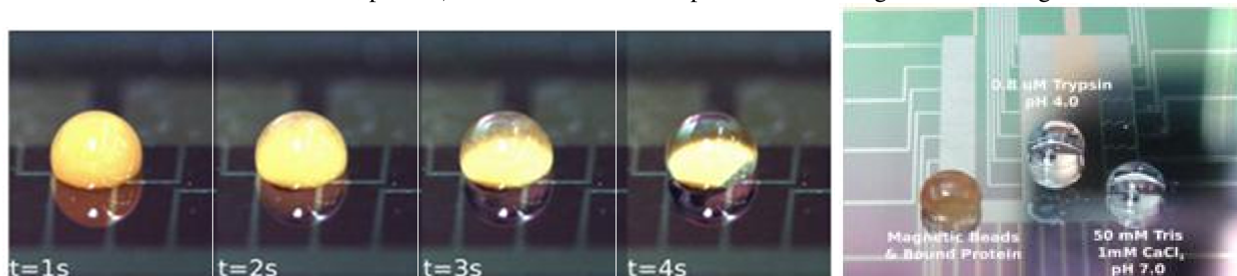

Figure 1: Magnetic bead enrichment using embedded electromagnet showing dispersed (yellow) beads pulled to the chip surface (*left*) for rinsing. On-chip tryptic digest of HIS-tagged BCP protein bound to magnetic beads (*right*).

used to introduce the eluted protein extract into a LC-FT-ICR mass spectrometer for intact protein analysis and top-down fragmentation. Separation was achieved on an Ultimate 3000 system (Dionex, CA) with a monolithic PS-DVB (500 µm × 50 mm). 20 s fractions of 6 µL each were collected in a 96 well microtiter plates and stored at -20 °C for further analysis. Data were acquired on a 12 T solariX FT-ICR mass spectrometer (Bruker Daltonics, MA) using a TriVersa Nanomate infusion robot (Advion BioSciences, USA) and analysed using DataAnalysis (Bruker Daltonics). Fragments were assigned using the Prosight PTM and Prosight 2.0 software packages. For MS/MS experiments, CID and ECD fragmentation was utilized.

For bottom-up analysis , BCP was digested on-chip, the DMF chip was used to combine and mix three 1 µl droplets: protein bound to magnetic beads, trypsin (0.8 µM, pH 4.0) and buffer solution (50 mM Tris-HCl, 1 mM $CaCl_2$ pH 7.0). Following combination and mixing, the sample was incubated on-chip for 1h at room temperature, during which small (200 nl) volumes of water were added using the liquid handling robot to counteract evaporation. On-line LC was carried out with a Dionex UltiMate 3000 HPLC using a Pepmap100 C18 precolumn (300µm x 5mm) and Pepmap100 C18 (150mm x 75µm) analytical column (LC Packings, CA). An HCT ion trap mass spectrometer, equipped with a nanoESI source (Bruker Daltonics), was employed, using data-dependent switching between MS and MS^2 and collision induced dissociation (CID) fragmentation. The resulting data were searched using Mascot (version 2.2; Matrix Science Ltd., U.K.).

RESULTS

The results of the top-down FT-ICR MS analysis of the intact protein are shown on the left side in Figure 2. The chromatogram and the charge state distribution of the predominant eluting protein are shown. The fraction containing the protein was then re-infused into the FT-ICR MS, and the $[M+24H]^{24+}$ charge state isolated in the mass resolving quadrupole, and fragmented using ECD. The resulting ECD fragments were assigned using Prosight 2.0 software, which provided unambiguous assignment of BCP.

The results of the bottom up experiment are shown on the right side in Figure 2. The predominant species observed was identified as *E. coli* BCP and the only other significant protein match was bovine trypsin. The BCP identification displayed a MASCOT score of 379 and total sequence coverage of 38%. Unambiguous identification of the BCP protein was achieved by accurate intact mass measurement and ECD fragmentation.

DISCUSSION

The focus of this work is on the integration of DMF technology with existing ESI mass spectrometry techniques for protein analysis. Sista *et al.*[8] have previously demonstrated that functionalized magnetic beads, suspended in a droplet, can be efficiently handled on DMF-devices; and they successfully demonstrate an on-chip immunoassay using this technology. In order to allow us to exploit functionalized magnetic bead technology, we incorporated an electromagnet into the design of our DMF platform, positioned directly under the DMF array (Figure 1). This allowed us to perform on-chip DMF-based protein enrichment.

On-chip enzymatic protein digestion has recently been demonstrated by other research groups, coupled with matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). [9, 10]. However, here we have used our low-voltage Si/Ta_2O_5-based DMF devices for this application. In addition, by utilizing a liquid handling robot, we integrate DMF-based

protein digestion with ESI-MS. This combination of DMF and ESI affords great flexibility in the analysis stage of our work-flow. Notably, ESI ionisation allows us to perform a range of tandem mass spectrometry experiments.

It seems clear that there will be increasing demand for more sophisticated sample preparation, sample enrichment and sample manipulation, in numerous fields of active research, such as the life sciences, biomedical sciences, food, beverage and environmental research, as well as military/defence. DMF devices offer one of the most promising routes for the delivery of a generic technology platform that can address these pressing areas of miniaturised exploratory measurement science

The results presented in this paper clearly demonstrate the utility of DMF devices for fluid handling and sample preparation technology to facilitate biological mass spectrometry. The DMF array utilised in this paper is a significant milestone for the development of larger arrays, which will permit processing of many samples in parallel and under full computer control.

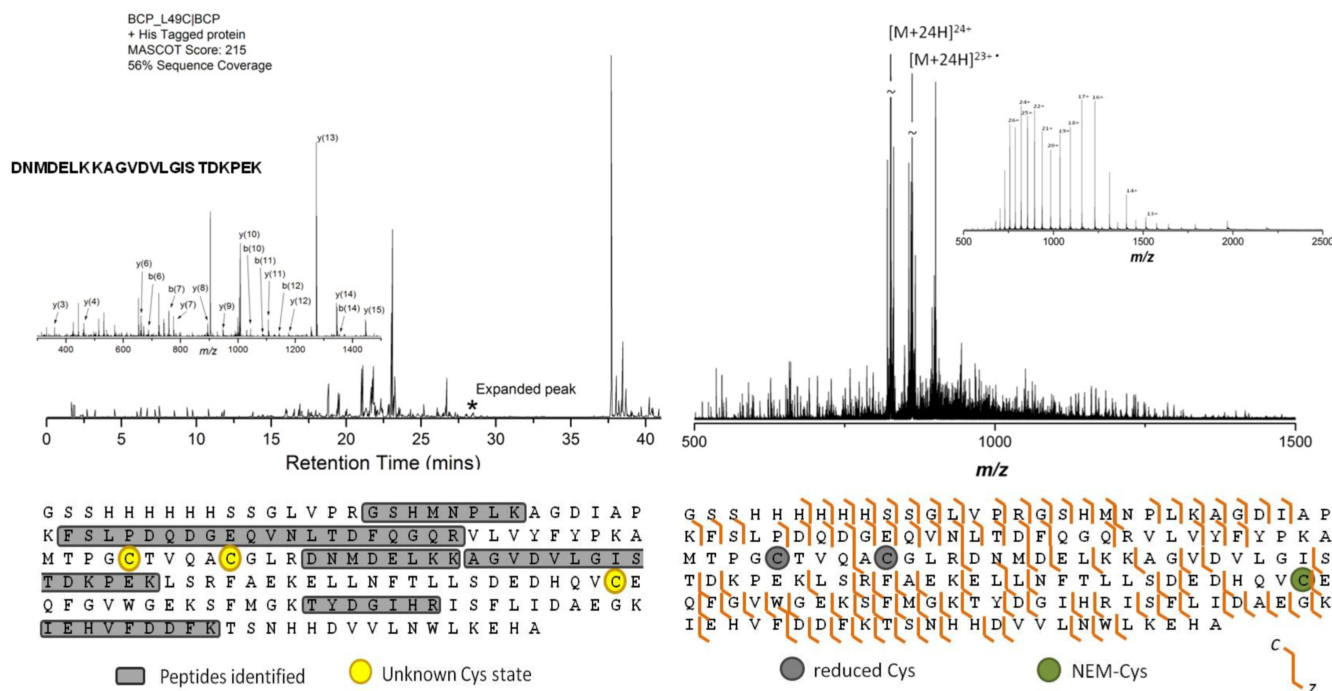

Figure 2: *Left*: Bottom-up MS Analysis. *Insert*; MS/MS spectrum for the highest scoring peptide from MASCOT: *Main*; LC chromatograph of BCP tryptic digest: *Bottom*; MASCOT results and sequence coverage achieved. *Right*: Top-down MS analysis: *Insert*; Intact protein MS after on-chip processing: *Middle*; Fragmentation of the 24+ charge state of BCP: *Bottom*; Fragmentation map for BCP.

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