Bioaffinity Mass Spectrometry Screening Using Droplet-Based Microfluidics

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Abstract: Bioaffinity mass spectrometry screening is a novel approach using non-denaturing electrospray ionization (ESI) mass spectrometry (MS) in identifying drug leads. This screening technique can detect and preserve noncovalent protein-active drug ligand complexes under different physiological conditions. Although there are many successful screening campaigns employing this technique, the big challenge of the screening is the reduction of sample volume needed. We demonstrate in this paper that analysis of samples can be performed using droplet-based microfluidics. Droplets of samples to be screened are formed and delivered directly into the electrospray emitter of a Fourier Transform mass spectrometer. The results show that a MS instrument with a conventional ESI source can clearly detect the samples and distinguish it with the separating oil phase. The proposed technique opens the possibility of bioaffinity mass spectrometry screening of small samples with a simple microfluidic device.

Keywords: Bioaffinity mass spectrometry, compartmentalisation, droplet-based microfluidics, ESI.

1. INTRODUCTION

Bioaffinity mass spectrometry screening is a simple mix-and-measure assay using mass spectrometry. This method identifies active compound ligands binding with therapeutic target proteins under different physiological condition. We previously applied this method to successfully screen crude natural product extracts and fragment compound libraries [1, 2]. Although this technique is a very efficient and powerful tool for preliminary screening processes, the consumption of limited target proteins and tested compounds still remains a big challenge. State-of-the-art automated systems such as Triversa Nanomate allows the direct transfer of samples to an array of electrospray ionization (ESI) nozzles [3]. However, the relatively large nozzle size increases the cost of consumables making it prohibitive for a large spectrum of researchers. Recent advances in microfluidics and lab-on-a-chip (LOC) technologies allow for the implementation of conventional lab protocols in a microchip. Efforts have also been made to use microfluidic technology for mass spectrometry based proteomics [4]. A sample-saving method is the use of droplet-based microfluidics, where samples are compartmented in droplets that are protected by an immiscible oil [5]. The droplets represent the reactors or the wells of the conventional microwell plates which reduce the volume consumption of samples.

The major challenge for the utilization of droplet-based microfluidic technology in mass spectrometry screening is the interface of the microfluidic device to a conventional ESI-MS system. Droplets can first be formed in a microfluidic chip using two basic configurations such as the T-junction or the flow-focusing junction [6]. The droplets then can be delivered directly to mass spectrometry for screening. Recently, Trivedi et al. proposed the use of off-the-shelf polytetrafluoroethylene (PTFE) tubing for the formation and handling of sample droplets [7]. With PTFE tubing as the storage and transport platform for droplets, a number of research groups have proposed automated robotic platforms that convert samples from a micro well array into a droplet train. Clausell-Tormos et al. used an autosampler to load samples from a microtitre plate into a plug train in a PTFE tubing [8]. The samples are encapsulated and separated by 2.5%(v/v) perfluoro-octanol in fluorinated FC40 oil. This system was used for kinetic analyse of compound libraries based on optical detection. Wu et al. applied the same concept of automated sampling to transfer a droplet train from a well plate to a PTFE tubing and then to a PDMS microfluidic chip for subsequent manipulation [9]. Gielen et al. reported a robotic sampling system using a rotating oil filled carousel [10]. Samples are transferred to a PTFE tubing from the bottom of sample tubes to minimize contamination. Perfluoro-octanol in FC40 with a concentration of 0.5%(v/v) was also used as the immiscible oil phase.

The sample droplets of the above mentioned works were analysed using simple optical absorbance detection or capillary electrophoretic separation [11]. A few attempts to interface droplet-based microfluidics with conventional mass spectrometry have been reported in the literature. Pereira et al. transferred a droplet train from the PTFE tubing to a matrix-assisted laser desorption/ionisation (MALDI) mass
spectrometry [12]. The droplets were deposited on the MALDI matrix, where the oil was removed by an oleophilic film. Fidalgo et al. were the first to couple droplet-based microfluidics to an ESI-MS system [13]. The droplets were transferred to a continuous aqueous stream before entering the emitter nozzle. Since the final stage of this concept is still based on continuous-flow microfluidics, the sample is diluted by Taylor dispersion resulting in low detection resolution. Zhu and Fang reported a similar concept of transferring sample droplets to a continuous flow and then to the emitter nozzle. Since the final stage of this concept is still based on continuous-flow microfluidics, the sample is diluted by Taylor dispersion resulting in low detection resolution. Recently, Smith et al. use a microfluidic device to deliver surfactant stabilised picoliter droplets to an ESI-MS system [17]. Four different protein samples compartmented in droplets and separated by FC-3283 could be detected without cross contamination.

In this paper, we demonstrate the formation of sample droplets and direct coupling of a droplet train to an ESI-MS system for identifying protein-ligand complexes. We successfully detected droplets with the protein and the protein-ligand complex using a microfluidic T-junction device. The results demonstrated that a simple and low-cost interface setup with common fluorinated oil is suitable for providing mass spectrometry data of proteins and protein-ligand non-covalent complexes. Successful detection of the complexes is an important step for designing and coupling new multiple channel devices with ESI-MS for the development of a high-throughput bioaffinity mass spectrometry screening.

2. EXPERIMENTAL

2.1. Chemical and Reagents

Fluorinated oil FC-40 and PFOH (1H, 1H, 2H, 2H-perfluorooctan-1-ol), bovine carbonic anhydrase II (bCAII, EC 4.2.1.1, 29,089 Da), and sulfanilamide (C6H8N2O2S, 172 Da) were purchased from Sigma Aldrich.

2.2. Sample Preparation

Bovine carbonic anhydrase II bCAII was used without further purification. The protein was dissolved in ammonium acetate (10 mM, pH 7) to generate the stock solution. The specific inhibitor of bCAII used in this study was sulfanilamide, which was dissolved in methanol to generate the stock solution. The protein was mixed with the inhibitor and incubated for one hour at room temperature. The final inhibitor: protein ratio was approximately 8:1.

2.3. Microfluidic Device

The microfluidic device was fabricated in Polymethylmethacrylate (PMMA) using standard laser ablation and thermal sealing technique. The widths of the dispersed phase and continuous phase channel are 450 µm and 800 µm, respectively. The microchannels have a depth of about 1500 µm. A Polytetrafluoroethylene (PTFE) tubing (Upchurch scientific, Australia) was inserted directly into the PMMA device through the end access hole and sealed using Ultraviolet (UV) glue to prevent any leakages.

2.4. Formation of Sample Droplets Using Capillary Tubings

Initial experiments were carried out on the formation of a droplet train in fluorinated oil using a capillary T-junction device. The fluorinated oil is a mixture of FC40 and 30% v/v PFOH to stabilize the droplets and to prevent unspecific adsorption of biomolecules to the surface of the fluidic system. PTFE tubings with a respective inner diameter and outer diameter of 1.56 mm and 0.15 mm, were purchased from Cole-Pamex (USA), and was used as the channels in our experiment. The two inlet tubings for the T-junction were 100 mm long. The outlet tubing for storage of the droplet train and interfacing to the ESI source was 400 mm long. The droplets were formed in a zero-dead volume stainless-steel internal T-junction fitting (ZT.5, Valco, Switzerland). The aqueous phase (protein sample) was delivered from a syringe (Hamilton, 250 µL, inner diameter of 2.30 mm). Another syringe (SGE, 500 µL, inner diameter of 3.26 mm) provided the oil phase to the tubing. Both syringes were run on the same pump (World Precision Instruments, model sp200i-z, USA). Thus, the ratio between the oil flow rate \( Q_{oil} \) and the sample flow rate \( Q_{sample} \) in our experiments were kept constant at \( Q_{oil}/Q_{sample}=2 \). The droplet formation process was observed and recorded by a high-speed camera (FASTCAM SA3, Photron, USA) mounted on an inverted microscope (ECLIPSE TS100-F, Nikon, Japan). Fig. (1) shows the setup of the capillary T-junction device for the formation of the droplet train.

![Fig. (1). Experimental setup for the formation of sample droplets in a T-junction device with Teflon tubings as microfluidic channels.](image-url)
tion. Each spectrum was a scan with 256,000 data points. All aspects of pulse sequence control and data acquisition were performed on an Intel® Xeon® CPU W3520@2.67 GHz Quad Core computer running Bruker Solarix control software under Windows operating system. The end plate voltage was biased at 500 V and the capillary voltage at 4000 V relative to the ESI needle during the data acquisition. Nebulizing nitrogen gas with a pressure of 1.5 bar and a counter-current drying nitrogen gas with a flow rate of 3 L/min were employed. The drying gas temperature was maintained at 180 °C.

2.6. Formation of Sample Droplets Using PMMA T-Junction Microfluidic Device

Similar experiments were carried out on the formation of a droplet train in a PMMA T-Junction microfluidic device. The device was made of polymethyl methacrylate (PMMA), which can withstand the organic surfactant perfluorooctanol. The droplets train was formed, observed in the device, and transferred to the ESI source using PTFE tubing connection with an inner diameter and outer diameter of 0.5 mm and 1.58 mm. The protein sample as the dispersed phase was delivered from a syringe (Hamilton, 2.50 mL, inner diameter of 7.28 mm). Another syringe (Hamilton, 5.00 mL, inner diameter of 10.30 mm) delivered the oil as the continuous phase fluid. The flow rate ratio \( \frac{Q_{\text{oil}}}{Q_{\text{sample}}} \) in the droplet formation experiments varied as 0.2, 0.25, 0.33 and 0.5.

3. RESULTS AND DISCUSSIONS

3.1. Optimization of MS Instrumental Parameters

Non-denaturing ESI-MS can preserve non-covalent protein-ligand complexes from solution phase into the gas phase. However the non-covalent complex is extremely fragile in the gaseous state. Excess heat or high collision energy in the atmospheric pressure or vacuum interface can dissociate the complexes. Instrumental parameters can be optimized to stabilize the non-covalent complexes. The first important parameter of the ESI source is the capillary temperature. At a capillary temperature of 100°C non-covalent complexes are poorly desolvated and are thus poorly observed. Increasing the capillary temperature to 200°C will improve the desolvation process but will destabilize the complexes. To get the best signal intensity of the noncovalent complexes, this work was carried out with the capillary temperature of 180°C.

The second important parameter of the ESI source is the skimmer voltage. A higher skimmer voltage generally accelerates ions from the source region to the high vacuum analyzer of the instrument, but also increases the internal energy imparted to the complex and can destabilize the complex. Experiments on different skimmer voltages were conducted to find the optimum value for the best protein-ligand complex signal. Fig. (2) shows the signal intensity of the protein-ligand complex decreases with increasing skimmer voltage from 50 V to 80 V. At a skimmer voltage of 50V, 60V and 70 V, the peak intensity of the \([\text{bCA-SFM}]\) complex at the charge state 11+ are \(2.5 \times 10^9\), \(2 \times 10^9\), and \(1 \times 10^9\), respectively. At a skimmer voltage of 80V, the intensity of the complex was almost at null. Fig. (3) shows the signal intensity of the protein-ligand complex at skimmer voltage of 50, 30 and 10 V. The protein complex signal had the best signal sensitivity at a skimmer voltage of 50V. When the skimmer voltage decreases to 30V or 10V, nonspecific bindings are apparent. Fig. (4) illustrates the effect of the skimmer voltages to the ratio between protein-ligand and the protein. In order to obtain the best protein-ligand non-covalent complex, a skimmer voltage of 50 V was chosen as the optimal skimmer voltage for all the experiments in this manuscript.

![Fig. (2). Mass spectra of the mixture of bCA II with sulfanilamide under skimmer voltages 50, 60, 70, and 80 V. [bCA II-SFM] complex is dissociated under high skimmer voltage.](image-url)
Fig. (3). Mass spectra of the mixture of bCAII with sulfanilamide under skimmer voltages of 10, 30, and 50V. [bCAII-SFM] complex was better with skimmer voltage of 50V.

Fig. (4). Effect of skimmer voltage to the protein-ligand complex.

3.2. ESI-MS Analysis of the Sample Train Formed in the PTFE Tubing of the T-Junction Device

The droplet train was formed after injecting both the protein sample (3.4 μM) and the fluorinated oil into the T-junction device. Fig. (5a) shows the total ion current (TIC) of the droplet train. Fig. (5b) shows the mass spectrum of the FC40 oil. Fig. (5c) shows the spectrum of the protein in three different charge states, \( m/z = 2424.9 \) at 12+, \( m/z = 2645.5 \) at 11+, and \( m/z = 2909.9 \) at 10+. Fig. (5d) shows the spectrum of the protein complex bCA-sulfanilamide at three different charge states, \( m/z = 2439.8 \) at 12+, \( m/z = 2661.4 \) at 11+, and \( m/z = 2927.3 \) at 10+. From the complex peak, the molecular weight of the binding inhibitor was identified:

\[
MW_{\text{inhibitor}} = (m/z_{\text{complex}} - m/z_{\text{protein}}) \times z
= (2661.4 - 2645.7) \times 11
= 15.7 \times 11
= 172.
\]
3.3. ESI-MS Analysis of the Sample Droplets Formed in the PMMA Microfluidic T-Junction Device

The protein sample and the oil were injected into the PMMA device using the same syringe pump. By keeping the dispersed phase flow rate constant at 500 µL/hour, mass spectra were acquired at different flow rate ratio (QD/QC) values. These ratios were 1/1, 1/2, 1/3, 1/4 and 1/5. With the ratio 1/1, the droplets were merged which is indicated by the peaks observed in the TIC. With the ratio 1/5, the droplets high intensity was small as the droplets are significantly smaller. Experiment results show that the best flow rate ratio was 1/4 with the dispersed phase flow rate at 500 µL/hour and the continuous phase oil flow rate at 2 mL/hour, Fig. (6). At these flow rates, the TIC peaks representing the sample droplets are distinctive and 8 droplets are detected during the scan.

Next, by keeping the flow rate ratio between the sample and the oil constant at 1/4, experiments were performed at five different sample flow rates ranging from 250 µL/hr to 750 µL/hr. Fig. (7) shows the TIC of protein samples with these different flow rates. At sample flow rates of 250 µL/hr and 375 µL/hr, the droplets were moving slowly and only a limited number of sample droplets were detected in a 3-minute scan. At the higher sample flow rates of 625 µL/hr and 750 µL/hr, the droplets were moving significantly faster than the MS scanning rate. Individual droplets cannot be detected clearly, Fig. (7). Therefore, these higher flow rates set the limit of the proposed system. The sample flow rate of 500 µL/hr was chosen for experiments with the protein-ligand complex (bCA-SFM) as it clearly detects the most number of sample droplets.

The complex bCA-SFM sample and the oil were injected into a similar PMMA T-junction device. The flow rate ratio is 1:4 with the sample flow rate of 500 µL/hr. Fig. (8) shows the TIC of the protein complex sample and its spectrum, indicating that the T-junction made in a PMMA device works well for this purpose.
CONCLUSION

This paper demonstrated a low-cost and simple method for compartmenting protein samples in fluorinated oil for subsequent analysis with a conventional ESI-MS system. The results show that the detection of a protein-ligand complex can take place in a short time on the order of seconds and with a small sample consumption. For the PMMA microfluidic device, a flow rate of 500 µL/hr and a flow rate ratio between sample and oil of 1:4 are the best values for the droplet train. A skimmer voltage of 50V was the optimal parameter of the ESI source in our microfluidic-MS configuration. The method presented here shows the proof of concept for droplet-based drug screening using mass spectrometry. In the future work, the sample droplets could be transferred to the tubing directly from a microwell plate using a robotic platform or a specially designed parallel to serial sampling microfluidic device. With the later concept, droplet merging and reaction could be integrated on the same chip for the ease of handling and the required high throughput.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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