Droplet-based compartmentalization of chemically separated components in two-dimensional separations†

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We demonstrate that nanolitre-sized droplets are an effective tool in coupling two-dimensional separations in both time and space. Using a microfluidic droplet connector, chemically separated components can be segmented into nanolitre droplets. After oil filtering and droplet merging, these droplets are loaded into a second dimension for comprehensive separations.

Chemical separation techniques play an important role in proteomics, genomics, metabolomics and a range of other biochemical fields.[1,2] In recent years, a diversity of two-dimensional (2D) separation methods have been developed in an effort to efficiently probe and resolve complex mixtures, such as proteins expressed by cells or organisms. By coupling orthogonal separation modalities, 2D separations provide enhanced analytical resolution and peak capacities defined by the product of the component peak capacities.[3] At the current time, 2D polyacrylamide gel electrophoresis methods are considered to be the gold-standard in separating complex protein mixtures. However, other 2D column-based separation techniques including LC-LC, LC-CE and CE-CE, have also been developed to provide automated analysis of a broader range of biomolecules.[4,5] Unfortunately, realizing high analytical performance is far from trivial, due to the inherent difficulties in spatially and temporally coupling two different separation techniques (using, for example, traditional valves) when only extremely small amounts of sample are available. The need to integrate multiple operations and achieve high throughput analysis suggests that chip-based or microfluidic platforms may be ideal in performing the comprehensive analyses demanded in -omics sciences.[6–12] However, transferring materials between two orthogonal dimensions has proved to remain a significant challenge. Herein, we describe a new approach for coupling two separation techniques (or mechanisms) using a dynamic micro-droplet interface. In theory, this approach does not sacrifice resolution in any single dimension and allows analysis of nanolitre to femtolitre volumes without the need for valves.

Droplet-based microfluidics has recently emerged as a valuable instrumental platform for performing high throughput chemical and biological experiments.[13] In such systems, droplets are made to spontaneously form when laminar streams of aqueous reagents are injected into an immiscible carrier fluid, either at a T junction or in a flow focusing geometry.[14,15] Analyte molecules can be encapsulated and stored inside the droplets, without evaporation or contamination between droplets. Moreover, recent advances in microfluidic control architecture have also facilitated the precise temporal and spatial manipulation of single droplets and functions such as sorting, splitting and merging[16–18] for complex analyses.[19,20] Edgar *et al.* have recently shown the compartmentalization of CE separated bands of amino acids into droplets with a flow focusing channel and the trapping of droplets for further analysis.[21] Our approach to efficiently integrate both separation dimensions utilizes droplet generation after the first dimension with oil depletion and droplet merging prior to the second dimension. This combination forms a fully functional droplet connector for two-dimensional separations.

In initial studies, two microfluidic devices were designed for analyte compartmentalization and oil depletion respectively (Fig. 1). Both devices were fabricated in polydimethylsiloxane (PDMS) using soft lithography. The first device includes a simple T junction, with an oil inlet, a sample inlet for effluent from the LC capillary (connected to an optional additive channel) and a droplet outlet channel where hydrophobic tubing can collect and transfer generated droplets in sequence. The second device shown in Fig. 1b is composed of channels with pillars at the junction area (Fig. 1c). Here pillar elements are crucial in evacuating oil and loading analyte droplets into the second CE channel. To minimize dead volume, surface adhesion of the molecules to channel and droplet poly-dispersity, all capillaries and tubing were inserted into the channels in a planar fashion. Fluidic channels are 120 μm wide (but dilate to 300 μm where capillary and tubing are inserted) and 150 μm high. The devices can be connected directly using tubing or a channel to provide on-line 2D separations, or a longer length of tubing can be used to collect all the droplets from the first dimension and store them in sequence (see ESI for experimental details†). This tubing can then be inserted into the second device for off-line separation. In the current proof-of-principle studies we adopt the off-line scheme. Moreover, it is important to note that no surface treatment is required and that the connection is valve-free.

Initial experiments involved a rapid LC separation of a peptide mixture on a capillary column packed with PS-DVB particles,[22] using AcN/phosphate (5 mM, pH 3), 30 : 70 v/v as the elution solvent. The separation was completed within 100 seconds. For these separation conditions only two peaks

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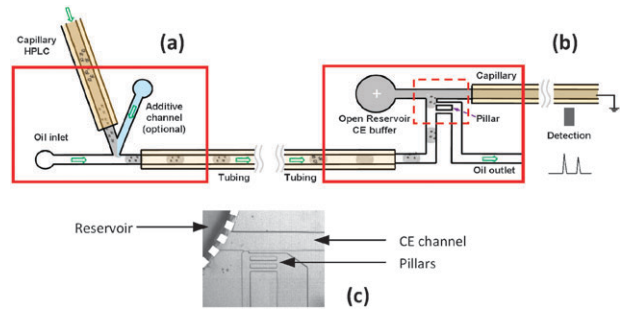

Fig. 1 (a) First dimension capillary HPLC separation and segmentation. (b) Droplet recombination to a continuous flow followed by the second dimension CE separation. (c) Image of fabricated device for droplet recombination.

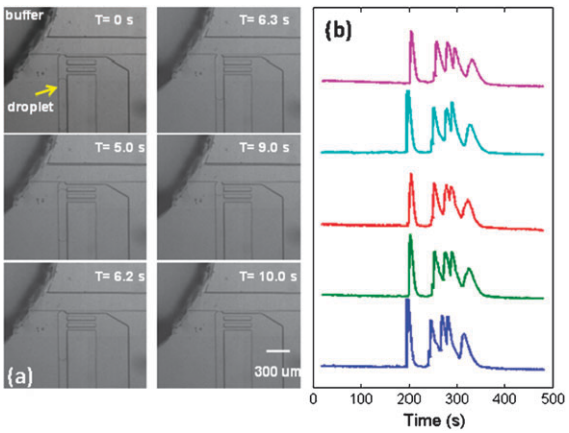

Fig. 2 (a) Complete merging of droplets with a continuous stream in the 2nd dimension CE separation and (b) five sequential CE separations.

and a low lying 'hump' were obtained for the five component mixture, as shown in the right inset of Fig. 3. The rapid elution of the peptides is primarily a result of their positive charge state which is buffered by the phosphate solvent at pH 3, and decreases their hydrophobic interaction with the stationary phase.

Subsequent to separation, the effluent was fractionated into droplets using the first device and stored sequentially in tubing for further analyses (Fig. S1†). Importantly, the fractionation resolution can be precisely adjusted by variation of the flow ratio and channel geometry.[15] According to *Giddings' Criterion* for two-dimensional separations,[3] which states that no resolution 'gained' in the first dimension may be lost in the second dimension, each peak in the first dimension should be sampled at least three times. Close inspection of the LC chromatogram in Fig. 3 shows that each peak extends over more than 12 s. Experimentally, the oil flow rate was maintained at 3.5 μL/min, with an aqueous eluent flow rate of 0.2 μL/min and the droplet generation frequency was 0.25 Hz. Within a time frame of 100 seconds, the HPLC effluent was thus converted into 25 droplets with individual volumes of approximately 10 nL. Repeated experiments with and without connecting the LC capillary to the chip or removing the collection tubing generate reproducible chromatograms, indicating that droplet generation does not affect the separation in the first dimension. Importantly, once the droplets are formed, encapsulated analytes cannot diffuse beyond the droplet boundary. Thus "re-mixing" effects, which are problematic in continuous flow based 2D separations,[10] are prevented and the tubing can be directly connected to the second device for separation or stored for further analysis.

Oil depletion and droplet merging are crucial processes prior to the second separation dimension using droplets, since it is recognized that oil contamination alters the surface chemistry of the capillaries/channels and defunctionalizes the separation.[19,20]

Here the second microfluidic device utilizes a passive pillar array to filter out the oil and induce merging between the droplet and the continuous CE channel flow.[18]

This droplet merging process can be visualized in Fig. 2a. An open CE buffer reservoir (5 mm in diameter) was punched directly on the microfluidic device, filled with buffer and connected to a high voltage power supply *via* a Pt electrode. The other end of the CE channel was connected directly to a fused silica capillary with an inner diameter of 100 μm. This silica capillary was immersed in a buffer reservoir connected to ground. Since the resolution of charged species in CE is related to both the time that the analytes experience the electrophoresis and the capillary length, a 20 cm long fused silica capillary was used to perform the CE separation.

Two syringe pumps were operated in constant volume mode to drive both the droplets and the continuous oil flow. During operation, one syringe pump allows fine delivery of single droplets downstream to the pillars, while the other is used to aspirate oil from the oil outlet. While the carrier oil phase is filtered sideways to the oil channel, the aqueous droplet containing sample is driven upwards, as shown in Fig. 2a (and a movie provided in the ESI†). Because no surfactant exists in the oil phase, droplets merge in a facile and automatic manner with the buffer in the CE channel. Upon merging, droplets still block the thin channels between the pillars due to surface tension, therefore further pushing of the oil flow manoeuvres the rest of the droplet into the CE channel, which is closely connected to the open buffer reservoir. Significantly, complete droplet injection occurs without any oil being introduced into the CE channel.

Once the sample droplet has been injected into the CE channel, a 10 kV high voltage is applied to initiate the electrophoresis, with UV absorbance detection (at 214 nm) being performed 20 cm downstream of the capillary. An evaluation of the reproducibility of the second dimension separation (using identical droplets segmented from the original peptide mixture) is shown in Fig. 2b. The excellent reproducibility of sequential droplet separations enabled the comprehensive analysis of all the fractions obtained from the first HPLC separation.

The droplet fractions stored in the tubing from the first dimension were then introduced into the CE column using the procedure defined above. Fig. 3 presents a 2D LC-CE pseudo-gel map of the peptide mixture (Fig. S2† shows the electropherogram of a representative droplet). Accordingly, all five peptides and possible isomers were resolved and displayed based on the orthogonal selectivity of LC and CE. The peak capacity achieved in the current system was only about 2000. This is due to the narrow elution window in the first dimension (leading to a peak capacity of only 25) while in the CE stage a reasonably high peak capacity of approximately 75 was obtained. However, the *droplet-on-demand* operation between the two dimensions detaches the separation stages both

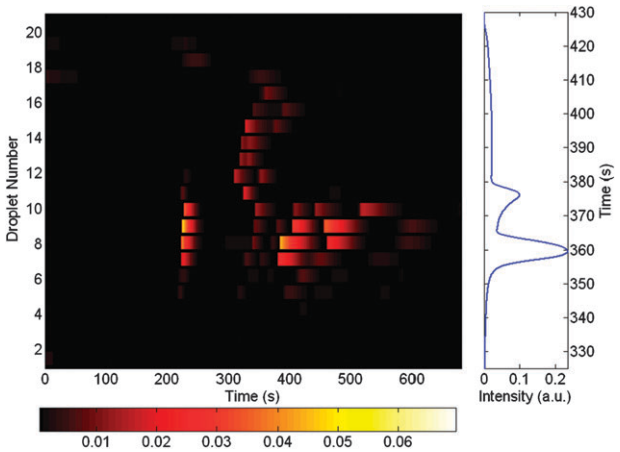

Fig. 3 Two-dimensional separation of a peptide mixture. The inset in the right is a chromatogram from the first dimension LC separation.

temporally and spatially, without sacrificing separation performance. This in turn allows independent optimisation of each separation dimension.

For more complex mixtures such as proteomic samples, a detailed analysis of certain sub-groups (*e.g.* proteins that are slightly different due to post translational modifications) is often more important than achieving a comprehensive 2D map. In this regard, we have pursued "heart-cutting" [23] 2D separations with the current platform. Here only certain fractions of interest are submitted to the 2nd dimension for further analysis (Fig. 4).

In conclusion, the studies presented herein describe the first proof-of-principle experiments on droplet-mediated two-dimensional separations. Further optimization of each dimension can be performed in isolation to increase component resolution and decrease separation time. The unique properties of droplet-based microfluidics provide significant advantages when interfacing orthogonal separation techniques. First is the ability to segment, encapsulate and manipulate the separated samples within nL-sized droplets. The generation and merging

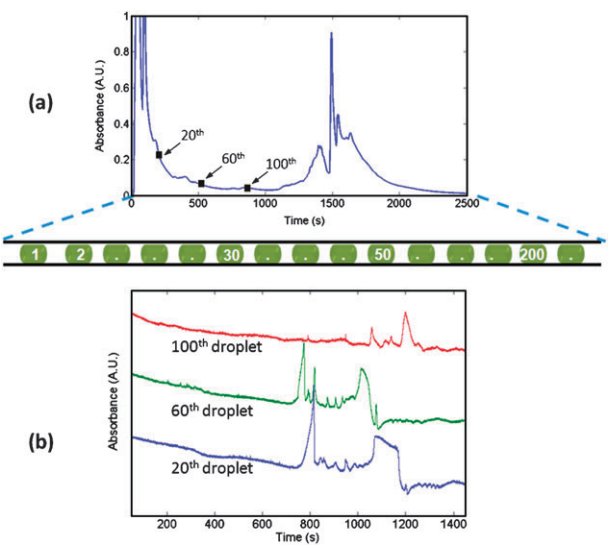

Fig. 4 'Heart cutting' separation of yeast cell proteins. (a) Chromatogram from a capillary HPLC separation of yeast cell lysate and the droplets segmented. (b) CE separation of the selected droplets.

of droplets occurs in a passive manner, without the need for valves or electrokinetic driven flows. The benefits of adjustable droplet volumes ranging from nanolitres to femtolitres are also significant. The ability to 'finely slice' peaks in the first dimension ensures that no chemical or biological information will be lost. Furthermore, the ultra small volume of samples that can be handled is especially important in many modern day applications such as single cell proteomics. Additionally, reagents can be added and mixed within droplets with ease, and therefore many sample preparation steps can be integrated into the droplet connector. Indeed, although we present a two-dimensional capillary HPLC-CE system in the current communication, we believe this approach can be widely adapted to couple a range of other separation techniques. Consequently, we expect that droplet-based interfaces could become key components in 2D or multi-dimensional separations.

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