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Capillary Liquid Chromatography Fraction Collection and Post-Column Reaction Using Segmented Flow Microfluidics

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Abstract

A challenge for capillary liquid chromatography (cLC) is fraction collection and manipulation of fractions from microscale columns. An emerging approach is use of segmented flow or droplet technology to perform such tasks. In this work, a fraction collection and post-column reaction system based on segmented flow was developed for gradient cLC of proteins. In the system, column effluent and immiscible oil are pumped into separate arms of a tee resulting in regular fractions of effluent segmented by oil. Fractions were generated at 1 Hz corresponding to 5 nL volumes. Fraction collection rate was high enough to generate over 30 fractions per peak and preserve chromatographic resolution achieved for a 5 protein test mixture. The resulting fractions could be stored and subsequently derivatized for fluorescence detection by pumping them into a second tee where naphthalene dicarboxyaldehyde, a fluorogenic reagent, was pumped into a second arm and added to each fraction. Proteins were derivatized within the droplets enabling post-column fluorescence detection of the proteins. The experiments demonstrate that fraction collection from cLC by segmented flow can be extended to proteins. Further they illustrate a potential workflow for protein analysis based on post-column derivatization for fluorescence detection.

Keywords

Capillary Liquid Chromatography; Post-Column Derivatization; Protein Separation; Segmented Flow Microfluidics

1. Introduction

Capillary Liquid Chromatography (cLC), compared to conventional scale HPLC, offers advantages such as reduced solvent consumption, better separation efficiency, and facile coupling to mass spectrometry (MS) [1, 2]. Despite these advantages, cLC was slow to be developed commercially. The advent of electrospray ionization (ESI)-MS has moved cLC to a commonly used technique for proteomics [3]. Along with this application, capillary LC instrumentation has matured so that now commercial instruments are available that have

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adequate pumping, injection, and detection capability to realize many of the benefits of cLC [4]. While instrumentation has been improved, it is still difficult to collect fractions and perform post-column processing of samples from cLC. A practical obstacle is the difficulty in manipulating small volume samples. Capillary LC columns with inner diameter less than 300 μm typically operate at $< 1 \mu\text{L}/\text{min}$ flow rate resulting in nanoliter volume fractions, which is far smaller than is suitable for conventional fraction collection systems. Lack of fraction collection limits the capabilities and workflow possible with cLC. It prevents use of slow secondary analytical techniques, like NMR, for identifying substances. It also restricts post-column sample manipulation such as digestion or labeling for detection.

Recently a novel approach to fraction collection from cLC, capillary electrophoresis, and microfluidic systems based on droplets has been reported [5–9]. In this method, microfluidic structures are used to compartmentalize column effluent into regular droplets or plugs with picoliter to nanoliter volume surrounded by oil. The advantage of this approach is that it is possible to store and manipulate low volume fractions reproducibly and at high throughput. This work has shown several potential uses of fraction collection from microscale separations. One application is off-line interface to ESI-MS which facilitates “peak parking” in which more time can be devoted to MS interrogation of particular peaks by slowing down the flow into the MS without disrupting the mobile phase gradient [6]. Another report demonstrated post-column digestion of proteins by adding enzymes to segmented flow fractions suggesting the potential of a novel top-down proteomic workflow at microscale [9]. Droplet fraction collection approach has been used for interfacing in 2-D separation at microscale [7]. A non-traditional use was creation of large concentration gradients from components [8].

In this report, we extend the utility of this approach by developing post-column derivatization for fluorescence detection of proteins. Derivatization for fluorescence detection is desirable to enhance detection sensitivity, especially in microscale formats where UV-absorbance is often inadequate because of short pathlengths. Pre-column labeling is problematic for proteins because incomplete labeling of all targeted functional groups, for example amines, on a macromolecule results in multiple species leading to multiple or broadened peaks for a single protein [10]. Post-column labeling is restricted to fluorogenic reagents, i.e. reagents must become fluorescent only after derivatization to avoid a high background in detection. Post-column labels must also react quickly to prevent extra column band broadening. These twin requirements greatly restrict reagents that may be used for post-column derivatization of proteins [11, 12]. Here we demonstrate collection of separated proteins and subsequent post-column derivatization with naphthalene-2,3-dicarboxaldehyde (NDA). In principle, the system can be adopted to other fluorogenic reactions with different excitation wavelengths and reaction kinetics. We also further evaluate conditions necessary for reproducible fraction collection. Technical difficulties such as protein adsorption and processing droplet data are also addressed.

2. Experimental

2.1 Chemicals and reagents

Chemicals and solvents were purchased from Sigma Aldrich (Saint Louis, MO) unless noted otherwise. 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) and NDA were from Invitrogen (Eugene, OR). HPLC grade water and methanol were from Burdick & Jackson (Muskegon, MI). Fused silica capillary was from Polymicro Technologies (Phoenix, AZ). Perfluorinated surfactant Rf-PEG, a mixture of $\text{CF}_3(\text{CF}_2)_m(\text{CH}_2\text{CH}_2\text{O})_n\text{H}$ variants, was extracted from Zonyl-FSO100, which is a water suspension of solid Rf-PEG, as described elsewhere [13]. Oil phases with additives used for segmenting flow were prepared fresh daily. All aqueous solutions were prepared with 18 M Ω resistivity water purified using a Series 1090 E-pure system (Barnstead Thermolyne Cooperation, Dubuque, IA). Protein stock solutions at 1 mg/mL were dissolved in water with 3% methanol and 0.1% trifluoroacetic acid (TFA), except insulin which was dissolved in 10% HCl for better solubilization.

2.3 Capillary LC separation and samples

Capillary LC columns were slurry-packed as described elsewhere [14]. Briefly, a frit was made by tapping nonporous silica (Micra Scientific, Inc., Northbrook, IL) into the outlet of a 75 μm inner diameter (ID) by 15 cm long fused silica capillary. The particles were briefly heated using butane lighter to sinter them in place. The slurry consisted of 1 mg of 3 μm C4 Prosphere particles with 300 $\text{\AA}$ pore size (purchased from Alltech, Deerfield, IL) suspended in 1 mL acetone. The capillary LC column was packed to 5 cm column bed length at 500 psi using a custom-made pressure reservoir.

Two Varian ProStar 210 (Varian, Palo Alto, CA) solvent delivery pumps were used to pump mobile phase. Mobile phases were prepared daily, purged with helium, and passed through 10 μm pore solvent filters (IDEX, Oak Harbor, WA). Flow rate was 0.5 mL/min and split to achieve 300 nL/min through the column. Mobile phase A and B were water with 0.1% (v/v) TFA and methanol with 0.1% TFA, respectively. Injection was accomplished using a six-port two-position injection valve (Valco, Houston, TX) and a stainless steel pressure bomb. Instead of using a loop injection, sample was pumped directly on to the capillary column through a piece of transferring capillary and inter-connected ports on the valve. During the sample loading step, gas pressure of 500 psi was applied for 20 s yielding an injection volume of 50 nL. After sample loading, the valve was switched so that sample was directed to waste and the column was connected to the pump. For separation, a linear gradient from 0% to 100% B was applied over 10 min. Then the system was held at 100% B for 5 min before re-equilibrating back to 100% A. A Spectra Series UV100 capillary UV detector from Thermo Scientific (Foster City, CA) was used to detect protein signal at 214 nm. The data was collected through NI USB-6008 data acquisition card (National Instrument, Austin, TX) and recorded by a home-written LabView program.

For all cLC experiments a test mixture of insulin, cytochrome C, bovine serum albumin (BSA), myoglobin, and carbonic anhydrase were used as sample. Proteins were diluted to 200 $\mu\text{g/mL}$ in 3% MeOH, 0.1% TFA from stock solution unless stated otherwise.

2.4 Droplet generation and fraction collection

Fractions from cLC were collected as segmented flow as shown in Figure 1A. A syringe pump (Fusion 400, Chemyx, Stafford, TX) was used to infuse perfluorodecalin with 1% perfluorooctanol (PFO) (v:v) and 0.5 mg/mL Rf-PEG at a flow rate of 0.3 $\mu\text{L}/\text{min}$ into a PEEK Tee union (C360QTPK4, Valco, Houston, TX). The cLC effluent was pumped into another arm of the tee at 90 degrees to the oil input. This arrangement resulted in LC effluent being segmented into plugs separated by oil. The resulting segmented stream flowed out the third arm of the tee into a 1.5 m length of 100 μm ID by 360 μm outer diameter (OD) perfluoroalkoxy (PFA) tubing (Upchurch Scientific, Oak Harbor, OR) where they were stored for later analysis.

For some experiments, a flow injection system was used instead of the cLC to test the effect of oil phase content on droplet uniformity and stability. In this system, the cLC was replaced with a 6-port, 2-position valve (Valco, Houston, TX) with flow driven by a syringe pump (Fusion 400, Chemyx, Stafford, TX) at 0.3 $\mu\text{L}/\text{min}$. The valve was used to switch between 50 $\mu\text{g}/\text{mL}$ myoglobin in 0.1% TFA with 3% methanol and the solvent alone. The protein solution also contained diluted blue food color to allow visualization of this stream. A 20 cm long fused silica capillary with 20 μm ID and 360 μm OD at the valve outlet was connected to the tee, replacing the column (Figure 1A), for flow segmentation. Movies of resulting droplets were recorded using a stereomicroscope (SMZ 745T, Nikon Instruments Inc., Melville, NY) equipped with a color CMOS camera (EO-1312C, Edmund Optics, Barrington, NJ). Resulting images were analyzed by Image J (US National Institutes of Health, Bethesda, MD).

2.5 Reagent addition and LIF detection

Reagent addition to droplet fractions was performed using a PDMS/capillary hybrid chip (Figure 2A) that was fabricated using soft lithography [15]. SU-8 2075 photoresist (MicroChem Corp. Newton, MA) was spin-coated onto a 3 inch silicon wafer (University Wafer, Boston, MA) at 4000 rpm. After pre-bake, the SU-8 coated silicon wafer was exposed to UV radiation for 13.3 s (365 nm mercury line, 60 mJ/cm^2 power, Optical Associates, Inc., Milpitas, CA) through a dark field mask (Fine Line Imaging Co., Colorado Springs, CO) to cross-link exposed features in the desired microfluidic pattern. After exposure the chip was post-baked and remaining photoresist treated with SU-8 developer (MicroChem Corp. Newton, MA). The resulting SU-8 features were 60 μm high and 150 μm , 60 μm , and 200 μm wide at the sample plug inlet, reagent inlet, and droplet outlet (PTFE tubing in Figure 2A) respectively. PDMS was cast over the mold to create channels. These channels served as guides for insertion of capillary connections to appropriate solutions.

Access holes were made by poking a blunt 18 gauge needle into the PDMS after removing from the mold. The PDMS with channels was sealed to a piece of unpatterned PDMS by plasma bonding followed by placing the combined device on a hot plate (temperature set to 75 $^{\circ}\text{C}$) for 10 min. The surface of the channels were derivatized by filling the channels with 1:10 (v:v) H₁₁H₂H₂H₂-perfluorooctyltrichlorosilane (PFOTCS) solution in anhydrous hexadecane via the punched access holes. The filled chip was placed on a hot plate (temperature set to 75 $^{\circ}\text{C}$) for 30 min. A 100 μm ID by 160 μm OD silica capillary, pre-

silanized by PFOTCS, was inserted from the side of the chip into the microfabricated channel to construct a segmented flow or sample plug inlet. The reagent inlet was made by inserting a piece of bare silica capillary that was 50 μm ID by 150 μm OD into the PDMS channel. The droplet outlet was made by inserting a piece of Zeus PTFE 38 gauge (approximately 100 μm ID and 220 μm OD) tubing (Amazon, Seattle, WA), which was glued in place using 5 min epoxy.

To derivatize separated proteins, segmented-flow fractions collected from the cLC column were pumped at 0.6 $\mu\text{L}/\text{min}$ into the reagent addition chip while reagent was pumped at 0.3 $\mu\text{L}/\text{min}$ using a syringe pump (Figure 2A). Derivatization reagent consisted of 1 mM NDA and 20 mM β -mercaptoethanol (BME) dissolved in 30% methanol and 70% 100 mM sodium tetraborate buffer at pH 9.3.

LIF detection was performed using an epi-illumination configuration based on a Zeiss Axiovert 35 M inverted microscope equipped with a 40 $\times$, 0.6 numerical aperture objective (Carl Zeiss, Inc., Thornwood, NY) and a Photon Technology International 814 photometer (Lawrenceville, NJ) [16, 17]. The photometer was fitted with 490 ± 30 nm and 530 ± 30 nm band-pass filters for excitation and emission, respectively. The excitation source was 20 mW of 488 nm from an optically pumped semiconductor Sapphire laser (Coherent, Santa Clara, CA). The laser was focused onto the PFA tubing that was 100 μm ID by 360 μm OD containing derivatized droplets. The droplets were pumped past the laser focus point at 0.9 $\mu\text{L}/\text{min}$ for detection. The reaction time was controlled by the flow rate and tubing length from reagent addition to detection point. Instrument control and data collection were performed using LabVIEW software written in house (National Instruments, Austin, TX).

2.6 Pre-column derivatization using CBQCA reaction

CBQCA was used to derivatize protein samples following the directions of the Invitrogen manual. The reaction was quenched and acidified with 12 M HCl before injection.

2.7 Data processing

Oil is non-fluorescent, therefore, single point detection of segmented flow result in a trace with individual peaks that correspond to the signal of aqueous drops and valleys indicating the oil segments. Chromatograms were constructed from this data using Igor Pro 6.01 (Wavemetrics, Inc., Lake Oswego, OR). Each droplet was identified as an individual peak and peak center and peak amplitude information extracted using a built-in macro. Fluorescent signal amplitude was plotted against peak center value for chromatograms. Origin 6.0 and Cutter 7.0 [18] were used to plot and process chromatograms.

3. Results and Discussion

3.1 Oil phase additive and droplet regularity

Capillary LC fractions were collected using a tee structure (Figure 1A) similar to other reports [5–7, 19]. This structure has been successfully used in many applications for generating reliable plugs; however, we found that when using perfluorodecalin as the oil, proteins in the segmented sample caused coalescence of plugs resulting in large and irregular

droplets. This is illustrated by flow injection data for myoglobin in Figure 3A. Droplets are irregular and bigger in peak width. These effects are detrimental to the separation because: 1) it reduces fraction collection frequency; 2) coalescence is variable, which makes downstream reagent injection and quantitation less reliable. We hypothesized that protein adsorption to the oil/droplet interface was causing this effect. Adsorption of proteins onto the aqueous/oil interface lowers the interfacial tension. With lower interfacial tension, droplets tend to be bigger [20, 21] and coalescence occurs more readily. Adding the fluorinated surfactant Rf-PEG to the oil phase tends to prevent protein adsorption to the oil/aqueous interface [13], which in turn improved droplet regularity and stability (Figure 3B). In the fraction collection experiments described in below, PFO was also added. We found that PFO did not improve droplet generation; however, it did reduce cross-contamination and droplet coalescence during reagent injection [22], so it was used in combination with Rf-PEG for all experiments that involved reagent addition.

3.2 Separation and fraction collection

When collecting fractions, it is important that samples can be collected at a rate that does not broaden peaks. Typically, collection of 8 -10 fractions across a single peak is considered to be sufficient to not cause undue extra column broadening. In practical work the number of fractions collected may be minimized because of the difficulty of manipulating many fractions; however, the segmented flow approach allows facile handling and manipulation of fractions so that collecting large number of fractions is not cumbersome.

To illustrate these points, we compared separations of a test mixture of 5 proteins on-column and after fraction collection. Figure 1B is a chromatogram of the test mixture by reversed phase LC at 300 nL/min and with on-line UV-absorbance detection. Figure 1C illustrates a trace resulting from collecting the same separation in 5 nL fractions, corresponding to 1 Hz collection rate (see Figure 1D) using the system in Figure 1A. The resulting fractions were pumped through the UV-absorbance detector to generate the trace. As shown, resolution is preserved despite fraction collection. For example, resolution for insulin and cytochrome C before and after fraction collection were both 2.0. Figure 1D shows that ~30 fractions were collected across the insulin peak. Thus, it was possible to collect well over the minimal 8-10 fractions needed to preserve resolution in this sample chromatogram.

These data also show that fraction size does not change significantly despite the change in solvent due to gradient elution. Droplet size was 4.4 ± 0.4 , 5.3 ± 0.5 and 5.7 ± 0.4 nL ($n = 5$ droplets) at 0%, 50% and 100% mobile phase B respectively. The slight increase in droplet size could be contributed to changes in methanol content.

3.3 Reagent addition and fluorescence detection

For fluorescence detection, it is necessary to add derivatization reagent to collected fractions and allow time for reaction before detection. Adding reagent to preformed droplets has been demonstrated previously [22–25]. A commonly used approach is to pump reagent continuously from a hydrophilic channel or capillary into a hydrophobic tee where sample plugs flow past. Reagent droplets that begin to form at the outlet of the hydrophilic channel

merge with sample droplets (cLC fractions in this case) and rapidly mix due to recirculation effects in the droplets as they flow. This approach has been used for enzyme assays [23–25].

We adapted this approach for our work using a PDMS/capillary hybrid device (Figure 2A). In this device, PDMS is used as a mold to allow a fused silica capillary to act as a hydrophilic channel for delivering reagent. Teflon capillary collects fractions with reagents added as shown in Figure 2A. For this system, fluorogenic reagent is added to cLC plugs at half the flow rate of segmented flow.

Cross contamination may occur in these devices because a portion of a droplet passing the reagent inlet can be left behind and then combine with the following droplet. Such effects could lead to extra column band broadening in this application. Using narrow bore tubing in the reagent inlet helps increase the Peclet number and keep the cross-contamination low [23]. Carry-over tests using food coloring confirmed the effectiveness of this design. Injection of yellow colored aqueous “reagent” into pre-formed dark blue fluid segment results in green colored droplets. As shown in Figure 2B, the yellow reagent inlet is barely tainted by the previous blue droplet thus low contamination is carried to the next droplet flow by. The lack of extra column broadening or tailing in chromatograms is further evidence that cross-contamination is inconsequential with respect to cLC (see below).

To test this system, we derivatized collected fractions with NDA/BME. NDA/BME has been shown to be effective for post-column derivatization in CE separation [26]. From kinetic tests using a fluorescence plate reader, we found that fluorescence intensity of NDA/BME derivatives of BSA reached a maximum within 10 s and began decreasing after 20 s which is consistent with other reports [27]. Reaction time was controlled to be 20 s by placing the detector an appropriate distance (4 cm) downstream of the reagent addition tee. Figure 4 shows the fluorescent signal trace of segmented flow coupled post-column derivatization with NDA reaction and LIF detection. Oil segments are non-fluorescent and appear as the signal baseline. Aqueous drops are detected as individual peaks. Fluorescent signal of droplets increases with protein collected from the cLC separation so that the chromatogram can be reconstructed. The use of fluorescence detection improved the detection limit by a factor of 10 and eliminated the drift due to mobile phase absorption. Figure 5C also shows that relative sensitivities of LIF detection of proteins are different from the UV detection. At same protein concentrations, the NDA reaction appears to be relatively less sensitive for cytochrome c and carbonic anhydrase compared to other proteins perhaps due to less accessible functional groups for derivatization or greater quenching of labels.

An alternative to fraction collection for analysis is post-column derivatization in a continuous flow reactor [28]. In continuous flow post-column reactors, it is important to keep the reaction fast and mixing efficient so that not much resolution will be sacrificed. Segmented flow eases this requirement because diffusion is limited to within each droplet and once the fractions are generated, resolution does not change over time. Therefore, in principle even reactions with relatively slow reaction kinetics, such as nano-orange, could be used post-column. Reaction time can be controlled by varying the length between the reagent addition point and the detection window. Off-line incubation [29] can be used if the reaction time is on the order of hours. Also, derivatization with segmented flow does not

require an on-line system, so collected proteins can be stored and derivatized later. For example, in these experiments proteins were typically derivatized and detected 1-2 h after the fractions were collected.

3.4 Comparison to pre-column derivatization

Compared to post-column derivatization, pre-column derivatization yields a more complex chromatogram as illustrated in Figure 5 which compares chromatograms for 5 underivatized protein standards (Figure 5A), the same standards after pre-column derivatization with CBQCA (Figure 5B), and after post-column derivatization with NDA (Figure 5C, reconstructed as described in experimental section). With CBQCA pre-column labeling, extra and overlapping peaks are found likely due to excess CBQCA and multiple labeling of proteins [30] making peak assignment and quantification difficult. On the other hand, the post-column approach circumvents the “multiple peak problem” by decoupling separation and labeling reaction and using the fluorogenic reaction simply as an indicator of protein.

In implementing a post-column reactor, attention is usually focused on reducing extra-column band broadening due to dispersion while maintaining good mixing of effluent and reagent. This is especially problematic in microscale systems. Segmented flow, however, provides an alternative to the traditional post-column reactor solutions. Operated at a high enough sampling frequency, segmented flow allows resolution to be maintained throughout post-column fraction manipulations such as fraction collection and post-column reaction. Thus, the resolution for insulin and cytochrome C peak from both on-line and off-line detection is 2.0 despite the storage and derivatization associated with off-line detection.

4. Concluding Remarks

In this work, we demonstrate that segmented flow microfluidics can be used to collect fractions and perform post-column reaction for cLC separation. Taking advantage of the capability of manipulating small volumes, we show that fractions can be collected at high enough frequency that chromatographic information is preserved. Fraction collection offers flexibility and versatility in post column sample processing. Aside from the LIF detection that we demonstrated, we believe this approach will enable more applications in capillary based separation techniques besides those already demonstrated [5–9]. For example, multiplexed detection, interface to NMR, or using post-column reaction to screening natural product mixtures.

References

1. Vissers JPC. *J Chromatogr A*. 1999; 856:117–143. [PubMed: 10526786]
2. Hernández-Borges J, Aturki Z, Rocco A, Fanali S. *J Sep Sci*. 2007; 30:1589–1610. [PubMed: 17623443]
3. Ishihama Y. *J Chromatogr A*. 2005; 1067:73–83. [PubMed: 15844511]
4. Rapp E, Tallarek U. *J Sep Sci*. 2003; 26:453–470.
5. Edgar JS, Milne G, Zhao Y, Pabbati CP, Lim DSW, Chiu DT. *Angewandte Chemie-International Edition*. 2009; 48:2719–2722. [PubMed: 19142923]
6. Li Q, Pei J, Song P, Kennedy RT. *Anal Chem*. 2010; 82:5260–5267. [PubMed: 20491430]

7. Niu XZ, Zhang B, Marszalek RT, Ces O, Edel JB, Klug DR, deMello AJ. *Chem Commun.* 2009;6159–6161.
8. Theberge AB, Whyte G, Huck WTS. *Analytical Chemistry.* 2010; 82:3449–3453. [PubMed: 20373759]
9. Ji J, Nie L, Qiao L, Li YX, Guo LP, Liu BH, Yang PY, Girault HH. *Lab on a Chip.* 2012; 12:2625–2629. [PubMed: 22695710]
10. Craig DB, Dovichi NJ. *Anal Chem.* 1998; 70:2493–2494. [PubMed: 21644770]
11. Nickerson B, Jorgenson JW. *J Chromatogr A.* 1989; 480:157–168.
12. Lee TT, Yeung ES. *J Chromatogr.* 1992; 595:319–325. [PubMed: 1577911]
13. Roach LS, Song H, Ismagilov RF. *Anal Chem.* 2004; 77:785–796.
14. Kennedy RT, Jorgenson JW. *Anal Chem.* 1989; 61:1128–1135.
15. Whitesides GM, Ostuni E, Takayama S, Jiang XY, Ingber DE. *Annual Review of Biomedical Engineering.* 2001; 3:335–373.
16. Lada MW, Vickroy TW, Kennedy RT. *Anal Chem.* 1997; 69:4560–4565. [PubMed: 9375517]
17. Hernandez L, Escalona J, Joshi N, Guzman N. *J Chromatogr.* 1991; 559:183–196.
18. Shackman JG, Watson CJ, Kennedy RT. *Journal of Chromatography A.* 2004; 1040:273–282. [PubMed: 15230534]
19. Edgar JS, Milne G, Zhao Y, Pabbati CP, Lim DSW, Chiu DT. *Angew Chem.* 2009; 121:2757–2760.
20. Cristini V, Tan YC. *Lab on a Chip.* 2004; 4:257–264. [PubMed: 15269790]
21. Wang K, Lu YC, Xu JH, Luo GS. *Langmuir.* 2009; 25:2153–2158. [PubMed: 19152256]
22. Li L, Boedicker JQ, Ismagilov RF. *Anal Chem.* 2007; 79:2756–2761. [PubMed: 17338503]
23. Song H, Li HW, Munson MS, Van Ha TG, Ismagilov RF. *Analytical Chemistry.* 2006; 78:4839–4849. [PubMed: 16841902]
24. Slaney TR, Nie J, Hershey ND, Thwar PK, Linderman J, Burns MA, Kennedy RT. *Analytical Chemistry.* 2011; 83:5207–5213. [PubMed: 21604670]
25. Sun SW, Slaney TR, Kennedy RT. *Analytical Chemistry.* 2012; 84:5794–5800. [PubMed: 22656268]
26. Ye M, Hu S, Quigley WWC, Dovichi NJ. *J Chromatogr A.* 2004; 1022:201–206. [PubMed: 14753787]
27. Kostel KL, Lunte SM. *Journal of Chromatography B: Biomedical Sciences and Applications.* 1997; 695:27–38. [PubMed: 9271126]
28. Deelder RS, Kroll MGF, Beeren AJB, Van Den Berg JHM. *J Chromatogr A.* 1978; 149:669–682.
29. Wang M, Slaney T, Mabrouk O, Kennedy RT. *Journal of Neuroscience Methods.* 2010; 190:39–48. [PubMed: 20447417]
30. Bardelmeijer HA, Waterval JCM, Lingeman H, van't Hof R, Bult A, Underberg WJM. *Electrophoresis.* 1997; 18:2214–2227. [PubMed: 9456036]

Figure 1.

(A) Illustration of fraction collection using segmented flow microfluidics. Picture shows the fractions generated from capillary LC that are oil immersed droplets stored in a piece of PFA tubing. (B) Chromatogram of reversed phase capillary LC separation of 5 protein standards: A is insulin, B is cytochrome C, C is BSA, D is myoglobin, E is carbonic anhydrase. All proteins injected at 200 $\mu\text{g/mL}$. Y-axis is in absorbance units (a.u.) (C) UV absorbance trace of the same separation but after fraction collection. Perfluorodecalin has strong UV absorbance at 214 nm and maxed out the detector when it reached the detection point. The valleys are the UV signal for the aqueous phase in this diagram. (D) Expanded view of insulin peak showing ~30 droplet fractions for this peak.

Figure 2.

(A) Reagent addition chip design. (B) Bright field image of a functioning reagent addition chip. Blue plugs represent fractions collected from a cLC separation. A drop of yellow reagent droplet is forming at the outlet of the reagent addition capillary which later merges with the incoming blue droplet and form a green droplet after the reagent addition step which enters the outlet that is made of a piece of PTFE tubing. Flow rates used here are identical to the fluorogenic reagent addition experiments. For scale, the fused silica capillary is 160 μm OD.

Figure 3.

Oil phase surfactant effect on droplet regularity observed using flow injection of myoglobin with blue dye. (A) is droplet trace for absorbance of blue food dye (to avoid absorbance of perfluorodecalin) using pure perfluorodecalin as the carrier phase following injection of myoglobin solution and (B) is droplet trace adding Rf-PEG as a oil phase additive for a repeated injection. Droplets that contain Rf-OEG are smaller and the size distribution is tighter compared to without.

Figure 4.
LIF trace of droplet fractions after NDA reagent is added for separation of 5 protein standards (same mixture and separation conditions as Figure 1B).

Figure 5.

(A) UV chromatogram of 5 protein standard mixture. (B) Chromatogram of the same five proteins that are CBQCA pre-column labeled. (C) LIF chromatogram reconstructed from Figure 4. Same standards and cLC conditions were used as in Figure 1B.