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Research Article

Capillary liquid chromatography fraction collection and postcolumn reaction using segmented flow microfluidics

A challenge for capillary LC (cLC) is fraction collection and the manipulation of fractions from microscale columns. An emerging approach is the use of segmented flow or droplet technology to perform such tasks. In this work, a fraction collection and postcolumn reaction system based on segmented flow was developed for the 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. The fraction collection rate was high enough to generate over 30 fractions per peak and preserve chromatographic resolution achieved for a five-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 postcolumn 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 postcolumn derivatization for fluorescence detection.

Keywords: Capillary liquid chromatography / Postcolumn derivatization / Protein separation / Segmented flow microfluidics
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1 Introduction

Capillary LC (cLC), compared to conventional-scale HPLC, offers advantages such as reduced solvent consumption, better separation efficiency, and facile coupling to MS [1,2]. Despite these advantages, cLC was slow to be developed commercially. The advent of ESI-MS has moved cLC to a commonly used technique for proteomics [3]. Along with this application, cLC instrumentation has matured so that now commercial instruments are available that have 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 postcolumn processing of samples from cLC. A practical obstacle is the difficulty in manipulating small-volume samples. cLC columns with an inner diameter <300 μm typically operate at <1 μL/min flow rate resulting in nanoliter-volume fractions, which is far smaller than is suitable for conventional fraction collection systems. The lack of fraction collection limits the capabilities and workflow possible with cLC. It prevents use of slow secondary analytical techniques, such as NMR spectroscopy, for identifying substances. It also restricts postcolumn sample manipulation such as digestion or labeling for detection.

Recently, a novel approach to fraction collection from cLC, CE, and microfluidic systems based on droplets has been reported [5–10]. 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 that facilitates "peak parking" in which more time can be devoted to MS interrogation of particular peaks by slowing the flow into the MS without disrupting the mobile-phase gradient [6]. Another report demonstrated postcolumn digestion of proteins by adding enzymes to segmented flow fractions, suggesting the potential of a novel top-down proteomic workflow at microscale [9]. The droplet fraction collection approach has been used for interfacing in 2D separations at microscale [7]. A nontraditional use was the creation of large concentration gradients from components [8].

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Abbreviations: **BME**, β-mercaptoethanol; **CBQCA**, 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde; **cLC**, capillary LC; **NDA**, naphthalene-2,3-dicarboxaldehyde; **PDMS**, polydimethylsiloxane; **PFA**, perfluoroalkoxy; **PFO**, perfluorooctanol; **Rf-PEG**, a perfluorinated surfactant

In this report, we extend the utility of this approach by developing postcolumn derivatization for the 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. Precolumn labeling is problematic for proteins because incomplete labeling of all targeted functional groups, e.g. on a macromolecule, results in multiple species leading to multiple or broadened peaks for a single protein [11]. Postcolumn labeling is restricted to fluorogenic reagents, i.e. reagents must become fluorescent only after derivatization to avoid a high background in detection. Postcolumn labels must also react quickly to prevent extracolumn band broadening. These twin requirements greatly restrict reagents that may be used for postcolumn derivatization of proteins [12, 13]. Here, we demonstrate the collection of separated proteins and subsequent postcolumn 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 Materials and methods

2.1 Chemicals and reagents

Chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless noted otherwise. 3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) and NDA were from Invitrogen (Eugene, OR, USA). HPLC-grade water and methanol were from Burdick & Jackson (Muskegon, MI, USA). The fused-silica capillary was from Polymicro Technologies (Phoenix, AZ, USA). Perfluorinated surfactant (Rf-PEG), a mixture of $CF_3(CF_2)_m(CH_2CH_2O)_nH$ variants, was extracted from Zonyl-FSO100, which is a water suspension of solid Rf-PEG, as described elsewhere [14]. Oil phases with additives used for segmenting flow were prepared fresh daily. All aqueous solutions were prepared with 18 MΩ resistivity water purified using a Series 1090 E-pure system (Barnstead Thermolyne Cooperation, Dubuque, IA, USA). Protein stock solutions at 1 mg/mL were dissolved in water with 3% methanol and 0.1% TFA, except insulin, which was dissolved in 10% HCl for better solubilization.

2.2 cLC separation and samples

cLC columns were slurry-packed as described elsewhere [15]. Briefly, a frit was made by tapping nonporous silica (Micra Scientific, Northbrook, IL, USA) into the outlet of a 75 μm id × 15 cm fused-silica capillary. The particles were briefly heated using a butane lighter to sinter them in place. The slurry consisted of 1 mg of 3 μm C4 Prosphere particles with 300 Å pore size (purchased from Alltech, Deerfield, IL, USA) suspended in 1 mL acetone. The cLC 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, USA) solvent delivery pumps were used to pump the mobile phase. Mobile phases were prepared daily, purged with helium, and passed through 10 μm pore solvent filters (IDEX, Oak Harbor, WA, USA). The flow rate was 0.5 mL/min and split to achieve 300 nL/min through the column. Mobile phases 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, USA) and a stainless-steel pressure bomb. Instead of using a loop injection, the sample was pumped directly onto the capillary column through a piece of transferring capillary and interconnected 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, USA) was used to detect the protein signal at 214 nm. The data was collected through NI USB-6008 data acquisition card (National Instrument, Austin, TX, USA) and recorded by a home-written LabView program.

For all cLC experiments, a test mixture of insulin, cytochrome C, BSA, myoglobin, and carbonic anhydrase was used as the sample. Proteins were diluted to 200 μg/mL in 3% MeOH/0.1% TFA from stock solution unless stated otherwise.

2.3 Droplet generation and fraction collection

Fractions from cLC were collected as a segmented flow as shown in Fig. 1A. A syringe pump (Fusion 400, Chemyx, Stafford, TX, USA) 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 μL/min into a PEEK Tee union (C360QTPK4, Valco). The cLC effluent was pumped into another arm of the tee at 90° to the oil input. This arrangement resulted in LC effluent being segmented into plugs separated by oil. The resulting segmented stream flowed out of the third arm of the tee into a 1.5 m length of 100 μm id × 360 μm od perfluoroalkoxy (PFA) tubing (Upchurch Scientific, Oak Harbor, OR, USA) 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 the oil-phase content on droplet uniformity and stability. In this system, the cLC was replaced with a six-port, two-position valve (Valco) with flow driven by a syringe pump (Fusion 400, Chemyx) at 0.3 μL/min. The valve was used to switch between 50 μg/mL myoglobin in 0.1% TFA with 3% methanol and the solvent alone. The protein solution also contained

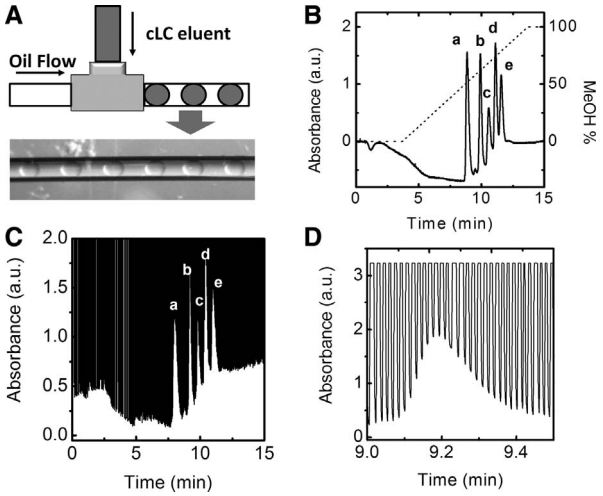

Figure 1. (A) Illustration of fraction collection using segmented flow microfluidics. The picture shows the fractions generated from cLC that are oil-immersed droplets stored in a piece of PFA tubing. (B) Chromatogram of the RP-cLC separation of five protein standards: (a) is insulin, (b) is cytochrome C, (c) is BSA, (d) is myoglobin, (e) is carbonic anhydrase. All proteins injected at 200 μg/mL. The *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.

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 (Fig. 1A), for flow segmentation. Movies of the resulting droplets were recorded using a stereomicroscope (SMZ 745T, Nikon Instruments, Melville, NY, USA) equipped with a color CMOS camera (EO-1312C, Edmund Optics, Barrington, NJ, USA). The resulting images were analyzed by Image J (US National Institutes of Health, Bethesda, MD, USA).

2.4 Reagent addition and LIF detection

Reagent addition to droplet fractions was performed using a PDMS/capillary hybrid chip (where PDMS is polydimethylsiloxane; Fig. 2A), which was fabricated using soft lithography [16]. SU-8 2075 photoresist (MicroChem, Newton, MA, USA) was spin-coated onto a 3 inch silicon wafer (University Wafer, Boston, MA, USA) at 4000 rpm. After prebake, 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, Milpitas, CA, USA) through a dark-field mask (Fine Line Imaging, Colorado Springs, CO, USA) to cross-link exposed features in the desired microfluidic pattern. After exposure, the chip was postbaked and remaining photoresist treated with SU-8 developer (MicroChem). The resulting SU-8 features were 60 μm high and 150, 60, and 200 μm wide at the sample plug inlet, reagent inlet, and droplet outlet [Poly-

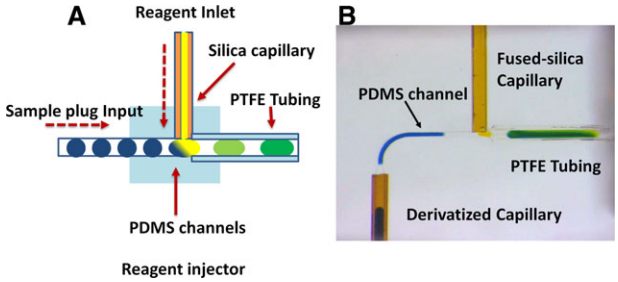

Figure 2. (A) Reagent addition chip design. (B) Bright-field image of a functioning reagent addition chip. Blue plugs represent fractions collected from a cLC separation. A drop of yellow reagent droplet is forming at the outlet of the reagent addition capillary that later merges with the incoming blue droplet and forms a green droplet after the reagent addition step that enters the outlet made of a piece of PTFE tubing. The flow rates used here are identical to the fluorogenic reagent addition experiments. For scale, the fused-silica capillary is 160 μm od.

tetrafluoroethylene (PTFE) tubing in Fig. 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°C) for 10 min. The surface of the channels were derivatized by filling the channels with 1:10 v/v H,1H,2H,2H-perfluorooctyltrichlorosilane solution in anhydrous hexadecane through the punched access holes. The filled chip was placed on a hot plate (temperature set to 75°C) for 30 min. A 100 μm id × 160 μm od silica capillary, presilanized by H,1H,2H,2H-perfluorooctyltrichlorosilane, 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 × 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, USA), 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 μL/min into the reagent addition chip while reagent was pumped at 0.3 μL/min using a syringe pump (Fig. 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×, 0.6 numerical aperture objective (Carl Zeiss, Thornwood, NY, USA) and a Photon Technology International 814 photometer (Lawrenceville, NJ, USA) [17, 18]. The photometer was fitted with 490 ± 30 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, USA). The laser was focused onto the PFA tubing that was 100 μm id × 360 μm od containing derivatized droplets. The droplets were pumped past the laser focus point at 0.9 μL/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).

2.5 Precolumn 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.

2.6 Data processing

Oil is nonfluorescent, therefore, single point detection of segmented flow results 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, Lake Oswego, OR, USA). Each droplet was identified as an individual peak, and the peak center and peak amplitude information were extracted using a built-in macro. Fluorescent signal amplitude was plotted against peak center value for chromatograms. Origin 6.0 and Cutter 7.0 [19] were used to plot and process chromatograms.

3 Results and discussion

3.1 Oil-phase additive and droplet regularity

cLC fractions were collected using a tee structure (Fig. 1A) similar to other reports [5–7, 20]. 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 Fig. 3A. Droplets were irregular and bigger in peak width than without protein (data not shown). These effects are detrimental to the separation because: (i) it reduces fraction collection frequency; (ii) 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 [21, 22] 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 [14], which in turn improved droplet regularity and stability (Fig. 3B). The advantage of using this surfactant is better illustrated by Fig. 3C, where the comparison of droplet size distribution shows that using Rf-PEG decreases average droplet size and makes the droplet size more uniform. 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 [23], so it was used in combination with Rf-PEG for all experiments that involved reagent addition.

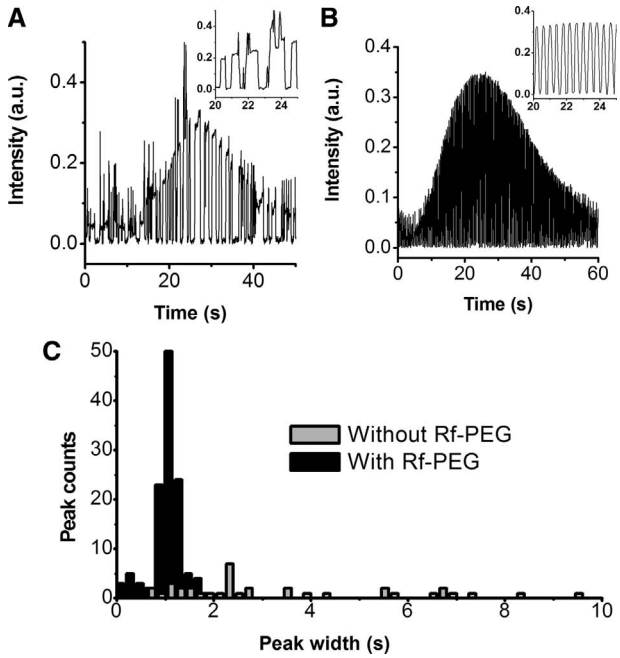

Figure 3. Oil-phase surfactant effect on droplet regularity observed using flow injection of myoglobin with blue dye. (A) is the droplet trace for absorbance of blue food dye (to avoid absorbance of perfluorodecalin) using pure perfluorodecalin as the carrier phase following injection of myoglobin solution and (B) is the droplet trace adding Rf-PEG as a oil-phase additive for a repeated injection. Droplets that contain Rf-PEG are smaller, and the size distribution is tighter compared to that without. (C) Histograms of droplet signal peak width distribution for myoglobin droplets with and without Rf-PEG added to the oil phase.

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 extracolumn 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 five proteins on-column and after fraction collection. Figure 1B is a chromatogram of the test mixture

by RP-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 a 1 Hz collection rate (see Fig. 1D) using the system in Fig. 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, the 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. The 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 a derivatization reagent to collected fractions and allow time for reaction before detection. Adding reagent to preformed droplets has been demonstrated previously [23–26]. 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 [24–26].

We adapted this approach for our work using a PDMS–capillary hybrid device (Fig. 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. The Teflon capillary collects fractions with reagents added as shown in Fig. 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 extracolumn band broadening in this application. Using narrow-bore tubing in the reagent inlet helps increase the Peclet number and keep the cross-contamination low [24]. Carry-over tests using food coloring confirmed the effectiveness of this design. Injection of yellow-colored aqueous "reagent" into preformed dark-blue fluid segment results in green-colored droplets. As shown in Fig. 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 extracolumn 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

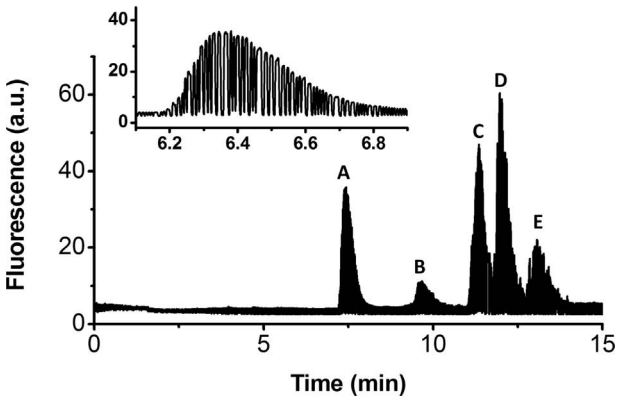

Figure 4. LIF trace of droplet fractions after NDA reagent is added for the separation of five protein standards (same mixture and separation conditions as stated for Fig. 1B).

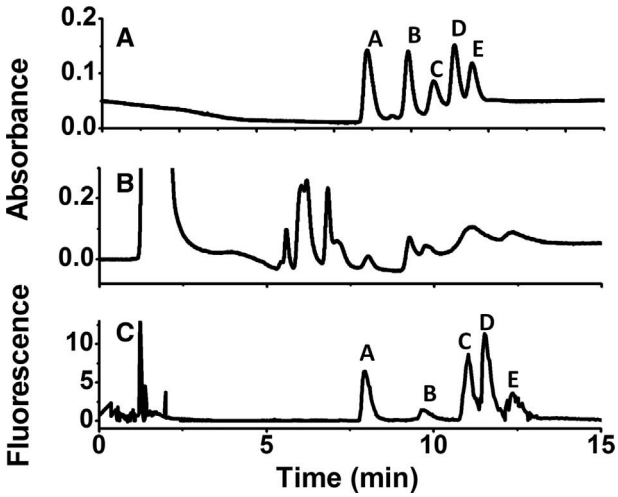

Figure 5. (A) UV chromatogram of five-protein standard mixture. (B) Chromatogram of the same five proteins that are CBQCA precolumn labeled. (C) LIF chromatogram reconstructed from Fig. 4. The same standards and cLC conditions were used as stated for Fig. 1B.

postcolumn derivatization in CE separation [27]. 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 [28]. The reaction time was controlled at 20 s by placing the detector an appropriate distance (4 cm) downstream of the reagent addition tee. Figure 4 shows the fluorescence signal trace of segmented flow coupled postcolumn derivatization with NDA reaction and LIF detection. Oil segments are nonfluorescent and appear as the signal baseline. Aqueous drops are detected as individual peaks. The fluorescence signal of the 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 the same protein concentrations, the NDA reaction appears to be relatively less sensitive for cytochrome c (peak B) and carbonic anhydrase (peak E) compared to other proteins perhaps due to less accessible functional groups for derivatization or greater quenching of labels. The reconstructed carbonic anhydrase peak was not very smooth due to coalescence of droplets, suggesting Rf-PEG is less effective for this particular protein.

An alternative to fraction collection for analysis is postcolumn derivatization in a continuous flow reactor [29]. In continuous flow postcolumn 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, the resolution does not change over time. Therefore, in principle even reactions with relatively slow reaction kinetics, such as nano-orange, could be used postcolumn. The reaction time can be controlled by varying the length between the reagent addition point and the detection window. Off-line incubation [30] can be used if the reaction time is in 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 precolumn derivatization

Compared to postcolumn derivatization, precolumn deriviatzation yields a more complex chromatogram as illustrated in Fig. 5, which compares chromatograms for five underivatized protein standards (Fig. 5A), the same standards after precolumn derivatization with CBQCA (Fig. 5B), and after postcolumn derivatization with NDA (Fig. 5C, reconstructed as described in Section 2). With CBQCA precolumn labeling, extra and overlapping peaks are found, likely due to excess CBQCA and multiple labeling of proteins [31], making peak assignment and quantification difficult. On the other hand, the postcolumn approach circumvents the "multiple peak problem" by decoupling separation and the labeling reaction and using the fluorogenic reaction simply as an indicator of protein.

In implementing a postcolumn reactor, attention is usually focused on reducing extracolumn 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 postcolumn reactor solutions. Operated at a high enough sampling frequency, segmented flow allows resolution to be maintained throughout postcolumn fraction manipulations such as fraction collection and postcolumn 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 postcolumn reaction for cLC separation. Taking advantage of the capability of manipulating small volumes, we show that fractions can be collected at a high enough frequency that chromatographic information is preserved. Fraction collection offers flexibility and versatility in postcolumn sample processing. Aside from the LIF detection that we have 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 spectroscopy, or using postcolumn reactions to screen natural product mixtures.

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The authors have declared no conflict of interest.

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