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Letters to *Analytical Chemistry*

Generation of Picoliter Droplets with Defined Contents and Concentration Gradients from the Separation of Chemical Mixtures

Ashleigh B. Theberge,[†] Graeme Whyte,[†] and Wilhelm T. S. Huck^{*,†,‡}

Melville Laboratory for Polymer Synthesis, Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, United Kingdom, and Radboud University Nijmegen, Institute for Molecules and Materials, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands

There has been an increasing drive toward miniaturizing and accelerating experiments with droplet-based microfluidics across the chemical disciplines. Current applications take advantage of the numerous techniques for manipulating nano- to femtoliter droplets within microfluidic devices. To expand the range of possible applications, we have developed a method for compartmentalizing pure compounds within droplets, at a gradient of concentrations, starting from chemical mixtures. In this technique, a mixture is injected into an ultra performance liquid chromatography (UPLC) system, and droplets are generated from the LC output at a frequency high enough to fraction each compound into $\sim 10^5$ droplets, compartmentalizing pure compounds into a sequence of droplets with a range of concentrations spanning 2–3 orders of magnitude. Here we used fluorescent dyes to quantify the concentration profile of the droplet collections, and to demonstrate the correspondence between the concentration profile of the droplets and the compound elution profile monitored with a UV absorbance detector, allowing the use of compounds that are not fluorescently labeled but show UV absorbance. Hence this technique is applicable to a wide variety of applications that require both compound purity and the ability to probe a variety of concentrations, such as drug screening and titrations.

Microfluidics has evolved as a powerful technology to dramatically reduce the amount of time and reagents required to conduct chemical and biological experiments compared to traditional benchtop methods.^{1–3} The emerging field of microdroplets (also referred to as plug- or droplet-based microfluidics) exploits

aqueous or organic droplets as discrete nano- to femtoliter reaction vessels, separated from each other by an immiscible continuous (carrier) phase and stabilized by surfactants.^{4–6} The compartmentalization introduced by the microdroplet approach offers several physical advantages over traditional single-phase flow microfluidics: dispersion of fluids in the microfluidic channel is avoided;⁷ reagents mix rapidly often leading to enhanced reaction rates;⁸ and the likelihood of channel blockage is reduced.⁹ Additionally, a wide array of techniques for droplet manipulation and interrogation have been developed enabling multistep experiments to be conducted using volumes much smaller than can be manipulated with conventional liquid handling. Once droplets are generated,^{10,11} they can be fused with other droplets,^{12–16} incubated,^{17–19} analyzed,^{20–23} and sorted according to their

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* To whom correspondence should be addressed. E-mail: wtsh2@cam.ac.uk, w.huck@science.ru.nl. Cambridge: fax, (+44) 1223-334-866; phone, (+44) 1223-331-797. Nijmegen: fax, +31-24-3652929; phone, +31-24-3652676.

[†] University of Cambridge.

[‡] Radboud University Nijmegen.

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contents.^{24,25} Microdroplets have great potential for high-throughput screening, and several applications of this technology have been demonstrated, such as protein crystallization, organic synthesis, and biological assays.^{4–6,26} However, nearly all experiments in microdroplets have started with purified samples loaded into droplets. The separation of complex mixtures using chromatography and electrophoresis are essential first steps for many biochemical and chemical experiments. Integrating these methods with droplet-based microfluidics could greatly expand possible applications of the microdroplet platform. Recently, Edgar et al. demonstrated the integration of capillary electrophoresis (CE) with droplet production, allowing for each separated component to be compartmentalized into droplets.²⁷ The droplet contents can then be analyzed further or subjected to a second-dimension separation without additional dispersion.^{27,28}

High-performance liquid chromatography (HPLC) is one of the most universal ways to separate small molecules for both preparative and analytical purposes as it can be used to separate complex mixtures of structurally similar compounds. Recent advances in this technology include the development of nanoflow ultra performance liquid chromatography (UPLC) which provides efficient separations, through the use of sub-2 μm -particle columns and pressure limits greater than 10 000 psi, at nanoliters per minute flow rates.²⁹ Reagent consumption is minimal due to low injection volumes (typically <10 μL) and reduced sample concentration, allowing application to precious or toxic samples, where using larger amounts of material is not possible or potentially dangerous.

Additionally, all of the aforementioned separation techniques inherently lead to elution concentration profiles that vary with time. Although this dispersion of the compounds is undesirable for many applications, droplet-based microfluidics could provide a means by which to harness the concentration gradient of each separated compound, providing an extra dimension of information. Producing large numbers of small-volume droplets from the elution of each analyte offers a method of sampling each compound across a wide range of concentrations, where sequentially produced droplets only differ in concentration incrementally. Hence, droplet-based microfluidics provides a platform for compartmentalizing two dimensions of information: analyte identity and analyte concentration. Here, we demonstrate that the nano-UPLC can be coupled with droplet-based microfluidics to split each compound eluted from the UPLC into 10^5 droplets, each at a different concentration. To follow the concentration profile of each population of droplets, we used fluorescent dyes that could be detected at single droplet resolution using laser-induced fluorescence measurements. These dyes serve as a model for

other small molecules, such as drug candidates, that could be separated and compartmentalized for further droplet-based high-throughput screening.

EXPERIMENTAL SECTION

Device Fabrication. Microfluidic channels (25 μm deep, 50 μm wide) were fabricated in poly(dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning) using soft lithography.³⁰ PDMS features were bound to a glass slide by exposing the surfaces to oxygen plasma and bringing them into contact. The channels were treated with Aquapel (U.K. trade name Duxback) and flushed with Fluorinert FC-77 (3M) after heating in a 100 °C oven for 30 min.

Fluidic Methods. The fluorinated oil continuous phase was loaded into a glass syringe (Hamilton) with a 25 G disposable needle (Terumo) attached. Polyethylene tubing (0.38 mm i.d., Becton Dickinson) was fitted over the needle, and the opposite end of the tubing was inserted into the oil inlet hole punched in the PDMS device. The oil flow rate (200 $\mu\text{L}/\text{h}$) was maintained using a Harvard PHD 2000 infusion syringe pump. The output of the nano-UPLC was interfaced to PEEK tubing (63.5 μm i.d.) using a zero dead-volume HPLC union. The end of the PEEK tubing was inserted into the aqueous inlet hole in the PDMS device.

Continuous Phase and Fluorescent Dye Solutions. Fluorinert FC-77 or FC-40 (3M) with 0.5 wt % EA surfactant (RainDance Technologies, Lexington, MA) was used as the continuous phase. Fluorescent dye solutions were prepared using deionized (DI) water from a Milli-Q purifier. Fluorescein and sulforhodamine 101 were purchased from Sigma-Aldrich. Stock solutions (600 μM) of each compound were prepared in HPLC grade methanol (Fisher), and these were diluted with DI to a concentration of 3.0 μM .

Nano UPLC. Chromatographic separations were performed using a NanoAcquity UltraPerformance LC (Waters) with an Acquity TUV detector (Waters). A Symmetry C18 trapping column (180 $\mu\text{m} \times 20$ mm, 5 μm , Waters) and a BEH130C18 analytical column (100 $\mu\text{m} \times 100$ mm, 1.7 μm , Waters) were used. Injection volumes were 2 μL . The analytical column was heated to 45 °C, and the samples were maintained at 4 °C. HPLC grade methanol (Fisher) and DI water from a Milli-Q purifier were used for the mobile phase, and the mobile phase flow rate was 0.8 $\mu\text{L}/\text{min}$. For the data shown in Figure 2, the mobile phase method was as follows: ramped from 35–50% methanol from 0–40 min, ramped from 50–90% methanol from 40–41 min, held at 90% methanol from 41–43 min to ensure any impurities were removed from the column, ramped from 90 to 35% methanol from 43.0 to 43.1 min, and held at 35% methanol until 55 min. The mobile phase method for the data in Figure 3 was altered such that gradient from 0 to 40 min ramped from 35 to 45% methanol. UV absorbance was monitored at 277 nm. To remove the background UV absorbance of the methanol gradient, the UV absorbance data were processed by averaging the UV versus time plot for two sample injections and subtracting from this the average of the UV plot for three blank injections, followed by a correction for linear baseline drift.

Detection System. Droplet fluorescence was measured using a bespoke laser-induced fluorescence microscope. Excitation of fluorescein and sulforhodamine 101 was achieved using DPSS

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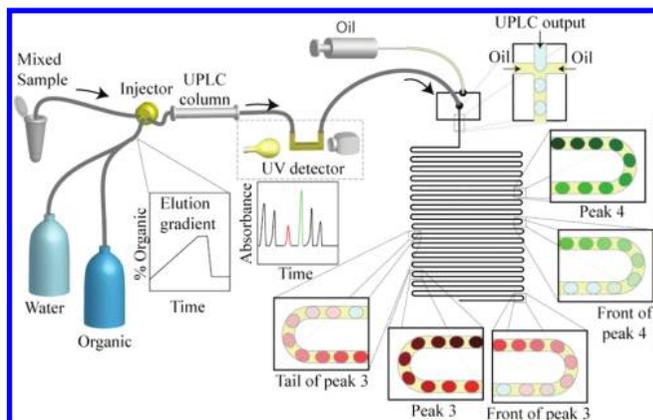


Figure 1. Schematic diagram of integration of nano-UPLC with droplet-based microfluidics. The chemical mixture is separated on the nano-LC column and analyzed with a UV detector. Droplets are produced using flow focusing within a microfluidic chip, allowing the concentration profile of the eluted compound to be compartmentalized into picoliter fractions. The concentration gradient within the droplets is represented by a gradient of colors; light blue droplets represent droplets containing methanol and water without analyte.

lasers at 488 (Cyan, Piccaro) and 594 nm (Mambo, Cobolt), respectively. The light was combined with dichroic mirrors

(Semrock) and fed into the fluorescence port of an inverted microscope (IX71, Olympus U.K.) and focused into the microfluidic channel using a 40 \times objective lens (Olympus). The emission light was separated using a multiband dichroic filter (Semrock), and the individual emission colors were further filtered and detected on two avalanche photodiodes (Newport). For detection of fluorescein alone (Figure 2), a photomultiplier tube (Hamamatsu) with increased sensitivity was used. Graphs of fluorescence versus time are the average over 5 s (~3800 droplets in Figure 2; 2500 droplets in Figure 3). Droplet size was measured by analyzing transmission images of droplets captured using a CMOS camera (Miro4, Vision Research). Analysis was performed using bespoke edge detection software written in LabView (National Instruments).

RESULTS AND DISCUSSION

As shown in Figure 1, our approach combines a well established separation technology, with microfluidic devices for droplet generation fabricated in poly(dimethylsiloxane) (PDMS). A mixture is introduced via an autosampler and separated on a nano-LC column. Each component elutes, separated in time, and also with a concentration profile in time which can be monitored with a UV detector. The flow can then be transferred to a microfluidic

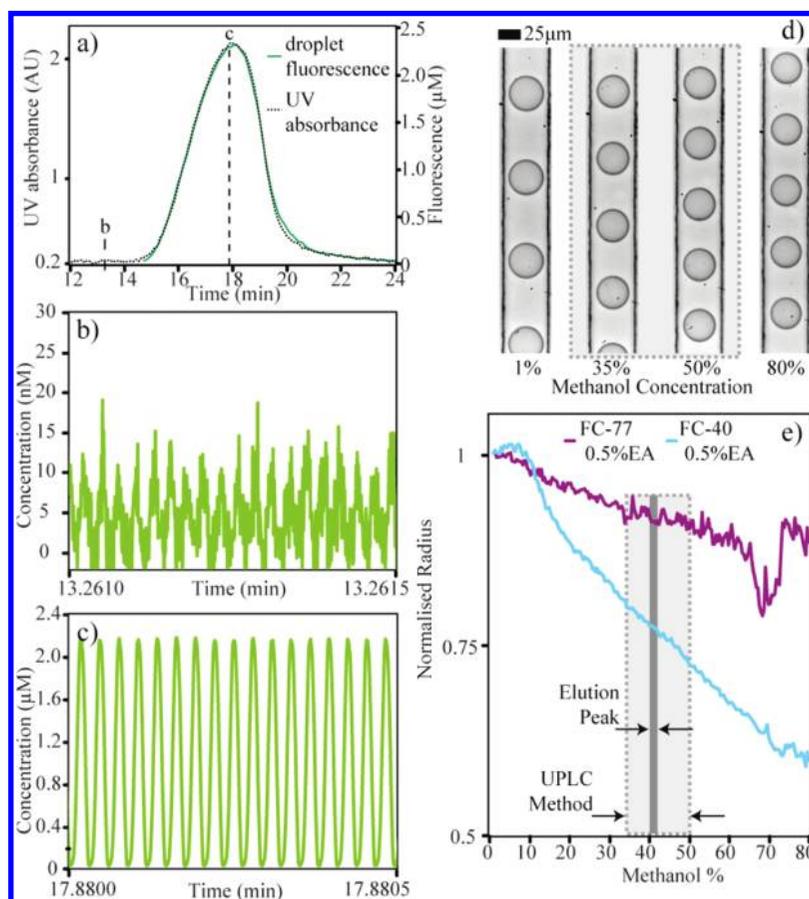


Figure 2. Compartmentalization of nano-UPLC output into microdroplets and characterization of the droplet concentration profile: (a) fluorescein elution with time monitored by a UV absorbance detector (dotted line) and average droplet fluorescence over time (green). Plots have been shifted in time so that the time indicated directly corresponds to the mobile phase method described in the Experimental Section. (b,c) Fluorescence intensities inside microdroplets formed from the UPLC output measured at the times indicated with the vertical dashed lines in part a. (d) Optical micrographs of water–methanol droplets in FC-77 with varied methanol concentrations. (e) Effect of methanol concentration on droplet radius for droplets formed in FC-77 or FC-40 fluorinated oils, each with 0.5 wt % EA surfactant (RainDance Technologies). The dark and light gray bands indicate the mobile phase composition over which fluorescein eluted and which was used for the entire gradient method, respectively.

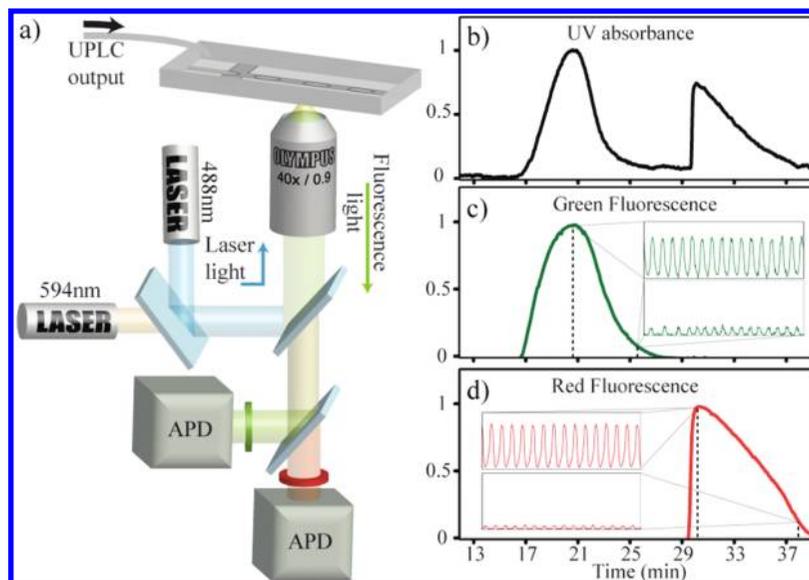


Figure 3. Separation of fluorescein and sulforhodamine 101 and compartmentalization into droplets: (a) optical setup used for laser excitation and independent detection of fluorescein and sulforhodamine 101; (b) chromatogram of the separation of fluorescein and sulforhodamine 101 as detected by UV absorbance after elution; (c,d) averaged droplet fluorescence with time for both green and red fluorescence, respectively. The insets show fluorescence with single droplet resolution for each analyte.

device where picoliter droplets are produced through flow focusing¹¹ of the aqueous sample with an immiscible fluorinated carrier phase. Hence the UPLC peak can be fractionated into isolated, discrete droplets, each individually addressable for further manipulations.

To quantify the concentration profile within individual droplets, a 3.0 μM fluorescein solution was injected into the nano-UPLC and eluted with a methanol–water gradient. A single peak was detected in the plot of UV absorbance over time (Figure 2a). Generating droplets from the UPLC output, the analyte was fractionated into 3.2×10^5 droplets. Example fluorescence traces of droplets produced at different times in the elution peak are shown in Figure 2b,c; droplets produced from the top of the peak contained 2.2 μM fluorescein, whereas the droplets toward the edge of the peak contained gradually lower concentrations, down to the detection limit of our optical setup, 9.6 nM. Hence, the sample is split into picoliter fractions, spanning a concentration gradient of 2–3 orders of magnitude. Furthermore, there is very close correspondence between the plots of UV absorbance and averaged droplet fluorescence with time (Figure 2a). Thus, the UV absorbance of the eluted compounds can be used as a measure of the concentration profile of each compound after compartmentalization into droplets, and this technique can be extended to compounds that are not fluorescently labeled but show UV absorbance. It should be noted that the distance between the nano-UPLC outlet and the droplet chip is approximately 65 cm. Although this could give rise to peak broadening, we do not see evidence for this under the conditions used. When working with separations that produce narrower peaks, the distance could be reduced to prevent broadening.

Compound elution was achieved by increasing the concentration of methanol through the LC column. Since droplet formation is influenced by the interfacial tension of the dispersed and continuous phases,^{31,32} we investigated the effect of changing

water–methanol concentration on droplet size. We monitored droplet size over the range of methanol–water concentrations used to elute fluorescein (indicated within the light gray band in Figure 2e) by using a fast camera to capture images of the droplets. Analysis of droplet radius indicated a change of $<2\%$ across the elution method and a negligible change across the methanol concentrations at which fluorescein eluted (corresponding to the dark gray band in Figure 2e). When working with other samples, it may be necessary to work in a different range of water/methanol concentrations in order to effect compound elution and separation. Parts d and e of Figure 2 show the effect of methanol concentration on normalized droplet radius for methanol concentrations ranging from 1 to 80%. Interestingly, the variation in droplet radius is lower when FC-77 is used as the continuous phase, rather than FC-40, underscoring the importance of a well chosen continuous phase. In order to provide the most consistent droplet size, FC-77 was used when quantifying the concentration profile of the droplets. Additionally, to ensure accurate quantification, we also measured the effect of methanol concentration on the fluorescence of fluorescein. Less than 5% deviation was observed over the concentration range used for the elution of fluorescein.

To demonstrate chemical separation, we used a mixture of two fluorescent dye molecules, fluorescein and sulforhodamine 101, with resolved spectral properties, allowing for selective excitation and detection of the molecules individually with a two-color laser detection system (Figure 3a). The separated compounds were compartmentalized into droplets, and the fluorescence in each droplet was monitored separately at wavelengths relevant for each dye. The two peaks in the plot of UV absorbance versus time (Figure 3b) were detected separately on detectors used to monitor green and red fluorescence as shown in parts c and d of Figure

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3, respectively. The initial sample mixture has now been separated into 7.7×10^5 droplets containing pure compounds at a gradient of concentrations.

In summary, we have demonstrated a technique that takes a chemical mixture and compartmentalizes the individual chemical contents of the mixture into a collection of picoliter droplets containing a gradient of concentrations of each analyte. Moreover, the droplets are produced within microfluidic channels, allowing not only for controlled, monodisperse droplets but also an environment for subsequent manipulation and detection. Here we used a mixture of fluorescent dye molecules as a proof of principle to enable quantification of the chemical concentration gradients within the droplet collections by laser induced fluorescence spectroscopy. We envision this technique to have widespread application to mixtures from synthesis (such as crude reaction mixtures or mixtures from combinatorial chemistry), natural product extracts, peptide mixtures, and biological samples. Furthermore, in UPLC separations with overlapping analyte elution profiles, cocompartmentalization of two or more analytes

in droplets at continuously varying concentrations leads to a “droplet library” containing additional information. The concentration gradients generated with this technique could enable efficient collection of data for dose–response curves and titrations, providing an extra dimension of data in a high-throughput manner.

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