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Simultaneous Generation of Multiple Aqueous Droplets in a Microfluidic Device

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Abstract

This paper describes a microfluidic platform for the on-demand generation of multiple aqueous droplets, with varying chemical contents or chemical concentrations, for use in droplet based experiments. This generation technique was developed as a complement to existing techniques of continuous-flow (streaming) and discrete droplet generation by enabling the formation of multiple discrete droplets simultaneously. Here sets of droplets with varying chemical contents can be generated without running the risk of cross contamination due to the isolated nature of each supply inlet. The use of pressure pulses to generate droplets in parallel is described, and the effect of droplet size is examined in the context of flow rates and surfactant concentrations. To illustrate this technique, an array of different dye-containing droplets was generated, as well as a set of droplets that displayed a concentration gradient of a fluorescent dye.

Keywords

droplet; microfluidic; gradient; on-demand generation

1. INTRODUCTION

There has been significant recent interest in the field of droplet-based microfluidics as evidenced by numerous applications covering a broad range of topics including nanoparticle synthesis [1,2], particle polymerization [3,4], chemical reactions [5], protein crystallization [6,7], biological assays [8–11], and cellular analysis [12]. Droplets provide a means to confine samples to a small volume for subsequent manipulation, reactions, and analysis [13,14]. In essence, droplets serve as reaction vessels enabling chemical and physical control of the contents [15,16].

Droplets are produced in microfluidic devices by a variety of methods, including the common continuous-flow methods such as flow focusing [17–20] or the T-junction method [21–23]. In these methods, droplet formation is usually continuous and fast, with a single flow-focusing system or T-junction producing many droplets sequentially over a period of time, typically in the range of Hz to kHz [24]. Flow rates are maintained for uninterrupted droplet formation, and are therefore well-suited for high-throughput processes. While the number of droplets produced can be quite large, there is to some extent a sacrifice of control of the droplets on an

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individual basis: specifically, gross sorting or encapsulation is possible, but retrieval and detection of specific individual droplets currently is not.

Other droplet formation methods have been developed for the production of discrete droplets on-demand, including using the electrowetting effect [9], electro-generation [25,26], and pressure-based techniques using microinjectors [14,24] or channel constrictions [12]. At the expense of throughput, exquisite control is afforded in these techniques, in which individual droplets are produced and are then used for the immediate task at hand. Using an on-demand droplet generation method such as these and arraying the droplet formation microchannels would enable the simultaneous generation of multiple droplets, thus maintaining precise control over droplet production and manipulation for more than one droplet at a time.

Additionally, certain applications, such as PCR analysis of a panel of DNA biomarkers from body fluids (e.g. plasma), often require different reagents (e.g. primers) to be contained in different droplets without the possibility of cross contamination. For these applications, high-volume throughput and sensitive detection would be more easily executed with a microfluidic chip that could: 1) create the necessary variety of droplets at the same time, while keeping the supply sources isolated, 2) move a generated set of droplets downstream for immediate utilization, so as to be ready for the next round of generation, 3) be integratable with other microfluidic designs and detection techniques.

Here we report the on-demand simultaneous generation of multiple droplets, providing a level of control not found with streaming techniques (e.g. those formed using T-junction or flow focusing), while also allowing for a degree of throughput generally not found in discrete droplet-generation techniques. Multiple aqueous channels are connected via channel constrictions (nozzles) to a large oil-phase channel. Droplets are formed by using the oil-phase flow to infuse and then pull the aqueous solutions out of each nozzle.

2. EXPERIMENTAL

CHEMICALS

Silicone oils used were AS 4, AR 20, and Dow Corning 200 Series (50 & 100 cSt) (Sigma-Aldrich, St. Louis, MO). The surfactant, Span® 80 (sorbitan monooleate), and the Polyethylene Glycol (PEG - average molecular weight 8000), were both purchased from Sigma-Aldrich. Fluorescein (Invitrogen-Molecular Probes, Carlsbad, CA) solutions were prepared in 1 mM phosphate buffer at pH 7.5. Porcine esterase was purchased from Sigma-Aldrich, and fluorescein diacetate was purchased from Invitrogen. 1X PBS (phosphate buffered saline) was purchased from Cellgro (Manassas, VA).

2.1 FABRICATION OF MICROFLUIDIC DEVICES

Two-layer photolithography was used to fabricate SU-8 patterned silicon masters. In the first photolithography step, a 7 µm thick layer of SU-8 (SU-8 2007, Microchem, Newton, MA) was prepared on a 3 in silicon wafer (Montco Silicon Technologies, Spring City, PA), exposed to UV through a transparency mask (Photoplot Store, Colorado Springs, CO), and developed to yield the first layer of microchannels. In the second step, a 50 µm thick layer of SU-8 (SU-8 2050, Microchem, Newton, MA) was spin-coated on the same wafer, and the wafer was exposed to UV through a second transparency mask, using a mask aligner to align the two patterned layers. Following development, the wafer was treated with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (United Chemical Technologies, Bristol, PA) for 2 h. Microfluidic devices were formed by replica molding PDMS (Sylgard 184, Dow Corning, Midland, MI) against a patterned silicon master; PDMS was cured for 2 h at 60 °C. Once cured, the PDMS replica was removed from the master and exposed to oxygen plasma, along with a glass microscope slide. The PDMS replica was then sealed against the glass slide. To revert

the PDMS device to the native hydrophobic state, the device was placed in a 120 °C oven for a minimum of 48 h.

2.2 FLOW CONTROL

Prior to sealing the PDMS replica to a glass slide, connections to the microfluidic device were made by punching holes at the ends of the microchannels with a blunt 16-gauge needle. Polyethylene tubing (PE100) was fitted into the punched holes in the PDMS and attached to 3 mL syringes via 16-gauge needles (tubing, syringes, and needles: Becton, Dickinson and Company, Franklin Lakes, NJ). Two syringe pumps (K&D Scientific, Holliston, MA) were used to control the flow of the aqueous and oil phases. Three 3 mL syringes, containing water, food coloring, or fluorescein solutions, were connected to the aqueous phase inlets, one for each inlet, and the syringe pump (KDS220) was set to infuse at $0.003 \mu\text{L min}^{-1}$. For the oil phase, a 3 mL syringe containing the selected silicone oil and surfactant was connected to the large horizontal channel in the microfluidic device and the second syringe pump (KDS210P), which is capable of pumping in both the forward (infusion) and reverse (withdrawal) directions. In addition, this syringe pump was programmed to repeat a three-step pumping program. A general pumping program for the oil phase consisted of: i) infuse at high flow rate; ii) withdraw at high flow rate; iii) infuse at low flow rate and could be repeated up to 100×.

2.3 ENZYME AND SUBSTRATE SOLUTIONS

Stock esterase solution (0.33 mg mL^{-1}) was made by dissolving lyophilized enzyme in 1X PBS. Substrate-containing oil and aqueous solutions were made by dissolving 6 mg of fluorescein diacetate in 50 mL of each solution. All solutions were filtered prior to use.

2.4 IMAGING AND ANALYSIS

Brightfield images were acquired with a Nikon SMZ1500 stereomicroscope (Tokyo, Japan), using a CCD camera (Cohu 4912, San Diego, CA). Fluorescent images were captured with a Nikon AZ 100 microscope (Tokyo, Japan), equipped with a Prosilica CMOS camera (Burnaby, Canada) or a Cascade 650 CCD camera (Roper Scientific, Tucson, AZ). Image analysis was performed using ImageJ (NIH). For plotting fluorescence intensity, the center one third (to avoid fringe effects) of each droplet was used to obtain an average intensity per pixel. Plot was then normalized to the buffer values.

3. RESULTS AND DISCUSSION

3.1 MICROFLUIDIC DESIGN

Figure 1 shows schematics of the microfluidic designs, which consist of five inlets ($100 \mu\text{m}$ wide by $50 \mu\text{m}$ tall) that feed aqueous solutions orthogonally into a larger ($750 \mu\text{m}$ wide by $50 \mu\text{m}$ tall) oil filled channel, where each inlet channel tapers down to a small nozzle ($7 \mu\text{m}$ wide by $7 \mu\text{m}$ tall) when connecting to the larger channel. The lengths of the inlet channels are of similar proportion, but were not exactly the same, because the primary contribution of the system's flow resistance is due to the small size dimension of the small nozzles.

For generating droplets with concentration gradients (Figure 1b), the microchannel network used was derived from previous designs [27,28], namely it consisted of a combination of a symmetric branched network used for the generation of aqueous concentration gradients and a horizontal oil-flow channel connected via small orifices for droplet generation. The gradient-network channels were all $100 \mu\text{m}$ in width and $50 \mu\text{m}$ in depth, with two inlets that branched multiple times to produce five outlets, which were used to introduce simultaneously aqueous phase into the oil phase through the corresponding five orifices intersecting the oil channel.

In order for the microfluidic device to be used for water-in-oil (w/o) droplet generation, the PDMS was used in a hydrophobic state to minimize interaction of water with the channel walls. As a result of the hydrophobicity, filling the gradient-network channels with the aqueous solutions required special attention. Because the hydrophobic walls were not easily wetted by the aqueous solutions and owing to the length of the gradient-network channels, a syringe pump with sufficient force (50 lbs) to counteract the back pressure of the system was needed to maintain the filled channels throughout the experiment.

For gradient droplet generation, we also found that the long serpentine channels with sharp turns, as used in the original work, were more likely to trap air bubbles during the initial filling of the aqueous solutions. Changing the serpentine channel design to have corners with larger radii of curvature decreased the incidence of trapped air bubbles. In addition to these concerns, the elastomeric nature of PDMS is also of importance to filling the chips because the pressure applied to fill the channels (flow rates up to $100 \mu\text{L min}^{-1}$ were often used) also was imparted capacitively to the PDMS. Once the channels have been filled, the device was allowed to relax back to the original state prior to attempting droplet formation; 10 min was sufficient.

Of the on-demand, discrete-droplet formation methods available, we decided upon a design that would utilize small ($7 \times 7 \mu\text{m}$ cross-section) orifices, or nozzles, which intersect with a relatively large oil channel ($750 \times 50 \mu\text{m}$ cross-section), similar to the channel constriction method described by He et al [12], which mimics bulk membrane emulsification methods [29–31]. Droplet formation occurs when the aqueous phase passes through the nozzle, rounds due to interfacial tension, and subsequently breaks off at the neck. The shape of droplets generated will be that of an oblate spheroid, as they are bounded by the top and bottom walls of the channel. The inset in Figure 1 is a schematic of the connection of the orifices and the oil channel, illustrating the difference in cross-section necessary for forming droplets with this approach. The advantage of using the nozzles is that droplets can be formed on-demand by infusing and/or withdrawing the aqueous phase through the nozzles. The difficulty lies with parametric coupling of the droplet generators, which necessitates control and balance of the pressure over the entire set of nozzles [19]. The oil channel was designed to have flow perpendicular to the aqueous-phase nozzles so that once droplets are formed they can easily be moved away from the nozzles allowing repeated droplet formation.

3.2 DROPLET FORMATION

In bulk membrane emulsification, a dispersed phase is pumped through membrane pores into a continuous phase flowing across a membrane surface to produce droplets. Similar concepts have been used in microchannels for droplet formation [32]. Applying this same scheme of simply pushing the aqueous phase into the oil phase in our design produced many droplets out of each nozzle in varying amounts, quickly leading to an inability to track each droplet and monitoring the contents. In addition to the lack of control over the number of droplets generated, it was also difficult to control the positions of the droplets after they were formed. The same desired effect of forcing the dispersed aqueous phase into the continuous oil phase also can be accomplished by pulling the aqueous phase using the oil phase. We found this approach offers excellent control by enabling the simultaneous creation of single droplets, which upon reducing or stopping of the oil flow, can be easily tracked, imaged, and correlated with the respective nozzles that gave rise to the individual droplets.

Initial experiments were conducted to determine the appropriate amount of surfactant to add to the oil phase, which determines both the ease of droplet formation and the subsequent stability of the formed droplets. Figure 2 is a series of images showing attempts at droplet formation in AS 4 silicone oil using Span 80 as the surfactant. The concentration of Span 80 was varied from 0.01% to 1%, using identical parameters for droplet formation in each case. At the lowest concentration, 0.01%, no droplet formation occurred and all of the aqueous phase

released from the nozzles coalesced into one continuous formation, due to the high surface tension. At the highest extreme tested, 1%, droplets formed rapidly and excessively, with minimal to no coalescence, but with pronounced flocculation. At the middle concentration of 0.1% Span 80, droplets were easily made, and the rate of formation was not too fast. As a result, a concentration of 0.1% Span 80 was determined to be optimal for AS 4 oil as well as for the other silicone oils we used in our experiments. This surfactant concentration was also optimal for subsequent droplet utilization: in addition to creating stable droplets by reducing interfacial tension, this concentration of surfactant was not too high to inhibit droplet fusion.

Flow of the oil phase was controlled by a programmable syringe pump. The pumping program consisted of three steps as illustrated in Figure 3a and 3b, which could be repeated for sequential droplet formation. The first step was to infuse at a relatively fast rate, flowing the oil forward by positive pressure. As the oil phase flowed forward in the horizontal channel it also flowed into the nozzles, forcing the aqueous phase up the nozzles (first 3 panels in Figure 3c). Because of the large difference in the cross-sections and thus volumes between the nozzle and the upstream channel, this displacement of the oil from the nozzle into the upstream channel did not result in noticeable displacement of the solution in the upstream channel. During this step, the nozzle walls were re-wetted with oil. The second step was to withdraw at an equivalent rate, pulling back on the oil phase to flow in the opposite direction. During this step, the aqueous phase flows back into and through the nozzles (middle 3 panels in Figure 3c). Once the aqueous phase entered the oil phase in the main channel, a droplet formed and detached from the aqueous phase to minimize surface tension (last 3 panels in Figure 3c). The third step of the program was to infuse, using a relatively slow rate. The purpose of this step was to allow droplet formation to finish and to prevent cross-over effects from having the pulses spaced too close together. As the cycle repeats, the fast flow rate of the first step serves to move the formed droplets away from the nozzle zone. The formed droplets remain stable because the inter-droplet spacing is maintained, which prevents droplet contact or flocculation. Wetting or sticking of the droplet to channel walls, however, can lead to droplet contact. When this sticking occurred, we believe it was caused by particulate contamination or by the presence of hydrophilic patches on the PDMS channel surface. We minimized both effects by filtering solutions and by baking extensively the PDMS chip to recover fully its hydrophobic surface. Although not implemented in our design, these formed droplets could be further manipulated downstream depending on the type of applications for which these droplets will be used.

The three steps of the pumping program span approximately 7 seconds. A period of 1 second each was used for step one and step two – the pulse steps. This is the minimum limit for the pump; longer periods caused more droplets to form. A time delay is apparent in the panels of Figure 3c as the aqueous phase did not start to move back into the nozzle until 1.9 seconds; this is most likely due to the elastomeric nature of the PDMS device, capacitively storing pressure, as the first three panels fit within the first 1 second infusion step, yet the aqueous phase did not reverse direction until almost 2 seconds. A period of about 5 seconds was typical for step three, and the magnitude of the flow rate varied in the range of about 1 to 7 $\mu\text{L min}^{-1}$.

Figure 4 shows the effects of varying the magnitude of the flow rates and consequently the applied pressure on droplet formation. Below a minimum flow rate (applied pressure), droplets rarely form. At higher flow rates, increasing the magnitude of the flow pulse serves to increase the size of the droplets as seen in Figure 4. The minimum optimal infusion/withdrawal flow rate was determined to be 250 $\mu\text{L min}^{-1}$. Using this pulse magnitude of 190 kPa, droplets were on average 85 μm in diameter. Increasing the rate to 300 $\mu\text{L min}^{-1}$ or 230 kPa increased the average droplet size to 95 μm , yet kept the size distribution to a minimum. Further increases to 350 and 400 $\mu\text{L min}^{-1}$ (270–310 kPa) produced droplets with an average size of ~ 120–125 μm with broader size distribution, as well as extra droplets that did not coalesce (see inset).

Because of parametric coupling of the nozzles during droplet generation, dust or debris can perturb generation when moving through the nozzle (Figure 4 top panel). This effect can be minimized by filtering solutions.

3.3 SIMULTANEOUS GENERATION OF MULTIPLE DROPLETS

Figure 5a and the inset of Figure 5b both show an image of five droplets being formed simultaneously by the device. The pumping program used in Figure 5a was as follows: i) infuse at $250 \mu\text{L min}^{-1}$ for 1 second; ii) withdraw at $250 \mu\text{L min}^{-1}$ for 1 second; iii) infuse at $1 \mu\text{L min}^{-1}$ for 5 seconds. Due to the high viscosity of food coloring, an additional transient infusion during the pressure pulse program iii) was manually added. While food coloring was used here to aid visualization, this experiment demonstrates that any diverse variety of chemicals may be used and be contained within the droplets. For Figure 5c inset, the aqueous phases ranged from 5 to 0 μM fluorescein in 1 mM phosphate and 0.125 w/w% PEG at pH 7.5. The pumping program used for the oil phase was as follows: i) infuse at $325 \mu\text{L min}^{-1}$ for 1 second; ii) withdraw at $325 \mu\text{L min}^{-1}$ for 1 second; iii) infuse at $1 \mu\text{L min}^{-1}$ for 5 seconds. Droplets were formed at each nozzle; the non-fluorescent droplet (0 μM fluorescein) was traced with dotted lines to aid visualization. The linescan of the fluorescent signal for the five channels is presented in Figure 5b, and shows the established concentration gradient of fluorescein, with Figure 5c demonstrating the average intensity of each droplet matched that of the corresponding “mother” channel as expected.

Figure 5d illustrates the potential to use this system for studying enzymatic activity, in which we monitored the activity of different concentrations of esterase using the fluorogenic substrate, fluorescein diacetate. Cleavage of the diacetate quenching groups by esterase results in the formation of the product fluorescein. For this experiment, we used the channel design in Figure 5a, in which five single droplets were formed simultaneously, after which all flow was stopped and the fluorescence from each droplet was monitored over a period of 70 min. The three esterase-containing droplets were interdigitated by two buffer-containing droplets with no esterase, which served as our internal calibration and control. The droplet containing the highest amount of esterase is labeled 75% esterase to denote the fact that this solution was 75% the concentration of the stock esterase solution (0.33 mg mL^{-1}). Similarly, 50% and 25% esterase denote the relative concentration of each solution with respect to the stock solution. The buffer droplet contained the same amount of substrate but no enzyme. In fact, the substrate has comparable solubility both in water and in oil, and thus the oil solution was also saturated with substrate to ensure a constant supply of substrate in the droplets. As anticipated the rate of fluorescence increase and thus enzymatic activity correlates well with the enzyme concentration contained within each droplet. Because of the long observation period (70 min), the droplets sometime started to wet the channel floor, which caused a slight flattening of their shape. Nevertheless, such long-time observations of simultaneously formed droplets are difficult to attain with the traditional methods of continuous-stream droplet generation.

4. CONCLUSION

This paper describes a method for generating multiple aqueous droplets simultaneously using programmed infusion and withdrawal of immiscible oils into and out of small nozzles where the aqueous solutions reside. This ability expands the current repertoires used for droplet generation, which mostly rely on the fast sequential formation of single droplets. We anticipate our method will find use in applications where different reagents and assays are to be performed in different droplets simultaneously and where the potential for cross-contamination between droplets must be avoided. Coupling previously described gradient-generation techniques with simultaneous droplet formation should facilitate studies where a defined concentration gradient

of molecules within droplets is used, such as in dose-response measurements or in the study of enzyme kinetics.

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Figure 1.

Schematic of microfluidic designs. (a) shows the design for generation of droplets with differing contents, while (b) shows the design for generating droplets with a gradient of chemical contents. Five syringes are intended for use with design (a), while two syringes are intended for design (b). For both designs, the aqueous-phase channels are 100 μm in width, while the oil phase channel is 750 μm wide. The system is fabricated to a height of 50 μm , except for the 7 μm wide and 100 μm long nozzles shown in the inset, which are 7 μm in height.

Figure 2. Effects of surfactant concentration on droplet formation. The concentration of Span 80 was increased from (a) 0.01%, (b) 0.05%, (c) 0.1%, (d) 0.2%, to (e) 1% in AS 4 silicone oil. A nine-outlet gradient-generator design was used. The oil-flow program used had a pulse magnitude of $350 \mu\text{L min}^{-1}$. The scale bar is $100 \mu\text{m}$.

Figure 3.

Droplet formation using programmed pulsed oil flow. (a) Schematic of oil flow controlled using a programmable syringe pump. The pulse illustrated has the following steps: i) infuse (pump pushes the oil phase) for 1 second at $250 \mu\text{L min}^{-1}$, ii) withdraw (pump pulls the oil phase) for 1 second at $250 \mu\text{L min}^{-1}$, and iii) infuse for 5 seconds at $4 \mu\text{L min}^{-1}$. (b) Schematic illustrating the corresponding movements of oil at the nozzle. (c) Sequence of images showing droplet formation using the oil-flow pulse in panel a. A nine-outlet gradient-generator was used, with the aqueous phase being $10 \mu\text{M}$ fluorescein in 1 mM phosphate; the oil phase was 0.1% Span 80 in AR 20. The white arrows point to the location of the water/oil interface. The scale bar for the panels is $50 \mu\text{m}$.

Figure 4.

Droplet formation as a function of applied pressure by way of oil-phase pulse magnitude. The applied pressure ranged from 190 kPa to 310 kPa. The corresponding pulse magnitudes are shown in the figure inset, with 190 kPa resulting from 250 $\mu\text{L min}^{-1}$ pulse and 310 kPa from 400 $\mu\text{L min}^{-1}$ pulse. A nine-outlet gradient-generator design was used. The aqueous phase was 10 μM fluorescein in 1 mM phosphate; the oil phase was 0.1% Span 80 in AR 20 silicone oil. The scale bar is 100 μm and applies to all panels.

Figure 5.

(a) Simultaneous generation of multiple droplets containing different dye solutions, for which a five-outlet parallel-channel design was used. (b) Linescan of the normalized intensity across five channels seen in the inset, which consisted of a concentration gradient of fluorescein, made with a five-outlet gradient-generator design. The inset is a fluorescence image of five droplets formed using the gradient device, with 5 and 0 μM fluorescein in 1 mM phosphate and 0.125 w/w% PEG being introduced into the two inlets of the gradient-generation network. Oil phase was a 1:1 mix of 50 and 100 cSt silicone oil with 0.1% Span 80. Scale bar is 65 μm . The channel and droplet that contained 0 μM fluorescein was traced with dotted lines to aid visualization. (c) Plot of the average normalized intensity for each droplet. (d) Plots of normalized

fluorescence intensities as a function of time for droplets containing different concentrations of esterase. The percent esterase refers to the concentration of each esterase solution with respect to the stock solution (0.33mg mL^{-1} esterase dissolved in 1X PBS buffer at pH 7.4), and thus 75% esterase denotes a concentration that is 75% of the stock solution: $\blacklozenge$: 75% esterase; $\bullet$: 50% esterase; $\blacksquare$: 25% esterase; $\blacktriangle$: buffer. The buffer solution contained the same amount of substrate but with no esterase. The substrate was dissolved in both the aqueous solution and oil solution, since it has comparable solubility in both solutions. Oil phase was a 1:1 mix of 50 and 100 cSt silicone oil with 0.1% Span 80. The inset shows a schematic of the channel layout as well as an image of each type of droplet. The scale bar for the images represents $75\text{ }\mu\text{m}$.