

Formation of Droplets of Alternating Composition in Microfluidic Channels and Applications to Indexing of Concentrations in Droplet-Based Assays

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For screening the conditions for a reaction by using droplets (or plugs) as microreactors, the composition of the droplets must be indexed. Indexing here refers to measuring the concentration of a solute by addition of a marker, either internal or external. Indexing may be performed by forming droplet pairs, where in each pair the first droplet is used to conduct the reaction, and the second droplet is used to index the composition of the first droplet. This paper characterizes a method for creating droplet pairs by generating alternating droplets, of two sets of aqueous solutions in a flow of immiscible carrier fluid within PDMS and glass microfluidic channels. The paper also demonstrates that the technique can be used to index the composition of the droplets, and this application is illustrated by screening conditions of protein crystallization. The fluid properties required to form the steady flow of the alternating droplets in a microchannel were characterized as a function of the capillary number Ca and water fraction. Four regimes were observed. At the lowest values of Ca , the droplets of the two streams coalesced; at intermediate values of Ca the alternating droplets formed reliably. At even higher values of Ca , shear forces dominated and caused formation of droplets that were smaller than the cross-sectional dimension of the channel; at the highest values of Ca , coflowing laminar streams of the two immiscible fluids formed. In addition to screening of protein crystallization conditions, understanding of the fluid flow in this system may extend this indexing approach to other chemical and biological assays performed on a microfluidic chip.

This paper characterizes a method of forming stable alternating droplets of two sets of aqueous solutions in microchannels. In addition to characterization, we describe an application of this method to indexing concentrations of the solutes in the droplets used for assays. Protein crystallization under microbatch conditions was used as an example. Indexing here refers to measuring the concentration of a solute by addition of a marker, either internal or external.

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Droplets in microfluidic channels allow transport in microfluidic channels with no dispersion¹ and allow rapid mixing using chaotic advection.^{1–3} In this paper, we use the word “droplet” to describe a plug—an aqueous droplet that is surrounded by an immiscible fluid and is in apparent contact with all four walls of a hydrophobic microchannel.⁴ Such droplets in microfluidic channels are being used as microreactors^{5–8} in chemical kinetics,⁹ chemical amplification,¹⁰ chemical and biological analysis,^{11,12} and protein crystallization.^{13,14} Tens to hundreds of crystallization trials or assays can be performed in the same channel inside droplets. We have recently used alternating aqueous droplets formed in a water-permeable carrier fluid to perform protein crystallizations under vapor diffusion conditions and to concentrate nanoliter volumes of solutions.¹⁴ In all of these experiments, each droplet contains solutes of unique concentrations, and the composition of each droplet needs to be known. Previously, we measured the composition of droplets by adding a different fluorescent dye to each of the solutions that were used to form droplets.¹³ The disadvantages of the method are the possible contamination of the sample and interference of the dye with the analyte, especially for assays that use fluorescence as the readout. Alternatively, we indexed the concentrations inside droplets by counting the droplets and keeping track of their sequential number during the experiment.

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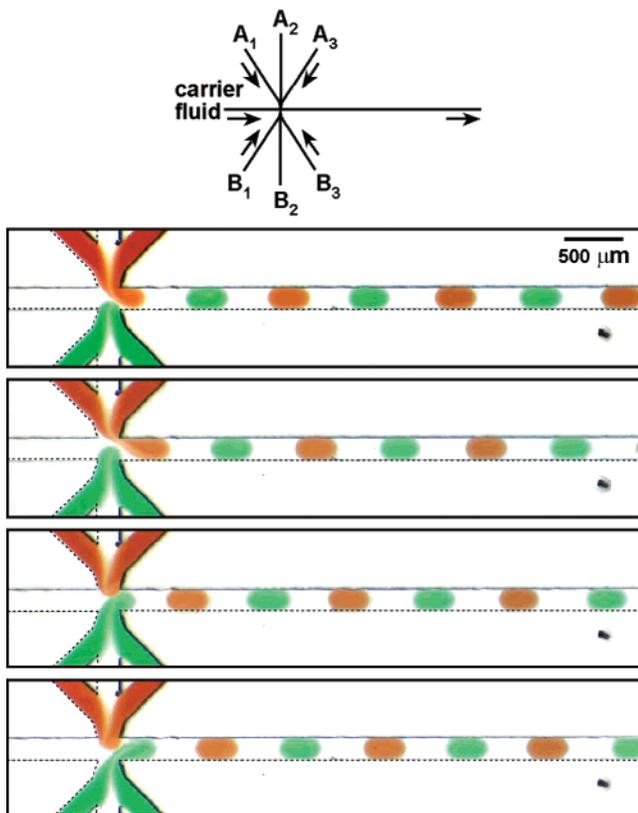


Figure 1. Formation of alternating droplets. A schematic illustration (top) of the experimental setup, and a set of four microphotographs showing the detailed process of the formation of the alternating droplets.

This method becomes less convenient for experiments when large numbers of droplets are used.

Here we describe an alternative approach to indexing the composition of the droplets. We generate droplets with alternating composition (ABAB...), with the first set of droplets (type A) containing the solutes of interest used in the reaction or assay and the second set (type B) containing markers, such as fluorescence indicators (Figure 1). The concentrations of markers in the second set of droplets are directly proportional to the concentrations of solutes in the first set of droplets.

In this paper, we carried out several investigations while implementing this idea: (i) characterization of the condition of forming stable alternating droplets; (ii) indexing compositions of droplets using fluorescent dyes; (iii) illustrations of the technique in protein crystallization.

EXPERIMENTAL SECTION

Microfluidic devices were fabricated using soft lithography in poly(dimethylsiloxane) (PDMS).^{15–17} The experiments were conducted in the same way as described previously¹⁸ except that the syringe pumps were computer controlled using a LabView program.

Formation of Alternating Droplets. To generate alternating droplets of two sets of solutions, we used a PDMS-based

microchannel with a channel geometry shown in Figure 1. A solution of $\text{Fe}(\text{SCN})_x^{3-x}$ (0.01 M) was used as streams A1 and A3. A solution of a green food dye (McCormick) was used as streams B1 and B3. Streams A2 and B2 were both water. The carrier fluid was 1*H*,1*H*,2*H*,2*H*-perfluoro-1-octanol (PFO, from Acros Organics) (30%, v/v) in perfluoroperhydrophenathrene (PFP, from Acros Organics). The flow rate of the carrier fluid was 2.0 $\mu\text{L}/\text{min}$, and the flow rate of each aqueous stream was 0.2 $\mu\text{L}/\text{min}$.

Characterization of Conditions for Generating Alternating Droplets. To study the formation conditions of alternating droplets of two sets of aqueous solutions, we used a PDMS-based microfluidic device. In this experiment, two combinations of carrier fluid and aqueous streams were used. In the first combination, both the carrier fluid and the aqueous streams were viscous. The viscous carrier fluid was PFO in PFP (10%, v/v; viscosity, 16 mPa s), and the viscous aqueous streams were glycerol (68%)/ $\text{Fe}(\text{SCN})_x^{3-x}$ (0.07 M) and glycerol (68%)/ KNO_3 (0.2 M). In the second combination, both the carrier fluid and the aqueous streams were nonviscous. The nonviscous carrier fluid was PFO in FC-3283 (from 3M) (10%, v/v; viscosity, 1.8 mPa s), and the nonviscous aqueous streams were glycerol (24%)/ $\text{Fe}(\text{SCN})_x^{3-x}$ (0.07 M) and glycerol (24%)/ KNO_3 (0.2 M). For fluorescence measurement, a Leica DMIRE2 microscope with a digital camera (ORCA-ER, Hamamatsu) was employed. GFP and DAPI filter cubes were used for observing the fluorescence of fluorescein and 1,3,6,8-pyrenetetrasulfonic acid, tetrasodium salt (PSS, from Acros Organics), respectively.

Comparison of Fluorescence of Two Sets of Droplets. In this experiment, two dyes, fluorescein (Molecular Probes) and PSS, were dissolved in PBS buffer (pH 7.2), respectively. The third aqueous solution was poly(ethylene glycol) (PEG) (average MW 6000, 20% w/v) in PBS buffer. The carrier fluid was PFO (25% v/v) in PFP. The flow rate of the carrier fluid was constant at 2.5 $\mu\text{L}/\text{min}$. The syringes containing fluorescein and PSS solutions were driven by the same syringe pump, and the syringes containing PEG solutions were driven by the second syringe pump. Both pumps were computer controlled, and the flow rates varied between 0.1 and 0.3 $\mu\text{L}/\text{min}$.

Indexing for Screening Thaumatin Crystallization Conditions. The protein solution was 50 mg/mL thaumatin (Sigma) in *N*-(2-acetamido)iminodiacetic acid (0.1 M, pH 6.5). The precipitant was 1.0 M sodium potassium tetraborate in *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES; 0.1 M, pH 7.0). Solutions of fluorescein and PSS in PBS (pH 7.2) buffer were used as fluorescence markers for the purpose of indexing. The carrier fluid was PFO in PFP (25%, v/v). Flow rate of the carrier fluid was constant at 2.0 $\mu\text{L}/\text{min}$. Thaumatin and precipitant solutions were injected from one side while the two dye solutions were injected from the other side of the main channel. The syringes containing thaumatin and fluorescein solutions were driven by the same syringe pump, and the syringes containing precipitant and PSS solutions were driven by the second pump. Both pumps were computer controlled, and the flow rates of the aqueous streams varied between 0.1 and 0.5 $\mu\text{L}/\text{min}$.

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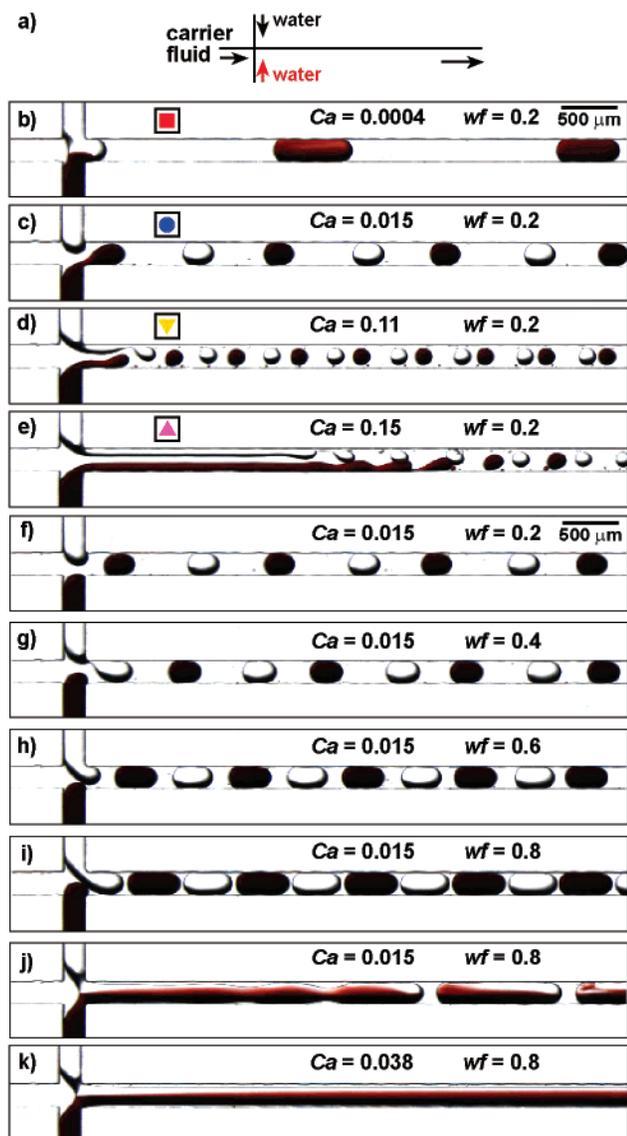


Figure 2. Four regimes observed for the formation of alternating droplets as a function of the capillary number Ca . (a) Schematic illustration of the experimental setup. (b)–(k) Microphotographs of the four regimes observed as the capillary number (Ca) and water fraction (wf) were varied (water fraction is defined in text).

RESULTS AND DISCUSSION

Formation of Alternating Droplets. We generated alternating droplets by using a microfluidic channel with the geometry shown in Figure 1. Two sets of aqueous solutions were used: set A, consisting of A1, A2, and A3, and set B, consisting of B1, B2, and B3. The mixture of A1, A2, and A3 was red, and the mixture of B1, B2, and B3 was green. At certain flow rates of the carrier fluid and the aqueous streams, alternating droplets with compositions of A and B were generated (Figure 1). These droplets did not appear to be cross-contaminated even though the solutions A and the solutions B came into proximity during the formation of the droplets. The alternation was not observed under all conditions, and the coalescence of droplets (Figure 2b) was a problem at low flow rates. To solve this problem, the mechanism of coalescence must be understood.

Coalescence of liquid droplets of the first fluid in a second immiscible liquid is complementary to the breakup of droplets

during the formation of emulsions.^{19,20} Coalescence has been extensively studied^{21–27} because it is important in the preparation and stability of emulsions, foams, liquid–liquid extractions, and many other industrial applications. Coalescence takes place in three steps. The first step is the approach and collision of droplets. The second step is the drainage of the film of immiscible fluid between the droplets. The third step is the rupture of the film and rapid coalescence. The rate of film drainage depends strongly on the interface mobility—higher interface mobility leads to higher rate of film drainage. This mobility is a function of two factors: the viscosity ratio of the internal and external fluids and the presence of surfactant at the interface.²² When the droplet has a higher viscosity, the interface is less mobile; therefore, it is more difficult for the two droplets to coalesce.²⁵ The presence of surfactant at the interface also causes the arrest of coalescence.²²

Characterizing the Four Regimes of Fluid Flow as a Function of the Capillary Number. We characterized the dependence of the formation of alternating droplets on two parameters: capillary number (Ca) and water fraction (wf). The capillary number “represents a measure of viscous stresses relative to interfacial tension stresses”,²⁴ $Ca = U\mu/\gamma$, where U (m s^{-1}) is the flow velocity, μ ($\text{kg m}^{-1} \text{s}^{-1}$) is the dynamic viscosity, and γ (kg s^{-2}) is the surface tension at the interface between the aqueous solution and the carrier fluid. It has been shown that Ca is the most important parameter describing coalescence of droplets.^{21–29} Water fraction (wf) is defined as the ratio of the combined flow rate ($\mu\text{L}/\text{min}$) of the aqueous phase to the total flow rate ($\mu\text{L}/\text{min}$) of the carrier fluid and the aqueous phase.

We found four regimes of fluid flow in the geometry shown in Figure 2a. Each regime is defined by Ca and its range is affected by wf . For simplicity, instead of six aqueous streams as in Figure 1, we used only two aqueous streams, a colorless one and a dark red one. When the carrier fluid and the aqueous streams were both viscous ($\mu = 16 \text{ mPa s}$) and wf was 0.2, all four regimes were observed (Figure 2b–e). In the first regime ($Ca < 0.001$, Figure 2b), surface tension dominates. The aqueous streams reached the inlet junction head to head and coalesced. Droplets containing a mixture of the colorless and dark red aqueous streams formed. In the second regime ($0.001 < Ca < 0.05$, Figure 2c), the aqueous streams snapped-off cleanly and did not coalesce. The two streams took turns to form droplets and a steady array of alternating droplets was generated in the main channel. In the third regime ($0.05 < Ca < 0.13$, Figure 2d), shear forces became larger and there was a competition between shear force and surface tension. Droplets formed with a diameter smaller than the cross-sectional dimension of the channel. We also observed

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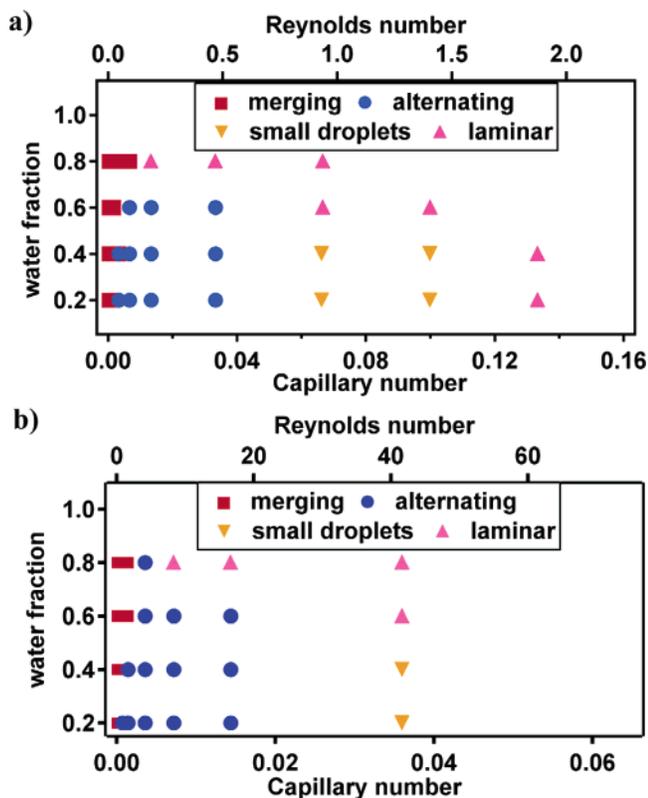


Figure 3. Phase diagrams characterizing the formation of alternating droplets as a function of capillary number (Ca) and water fraction (wf) for the flow of viscous fluids ($\mu = 16$ mPa s) at low values of Re (a) and for the nonviscous fluids ($\mu = 1.8$ mPa s) at intermediate values of Re (b).

this regime at similar values of Ca in a simple cross-flow geometry where droplets of a single aqueous stream formed.³⁰ While this regime is interesting from the pattern formation point of view,³¹ it is less useful for the indexing of assays described here, because these droplets are not plugs, and it is more difficult to transport such droplets without merging and more difficult to induce rapid mixing inside these droplets. In the fourth regime ($0.13 < Ca$, Figure 2e), shear force dominated and induced laminar flow, and the two aqueous streams formed laminar segment beyond the junction before droplets could form. Small alternating droplets formed (similar to the droplets formed in the third regime) and often merged as they moved along the channel. The size of droplets could be increased and spacing between them decreased in a predictable way by increasing the water fraction (Figure 2f–i).

The range of Ca describing each regime varies as water fraction changes (Figure 3). For water fractions below 0.4, the region of stable alternating droplets is the widest. As the water fraction increases, the region of stable alternating droplets shrinks. At wf of 0.8, it is difficult to form stable alternating droplets at any Ca . Even at the optimal value of Ca of 0.015, the system appeared bistable—either alternating droplets (Figure 2i) or laminar flows of the carrier fluid and water (Figure 2j) were

observed. We do not know if this behavior is a true bistability or if it is caused by slight variations in the experimental conditions. At high wf of 0.8 and slightly higher Ca of 0.038, laminar flow completely dominated (Figure 2k).

The capillary number is directly proportional to the Reynolds number,³² Re , if only the flow velocity U is changed during the experiment. By changing the viscosity of the fluids we demonstrated that it is Ca and not Re that describes the fluid flow in this system, as long as Re is not too high. Viscosity is the most indicative parameter because the decrease in viscosity of fluids decreases Ca but increases Re . We made the carrier fluid and aqueous streams both nonviscous ($\mu = 1.8$ mPa s) and observed the same four regimes for the formation of alternating droplets (Figure 3b). The ranges of the four regimes were very similar (within a factor of 2) to the ranges observed for the more viscous fluids in terms of the capillary number but very different (a factor of 50) in terms of the Reynolds number.

Indexing Compositions of Droplets. Detailed characterization of the multiphase fluid flow shown in Figure 2 enabled us to use alternating droplets for indexing. To index compositions of droplets, we need to correlate the concentrations of two solutes in two adjacent droplets. Previously we established that the concentration of a solute in a droplet is determined by the instantaneous relative flow rate of the aqueous stream that contains the solute.¹³ Relative flow rate of a stream refers to the ratio of the flow rate of the stream to the total flow rate of all aqueous streams. To correlate the concentration of two solutes in adjacent droplets, we need to correlate the flow rates of the two streams that contain the solutes. The flow rates of the two streams can be correlated, for example, by using the same syringe pump to drive the two syringes that contain the solutions.

This idea is illustrated schematically in Figure 4a and b, showing the formation of alternating droplets containing variable concentrations of a dye A and a dye B. Stock solutions of dye A and dye B of the same concentration are injected into the device at the same low flow rate. The flow rates of the two buffers are also equal and high, leading to the formation of droplets containing solutions of the dyes of the same low concentration (Figure 4a). Then the flow rates of the two buffers are decreased, and the flow rates of the two streams of dyes are increased, leading to the formation of alternating droplets containing the two dyes at the same high concentration (Figure 4b).

To prove experimentally that the compositions of the adjacent droplets are linearly related, we added PSS (as dye A) to the aqueous solution to form droplets A and added fluorescein (as dye B) to form droplets B. The flow rates of the two solutions of the dyes were kept the same during the experiment. The flow rates of the two buffers were also the same, and the ratio of the flow rates of the buffers relative to the flow rates of the dye solutions was changed during the experiment. As shown in Figure 4c, in the beginning of the experiment (the first 15 droplets), the flow rates of the solutions of the dyes were kept constant and lower than the flow rates of the buffers. Therefore, the concentrations of the dyes in the resulting droplets were low (as in Figure 4a). For the next 25 droplets (from droplets 16 to 40), the flow rates of the dye solutions were gradually increased over the flow

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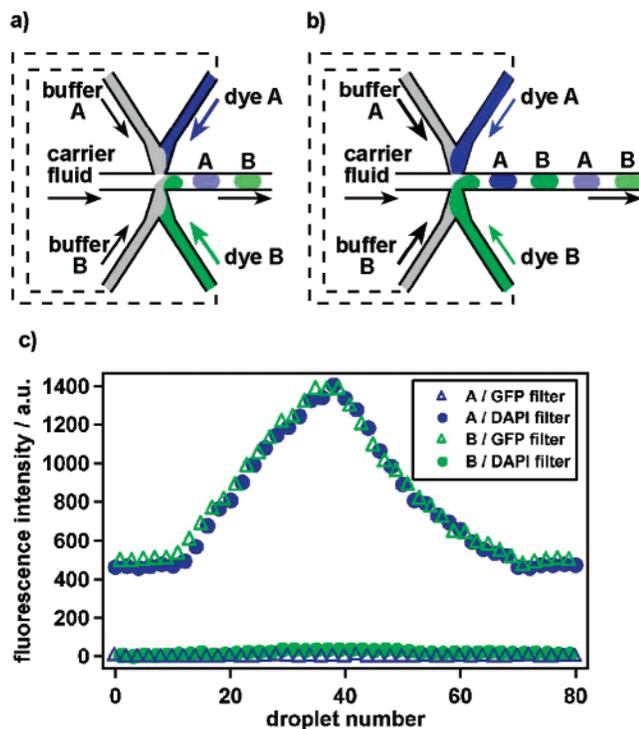


Figure 4. Indexing concentrations using alternating droplets by correlating concentrations of two solutes in two adjacent droplets. (a, b) Schematic drawing of the experimental setup. The dashed lines indicate that the two syringes were driven by the same syringe pump at the same flow rate. Droplets A were formed from the solutions of the blue PSS fluorescent dye and buffer. Droplets B were formed from the solutions of the green fluorescent dye and buffer. Changing the relative flow rates induced the formation of droplets with low (a) and high (b) concentration of the dyes. (c) Plot of fluorescence from each droplet vs the sequential number of the droplet. Blue ● and Δ symbols represent fluorescence intensity from droplets A using filter set for PSS and fluorescein, respectively. Green Δ and ● symbols represent fluorescence intensity from droplets B using filter set for fluorescein and PSS, respectively.

rates of the buffers (as in Figure 4b). The concentrations of the dyes in the droplets increased accordingly. In the third stage (from droplets 41 to 65), the relative flow rates of the solutions of the dyes were gradually decreased and the concentrations of the dyes decreased to the starting value. We found that the fluorescence intensity of droplet B followed linearly the fluorescence intensity of its adjacent droplet A (Figure 4c). We have found no evidence of cross-contamination between droplets A and droplets B. No fluorescein from the droplets B was detected in the droplets A, and no PSS dye from the droplets A was detected in the droplets B (Figure 4c). Very slight increase of fluorescence above the baseline (observed in Figure 4c) was presumably due to the bleed-through of the fluorescence of fluorescein through the DAPI filter we used. This result is consistent with the presence of a slowly draining thin film of carrier fluid separating the two droplets, even when the droplets appear to be in contact as in Figure 1.

Application of Alternating Droplets to Protein Crystallization. We illustrated this technique by using it to index concentrations during screening of protein crystallization conditions under microbatch conditions. Screening crystallization conditions is important for determining structures of proteins by X-ray crystallography. Generally it involves tens to hundreds of trials with

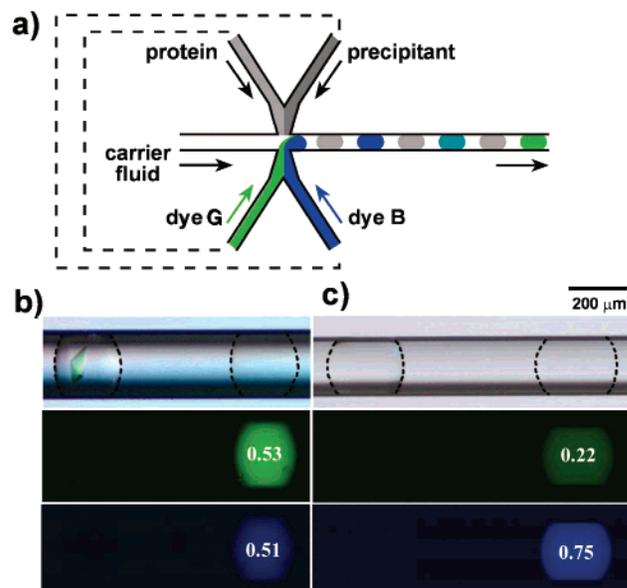


Figure 5. Indexing composition of droplets during crystallization of thaumatin. (a) Schematic drawing of the experimental setup. The dashed lines indicate that the syringes holding thaumatin and dye G solutions were driven by the same syringe pump, and the syringes holding the precipitant and dye B solutions were driven by the other syringe pump. (b) Droplets formed under conditions that yielded thaumatin crystal. (c) Droplets formed under conditions that did not yield thaumatin crystals. (b), (c), top row: a polarized light micrograph showing one droplet (left) containing mixture of thaumatin and precipitant next to a droplet (right) containing mixture of dye G and dye B. The edges of the droplets are highlighted by dashed lines. (b), (c), middle row: fluorescence images of the corresponding droplets obtained through the GFP filter. Green represents fluorescence from dye G (fluorescein), and the intensity of this fluorescence indicates the relative concentration of thaumatin. The number on each droplet represents the relative fluorescence intensity. (b), (c), bottom row: fluorescence images of the corresponding droplets obtained through the DAPI filter. Blue represents fluorescence from dye B (PSS), and the intensity of this fluorescence indicates the relative concentration of the precipitant.

different concentrations of protein and precipitant. We recently developed a screening method based on microfluidic channel using droplets as microreactors.¹³ Each droplet contained a mixture of protein and precipitant at a different concentration and represented a unique crystallization condition. To identify the conditions for crystallization, it is necessary to read out the concentration of the protein and the precipitant in each droplet.

We performed a simple crystallization experiment where we screened the crystallization conditions by varying the ratio of the protein concentration to precipitant concentration in each droplet. We showed that the concentrations of thaumatin and the corresponding precipitant in the droplets could be measured by using the technique of alternating droplets. Droplets containing thaumatin and precipitant and droplets containing fluorescein and PSS were generated alternatively in the microchannel (Figure 5a). Fluorescein (dye G) was used in this experiment to index thaumatin. Another dye, PSS (dye B), was used to index the precipitant. We have not characterized formation of alternating droplets from two aqueous streams with very different viscosities. This situation is important in protein crystallization because the precipitant may contain viscous PEG. Here we circumvented this problem by adding PEG to the solution of PSS to make the

viscosities of the two streams match. We have confirmed that PEG does not affect the fluorescence of the dyes we used.

We screened protein crystallization conditions by varying the relative flow rates of the solutions of thaumatin and precipitants (Figure 5a). Each resulting droplet contained thaumatin and precipitant at a different ratio of the concentrations. Only droplets containing the optimal 1:1 ratio of the two concentrations yielded crystals of thaumatin (Figure 5b). This ratio could be read out by measuring the \sim 1:1 ratio of the fluorescence of fluorescein to the fluorescence of PSS in the adjacent droplet and was consistent with the previously obtained result.¹³ Droplets containing other ratios of the concentration of thaumatin to the concentration of the precipitant did not yield crystals, for example, the droplet shown in Figure 5c, with the \sim 1:3 ratio readout again by measuring the ratio of the fluorescence of fluorescein to the fluorescence of PSS in the adjacent droplet.

CONCLUSIONS

We have characterized the formation of alternating droplets (plugs) in microchannels for aqueous streams of matching viscosities and observed four regimes characterized by different values of the capillary number Ca . The formation of alternating droplets is more difficult than the formation of droplets of a single aqueous phase. Droplets can be formed from any aqueous stream as long as the aqueous phase does not wet the walls of the channel and the value of Ca is low enough.³⁰ Alternating droplets also require low values of Ca to avoid transition to laminar flow. In addition, if the value of Ca is too low, the two aqueous phases coalesce and no alternating droplets form. This observation is consistent with previous studies of droplets coalescence.²⁵ It has been shown that two droplets with a nonzero offset coalesce from head-on collision if the capillary number is below a critical value. The maximum Ca for coalescence of the two aqueous streams that we determined ($Ca \sim 0.001$) is consistent with the one measured by Ha et al. ($\sim 5 \times 10^{-3}$ in the absence of polymeric surfactant and $\sim 1 \times 10^{-3}$ in the presence of the surfactant).²⁸

However, the geometry of the channel in the current study is different in that the two aqueous streams collide head-on and the resulting droplets escape in the direction perpendicular to the line of collision. Therefore, a model taking into account the specific geometry used in the experiments described here is needed to accurately describe the four regimes that we observed. For experiments with low water fractions, the window of Ca ($0.001 < Ca < 0.05$) where alternating droplets form is wide. However, at water fractions higher than 0.8, it may be impossible to form alternating droplets at any value of Ca . These phase diagrams are important to understand when alternating droplets are used. The regimes observed at higher values of Ca resembled several previously described phenomena: formation of patterns of smaller droplets^{31,33,34} and laminar coflow of immiscible fluids.^{35,36}

To calculate the value of Ca in this paper, we used the interfacial tension between the aqueous phase and the carrier fluid measured at equilibrium. During the formation of the droplets, the interfacial tension is probably higher because the instantaneous concentration of the surfactant at the interface is lower than the concentration at equilibrium. Dynamic measurements of surface tensions should be used to fully characterize this process. In addition, the formation of alternating droplets of aqueous solutions with contrasting viscosities still remains to be characterized.

We only indexed concentrations of two solutes in the protein crystallization described here, but forming alternating droplets of three or four solutions each is straightforward, and even more solutes may be indexed using sets of fluorescent labels that are being developed.^{37,38} Alternating droplets may be useful in applications beyond indexing. Previously, we have also used alternating droplets inside water-permeable fluid to concentrate aqueous solutions by diffusion of water between droplets driven by osmotic differential between the droplets.¹⁴ This method was useful for inducing formation of protein crystals.¹⁴ This method allows precise control of both the rate and the final extent of concentrating solution and will complement the methods based on the use of water-permeable PDMS^{13,39} or water-absorbing carrier fluid.⁴⁰ We believe that the phenomena that we have characterized in this paper will find a range of applications in chemical and biological analysis.

ACKNOWLEDGMENT

This work was supported by the NIH (R01 EB001903) and was performed at the MRSEC microfluidic facility funded by NSF. J.D.T. is a Beckman Fellow. We thank Wendy Zhang for helpful discussions.

Received for review March 18, 2004. Accepted June 9, 2004.

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