Monodisperse alginate microgel formation in a three-dimensional microfluidic droplet generator

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Droplet based microfluidic systems provide an ideal platform for partitioning and manipulating aqueous samples for analysis. Identifying stable operating conditions under which droplets are generated is challenging yet crucial for real-world applications. A novel three-dimensional microfluidic platform that facilitates the consistent generation and gelation of alginate-calcium hydrogel microbeads for microbial encapsulation, over a broad range of input pressures, in the absence of surfactants is described. The unique three-dimensional design of the fluidic network utilizes a height difference at the junction between the aqueous sample injection and organic carrier channels to induce droplet formation via a surface tension enhanced self-shearing mechanism. Combined within a flow-focusing geometry, under constant pressure control, this arrangement facilitates predictable generation of droplets over a much broader range of operating conditions than that of conventional two-dimensional systems. The impact of operating pressures and geometry on droplet gelation, aqueous and organic material flow rates, microbead size, and bead generation frequency are described. The system presented provides a robust platform for encapsulating single microbes in complex mixtures into individual hydrogel beads, and provides the foundation for the development of a complete system for sorting and analyzing microbes at the single cell level. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4765337>]

INTRODUCTION

Droplet based microfluidic systems, or multiphase fluidic systems, have been used extensively for the study of chemical and biochemical reactions. The precise and repeatable control of droplet size combined with the commensurate control of droplet composition provides a model platform for studying reactions in confined volumes. Moreover, the ability to manipulate these droplets on chip, combining them and mixing their contents,¹ subjecting them to local changes in temperature and pH,² and/or exposing them to nonlinear electromagnetic fields,³ makes the approach powerful and broadly applicable.

Notably, recent work has highlighted the use of these systems to manipulate and interrogate biological systems at the single cell level.^{4–6} Processes including cell lysis,⁷ DNA purification,⁸ protein synthesis,⁹ and enzymatic conversion¹⁰ have been carried out in microfluidically generated emulsions, allowing quantitative classification of intracellular chemical components and cell metabolic characteristics. The small micro-droplets generated within these systems, having diameters of tens to hundreds of micrometers, serve as ideal platforms for the segmentation and isolation of cells from complex mixtures into isolated femto- to nanoliter compartments.¹¹ Monodisperse populations of microdroplets containing single cells can be readily created¹² and allow the dynamic

and precise control of reagent concentrations. This provides a predictable microenvironment for cell growth, differentiation, and analysis of monoclonal cell populations.¹³

Encapsulation of microbial cells in hydrogel beads offers significant advantages over conventional aqueous emulsion systems. Chemically crosslinked hydrogels provide a stable physical network that prevents microdroplet coalescence and serves to keep microbial colonies confined. Once crosslinking has occurred, the microbe-loaded beads can be resuspended or washed with aqueous fluids and further cultivated and treated for analysis.¹⁴ A variety of hydrogel materials have been used in multiphase fluidic systems for cell encapsulation with crosslinking being controlled via changes in temperature, ionic concentrations, pH, and introduction of UV radiation.^{2,15–17} Alginate hydrogels have proven particularly useful in microgel synthesis due to their biocompatibility and easy crosslinking with divalent ions such as calcium.¹⁸ Premixing of alginate and calcium streams with immediate droplet breakup,^{19,20} release of calcium ions from encapsulated nanoparticles induced by pH variation,^{21,22} use of microchamber arrays,²³ and droplet coalescence^{24,25} have all been used to control the introduction of divalent calcium into these systems as a means of controlling alginate hydrogel stability.

Droplet size and generation rates depend on flow instabilities stemming from nonlinearities in multiphase systems. The interplay of interfacial tension, shear stress, inertia, and channel wall wettability all contribute to the droplet formation process. Approaches to generating micro-droplets typically include the variation of two-dimensional channel geometries and/or control of fluid flow rates to manipulate shear stress. Multiple studies have described the conditions, or parameter space under which droplets are generated and provide an empirical and theoretical basis for predicting droplet generation.^{20,26,27} T-junction and flow-focusing channel geometries are the most common designs used in droplet generation.^{28,29} Concentric glass capillary microfluidic devices have also been used.^{30,31} Both T-junctions and flow-focusing geometries can be operated to allow segmentation of fluid by dripping or jetting mechanisms.^{32–34}

Despite the strong foundation of work in this area, real-world application of digital microfluidics can be significantly hindered by inconsistent droplet generation that results in sample loss or “streaming” of reagents which damage down-stream processes.²⁷ For biological applications, such as the isolation and encapsulation of single cells from complex populations, robust and consistent droplet generation and gelation are essential. “Streaming” of reagents in such a case could lead to the loss of rare cells from a mixed population, or release of large hydrogel plugs into downstream channels, resulting in irreversible device failure. Inconsistent generation of droplets rarely stems from a lack of understanding of the conditions under which droplets should be generated. Often, seemingly minor defects in device geometry, sealing, or the presence of particulates and/or air bubbles can significantly disrupt flow conditions within a microfluidic system, and may alter the time required for the system to come to equilibrium. Thus, innovations in device design and operation that expand the conditions under which microbead generation and gelation occur minimize the time needed to reach equilibrium, and generally enhance robustness, dramatically improve translation of this technology.

Here, we present a novel pressure driven flow-focusing system that enhances the range of operating conditions across which hydrogel droplets can be consistently created. A three-dimensional microfluidic channel arrangement is used to create an abrupt height change at the junction of the immiscible aqueous and organic carrier solutions resulting in a substantial expansion in the parameter space where stable droplets can be formed in comparison to conventional two-dimensional designs. The 3D system is fabricated using the same techniques as conventional 2D devices, and produces droplets over a wider range of input pressures. This allows droplet size, and generation frequency to be modulated. Operation under constant pressure conditions leads to rapid equilibration of the system, and when combined with the three-dimensional system design, minimizes sample “streaming.” Gelation of droplets was carried out downstream of the 3D channel junction by introducing an ionic calcium source dissolved in the organic carrier phase. Control of microbead spacing and gelation time facilitated the production of stable alginate microspheres with intact boundaries, even in the absence of surfactants. Green fluorescent protein (GFP) expressing *Escherichia coli* (BL21) was encapsulated using this

platform over a 10 h period, with single cell encapsulation achieved by appropriate control of input bacteria concentration and microbead diameter.

MATERIALS AND METHODS

Microfluidic device fabrication and assembly

SU-8 masters were fabricated using conventional photolithography techniques. SU-8 2015 (Microchem, MA) negative photoresist was spin coated on 4-in. silicon wafers, soft-baked, exposed, baked, and developed according to manufacturer's specifications to form the first 12 μm thick channel layer. This channel is ultimately used to introduce the aqueous phase containing the alginate/cell solutions (Figure 1). Deposition and patterning of a second layer of SU-8 2050 (Microchem, MA) are then performed following a 30 min dehydration bake. The 50 μm thick channels ultimately form the remaining organic phase and downstream channel networks. The taller channel widths are 60 μm for the main/organic carrier phase channels, and 12 μm for the aqueous alginate solution channel. SEM micrographs (Figure 1, inset) show the patterned SU-8 mold at the 3D channel junction.

SU-8 masters were treated in a vapor of trimethylchlorosilane (Aldrich) for 1 h prior to polydimethylsiloxane (PDMS) molding to facilitate the removal of the PDMS replica. Sylgard 184 PDMS pre-polymer and the crosslinker were mixed at the recommended 10:1 w/w ratio. After pouring the mixture onto the SU-8 master and degassing for 1 h, the mixture was cured at 75 °C for 2 h. PDMS moldings were cut and peeled off the masters. Inlets and outlets were stamped using a Harris Uni-core™ punch (o.d. = 0.75 mm) (Ted Pella, Redding, CA). The chip was then placed into air plasma for 10 s. The same surface treatment was applied to a microscope slide with 10 μm thick, spin-coated layer of PDMS. The assembled device was cured at 120 °C for 48 h to regain hydrophobicity.

Flow control and bead generation

Five voltage-to-pressure transducers (Marshbellofram T2000) were assembled in an aluminum metal frame to regulate pressure from a compressed nitrogen tank to the fluid reservoirs. Pressure was controlled by adjusting the supply voltage within a range of 0 to 10 V corresponding to a pressure output of 0 to 210 kPa. The accuracy of the pressure transducers is ± 0.01 V (0.21 kPa) based on the manufacturer's specifications. A MATLAB™ interface was written for the USB voltage controller (USB3103, Measuring Computing, MA). In fluid reservoirs, the input pressure pushes the fluid into the outgoing 24-gauge Teflon tube. At the end of the Teflon tube, a 23-gauge blunt metal tube is inserted. The metal tube is then inserted into a hole punched in the PDMS microfluidic device. Fluid flow within the system is regulated via the control of pressure within each reservoir.

FIG. 1. (a) Schematic of a complete microfluidic platform for the isolation and encapsulation of microbes from mixed populations into alginate microbeads. Insets show the SEM image of a SU-8 mold of a two fluid phase junction, calcium stream introduction, and a two-layer aggregation of alginate beads in storage channel (from left to right). Scale bar: 60 μm . (b) Schematic showing the process of droplet formation at the two-phase junction site.

Bead generation and gelation

Beads were generated using oleic acid (Fisher Scientific) as the continuous/organic phase and 2% (w/w) sodium alginate (Sigma Chemical) in deionized water mixed with various concentrations of microbial cells as the aqueous phase. The viscosity of 2% alginate solution was approximately 250 Cp. Oleic acid was loaded into the inlet of the larger continuous phase channel (Figure 1), and the aqueous alginate/cell mixture was loaded into the inlet for the smaller channel. A phase diagram that describes the flow conditions that yield droplet generation in two different device designs was created by flowing the organic “continuous” phase with a constant backpressure. The two designs consisted of a 2D flow focusing system and the 3D flow focusing system described above. The backpressure of the alginate channel was increased by adjusting the output voltage to the corresponding transducer while the flow of the oil channel was kept constant. The pressure at which different regimes of droplet formation occurred was recorded. A stereo microscope (Leica zoom 2000, Leica) and Motic CMOS camera (Motic) were used to observe and record videos of droplet formation. Fluid flow was stopped after counting the generating frequency in order to capture clear images of multiple droplets in the channel. Analysis of the droplet size, shape, and spacing was carried out using Motic ImagePlus 2.0 (Motic).

To initiate crosslinking of the alginate hydrogel, oleic acid loaded with calcium chloride was added through a channel downstream to the droplet generation site (Figure 1). The calcium chloride solution is prepared by mixing CaCl_2 in 2-methyl-1-propanol at 3% w/v ratio. The mixture is added to an equal volume of oleic acid and placed on a 120 °C hot plate to evaporate the organic solvent.²⁸ Filtration using a filter unit with 5 μm holes (Sartorius Stedium Biotech) is carried out to remove solid residues. A serpentine gelation channel with varying numbers of loops (1, 4, and 8, see Figure 4) was used to examine the gelation time required to create well-separated solidified microgel beads without the use of surfactants.

Bacterial cell preparation

Demonstration of cell encapsulation using the 3D droplet generation system was carried out by adding *E. coli* (BL21) cells expressing GFP into the aqueous phase solution prior to bead generation. Solutions were prepared using standard microbial culture protocols. Colonies were picked from an agar plate and put into a 50 ml centrifuge tube containing 5 ml of LB media with 50 $\mu\text{g/ml}$ ampicillin. The growth of *E. coli* reached the exponential phase after approximately 4 h of incubation at 37 °C. The optical density as measured via absorbance at 600 nm was between 0.4 and 0.6 and corresponded to a concentration of 0.4 to 0.6×10^9 cells per milliliter. The cell solution was mixed with the 2% alginate solution at different volume ratios and used in bead generation experiments. Fluorescent and bright field images of the beads were captured and analyzed. The number of cells per bead was recorded for approximately fifty beads.

RESULTS

Definition of droplet generation parameter space

A phase diagram describing the regimes under which droplets are formed is shown in Figure 2. This phase diagram demonstrates the broad range of conditions under which the 3D flow focusing system produces droplets in comparison to a comparable 2D flow-focusing device. Within Region I of Figure 2, consistent streaming of the aqueous phase is observed. This is also referred to in the literature as the annular flow region.²⁷ Plug generation, where the droplet length is more than twice the channel diameter, is observed in region II. Droplet generation in the 3D flow focusing device was recorded within region III. Region III overlaps region IV, which represents operational conditions where droplets were formed in the 2D flow focusing system (marked as “Droplet-2D” in figure). For both 2D and 3D devices, a minimum alginate flow rate is required in order to advance the aqueous tip in hydrophobic PDMS and

FIG. 2. Droplet generation phase space plotted as a function of aqueous and organic carrier phase backing pressures for 3D (Regions I, II, III) and 2D devices (Droplet 2D).

overcome the backpressure from oil to penetrate into the main channel. For comparison with other work in this area, this phase space was examined as a function of oil pressure and capillary number of the continuous organic phase.³⁵ The capillary number is defined as $Ca = \mu U / \gamma$, where μ is the fluid viscosity, U is the average laminar velocity, and γ is the surface tension between two fluids. Both plots show a similar expansion of the conditions under which microdroplets could be readily formed.

Droplet size and generation frequency

Droplet size and generation frequency were analyzed as a function of applied backing pressures for the aqueous and organic carrier solution reservoirs. Under conditions in which droplets are formed, increases in either aqueous or organic carrier phase backpressure lead to a higher frequency of droplet generation. A maximum of 160 droplets per second was recorded at the maximum pressure output of the voltage to pressure transducers. In Figure 3, we have applied low oil and alginate pressures in order to understand the combined effect of surface tension and shearing force. Figure 3 shows trends relating droplet size and generation rate at three different backing pressures for the organic carrier phase as the backing pressure of the aqueous reservoir was increased. Figure 4 shows the histograms of droplet size generated at three aqueous alginate back pressures while oil pressure is kept constant ($P_o = 15$ kPa). All cases show narrow expanded histograms with an average coefficient of variation less than 5%, which is indicative of “monodispersity.”^{20,26} Images of droplets travelling within the device are shown next to their respective histograms.

The impact of gelation time on droplet stability was examined by varying microchannel length following the initiation of crosslinking. Figure 5 illustrates the effects of gelation time on droplet stability. For short residence, or gelation times, droplets coalesce to form a large pool of fluid or semi-solidified aggregate clumps (Figures 5(a) and 5(b)). For channels providing adequate gelation time, monodispersity of droplets is maintained. The two groups of beads in Figure 5(c) have a distribution of $14.55 \mu\text{m} \pm 1.13 \mu\text{m}$, and $27.6 \mu\text{m} \pm 0.69 \mu\text{m}$ (top and bottom, respectively). Reductions in droplet size due to calcium-induced alginate crosslinking and aqueous phase evaporation were observed during experiments.

Microbe encapsulation

Once droplet generation and gelation were adequately characterized, encapsulation of model microbes into alginate microbeads was performed. In order to achieve the goal of single microbe encapsulation, a solution of cultured microbes was mixed with alginate solution at different ratios. Figure 6 shows the Poisson distribution for the encapsulation of GFP expressing *E. coli* in hydrogel beads within the 3D flow focusing system having a serpentine channel

FIG. 3. Droplet generation frequency (upper) and droplet size (lower) are shown as a function of aqueous phase backing pressure for three different organic phase backing pressures.

length of 100 mm. $f(x)$ stands for the probability that the number of fluorescent bacteria is encapsulated in a single bead. The device was operated with the backing pressure of the organic carrier, aqueous/alginate/*E. coli* solution, and calcium loaded organic carrier pressures all held constant at 30 kPa. The generation frequency was approximately two droplets per second. For an initial concentration of 2.5×10^6 cells/ml, single GFP expressing *E. coli* encapsulation is realized.

DISCUSSION

The 3D pressure controlled droplet generation system described here allows for repeatable and robust generation of alginate hydrogel droplets for microbial encapsulation. Figure 2 shows that the 3D flow focusing device presented here is capable of generating droplets over a broader range of backing pressures than its two-dimensional equivalent. Even at high alginate flow rates, the droplet break-up location is maintained at the junction of the aqueous and organic

FIG. 4. Histograms showing the size distribution of droplets generated at three different combinations of operating pressures. Scale bar: 60 μm .

FIG. 5. Alginate microbeads imaged following collection from a “gelation” channel of different lengths. Droplet stability was examined as a function of channel length and approximate gelation time. Completion of gelation is illustrated by intact boundaries of micro-beads in collection channel. Scale bar: $60 \mu\text{m}$.

channels. In comparison, when using 2D “T-junctions” (data not shown) or flow-focusing devices, a “jetting” region is often seen at high disperse phase flow rates. This was not observed in experiments using the 3D flow-focusing devices. Within 2D systems, a random breakup of droplets with small satellite droplets due to Rayleigh-Plateau instabilities has been reported.^{22,27} The 3D flow-focusing platform described here utilizes an interfacial tension aided mechanism to produce monodisperse droplets on-demand. The abrupt height difference between the aqueous inlet and the junction of fluidic channels causes local expansion of the aqueous tip at the junction area in all directions, followed by an increase in the radius of curvature between the

FIG. 6. A Poisson distribution fitted to a histogram representing the number beads found with different numbers of cells per bead. The mean is 0.68. About 83% of the beads contain either one or no cell. Scale bar: 15 μm .

alginate tip and the neck which connects to the main stream. Increased velocity and corresponding shear at the junction site may also contribute to droplet pinch-off.²⁹ This is similar to the droplet formation mechanism described by Jung *et al.*³⁶ where the creation of discrete water-in-oil droplets by using a 1 μm tall aqueous channel that intersects with an 18 μm tall organic-phase channel was demonstrated. In that work, droplet formation is solely governed by the instability from self-shearing stress as a result of changes in interfacial tension at the junction of channels with different heights. At the junction, surface tension develops and continues to thin the neck so that a spherically shaped aqueous tip with a minimal energy state is achieved. In the 3D flow focusing geometry, this mechanism contributes to the pinch-off at the junction with additional external shearing caused by the flowing organic phase. This allows more precise control over droplet size, spacing, and frequency. Similar work using expanded channels or “steps” at the junction of organic and aqueous phases to facilitate droplet formation has also been implemented.^{37,38} Here, we highlight the impact of 3D channel architecture on the repeatable and predictable generation of monodisperse alginate hydrogel beads across a broader range of flow conditions than is feasible using purely two-dimensional fluidic architectures.

The phase diagram for the 3D flow-focusing platform shows trends consistent with observations in related systems.^{20,26,39} The region in which droplets are generated steadily widens as the input pressure of the continuous phase increases. This region is confined by linearly increasing organic and aqueous phase backing pressure boundaries. Choi *et al.*²⁰ utilized an angled geometry for flow focusing that combines two aqueous phases prior to pinching off at the cross-flow of an oil channel. In that work, stable droplet formation was obtained for only a limited number of conditions. Fluctuations in plug generation and non-reproducible sizes were observed at low aqueous flow rates (<0.6 $\mu\text{l}/\text{min}$) regardless of oil flow rates. These instabilities were not observed using our 3D flow-focusing platform.

Our experiments demonstrate that sufficient aqueous backing pressure is required to overcome the surface tension from the hydrophobic channel wall and the capillary pressure from the organic phase so that the tip of aqueous phase is able to advance into the main channel. This minimum pressure increases in conjunction with the organic carrier phase backing pressure. Onset

of consistent droplet formation using the 3D system begins at input pressures, $P_{oil} = P_{alg} = 4$ kPa, corresponding to a capillary number of 0.001 and an aqueous flow rate of 10 nl/min. At such low pressures, the spacing and droplet size can be precisely controlled, providing more freedom for the manipulation and interrogation of single droplets. For applications where high frequency generation is desired, the surface tension induced stress is effective in pinching-off droplets at high aqueous flow rates. The 3D platform presented here can be operated at aqueous backing pressures ten times higher than the backing pressure of the organic carrier phase. Effective pinch-off and plug formation are maintained. Consequently, we anticipate our devices to be fully functional at even higher pressures and generation frequencies.

Figure 3 describes the relationship between droplet generation frequency and size under different pressure-controlled flow conditions. Identical experiments were repeated five times with variations in results being attributed to minor device-to-device variation (surface roughness, fabrication artifacts, etc). Ward *et al.*,⁴⁰ using a 2D pressure controlled system, found that frequency of generation and droplet size appear to have a linear dependence on the ratio of aqueous to organic phase pressure (P_w/P_o), only for ratios less than 0.5. Within the 3D droplet generation system described here, linear increases in frequency and droplet size were observed for ratios as high as 2. A linear pattern is observed between alginate pressure and the frequency of generation and droplet size for all ratios of P_w/P_o that were tested. It can also be noted that droplet generation is not hindered at low organic phase backing pressures. In 2D devices, it is common for jetting of the aqueous stream to occur downstream of the junction site, protruding into the main channel. This was not observed in the system described here. Efficient thinning of the alginate neck at the 3D channel junction results in pinch-off precisely at the junction site.

Droplet generation in the 3D microchannel system results from competing processes that include the external shearing from the continuous organic phase flow and surface tension induced stretching that results from the geometric expansion. The surface tension between two immiscible phases is inversely proportional to the radii of curvature of the extruded aqueous tip. The further the tip enters and expands in the main channel, the more easily it breaks up into separate segments. At low organic phase flow rates, resulting from low backing pressures, shear is minimal. Surface tension is the primary factor in droplet formation. If the aqueous/alginate backing pressure is also low, the break up may occur prior to full occlusion of the main channel by the aqueous stream. This results in low frequency generation of droplets. Also, droplet size becomes more dependent on the aqueous backing pressure than the other two cases (Figure 3), suggesting a dominating surface tension induced break-up. Increasing the organic phase backing pressure and flow slightly increases shear force, but also allows aqueous/alginate flow to increase without the risk of streaming. Under such conditions, an increase in the aqueous/alginate flow rate allows complete formation of the aqueous tip, which leads to formation of larger droplets at high frequencies (shown by squares in Figure 3). As the backing pressure of the organic phase is increased further, shear stress increases and droplets are again generated prior to full expansion of alginate tip, causing smaller break-ups at a faster rate (shown by triangles in Figure 3).

Figure 5 indicates that sufficient gelation time is required to create stable microgels.²⁸ The time required for adequate gelation can be modulated via changes in fluid velocity or channel length. Modulation in Ca^{2+} concentration as well as alginate concentration and type might also affect stability. Moreover, variations in droplet size may affect mixing and diffusion of calcium ions into the droplets. A thorough analysis of this parameter space is beyond the scope of this investigation; however, the range of conditions under which this system can be operated suggests that appropriate changes to channel length, and flow rates can be made to optimize the gelation of a variety of hydrogels for different applications.

For devices in which the resistance to flow and compliance are high, relying on volume-displacement to induce flow can result in a significant lag in flow rate as pressure builds within the device. As a result, droplet production can take a significant time to come to equilibrium resulting in reagent streaming, and potential loss of sample or clogging. The system presented here addresses the need for minimizing sample loss and improving device reliability, providing

consistent and instant start-up as well as long-term stability through the use of constant pressure operation. Successful long-term operation of the device was carried out for greater than 10 h with no interruption or failure due to clogging or pressure instabilities, which makes the device applicable for single cell encapsulation. Individual bacterial cells were captured in alginate beads once appropriate cell concentrations were determined. Large volumes of cultured cells or environmental samples can be processed, collected, washed, and cultured on-chip for subsequent testing and analysis. Future work will include the incorporation of on-chip washing and labeling protocols as well as on-chip sorting techniques to eliminate the need for manipulating beads externally.

CONCLUSION

Here, we describe the development of a robust platform for the formation of monodisperse alginate microbeads over a broad range of flow conditions. The 3D flow focusing design, operated under constant pressure control, enables surface tension enhanced self-shearing of droplets, at a precise location, for extended periods. Because of the expanded parameters under which microbeads are formed and the rapid equilibration of the pressure controlled system sample streaming at start-up is minimal. This significantly reduces sample loss, waste of reagents, and risk of device contamination or clogging. Furthermore, flexibility of the operation parameters allows tuning of droplet size and generation frequency while controlling total flow within the system. With the incorporation of additional washing, labeling, and sorting functionality, this platform should provide a robust system for the isolation, culture, and characterization of diverse microbial samples.

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