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Review Article

Droplet-Based Microfluidics: Formation, Detection and Analytical Characterization

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Abstract

Microfluidics has become attractive in the recent decade due to its ability to offer low-cost, robust and miniaturized assays at a small cost as compared to the bulk tests. The credit mainly goes to the advancement in miniaturization technologies allowing cheaper and faster fabrications of microfluidics devices. Continuous microfluidics has been developed for the study of chemical and biochemical reactions inside microchannels, but the Taylor dispersion, sample contamination on the channel walls and less mixing efficiency has offset the benefits of microfluidics. Droplet-based microfluidics, on the other hand, has emerged as a powerful tool to encapsulate chemical and biological samples in discrete droplets and has generated a diverse array of applications including biochemical reactions, chemical synthesis, drug delivery, and point-of-care diagnostics. This review will outline the droplet generation, mixing, merging and detection methods, and characterization of droplet contents.

Keywords: Droplet microfluidics; Miniaturization; Analytical chemistry; Optical detections

Introduction

Microfluidics has been a critical technology for over two decades to study and manipulate fluids in microstructures. It has the potential to provide smart microdevices, which can change how modern biology, chemical synthesis, and point-of-care diagnostics are performed [1]. Microfluidics offers many advantages, including minute quantities of samples and reagents, compact ability, low cost, rapid, high resolution and sensitive analyses. Continuous microfluidics typically involves single-phase flow in microchannels, although traditionally liquids with macromolecules, microparticles or cells are also categorized in continuous microfluidics. Due to the small Reynolds number (0.01-100), Re= ULρ/μ, the flow is laminar in microfluidic devices, where U, L, ρ and μ stand for the velocity of the flow, the diameter of the capillary, density and viscosity of fluid flow respectively [2]. Continuous microfluidics suffers from less efficient and slow mixing in microchannels, molecular contamination of loss on the surface of the channels and Taylor dispersion of molecules alongside the microchannels. The Taylor dispersion leads the parabolic velocity movement of liquid inside microchannels, which involves two velocity regimes, i.e. at the walls and in the middle of the microchannel [2,3].

As an alternative to continuous microfluidics, droplet-based microfluidics encapsulates molecules into separate droplets along the microchannel, and hence, eliminate the Taylor dispersion or contamination of the analytes. Analytes of interest are compartmentalized into droplet plugs, and the carrier phase prevents the sample from contact with the surface wall and hence, eliminates sample loss/cross-contamination between droplets. Therefore, it offers generation and storage of millions of droplets for transportation, incubation and analysis in a single device whereas, continuous microfluidics can only acquire multiple samples analysis either by separating microchannels for each sample or *via* miniaturized valves [4].

Droplet generation

Droplets are generally formed with two different techniques known as T-junction [5] and flow focussing [6]. In the T-junction technique, dispersed phase flow intersects perpendicularly with the carrier phase flow. The tip of the dispersed phase enters the carrier phase as the flow continues and elongates along the channel due to the interfacial tension and shear stress present at the interface. Finally, the interfacial tension increases and breaks the

dispersed phase into a droplet stream. Droplets can be generated with different sizes, frequencies by altering the physical parameters namely flow rates, the ratio of the carrier, and dispersed phases flow rates, the viscosity of aqueous phase [7], microchannel dimensions [8], and interfacial tension [9]. Ismagilov's group [10] is among the first to demonstrate that droplets can also be generated with multiple aqueous phase streams and oil carrier phase to initiate reactions inside droplet plugs. Reactions can also be initiated by injecting gas as a carrier phase. In the latter case, gas bubbles separate aqueous slugs (water in gas plugs are known as slugs) where reaction happens [7].

Another droplet generation technique is flow focusing, in which two opposite carrier phase streams intersect through a narrow opening with the centrally dispersed phase stream. As the dispersed phase passes through the intersection, it elongates due to the increased shear stress and interfacial tension and finally, the dispersed phase breaks into droplets. In this configuration, the shear stress and interfacial tension is symmetrical from both sides of the carrier phase and thus form more stable droplets. Levent et al. [6] fabricated a nozzle like an orifice with extreme shear stress and generated very uniform droplets and controlled the size of the droplets by changing carrier phase flow rates. The droplet size decreased with increase in oil flow rate, and the frequency of droplet generation also increased with an increase in oil flow rate. Note that flow focusing and T-junction technique work on a similar principle utilizing shear stress and interfacial tension. Therefore, the droplet generation is determined/affected by the same set of physical parameters.

Except for the flow rate and ratio mentioned above, surfactants, viscosity of the liquids and hydrophobic and hydrophilic nature of the channel surfaces also affect the droplet generation and their stability in microchannels. The dynamics of droplet formation are determined by capillary number (*Ca*), which is defined as $\eta v/\gamma$, where η is the viscosity of dispersed phase, v is the velocity of the carrier phase and γ is interfacial tension between the carrier and dispersed phases. *Ca* is primarily used to compare the strength of viscous and interfacial tension in a multiphase microfluidic system. During droplet generation, the shear force at the oil-water interface causes the elongation of the droplet, and the interfacial tension causes the contraction of the surface area, with the result that, droplet generation occurs.

Both water-in-oil and oil-in-water droplets can be produced by injecting oil or water as a carrier phase. In any case, the carrier phase should have lower interfacial tension (wet) with channel walls as compared to the dispersed phase. In water-in-oil droplet systems, usually, fluorocarbons such as FC-40 are used as carrier phase fluids. FC-40 is an inert oil because of the extremely stable bond between carbon and fluorine atoms. Moreover, fluorocarbons have weak intermolecular forces, high density, and low surface tension, which make them suitable to be used as a carrier fluid. Besides, surfactants are also added in the carrier phase to adjust surface tension or prevent droplet coalescence after generation. Surfactants are a class of compounds known as amphiphilic molecules [11] consisting of hydrophobic and hydrophilic groups on each end which drive the molecule towards the interface between water and oil layers. In droplet-based microfluidics, surfactants are added in the carrier phase, which lowers the interfacial tension between the carrier phase and continuous phase and helps stabilize the droplet generation. Moreover, surfactant makes a layer on the liquid/liquid interface, which prevents coalescence of the droplets or leakage of molecules from the droplets [12]. Daniel *et al*. [12] synthesized twelve different surfactants and tested the stability of droplets in microfluidic systems. The hydrophobic tails were perfluoropolyether (PTFE) and perfluoroalkyl (PFA) alkyl groups and hydrophilic ends comprised of carbohydrate, crown ether, and hexaethylene. Interfacial tension was measured by pendant drop technique, and it was found that surfactant with hexaethylene hydrophilic groups had the smallest interfacial tension (<5mN/m) and hence, a most satisfactory surfactant for stabilizing droplets. On the molecule, hydrophilic heads have highly electronegative hydroxyl groups which make a strong interaction with an aqueous phase at the interface and decrease the interfacial tension.

Apart from the aforementioned sequential droplet generation with T-junction or flow focusing, there are parallel droplet generation techniques, for example, Slipchip first developed by Ismagilov's group [13]. Slipchip is a simple device consisting of two plates with microwells fabricated on each surface of the plates. The wells can be filled with different reagents, and upon sliding one layer relative to the other, droplets can be generated in parallel or fused to initiate chemical reactions. The Slipchip is ideal for integrating multiple samples handling steps into a single chip enabling critical processes for droplet-based microfluidics, for example, parallel droplet generation, and manipulation, or sample washing steps [14,15]. Microfluidic channels in Slipchip are controlled by positioning the wells and ducts on two separate plates. Hence, these two plates are not bonded to each other, unlike other bonded microchips. For this reason, the liquid can leak out from the wells if the pressure on the two plates is not enough or non-uniform and hence, can contaminate the sample. The platform offers the ability to perform parallel and multiple processes on a single microchip. High-throughput bead-based heterogeneous immunoassay on Slipchip has been carried out for 48 samples in parallel [16]. Ismagilov's group performed many operations using Slipchips in a high throughput format [14-20], and the Slipchip format can be a step forward in parallelization of biochemical assays [21]. Zhao *et al*. [22], Shujun *et al*. [15] and later Hassan et al. [23] demonstrated that this Slipchip format has the potential to be used for separation science, e.g. for segmenting separated samples after isoelectric focusing (IEF) into microdroplets to avoid any sample remixing during post-separation sample collection and to inject multiple sample droplets into separation channels.

Droplet mixing, merging and reactions

Mixing inside microfluidic channels between two co-flowing continuous streams of liquids is very weak and slow because of the dominance of laminar flows in continuous microfluidics [3,24]. The molecules mix *via* diffusion along the flow streams in the

microchannels and require longer times to mix thoroughly even though the microchannel dimensions are small. To be able to study the chemical kinetics and essential biological reactions, fast mixing in microfluidic systems is required. Mixing in droplets is enhanced by circulating flow and chaotic advection. For a droplet plug flowing in a channel, circulation flows are produced by shear for the droplets touching the solid channel walls. In a straight channel, the opposite sides in a droplet remain unmixed [24]. However, this symmetry can easily be broken up and chaotic advection induced by flowing the droplets *via* bends and turns geometries in the microchannels. These geometries introduce stretching and folding of the fluid halves inside the droplets and advance mixing [3]. Serpentine microchannels are designed to bring in chaotic advection and uniform mixing inside the droplets after few turns [25]. Sharp bends can also be used to reorient the droplets and to promote mixing; however, sharp bends increase the chance of further breakup of droplets where the surface of droplet experience the strongest shear force. Mixing in such serpentine channels is very efficient, and the extent of mixing can easily be quantified with the length of the microchannels [26]. It is reported previously in one of the studies [25] that only 0.72ms of time is needed for a uniform mixing in a droplet (to measure the kinetics of Ribonuclease A). This kind of fast passive mixing (without active actuators) in droplets is in strike contrast with the continuous microflows where mixing is relatively slow, making droplets an ideal tool for the study of fast reactions like nanoparticle synthesis. There are other geometries studied to promote mixing in droplet such as coalescence geometry in which droplets are mixed perpendicularly [27], protrusion in the serpentine channels for mixing viscous biological reagents such as Bovine serum albumin (BSA) [28], serpentine geometry to decrease distance between the droplet and channel wall, which increases chaotic advection [29].

Droplet merging or fusion is an important unit of function for the study of chemical/biological reactions, synthesis of nanoparticles/biomolecules and their kinetics in a controlled manner. The compartmentalization of specific reagents allows merging reagents when the reaction conditions are available. For the fusion to start, the droplet interfaces need to come in contact with each other with a thin layer between them. When the pressure increases on the thin film between the droplets, then the imbalance in interfacial tension breaks this thin layer merging two droplets into a single one such as a wide channel geometry allows the drainage of the carrier phase and decreases the droplet flow rate [30,31]. The droplets redirected into the narrow channel where the pressure increases due to an increase in flow rate, and hence, the droplets coalesce. However, this approach is less useful because multiple droplets can merge inside microchannels and requires extremely precise control of flow rates and droplet sizes. Pillar array structures have also been designed to merge droplets in a selective manner by Niu *et al*. [32]. The droplet merging is achieved by injecting the droplet in a pillar-based chamber where it slows down and waits for the other droplet to come into the chamber. The pillars are fabricated at a distance smaller than the droplet size. Therefore, the carrier phase is drained, and the droplet slows down or even be trapped. The second droplet approaches the pillar and merges with the first droplet because of the pressure generated by the flow. The electric field can also be applied to facilitate droplet merging, especially when the surfactant is mixed in the oil to stabilize the droplets, such as a pair of electrodes were built at the bottom of the chamber [33]. The pillars help to drain the oil, slow down the droplet and bring both droplets in contact with each other. Upon application of the electric field *via* built-in electrodes in the chamber, the thin oil film between the droplet breaks and the second droplet merges with the first droplet. This type of geometry can be beneficial for the selective merging of chemical and biological reagents. Droplet fission [34] and droplet sorting [35] are other droplet manipulation techniques, which are beyond the scope of this review.

Reactions in droplets offer robust mixing, shorter distances between molecules for diffusion, fast mass, and heat transfer, reduced hazardous material exposure and requirement of small quantities of precious and expensive reagents [27]. Reactions such as gene expression analysis, drug discovery require high-throughput parallel reactions to be performed at the same time, and droplet-based microfluidics fulfils this requirement by fabricating parallel microchannels with inexpensive fabrication techniques such as soft lithography or plastic micromachining. Multiple reactions can be performed within droplets by changing reaction conditions such as temperature, concentration or catalysts. Diffusion occurs in microchannels before reaction products are detected which can be overcome by segmenting into droplets. Droplets can also be stored for longer times in microchannels without evaporation of the reagents and transported whenever required for detections or further reactions. For the reactions to be performed in microchannels, conditions such as controlled reagent addition, mixing and reaction times, and analysis must be similar to the macroscale reactions. High-throughput reactions also require accurate indexing of the droplets and analysis in a shorter time [36].

Multiple reagent droplets can be stored in a capillary or tubing to serve as a cartridge and used as plug and play devices [36,37]. Song et al. [37] generated sample blood droplets and injected controlled volumes of calcium chloride to study the clotting effect. Droplets were formed in Teflon coated microchannel, and calcium chloride was pumped from the hydrophilic channel. The fluorogenic substrate was used to assess the reaction *via* fluorescence microscopy for thrombin formation, and bright field microscopy was used to analyze fibrin clots inside droplets. In contrast, bromination reaction has also been studied inside droplets to explore the effect of organic phase on swelling of the polymeric devices [38]. A colourless solution of styrene and orange solution of bromine was added to the droplet, and the colour change was observed. The droplet becomes faded as the reaction proceeded towards the formation of a colourless product. Ahmed *et al*. [39] performed a hydrolysis reaction inside droplets and increased the product yield by changing flow rates. Segmented flow at 50 °C under microwave irradiation produced higher amounts of product as compared to the bulk reaction inside the beaker.

Optical detection of droplet contents

Qualitative and quantitative analysis of droplet contents is an essential requirement for the development of droplet-based systems. Physical and biological behaviors of droplets, chemical reactions inside droplets, droplet generation, and manipulation functions are widely studied by Bright-field microscopy [25,31,34-36]. These processes are very fast, and bright field microscope equipped with a high-speed camera is used for studying dynamics inside droplets. Reactions with colour formation are easily detected by microscopes such as the reaction of Fe3+ with SCN- [40]. Droplets of KCN and ferric solution were generated as water-in-oil emulsions and directed towards a trap where the electric field was applied to merge these droplets. The thin film between the droplets took about 100μs to break completely, and the reaction started. Bright-field microscope attached to the high-speed camera was used to study the kinetics of the reaction, which completed in 1166ms. Kim *et al*. [41] generated double emulsions with colour coded droplets and solidified using UV polymerization. These transparent microspheres were coded with single, double and triple colours and were optically identified. This type of coding can be advantageous to perform multiple chemical and biological analyses such as multiplex immunoassays for immobilizing target biomolecules on the surfaces of microspheres. Bright-field microscopy can also be used for heterogeneous reactions, such as screening droplets composed of protein, precipitants, and additives can optimize crystallization in droplets [42,43]. The colour difference of proteins makes it easy to be distinguished from reagents when the protein crystals precipitate out in the droplets. Bright-field microscopy also allows for the study of kinetics for nucleation of protein crystals within droplets [44] and colour detection of changes in BZ reaction in droplets [45]. Absorbance based detections of droplets are also carried out and provide robust and low-cost detections for colourimetric assays such as glucose, lactate and nitrate assay kinetics [46-51].

Fluorescence microscopy is a powerful technique to quantify very low concentrations of biomolecules in droplets, and its sensitivity can be very high with excellent light sources and optics. Fluorescence is based on excitation and emission of light from the molecule [52]. Most of the proteins, nucleic acids, antibodies and other biomolecules require derivatization with fluorescent dyes. Derivatization has been successfully applied to biomolecules using fluorescein isothiocyanate (FITC) [53], naphthalene-2,3-dicarboxyaldehyde (NDA) [54], nano-orange [55] etc. The fluorescence efficiency of derivatization dyes is measured by quantum yield (Φ) which is known as the ratio of a number of photons emitted to the number of photons absorbed. Extinction coefficient (ε) also determines the fluorescent efficiency and measures how strongly the molecule absorbs the photons. For example, FITC, a strong fluorescent molecule has an extinction coefficient of 76,000$M^{-1}cm^{-1}$ and quantum yield of 0.93 [56]. Detectors known as sensors capture the emitted photons from the molecule. The sensors are of three types briefly: 1) a photomultiplier tube (PMT). It works in two stages. Firstly, the electrons are produced when photons absorb on the cathode. Secondly, the dynodes (electron multipliers) present in between the cathode and anode, multiply the electrons exponentially while they reach the anode [57]; 2) avalanche photodiode (APD) that works on the principle of generating electron-hole pairs [58] and 3) charge-coupled device (CCD). CCD is a versatile detection method as it allows the position of the light to be measured as well as intensity. The images can be recorded and analyzed by imaging software [59]. An example of studying single-cell kinetics with a fluorescence microscope and optical trapping and pulsed laser has been demonstrated here [60]. A single cell is trapped by using Nd: YAG2 laser at a wavelength of 1064 nm and moved to the oil-water interface. Once the cells are confined inside droplets, a laser at a wavelength of 355 (Nd: YAG1) is used to lyse the cell with a pulse of 5ns duration. The enzyme activity is immediately measured from the changes of fluorescence intensity inside the droplets with an Ar+ laser at a wavelength of 488nm. At 0s, no fluorescence signal is present because of the intracellular enzyme β-galactosidase was separated by the cell wall from FDG. After laser pulse had induced lysis, enzyme catalyzed the production of fluorescein, which was observed by a high fluorescence signal. Another example shows that surface properties of the droplet can be studied by detecting protein adsorption on the surface. Protein adsorption was controlled with the addition of surfactants miscible in carrier phase, and Alexafluor-fibrinogen compound was detected under the fluorescent microscope [61]. This example shows that the adsorption of protein on channel surfaces can be prevented by encapsulation of droplets, and non-specific protein adsorption can be quantified and controlled *via* fluorescent microscopy. In a study from Ismagilov's group [62], chemical kinetics of ribonuclease A (RNase A) were measured in milliseconds within the droplet-based system. Fluorescent intensity at different points along the channels was measured to assess enzyme activity.

Laser-induced fluorescence (LIF) is the most common technique used for analytical detections and offers the best signal to noise ratio among the other optical detection methods [63,64]. LIF can be employed for high-throughput screening and single-molecule detection in droplets [65]. For example, the droplet-based system has been used previously to detect fluorescence-labelled single DNA molecules by LIF [66]. Droplets were squeezed at the detection window to increase the detection efficiency of the LIF systems. Confocal based LIF detection is the most sensitive technique and provides on-chip detection of the biomolecules with a very low LOD in the range of picomolar concentrations [67,68]. However, due to the complexity and intricate structure of the LIF setups, it is challenging to be miniaturised for portable applications. Light Emitting Diode-Induced fluorescence (LED-IF) is an alternative method to miniaturized detectors for lab-on-a-chip systems. LED-IFs offer reasonable sensitivity and perform much better when combined with optical fibres [69]. LED-IF systems can be very compact and cheap. An array of LEDs can be integrated into multiple sample detections. LEDs are available in single wavelengths, and therefore, the sensitivity can be increased by matching the maximum absorption wavelength with test samples. For instance, Boutonnet et al. [70] used LEDs with matching

maximum absorbance of fluorescent dyes and achieved six times lower LODs as compared to the conventional LIF detections.

Analytical characterization of droplet contents

Apart from imaging techniques, the composition of droplets can also be quantified using analytical techniques such as absorption spectroscopy immunoassays and Mass Spectrometry (MS). Immunoassay is a widely used technique to detect and quantify proteins based on the reactivity of antibody with specific proteins. Immunoassays in droplets have been performed to quantify the particular biomarkers such as Guo et al. [71] showed the generation of droplet libraries for high-throughput immunoassay in droplets. Droplets are generated in parallel from individual vials and are labelled with different colours to separate them from the others. This method of droplet injection allows the high-throughput screening of droplets for suitable antimicrobial drugs. Magnetic bead-based immunoassays using droplets have also been developed, and a typical droplet-based immunoassay on a digital platform works in multiple steps [72]. Firstly, droplets of sample and reagents (containing capture antibodies with magnetic beads and secondary antibodies) are dispensed on the platform. The droplets are then merged, mixed and incubated for a specific time. Those droplets containing antibody-antigen complex are immobilized by a magnet, washed and split to remove unbound species. Finally, the detection is achieved by the addition of reagents.

Mass Spectrometry is another powerful technique to identify molecules based on their mass-to-charge ratios. Droplet-based chips have been integrated with electrospray ionization-mass spectrometry (ESI-MS) detection. Zhu et al. [73] integrated droplet generation, droplet extraction and ESI emission on a single chip and used the hydrophilic tongue to extract the sample droplet while the oil flowed to the waste reservoir. In another study done by Kennedy's group [74], a simple ESI-MS method was developed by directly injecting droplets into the commercially available ESI emitter and eliminated the need for extraction. The droplets were loaded in a cartridge segmented with oil and gas and were analyzed without dilution of the sample. This method of droplet injection can detect the complex mixtures of reagents, and also be combined with other analytical techniques such as electrophoresis or high-performance liquid chromatography (HPLC) to enhance the capability of droplet-based systems. Research efforts have also been devoted to study the contents of droplets by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS). For example, Fiona et al. [75] developed a method to generate droplets containing separated biomolecules from the nano-HPLC column. The droplets were deposited onto the MALDI-MS plate for further MS analysis. The authors used an oleophilic film to remove the oil before the deposition. This method achieved 50% enhancement of the performance when compared with conventional nano-LC MALDI-MS using the digests of BSA and Cytochrome C, due to the in-situ encapsulation of the separated eluent from nano-LC column. The platform has the potential to be interfaced with other analytical techniques such as MCE or solid-phase extraction.

Conclusion

This paper reviewed the droplet generation and merging techniques along with reactions in droplets, and droplet content detection techniques. In summary, droplet-based microfluidics offers robust mixing inside droplets reducing the reaction time, eliminates Taylor dispersion, band broadening and contamination usually encountered in continuous microfluidics. This review mainly discussed bench top techniques and paves the way for the development of droplet-based portable devices benefitting from the high-throughput nature of the droplet-based microfluidics.

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