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Micro segmented flow-functional elements and biotechnical applications

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1. ABSTRACT

Microdroplets are widely used for industrial applications such as food, drug, biotechnology and new materials. This review will summarise the key development in microdrop technologies, especially double-emulsion droplet technologies, with a focus on microchip-based techniques. For completeness, the key topics in single emulsion droplets such as generation, control and application will be briefly presented first. Then the current microfluidic techniques for double emulsion generation will be reviewed. Several techniques for increasing the instability of double emulsions are discussed, followed by methods for measuring double emulsion properties such as stability, size and mass transfer between phases. Double emulsions have found use in many biomolecular applications that include drug delivery and protein engineering. A range of methods, e.g. liposomes, polymerosomes, polymer beads and colloidosomes-based emulsions, have been developed for drug delivery applications. The future work in the area would be the development of novel partition system that encloses the chemicals and biologicals effectively and novel control mechanism for advanced sorting and selection of droplets.

2. INTRODUCTION

Micro segmented fluids refer to multiple fluid compartments of micron scale that are divided by a second fluid phase. The fluid compartments are separated from each other due to the partition capability of the second phase so that each compartment can be used for independent chemical and biological applications. These segments could be in the form of long fluid slugs with a length scale several times of the width or microdroplets/microbubbles with a length scale comparable to the width. The focus of this chapter is mainly on the droplets and its application for biomolecular applications.

Droplets are liquid based dispersions of two immiscible fluids such as oil and water. These droplets have been used extensively in many industrial applications such as food, cosmetics, coating, pharmaceuticals, chemicals and biotechnology. The single emulsion droplet is one phase dispersed into another such as water in oil droplets (Figure 1a). Double emulsions, on the other hand, are highly structured dispersions consisting of emulsion droplets that enclose finer droplets inside (Figure 1b). There are two major types of double emulsions, i.e. water-

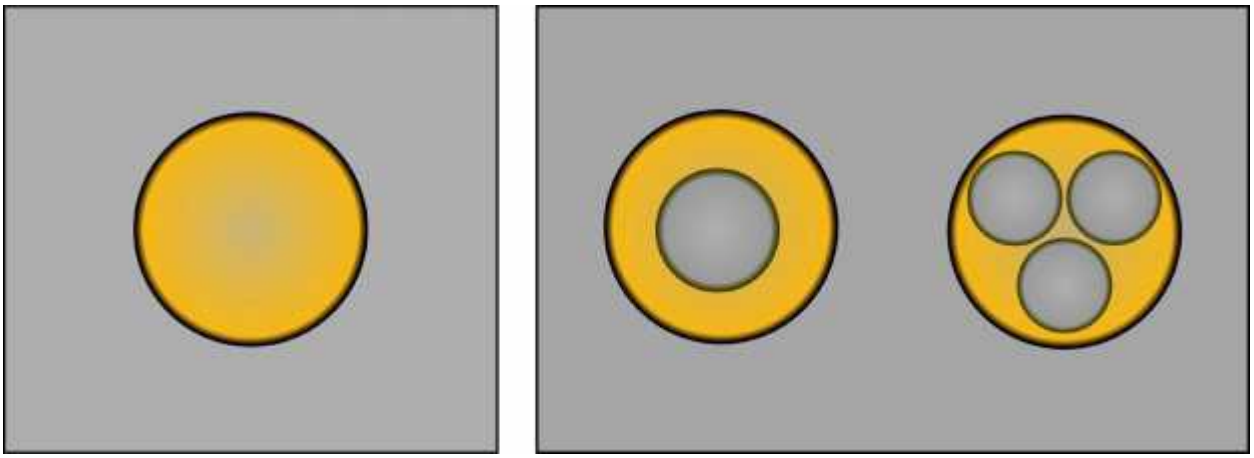

Figure 1. Emulsion droplet type. (a) single emulsion (b) double emulsion with one and three inner droplets.

oil-water (W/O/W) and oil-water-oil (O/W/O) double emulsions. Double emulsions were first described by Seifriz in 1925 (1). Since then, there has been considerable attention in the research and development due to their wide range of applications. For example, double emulsions can form a vesicular structure with the innermost compartments separated from the outer phase by a layer of immiscible intermediate phase. Such emulsions are advantageous over single emulsions for encapsulation, such as the ability to carry both polar and non-polar cargos (2), and protection of active and sensitive components from the attack of the environment. This structure can also make double emulsions highly desirable for applications in controlled release of substances (3) such as drug molecules (4-6) and the aroma and flavor for food additives (7-9), separation techniques (10-12); preparations of various personal care products (13, 14) and biodegradable microcapsules loaded with bioactive polymers (15).

The recent advance in microfluidic technologies offers new opportunities for droplet-based system development. Microfluidics was originated from integrated circuit (IC) industry in the early 1990s by Manz *et al.* (16). It involves the transport, manipulation and analysis of fluids at small scales with a channel size of a few to a few hundred micrometers. The reduced dimensions provide a much larger surface-to-volume ratio, smaller volume and better control of hydrodynamics in comparison with conventional fluidics technologies. Such changes could significantly enhance heat and mass transfer efficiencies and intensifies reactions, thus offering unprecedented opportunities for chemistry and biology applications. The field has undergone a very rapid development in many areas such as single molecule analysis (17), single cell processing (18), chemical biology (19), point of care testing (20, 21), drug discovery (22) and so on.

As one subcategory of microfluidics, droplet-based microfluidics focuses on creating discrete volumes with the use of immiscible phases, such as water-oil or air-water systems. These systems can produce highly monodispersed droplets in the range of a few to a few hundred of micrometers. These are much more superior to the conventional systems in which emulsion droplets were prepared by techniques such as homogenizers, stirrers or extruding devices in which only polydispersed droplets can be produced. In microchip-based droplets, each generated droplet is isolated from the surrounding immiscible fluid so that parameters of the reaction such as reagent contents and concentration, mixing, heat and mass transfer can be precisely controlled (23-27). Further, the droplets can be manipulated individually to achieve fusion (28-30), splitting (31-33), sorting and probing (34-38). Such droplet-based reactors have been used to form nanoparticles (39-41), polymer particles (42, 43) and double emulsions (44, 45) for applications ranging from drug delivery to chemical synthesis.

Since the last ten years, the development of microfluidic droplet technology has attracted a great deal of attention. A number of review articles have been published in droplet-based microfluidic devices (24-27, 46-49). This chapter will address specifically microfluidic double emulsions and the applications for biomolecular applications. The current understanding and progress in single emulsion will be introduced first, which include formation, control and application. Double emulsion droplets will be then be discussed in terms of formation techniques and stability. The recent analytical techniques for double emulsions are also outlined. Finally, the application of double emulsion for biomolecular applications will be reviewed. The main focus will be on drug delivery, directed evolution and protein expression. The chapter is concluded with brief summary and discussion on future prospects.

3. SINGLE EMULSION DROPLET

3.1. Droplet formation

There are two most commonly used networks of microchannels to achieve droplet formation, i.e., T-junctions (50, 51) (Figure 2a) and flow-focusing geometries (52-55) (Figure 2b). In the simplest form of a T-junction microfluidic system the dispersed phase is injected perpendicularly into a continuous phase. The shear force

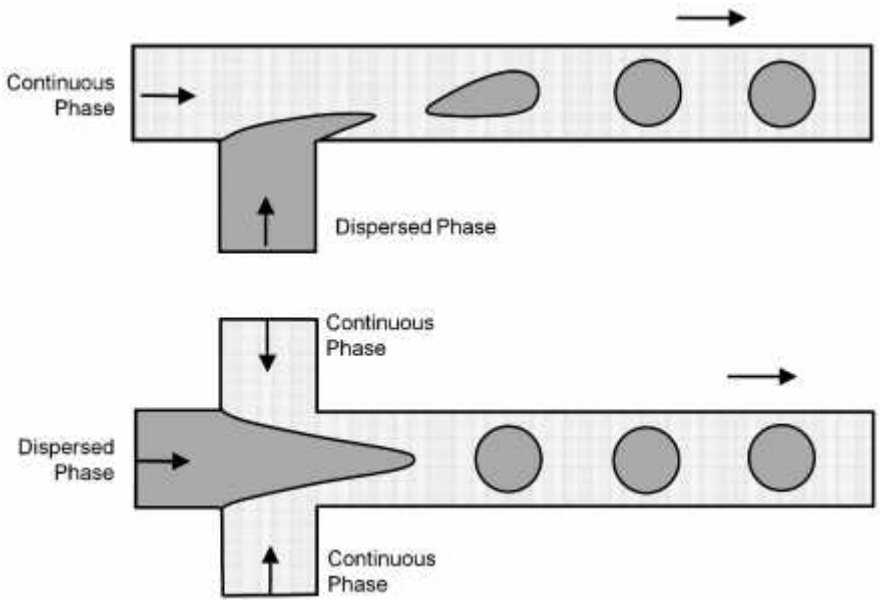

Figure 2. Geometries of microchannel networks for droplet formation. (a) Cross-channel geometry, (b) T-junction geometry.

generated by the continuous phase and the subsequent pressure gradient cause the aqueous stream to elongate in the continuous stream until it breaks into droplets. A double T-junction microchannel has been used to generate alternating droplets (56). Such systems have been used for a wide range of applications, such as chemical reactions (57), protein crystallisation analysis (58), blood typing (59) and so on. In the flow-focusing geometry, the dispersed phase is forced through an inlet channel while the continuous phase is added through two symmetric side channels perpendicular to the inlet channel. The pressure and viscous stresses exerted on the disperse phase cause it to thin to a narrow thread and then break into droplets inside the orifice. By varying the flow rates of the two phases, droplets of a diameter smaller than the orifice width can be produced. The third format of droplet formation is the microchannel plate which used a microfabricated channel array (60). The device essentially arranged the single nozzle focusing set-up in parallel in large numbers with common oil and water phase inlet. For example, 4x400 flow focusing channels were used with a single inlet channel for high throughput production of oil-on-water droplets (61-63).

The most widely used microchip material reported in public domain is polydimethylsiloxane (PDMS) since it is easy to pattern, optically transparent, flexible and biocompatible (64). However, due to the drawbacks such as absorption and inability for mass production (65), many alternative polymeric materials are used for fabrication of microchips, such as polymethylmethacrylate (PMMA) (65-68), polyester (69) and cyclic-olefin-copolymer (COC) (70). Besides polymeric materials, glass (44) and silicon (71) have also been widely used as microchip material for biological studies. These latter materials should provide better mechanical properties. However, unlike IC chips, microfluidic microchips using these materials are very expensive. Increasing effort is being devoted to developing polymeric microchip which can be suitable for mass production using techniques like injection moulding.

3.2. Droplet control

Droplet control techniques are required to manipulate droplets to perform certain tasks in the microchip. These may include fusing the droplets with different reagents, mixing the reagents to improve reaction efficiency, probing the content of droplets for analysis, and sorting the droplets for desired properties. The control techniques can be achieved either passively or actively. Passive controls are usually achieved through altering the geometries of the microchannels (72, 73) or surface wetting properties (50, 74, 75). For example, two adjacent droplets could come into contact for fusion by speed tuning with use of different widths of microchannels (57) (Figure 3a). To improve the mixing within droplets, channel geometries can be altered to create chaotic advection by the use of winding channels (Figure 3b) (76). Active controls utilise local forces to direct droplet movement and allow each droplet to be individually manipulated in desired ways. Many techniques including electrowetting (71, 77-82), dielectrophoresis (DEP) (83, 84), electrostatic forces (85), pneumatic pressure (86), and thermocapillary actuation (87) have been demonstrated to enable active controls of droplets inside microchips. For example, Figure 3c presents droplet fusion by use of electrocoalescence (88). One disadvantage of these latter techniques is the possibility of contaminating reaction droplets with the products of electrochemical reactions on the electrode surfaces. This can be minimised by introducing a dielectric layer between the fluids and the electrodes (83, 89, 90). It has been reported that microelectrodes placed underneath a PDMS channel produced dielectrophoresis (DEP) forces of more than 10 nN on a water drop in inert oil, resulting in sorting rates greater than 1600 droplets per second (Figure 3d)

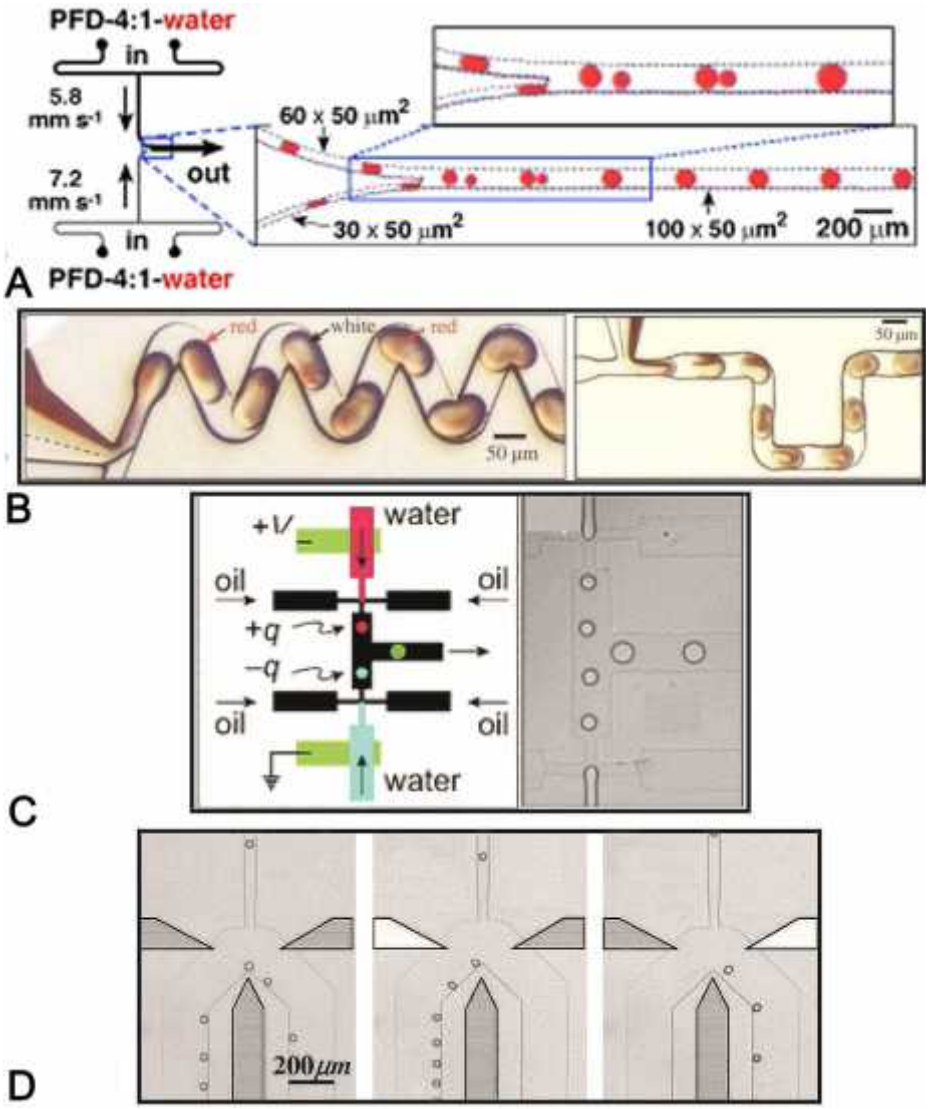

Figure 3. Active and passive droplet control techniques. (a) A passive droplet fusion technique. The fusion is achieved by flowing two streams of droplets and merging in a main microchannel (From ref. (58), reproduced by permission of John Wiley & Sons Australia, copyright 2003); (b) A passive technique for mixing inside droplets. A winding channel was designed for induce chaotic motion inside droplet to achieve mixing (From ref. (77), reproduced by permission of the Royal Society, copyright 2004). (c) An active control of droplets to achieve fusion by electrocoalescence technique. A schematic drawing of the set-up is shown in the left picture. Then right photograph shows the demonstration of droplet fusion. (From ref. (89), reproduced by permission of John Wiley & Sons Australia, copyright 2006); (d) An active control for droplet sorting. The three pictures show the dielectrophoretic bidirectional manipulation of droplets. The flow direction of the droplets is controlled by alternately energising the two symmetric electrodes (From ref. (38), reproduced by permission of the American Institute of Physics, copyright 2006).

(37). More complicated controls (91, 92) incorporating washing steps, incubation, delay-lines, or presentation to analytical instrument into droplet microfluidics have been developed for complex systems.

3.3. Application of single emulsion droplets

Due to the advantages of high production rate, monodispersity and controllability, microfluidic droplets have been used as bioreactors for many biotechnology applications (65, 93-99). For example, the introduction of *Danio rerio* eggs for embryo screening and development in microfluid segments has been reported for toxicological and drug screening studies (100-102). Fluorescence resonance energy transfer (FRET) technique combined with microfluidic droplet has been explored (103) for the detection of DNA molecules. In another example for cell biotechnology, mammalian cells and multi-cellular microorganisms were encapsulated in droplets using biocompatible surfactants and gas-permeable storage system and their viability was maintained within droplets during a range of analyses and manipulations (104-106). A highly parallelized cultivation of monoclonal cell

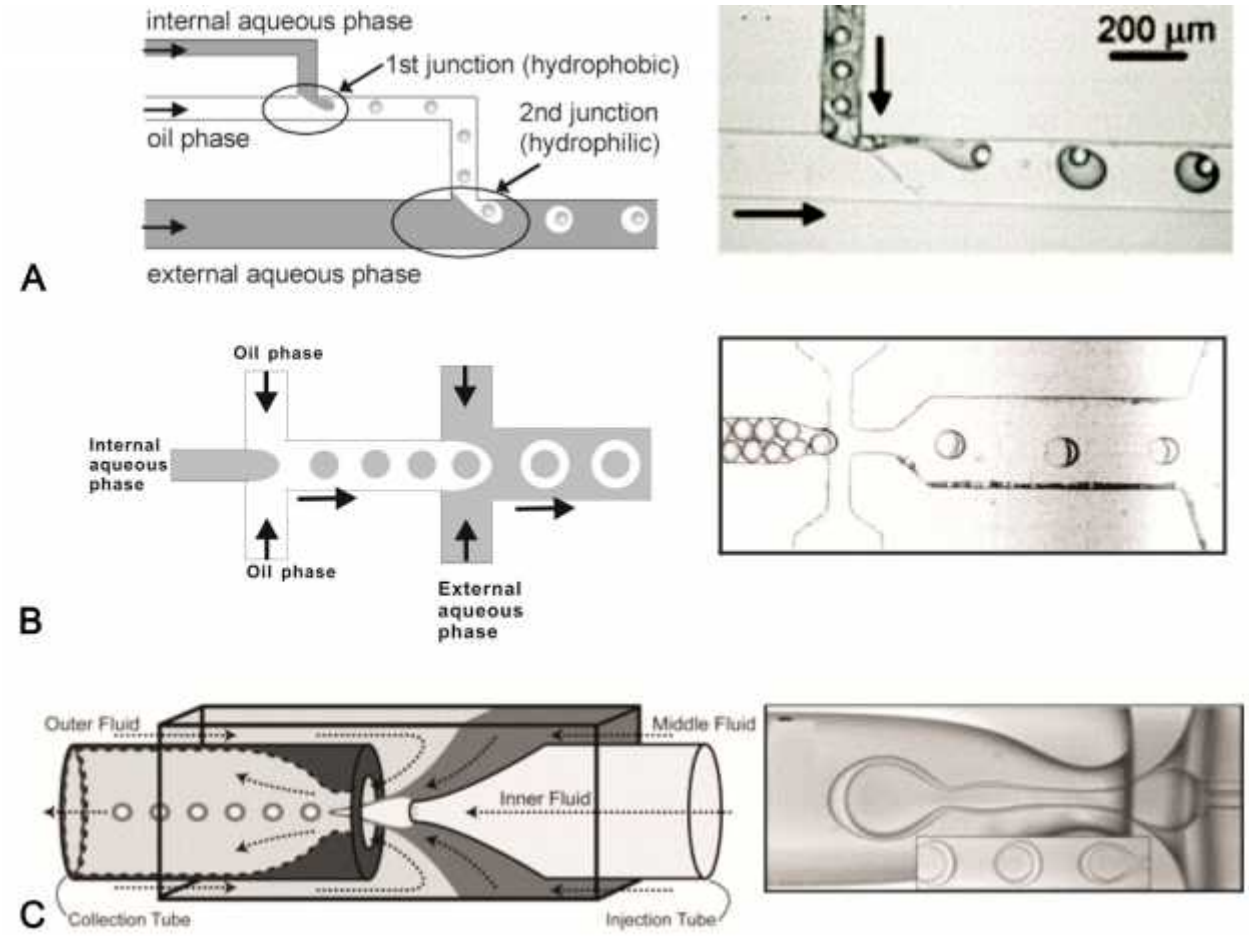

Figure 4. Three fluidics techniques for double emulsion generation. (a) A T-junction microchannel device: a schematic drawing of the microchip (left) and a photograph of generated double emulsion droplets (right) (From ref. (118), reproduced by permission of American Chemical Society, copyright 2004) (b) A flow-focusing microchannel device: a schematic drawing of the microchip (left) and a photograph of generated double emulsion droplets (right). Two steps were applied to the system including production of single emulsion droplets on a PMMA microchip and the subsequent generation of double emulsion droplets on a PDMS microchip with the nozzle width and depth of 70 µm, respectively. The flow rates of the aqueous phases for single and double emulsion droplets were 24 µl/h and 250 µl/h, respectively. The oil flow rate was 50 µl/h. The experiments were carried out in the Microfluids Lab at CSIRO Division of Materials Science and Engineering, Highett, VIC 3190, Australia. (c) A microcapillary device for generating double emulsions from coaxial jets. (From ref. (44), reproduced by permission of the American Association for the Advancement of Science, copyright 2005).

populations by use of microchip segmentor modules has been developed basing on determination of the green fluorescence of constitutively expressed GFPuv in the cells (107). For DNA amplification, a thermocycler made of silicon and glass chips has been developed for miniaturized rapid PCR by use of flow-through two phases system, in which system the amplifications of various DNA templates of different sources and properties were achieved in less than half an hour (108). A novel microfluidic segmented flow system for reverse transcription-polymerase chain reaction (RT-PCR) has been demonstrated by the amplification of cell-isolated, HPV 16 target ontogeny expressing RNA (109). Transcripts of measles and human papilloma virus (HPV) by reverse transcription (RT) and amplification of cDNA have been successfully complemented and detected in droplet based microfluidic system which holds a great promise for portable diagnostic biomedical applications (110).

Single emulsion droplets have also been used for directed evolution of protein molecules. Heterologous expression of large libraries of genetic variants of particular proteins in systems allows for rapid screening and detection of variants with improved ability to carry out a desired reaction (111). The use of cell-free droplet-based microfluidics offers the prospect of increasing the scale of the libraries and hence the power to find better variants by many orders of magnitude (112). Cell-free transcription and translation also allows the use of modified translation systems that can incorporate non-natural amino acids into the protein, which also has the potential to greatly expand the possible utilities of the variant proteins (113).

4 DOUBLE EMULSION DROPLET

4.1. Preparation of double emulsions

The double emulsions are normally produced by conventional emulsification devices like stirring apparatus, rotor-stator system, homogenizers and membrane emulsifiers (114, 115) and the recently commercial available microtechnology-based emulsifiers. These techniques produce poly-dispersed emulsions. To achieve monodispersed double emulsion, microfluidics-based techniques are typically used, as in the case for single emulsion. The generation of double emulsion droplets is fundamentally the same as that for single emulsion droplets, i.e., either by the cross-channel or T-junctions geometry, as shown in Figure 2 (27, 65, 98). A slight variation of the T-junctions geometry is the Y-junction geometry (116). However, the generation needs to be repeated with phase inverted to produce double emulsions. Figure 4 shows a few examples of double emulsion droplets generation in microfluidic chips using the cross-channel and T-junction geometries. In the T-junction technique, to generate water-in-oil-in water droplets, the water in oil (W/O) single emulsion droplets in disperse phase is directed towards a second T-junction placed downstream to form monodisperse organic drops containing aqueous droplets within an external aqueous phase (117, 118). More elaborate T-junction systems have enabled separate addition of different reagents (57) and generate alternating droplets with different compositions (56, 117). By integrating moving-wall structures beside the secondary T-junctions within the microchip, up to ten internal droplets of W/O/W emulsions has been achieved by the active control (118). The similar approach has also been applied to fine-tune the internal/external droplet size of double emulsion droplets using compressed air to control the moving-wall structures (119).

In the cross-channel geometry, the dispersed liquid droplets are created by flow-focusing method, where the disperse fluid is squeezed by the immiscible continuous phase from two symmetric perpendicular channels (Figure 4b) and forced through a small orifice to break into droplets. The similar process is repeated as a second step to achieve double emulsion droplets when the single emulsion fluid is used as disperse phase. (55, 65) The picture on the right-hand size of Figure 4(b) shows an example of double emulsion generation at the second stage using a microfluidic microchip. The droplet size was about 70 μm. Microcapillaries have also been used as 3d flow focusing device for double emulsion generation. (44, 120)

In addition to the repeated steps of droplet generation for producing double emulsion droplets, the main difference of double emulsion generation to single emulsion droplets is the requirement of surface property of microchannels. The surface property needs to be altered for the second step due to the reversion of the fluid phase. The nature and stability of emulsion droplets are significantly affected by the wetting properties of microchannel (54, 121, 122). Seo *et al* (123) have investigated the effect of surface energy of microchannels on microfluidic emulsification and generated different types of double emulsions with precisely controlled size and morphology by using two consecutive flow-focusing devices. Depending on the wetting properties of microchannels, stable O/W/O (124), W/O/W (125) and O/O/W (45) droplets have been generated. Generally, it is difficult to achieve a long-lasting stable surface after modification of microchannels either by exposure to oxygen plasma or dynamic coatings. A method has been described to pattern and modify the hydrophobic/hydrophilic surface of PDMS using plasma polymerization. The channel surface property in the microchip could be stable for seven days and thus enable the preparation of double emulsion droplets (126).

Since most of double emulsifications on the planar microchips are performed in a two-step process and the channel surface is required to be modified, Utada *et al* has developed a different strategy to fabricate double emulsions in a single step and without modification using a capillary device (44). In the device illustrated in Figure 4c, two cylindrical glass capillary tubes were coaxially nested within an outer square glass tube. All the fluids were pumped into the system simultaneously at controlled flow rates. The droplet size could be quantitatively predicted and tuned by altering the flow rates. Chang *et al.* used a two-step co-axial microcapillary device to produce double emulsions for synthesis of polymer shell particles (127). A similar device was also developed for microcapsule fabrication (128). A planar microfluidic flow-focusing device with three-dimensional orifices was developed (129). Three immiscible fluids can be focused through the coaxial orifices, producing monodisperse double emulsion droplets with a coefficient of variation of less than 4.1%. The method may overcome problems of alignment, assembly and size of orifice based on capillary tubes.

In the process of microfluidic emulsification, the thickness of the intermediate layer or shell thickness can be varied by changing the flow rates of the external phase or designing a novel structure of microchannel to remove the intermediate phase without the requirement of local surface modification (130). Off-centering of the internal droplets with respect to the intermediate layer is controlled by varying the shell phase viscosity (131). Regardless of the device format being, either chip-based (132, 133) or capillary-based (43, 134), such a bottom-up method of emulsion preparation has brought the success in the work at the “single droplet level” rather than a large population of droplets. With the possible parallelization of large numbers of devices (135), production of double emulsion could be intensified for industrial use in the near future.

4.2. Stability of double emulsions

The stability of dispersions typically describes the extent to which small emulsions making up the dispersion maintain their uniform distribution over time (136). The development of a stable double-emulsion system is essential for successful commercial use (137). Therefore, it is important to understand the conditions of the emulsion stability. There are several factors to affect the stability of double emulsions which include composition of the three phases, process of fabrication and mechanism of release of

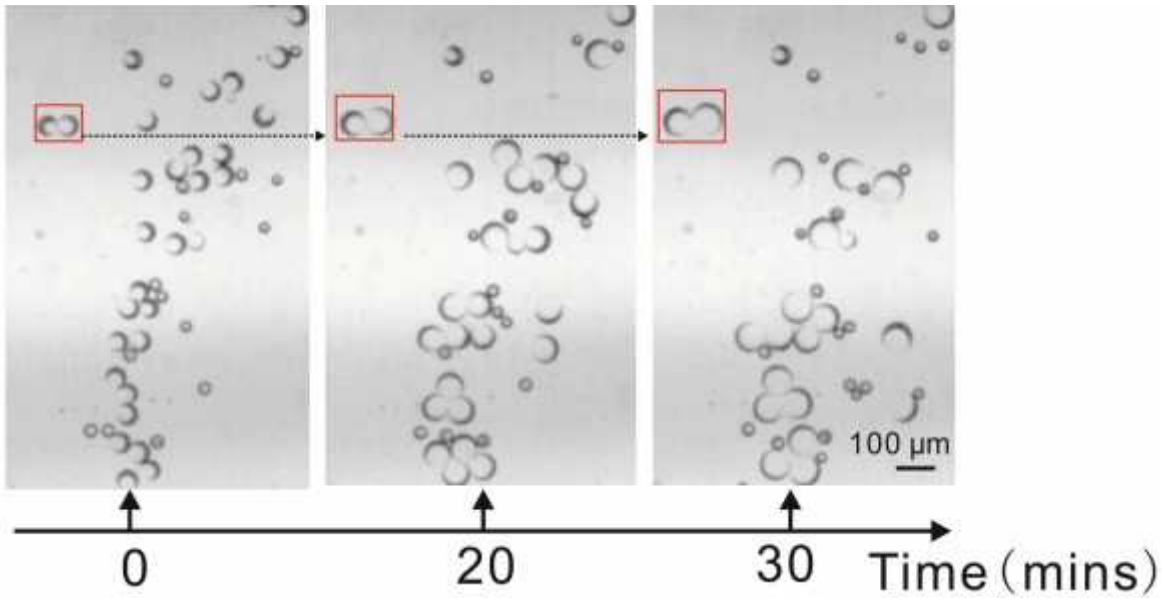

Figure 5. Storage of double emulsion droplets with time in the chamber of the PDMS microchip. The internal droplets of these double emulsions contained the *in vitro* transcription and translation reagents for OpdA expression. The experiments were carried out in the Microfluids Lab at CSIRO Division of Materials Science and Engineering, Highett, VIC 3190, Australia. A microscope (NIKON Eclipse TE2000-U) was used for imaging the droplets.

materials from inner droplets (138). For oil-water system, the instability usually occurs due to the coalescence of the oil phase, the coalescence of inner aqueous droplets or the osmotic balance between the inner and the outer aqueous phases in W/O/W emulsion system (114, 139-141). Driven by the osmotic gradient, water and water-soluble substances can permeate through the oil phase via reverse micellar transport or diffusion across the thin lamellae of surfactant formed. The differences in the osmotic pressure can be regulated by addition of the salt with the right concentrations or other ingredients in the internal aqueous phase (142). The permeation is also affected by the thickness of the oil film (143).

The mechanism of mass transport in and out of the droplets observed in single emulsions also applies to double emulsion system. Figure 5 shows an example of experimental observation in which a series of photographs of double emulsions stored over time inside the reservoir of a microchip were taken. The concentration gradient exerted by the whole set of various molecules (e.g., electrolytes, water-soluble materials) between the internal aqueous droplet and the external aqueous phase resulted in a difference of the osmotic pressure which subsequently drove the transport of water molecules from outer phase into the inner aqueous droplets (144). The substances encapsulated in the internal aqueous droplets were the machinery for the expression of OpdA enzyme. The fast ripening of the double emulsion within half an hour demonstrated the instability of the emulsion system.

To increase the emulsion stability, emulsifiers can be used (145). In the majority of the reported studies, double emulsions are prepared using two kinds of emulsifiers, a hydrophobic emulsifier used to stabilize the interface of the W/O internal emulsion and a hydrophilic emulsifier for the external interface of the O/W emulsions. Ionic and non-ionic low molecular weight surfactants have been traditionally used for stabilization of multiple emulsions. For example, the surfactants in oil phase can form reverse micelle vesicles which can solubilize water molecules and carry them through the oil layer. It has been demonstrated that with the increase of oil soluble surfactant concentration, the permeation coefficient of water molecule increases whereas with the increased volume of internal droplets, the permeation coefficient of water molecule decreases significantly (146). In addition to the traditional monomeric surfactants, natural macromolecules or synthetic amphiphilic polymers such as proteins (147, 148), hydrocolloids, polymerizable surfactants and copolymers (149) have been used as emulsifiers to form polymeric thick films in order to improve the stability. For example, bovine serum albumin (BSA), a polymeric emulsifier (biopolymer amphiphile), can be adsorbed onto the O/W interface (150). Furthermore, hybrids (144), complexes (151), or adducts (152) between the amphiphiles and co-emulsifiers as co-solvents have been demonstrated to improve the emulsion stability distinguishably and decrease the release rate from inner aqueous phase. In addition to emulsifiers, several other approaches have been demonstrated to regulate the stability which includes reducing the droplet size, increasing the viscosity of the inner water phase or the intermediate oil phase (3), the use of colloidal solid particles to form a rigid film at the interface (153, 154) and so on.

In many of the double emulsion generation systems, either microchip-based or microcapillary-based (98, 127, 131), the internal number of droplets encapsulated in double emulsions can be precisely controlled. Therefore,

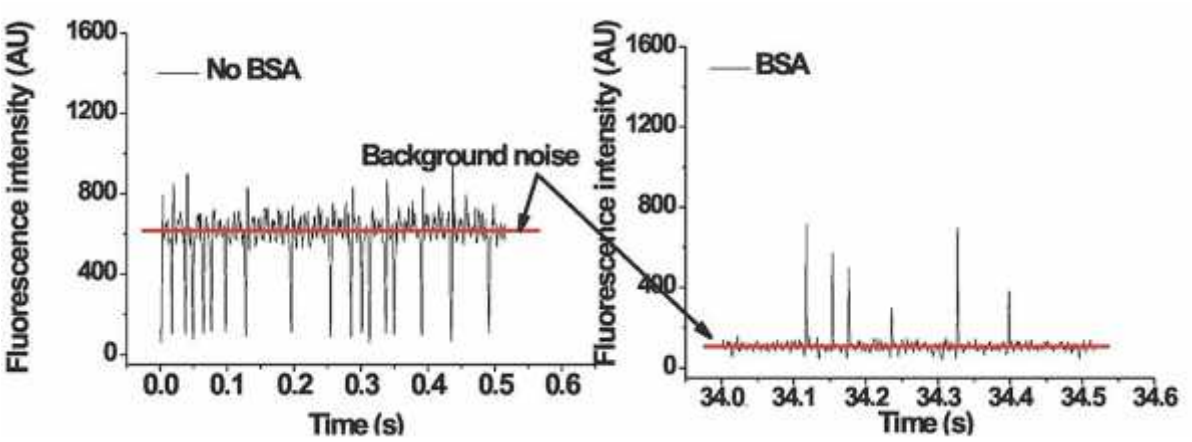

Figure 6. Detection of 4-MU inside the internal aqueous droplets of double emulsions by laser induced fluorescence. The droplets were prepared with 2.5% (w/v) (right) and without BSA (left) in the continuous aqueous phase. The concentration of 4-MU in aqueous droplets was 200 µM. The 4-MU solution was pumped to the microchip by a syringe pump at a flow rate of 70 µL/h while the oil flow rate was 20 µL/h. The external aqueous phase was pumped at a flow rate of 300 µL/h. (From ref. (156), reproduced by permission of John Wiley & Sons GmbH, copyright 2010).

the coalescence derived from the inner droplets can be circumvented by encapsulation of only one individual droplet inside double emulsion droplets. For example, Wu *et al* investigated the diffusion of 4-methylumbelliferone (4-MU) from the internal aqueous phase of W/O/W emulsions in microfluidics and discussed the effect of bovine serum albumin (BSA) on the diffusion of 4-MU. A proof-of-concept of the reaction of the enzyme OpdA with the substrate coumaphos in W/O/W droplets was demonstrated allowing the coumaphos to diffuse from outer aqueous phase to inner aqueous droplets across the oil phase (155).

4.3. Measurements of double emulsions

For monitoring the stability and release properties of double emulsions, several modern techniques have been developed to determine when the inner droplets tend to rupture or to assess the dynamic behavior of multiple emulsions, e.g. freeze-etching (156) and rheology measurements (157, 158). The emulsion stability and size changes can be observed by capillary microscopic observations (138). This method uses microcapillaries to trap certain sized double emulsion droplets and the microcapillaries are usually attached to a video camera and image analysis system. The droplets within the microcapillaries can be examined under light microscope. Such a system can be used to investigate the stability parameters such as the nature of the surfactant, pH and salinity (159). Furthermore, phase contrast microscope is also commonly used to observe the microstructure of double emulsions. The diffusion behavior of different components of the emulsions can be determined by a pulsed-field-gradient NMR technique (160). This technique can discriminate the water within the W/O/W emulsion droplets from water in the continuous phase which allows for the individual study of the two different classes of water.

In addition to the above-mentioned tools, microtechnology has enabled the highly precise control on individual double emulsion droplet and thus the direct visualization of individual double emulsion droplets for their entire life span. Moreover, combined with laser induced fluorescence, entrapped active matter of double emulsion droplets in microfluidics can be quantitatively determined so as to manage the diffusion of active matter (155). For example, Figure 6 shows the detection of 4-MU inside the internal aqueous droplets of double emulsion with and without BSA in the continuous aqueous phase by laser induced fluorescence. Each spike corresponds to an individual double emulsion droplet. The reduction of the diffusion of 4-MU out of emulsions as a result of the addition of BSA has been clearly demonstrated.

Molecules transported from the inner phase to the outer phase and vice verse can be quantified using fluorescent probes (3). Several approaches have been reported to evaluate the release of fluorescent probes, such as liquid scintillation (161), luminescence spectroscopy and HPLC (162). Fluorescence-activated cell sorting (FACS) technique can also be used to determine the yield of entrapment and release of the solute simply and fast (163). This technique is based on flow cytometry and typically uses fluorescent probes that bind specifically to cells associated with various phenotypic, genotypic and other biochemical and molecular characteristics suspended in a fluid stream. The fluorescence of cells can be measured when they flow and pass through a sensing spot. Similar to cells, the fluorescent marker was added to the internal aqueous droplet of W/O/W emulsions and its fluorescence and size of each droplet can thus be measured. FACS has been widely used for directed evolution in format of double emulsions, which will be discussed in the following section.

5. DOUBLE EMULSIONS FOR BIOMOLECULAR APPLICATION

5.1. Double emulsions in drug delivery

Drug, as one format of therapeutic agents, has been widely applied to treat different kinds of diseases through ingestion or injection. Successful medical treatment depends not only on the pharmacokinetic/pharmacodynamic activity of the drug, but also on its bioavailability at the site of action in the human system (164). Reducing local side-effects, controlled release, site-specific delivery and targeting have been the focus of drug development (165, 166). Double emulsion droplets have been suggested for drug encapsulation and controlled release (167). These double emulsion droplets can be used to contain various drugs, such as vaccines, vitamins, enzymes and hormones (168, 169). The effectiveness of double emulsions has already been proven in both topical and oral administration (4, 170). In this section, several microfluidic double emulsions will be discussed as carriers for drugs.

5.1.1. Liposomes based carriers

Liposomes are phospholipid bilayer membranes which surround aqueous compartments. The partitioning capacity of the bilayer allows for encapsulation of hydrophilic solutes (e.g., drugs, enzymes) since these chemicals cannot readily pass through the hydrophobic lipids. Hydrophobic chemicals can be dissolved into the membrane. In this way, liposome can carry both hydrophobic molecules and hydrophilic molecules.

Liposome can be formed by several techniques such as hydration (171), electroformation (171), sonication (172), extrusion (173) and so on. These conventional methods typically lead to the formation of liposomes that are non-uniform in both size and shape and unsatisfactory encapsulation efficiency (174, 175). To overcome these shortcomings, microfluidics can be used to generate double emulsions as templates to from liposomes (176-180). For example, Pautot *et al.* (179) demonstrated simple water-in-oil emulsions could be used to improve the quality of the liposomes. Shum *et al.* further improved the system by using controlled double emulsions. Monodispered liposomes with high encapsulation efficiency could be produced using a glass microcapillary device (181). A pulsed jet in a microfluidic device was used to break lipid membranes into liposomes (182).

5.1.2. Polymerosomes based carriers

Polymerosomes are polymer vesicles generated from amphiphilic diblock copolymers by self-assemblies (183). Similar to liposomes, polymerosomes also consist of compartments which can be used for encapsulating drugs. However, the polymer membrane is advantageous to the phospholipid bilayer since it can offer enhanced mechanical and structural stability. Polymerosomes are typically formed using the same techniques for forming liposomes and thus suffer from the same shortcomings of polydispersity and low encapsulation efficiency. Microfluidic devices have also been proposed to improve such a formation. Lorenceau *et al.* (184) have developed a new method to create highly uniform polymerosomes using a microcapillary device. This technique generates monodisperse double emulsion droplets consisting of water droplets surrounded by a layer of organic solvent dispersed in a continuous water phase. The diblock copolymers poly(normal-butyl acrylate)-poly(acrylic acid) (PBA-PAA) were dissolved in the organic solvent and formed polymerosomes followed by completely evaporating the organic solvent from the shell. The size of polymerosomes can be precisely controlled. Using the same set-up, Shum *et al.* (185) fabricated monodisperse biocompatible and biodegradable polymerosomes from block copolymer of poly (ethylene-glycol)-b-polylactic acid (PEG-b-PLA). The high encapsulation efficiency was demonstrated by encapsulating the 1 μm yellow-green fluorescent latex particles. Thiele *et al.* (186) further improved the system by introducing additional solvent stream to allow for independent injection and mixing of two organic phases which also prevented the formation of precipitates and fouling. Perro *et al.* (187) reported a two-step microfluidic device for production of complex polymersomes. By simply varying the flow rates of the three fluids, a controlled number of aqueous droplets could be formed inside the oil droplets. Such a behavior has also been reported in Wu *et al.* (98) where GFP molecule is synthesized in a double emulsion.

5.1.3. Polymer beads/capsules based carriers

Polymer beads, especially porous beads, can be used as carriers for drug delivery. There have been extensive studies in the fabrication of such materials. Significant effort has recently been devoted to microfluidics-based technology development for producing well-controlled porous beads. For example, the fabrication of mondispersed polymer microspheres has been demonstrated in many studies (127, 128, 134, 188, 189). The fabrication of macroporous polymer microspheres was reported in a double emulsion microfluidic device using simultaneous reactions within single droplets induced by UV irradiation (132).

Double emulsion droplets were also used in a capillary fluidic device by, Choi *et al.* (188) to produce microbeads with a controllable hollow interior and porous wall. The porous wall was obtained with water-in-oil (W/O) emulsions as the middle phase instead of the pure oil phase. After the organic solvent in the middle oil phase evaporates, Poly($_{D,L}$-Lactide-co-glycolide) (PLGA) microbeads with a hollow interior and porous wall were produced. Microfluidic double emulsion technology has also been used for producing microcapsules with gel, oil or water as inner cores (128, 131, 133, 190-195). For example, a capillary microfluidic device has been developed using oil-in-water-in-oil (O/W/O) double emulsions as templates to fabricate alginate microcapsules containing oil cores that have the potentials for encapsulating lipophilic drugs. W/O/W double emulsion template can also be applied to fabricate functional microgel particles using a capillary microfluidic technique (131). Janus microparticles have also been fabricated using microfluidic double emulsion technology (42, 196-199). The Janus particles consisted of

half hydrophilic based polymer and half organophilic based polymer, which can be used for a range of applications such as emulsion stabilization (199, 200), sensing (201, 202), optical control (203) and so on.

5.1.4. Colloidosomes based carriers

Colloidosomes are types of multiple emulsions in which the shells are formed by densely colloidal particles instead of bilayers as in liposomes or polymer bilayers as in polymerosomes. The structure of colloidosomes also allows for their applications in drug delivery, encapsulation and controlled release. Colloidosomes are typically prepared by creating particle-covered water in oil emulsion droplets dispersed in a continuous phase (204). A new strategy has been developed to fabricate non-spherical colloidosomes using microfluidic double emulsion template in order to potentially improve the flow properties of these capsules through constrictions mimicking the non-spherical structure of red blood cells (205).

5.2. Protein expression and directed evolution

Directed evolution is a method used in protein engineering to evolve proteins or RNA with desirable properties for use in agricultural, medical and industrial applications (206-211). The technique involves the steps of generating a library of mutant genes from the target gene, translating the DNA into proteins, screening and selecting of the presence of mutant genes with desired property. The process is repeated until the goal is achieved. Directed evolution like natural evolution lies in a link between genotype and phenotype to perform the selection. The technique has been developed at a huge pace and many impressive research results have been generated in the functional adaption of biomolecules to artificial environments (212-214).

Directed evolution can be performed in living cells (*in vivo* evolution) or without living cells (*in vitro* evolution). It is known that the probability to discover truly superior mutants is positively correlated with population size, which addresses the demand of high-throughput production. Conventional laboratory method employs the microtitre plates to perform high-throughput selection which could be a time-consuming and high cost task if the huge number of potential protein-protein and protein-nucleic acid interaction in the expressed genome is to be investigated (215). Furthermore, in drug discovery and clinical diagnostics, a low volume of reaction by a billion fold in comparison to the conventional microtitre-plates based approach is preferred. Therefore, it is suggested an 'evolution machine' should be capable of automation, miniaturization, integration and disposability (216). Emulsion droplets-based microfluidics can address these demands.

In addition to the high-throughput production, the selection and screening to identify desirable variants have also attracted much attention. The variant selection strategies are required according to the desired function of biomolecules. For example, based on the physical link (217, 218), the covalent binding (219) or noncovalent binding (220) to the gene, DNA-modifying enzymes and peptides could be selected. Selection procedures operate on the population in bulk which usually includes selective bacterial culture, affinity chromatography, and electrophoretic separation. For screening steps, each variant individually is assessed and sorted according to their parameters using techniques such as bacterial plates, microplate reader, cell sorters and so on. Microchip based sorting technique has been demonstrated using electric steering (88), heating (221), and acoustic waves (222). To date, the method of electric steering combined with droplet fluorescence detection can allow for the sorting frequency up to several kHz which is only 1-2 orders of magnitude slower than conventional fluorescence activated cell sorter (FACS) (223).

With the advances of droplet microfluidics, many biological applications such as cell based assay (224), *in vitro* protein expression (65, 225), and PCR (226) have been achieved. For example, Köster *et al.* has developed a highly flexible and adaptable device for a variety of cell-based assay using droplet-based microfluidic system (227). Individual devices are combined to fulfil cell encapsulation, incubation, and manipulation in picodroplets. The power of these devices has been successfully demonstrated through the detection of the antibodies secreted from the single mouse hybridoma cells in droplets after six hours. Schaerli *et al* reported a high throughput microfluidic device for continuous-PCR in water-in-oil droplets of nanoliter volumes (228).The novel device with the circular design allowed the droplets continuously transporting between two temperature zones. This system has successfully performed the amplification of an 85 base-pair long template from four different start concentrations with a specificity and high efficiency.

To carry out more complex biological experiments, especially for a complete directed evolution in microfluidics, different formats of controlled modules are required to be integrated in a device. Paegel *et al.* (229) has developed a microfluidic compartmentalized evolution system for evolving RNA enzymes with RAN ligase activity, i.e., the selected enzymes could resist inhibition by neomycin (Figure 7). The device consists of a circular nozzle array with 110 microfluidic channels that fan out from a central aqueous input reservoir. The microdrops are formed at the nozzle tips and the reported production rate is in the range of 1.9 ~ 40 kHz (i.e., 10^7~10^8 droplets per hour). The system was used to compartmentalize single catalytic RNA parent molecules, an oligonucleotide substrate for RNA-catalyzed ligation and enzymatic replication reaction mix. The selection bias can be prevented in this experiment allowing all antibiotic-resistant variants to persist and multiply. Mazutis *et al.* (230) have developed a high-throughput and on-chip screening system which can not only perform the isothermal amplification of single DNA molecule in each picodroplet but also measure the activity of the encode enzymes after fusion with another picodroplet containing an *in vitro* translation system. The system significantly increases the range of potential applications of microfluidics in directed evolution.

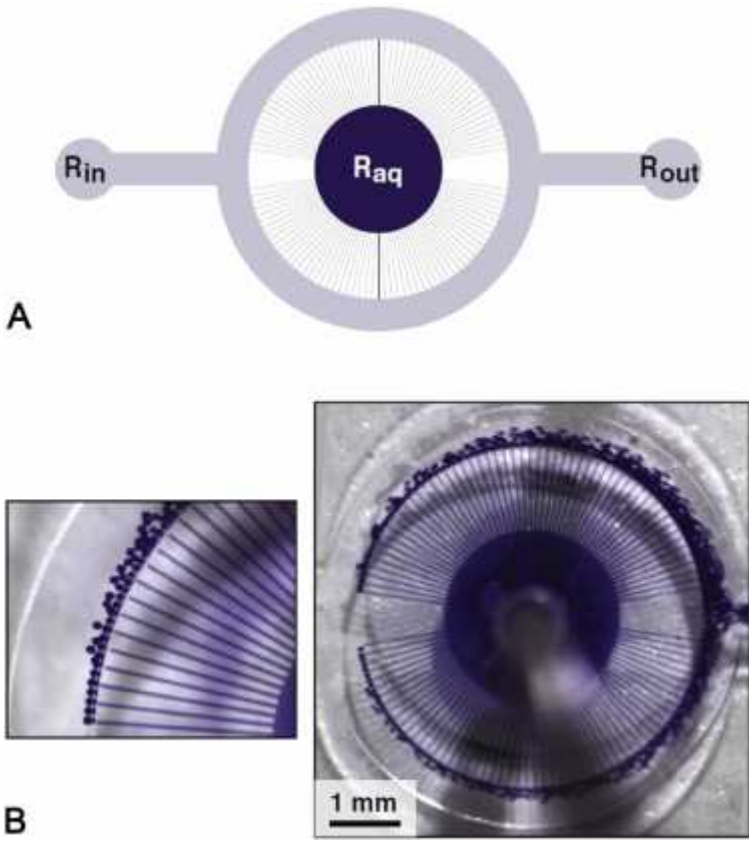

Figure 7. A microfluidic device for generating uniform water-in-oil emulsions. (a) A schematic drawing of the device (b) A photograph of the device with magnified view. Raq is the central aqueous input reservoir with 110 radical nozzles that empty into an annular oil flow channel. Rin is the oil input reservoir. Rout is the reservoir to collect the emulsion. (From ref. (229), reproduced by permission of Elsevier Ltd, copyright 2010).

Most work related to directed evolution in microfluidics are based on water-in-oil single emulsion system. The organic phase prevents the use of FACS machine for high throughput sorting and screening. In microfluidic systems, various on-chip techniques have been developed for sorting and selecting. To leverage the existing powerful FACS machine for the job, one has to use double emulsion in which the outer phase is aqueous. This is compatible with the requirements of the machine. The contribution of double emulsions to *in vitro* directed evolution has been demonstrated in conjunction of FACS technique for high-throughput screening and sorting. For example, Bernath *et al.* (231) used FACS to isolate and enrich genes with a fluorescent marker from those without a fluorescent marker. The selection for catalysis among large enzyme libraries has been reported in cell-based (232) or cell-free W/O/W double emulsions (233). However, little progress has been made in microchip-based double emulsion system for directed evolution.

6. CONCLUDING REMARKS

Droplet based microfluidics is a fast growing multidisciplinary field of research and development. It involves physics, chemistry, biology, material science, microfabrication and fluid dynamics. Microdroplets are microfluid segments that are independent from each other and have the capability of high throughput and high level of controllability. They can be used for a range of applications, ranging from material science, biotechnology, chemical engineering to detection of molecules.

In this chapter, the generation and application of double emulsion droplets were discussed. For completeness, the key topics in single emulsion droplets such as generation, control and application were also presented. The development of double emulsion droplets is promoted from the demand from food and drug industry. The recent development of single emulsion droplet also reveals the need for developing double emulsions to increase the partitioning capacity of interface and enable the handling of emulsions for existing technologies. The physics of double emulsion generation remains the same as single emulsion droplets. However, a two-step method is usually required and this also requires the modification of surface property. Due to the more complicated structure of double emulsions, the stability is much more challenging. A range of methods have been studied to achieve the stability. Such studies are especially reflected in the development of drug delivery platforms. Liposomes, polymerosomes, polymer beads and colloidosomes-based emulsions are examples of different delivery platforms that

are developed to increase the stability of the emulsion (thus stable encapsulation of drugs) and controllable release of substances.

In addition to drug delivery and other applications such as food, cosmetics, pharmaceuticals, printing, textile industries and so on, double emulsions have also been used in biotechnology applications such as protein expression and directed evolution. However, the latter development is still in its infancy, especially for chip-based technologies. No integrated system has been reported which is capable of protein evolution. In spite of the progress made so far, the main challenges still remain unresolved, i.e. stability of emulsions, high throughput production of double emulsions and control of the emulsions. For biotechnology applications, these would remain the focus of research in the future. For protein evolution, in particular, the focus would be the development of novel partition system that encloses the chemicals and biologicals effectively. In the meantime, novel control mechanism is also required for advanced sorting and selection of droplet and the utilization of existing technologies such as FACS.

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Abbreviations: IC**:** integrated circuit, IVTT: *in vitro* transcription and translation, W/O/W: water-in-oil-in-water, O/W/O: oil-in-water-in-oil, 4-MU: 4-methylumbelliferone, FACS: Fluorescence-activated cell sorting, BSA: bovine serum albumin,

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