Multiphase Microfluidic Processes to Produce Alginate-Based Microparticles and Fibers

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With the recent developments in microfluidic instruments and devices, various types of micrometer-sized materials have been produced by employing multiphase flow patterns formed in the microchannel. In particular, microparticles and microfibers, which are compatible with biomolecule incorporation or living cell encapsulations, have been gaining significant attention as new tools for biochemical analysis, cellular physiological studies, tissue engineering, cell transplantation, and controlled drug delivery. Herein, we introduce recent developments in microfluidic systems to produce alginate-based hydrogel microparticles and microfibers. By utilizing droplet dispersions either in equilibrium or non-equilibrium states, or by employing parallel laminar flows, microengineered functional materials that are difficult to generate using conventional devices and operations can be obtained. New and interesting multiphase phenomena are reviewed, together with the pros and cons of these systems and their applications. Furthermore, the fundamentals of multiphase microfluidics and the materials used to prepare particles and fibers are briefly introduced.

Introduction

Since the emergence of microfluidic technologies in 1990s, miniaturized systems for chemical, biological, and biomedical research have been intensively developed (Sackmann *et al.*, 2014). Microfluidic systems have become a standard technique used in chemical/biological laboratories, and have been incorporated in biochemical research equipment, analytical devices, and small-scale chemical synthesis processes. Numerous studies on multiphase microfluidic processes have been reported, ranging from fundamental studies of fluid dynamics to micrometer-sized material production. The representative examples of multiphase flows operated in microfluidic systems include parallel liquid–liquid flows, droplet dispersion, gas–liquid–solid systems for efficient chemical conversion, microbubble flows, particle suspension, and biological samples containing living cells and/or biological particles. One of the factors contributing to the acceleration of microfluidic research is the development of microfabrication technologies, as represented by the replica molding and soft lithography processes to prepare polydimethylsiloxane (PDMS)-based microfluidic devices (Duffy *et al.*, 1998). Other types of fabrication techniques have also been used, including wet and dry etching (Iliescu *et al.*, 2012), direct machining (Kitagawa *et al.*, 2014), deep X-ray lithography (Matsui *et al.*, 2007), and stereo-lithography using 3D printers (Waheed *et al.*, 2016).

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Microfluidic systems offer several advantages over other conventional processes, such as improving the controllability of multiphase flow patterns. When two (or more) types of immiscible fluids are continuously pumped into a microchannel, microdroplets, plug flows, or parallel laminar flows are formed, depending on the physicochemical properties of the fluids, operation parameters such as flow rates, and the geometry and surface wettability of the microchannel (**Figure 1**). In particular, in contrast to conventional bulk-scale operations, microfluidic processes are capable of producing highly monodisperse droplets or bubbles (Figure 1(a), (b)) (Teh *et al.*, 2008; Satoh *et al.*, 2014). The typical value of the coefficient of variation (CV) in droplet size is less than 10% when microfluidic processes are employed, and this value is significantly smaller than that of bulk-scale mixing techniques and even the sophisticated membrane emulsification process (typically the CV value in droplet size of ~10%). Furthermore, the formation of engineered droplets has been demonstrated, including double emulsions, droplets composed of hemispheres with different compositions, and non-spherical droplets (Sun *et al.*, 2014). Many review articles have been published on the microfluidic processes used to produce droplets (Tumarkin and Kumacheva, 2009; Vladisavljević *et al.*, 2012; Wang *et al.*, 2014; Choi *et al.*, 2017; Huang *et al.*, 2017; Shang *et al.*, 2017; Wang *et al.*, 2017). The formed droplets have been utilized to produce microparticles; when the droplets formed in microfluidic devices are solidified, one can obtain micrometer-scale particles with highly controlled sizes and morphologies (Choi *et al.*, 2008; Nakatsuka *et al.*, 2016).

On the other hand, the introduced fluids form stable parallel flows, especially when the physicochemical properties of these two types of fluids are similar (Figure 1(c)). In such parallel flows, the flow patterns, i.e., the width of the flow

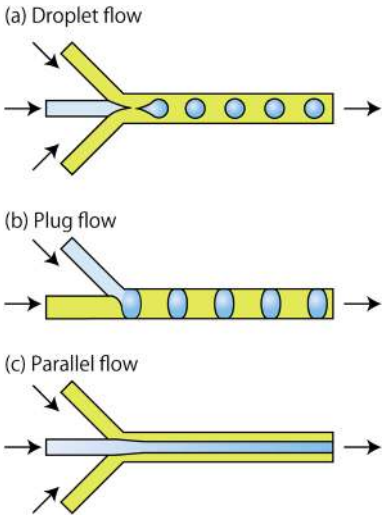

Fig. 1 Schematic images of the flow patterns in microfluidic devices. (a) Droplet flow, (b) plug flow, and (c) parallel flow

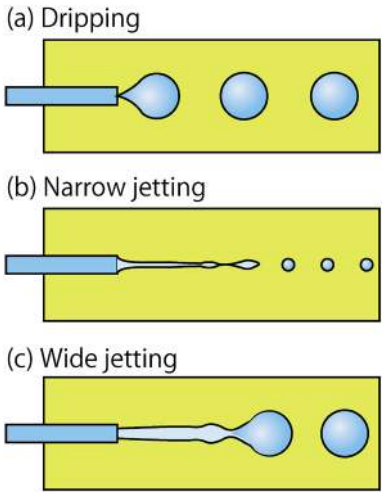

Fig. 2 Droplet generation schemes in a double-capillary device (reproduced from images in a literature (Utada *et al.*, 2007))

region of each fluid, can be accurately controlled using the operation parameters, including the volumetric flow rates of the fluids, microchannel width/depth, and viscosities of the fluids. This parallel flow has been adopted in various chemical processes, an example of which is the continuous-flow chemical processing accompanied by diffusion-based molecular transport (Tokeshi *et al.*, 2002). Furthermore, the parallel flow system has been utilized to produce microfibers with controlled shape and size; when the core fluid is continuously solidified by the interaction with the surrounding flows, micrometer-scale fibers can be easily generated.

Among various types of materials used for microparticle/fiber production, we focus on the production of alginate-based biomaterials, which have been mainly used for cellular studies, biomedical research, and biomolecule immobilization. Alginate-based hydrogels have remarkable advantages, i.e., intact biomolecules or cells can be encapsulated into the hydrogel matrices. In this article, we briefly review the recent research progresses on the microfluidic multiphase processes used to produce alginate-based microparticles and fibers. First, the fundamentals of fluid mechanisms specific to microfluidics are explained, and subsequently, the characteristics of alginate-based biomaterials are described. Then, droplet-based processes used to produce microparticles and parallel flow-based processes used to produce microfibers are introduced, together with their biological and biomedical applications.

1. Fundamentals of Liquid–Liquid Multiphase Microfluidics

When a non-compressive viscous fluid (e.g., water) is continuously introduced into a microchannel with a width and depth of 100 μm, laminar flow is stably formed owing to the low *Re* value, which is typically less than 1. If multiple types of fluids are continuously introduced into a microchannel, they form droplets or parallel flows, depending on the types of fluids and the operation conditions. In the case of immiscible two-phase flows, the parallel flows are stabilized when the following conditions are satisfied: the interfacial tension between the two phases is low; the difference in wettability of the fluids on the surface of the microchannel is small; the flow rates of these fluids are well balanced. For two types of miscible fluids, it is usually difficult to produce droplets; they form parallel flows, which are gradually mixed owing to the molecular diffusion in a direction perpendicular to the flow.

In contrast, when the interfacial tension between these fluids becomes relatively high, droplets are generated in many cases, depending on the physicochemical characteristics of the fluids and the operation parameters. The droplet generation schemes are classified into several types; for example, when double-nozzle microcapillaries are used as the microfluidic device, the droplet generation pattern shows (i) dripping, (ii) narrow jetting, or (iii) wide jetting (**Figure 2**) (Utada *et al.*, 2007; Shang *et al.*, 2017). In the dripping scheme, the shear stress exerted on the thread of the inner fluid is constant and well controlled, resulting in the formation of monodisperse droplets. On the other hand, the droplet formation behavior in the jetting schemes is mostly dependent on the Rayleigh–Taylor instability, and hence, droplets with sizes significantly smaller than microchannel/microcapillary sizes can be generated, even though the droplet sizes become non-uniform. Utada *et al.* (2007) demonstrated that the transition from dripping to jetting schemes mostly depends on the Capillary number of the outer fluid and the Weber number of the inner fluid.

In a microchannel with a rectangular cross-section, prepared by using lithography or micromachining, the wettability of fluids on the surface of the microchannel also critically affects the droplet generation behaviors. Oil-in-water (O/W) droplets are usually formed in hydrophilic microchannels (e.g., glass microchannels), whereas water-in-oil (W/O) droplets are generated in hydrophobic microchannels (e.g., PDMS microchannels). Chemical modification techniques

of the surface of the microchannel have been developed and utilized for controlling multiphase fluids; in particular, selective control of the surface of the microchannel is required to produce double emulsions (Shang *et al.*, 2017). Furthermore, the usage of a surfactant or polymeric stabilizer assists the generation of more monodisperse and smaller droplets because the interfacial tension between the two phases is decreased. Surfactants or stabilizers also increase the stability of the formed droplets, which prevents the coalescence of the generated droplets and improves the uniformity of droplet size. In summary, when attempting to control the microchannel patterns from droplet flows to parallel flows, these factors, including the operation conditions, wettability of the microchannel, and usage of surfactants or stabilizers, should be carefully considered depending on the applications/purposes of the system.

2. Materials for Particle/Fiber Production

In order to produce particles and fibers in microfluidic devices, various types of natural and synthetic materials, mostly polymers, have been used. The synthetic polymers include, but are not limited to, polystyrene, polylactic acid, poly(lactic acid-co-glycolic acid), poly methyl methacrylate, polyacrylamide, and polyethylene glycol (PEG). Among them, PEG is often used to produce hydrogel materials that can encapsulate living cells in the matrix. PEG is soluble in water, and hence, PEG-based hydrogel particles are generated using W/O emulsions. Polyethylene glycol diacrylate (PEGDA) is the most common form of PEG used to produce hydrogel particles. An aqueous solution of PEGDA is transformed into hydrogel mainly by using photoinitiators and photocrosslinking processes (Yeh *et al.*, 2006; Kantak *et al.*, 2012; Akbari *et al.*, 2017). In addition to the multiphase process, photocrosslinking technologies with micrometer-sized predefined patterns have been developed to produce particles with highly complicated morphologies, in which living cells have been encapsulated (Chung *et al.*, 2008; Panda *et al.*, 2008).

Proteins and polysaccharides are frequently used as the natural biopolymers. For cell culture operations, extracellular matrix proteins such as collagen and its denatured form, gelatin, have been used to produce microparticles (Matsunaga *et al.*, 2011) and fibers (Haynl *et al.*, 2016). Collagen hydrogels are stable at 37°C—the optimal temperature for mammalian cell culture. In contrast, gelatin hydrogels are soluble in water at this temperature, and hence, photocrosslinkable gelatin, modified with methacrylate group, has been widely employed (Kim *et al.*, 2016). The representative examples of the polysaccharides used include alginate, chitosan, agarose, and dextran. The applications of these biological polymer-based microparticles and fibers are mostly associated with cell cultivation, biological studies, or biomedical research fields, owing to the biocompatible nature of these materials. Furthermore, most of these natural biopolymers are biodegradable and relatively inexpensive except for collagen, rendering them useful for such applications.

Among these synthetic and natural polymers, alginate has been gaining significantly increased attention, and the number of research papers on the microfluidic production of alginate-based materials is increasing. Alginate is a natural biopolymer found in all the species of brown algae (Aslani and Kennedy, 1996) and usually obtained in the form of sodium alginate (Na-alg). Alginate polymers are safe and highly biocompatible, and hence, they are widely used as a food additive as a thickener, an impression-making material in dentistry, immobilization matrices for cells and biomolecules, skin wound dressings, immunoprotection material for cell transplantation therapies, etc. The aqueous solution of Na-alg is rapidly gelled in the presence of multivalent cations, including Ca^{2+}, Mg^{2+}, Ba^{2+}, and Fe^{3+}. For example, when droplets of Na-alg are dripped onto an aqueous solution containing these cations, Ca-alginate hydrogel beads are formed. In contrast, when an aqueous solution of Na-alg is continuously extruded into a $CaCl_2$ solution through a nozzle (e.g., syringe needle), fibers are obtained.

There are several advantages in using alginate-based materials for microparticle/fiber production. (i) The gelation process is mild compared to the formation of other polysaccharide-based hydrogels, such as agarose hydrogel, because temperature change is not required, rendering the operation system simple. Furthermore, potentially cytotoxic chemicals such as reactive crosslinkers are not required, and hence, living cells can be encapsulated in the hydrogel matrices without causing significant damage to the cells. (ii) The gelation speed of alginate is rapid. (iii) Alginate polymer is relatively inexpensive. (iv) Alginate hydrogels can be digested or removed simply by treating the hydrogel with chelators such as citric acid or EDTA, or by employing specific enzymes (alginate lyase). (v) Chemical functionalization of the alginate polymer is possible. For example, bio-functional peptide-conjugated alginate polymers are widely employed and some of them are commercially available. (vi) The mechanical property of the hydrogel can be tuned. The alginate polymer is composed of two types of sugars—guluronic acid (G) and mannuronic acid (M)—and the hydrogel of high G-content alginate becomes stiffer than that of high M-content alginate. The nature and characteristics of alginate-based materials and their biological applications have been documented in detail in literatures (Wee and Gombotz, 1998; Zimmermann *et al.*, 2007; Huang *et al.*, 2012; Lee and Mooney, 2012, Leong *et al.*, 2016).

Owing to these characteristics, alginate-based hydrogels have been widely used for biomedical research fields in the past few decades. Not only homogeneous particles, but also hollow capsules have been developed (Koyama and Seki 2004a, 2004b). However, it was not easy to produce alginate-based particles with sizes less than $\sim 100\,\mu m$, mostly owing to the difficulty in producing small droplets of highly viscous Na-alg solution using conventional techniques. Attempts have been made to produce small materials that are advantageous in terms of high surface-to-volume ratio and the enhanced transport of molecules through the ma-

trices, especially for biological encapsulation experiments. As mentioned above, microfluidic technology is a powerful tool to generate and manipulate microdroplets and parallel flows, which have been used to produce alginate-based microparticles and fibers. The sizes of cultured mammalian cells typically range from several micrometers to tens of micrometers, and these values are comparable to the sizes of typical microchannels and the materials produced by using microfluidic devices. In the following sections, the details of the production processes of alginate-based micrometer-sized materials are reviewed.

3. Droplet Microfluidics to Produce Alginate Particles

3.1 Production of alginate hydrogel microparticles using immiscible water–oil two-phase flows

In droplet microfluidics studies, droplets that are in an equilibrium state with the surrounding continuous phase are formed in most cases, by using two types of immiscible fluids (e.g., water and oil). When droplets of an aqueous solution of Na-alg are generated and subsequently transformed into hydrogel, we can obtain microparticles. However, a proper strategy to transform the precursor droplets of Na-alg into hydrogel microparticles is required, because the gelation speed of alginate is generally very high, and hence, multivalent cations should be supplied to the droplets in a controlled manner. The gelation methods of alginate-based droplets can be categorized into several types; (i) coalescence of a Na-alg droplet with a droplet of a gelation agent, (ii) off-chip gelation of Na-alg droplets using a bath containing a gelation agent, (iii) external gelation using an oil phase containing a gelation agent, (iv) internal gelation using a poorly soluble salt of a gelation agent, (v) rupture of double emulsions in the continuous phase of a gelation agent, and (vi) ion exchange and/or *in-situ* mixing in droplets (**Figure 3**).

One of the early studies on the microfluidic production of alginate particles was based on scheme (i), which was first reported by Sugiura *et al.* (2005). In this process, microdroplets of Na-alg and $CaCl_2$ solutions were formed at different sites of a microchannel array device, which flowed downstream and coalesced together to form alginate microparticles. Although the controllability of the size of the particles was not high, this report provided a new insight in the field of alginate-based microparticle production. In order to improve the coalescence efficiency of two types of droplets, microchannels with partially broadened regions were employed. Liu *et al.* achieved 1 : 1 coalescence of droplets to form alginate particles (Liu *et al.*, 2006; Liu *et al.*, 2012a) (**Figure 4**). The authors also demonstrated that the gelation in a shallow channel enabled the formation of disc-shaped alginate hydrogel particles from the deformed droplets. Furthermore, a method to coalesce a pair of aqueous core droplets in a water-in-oil-in-water double emulsion system was reported by Lee *et al.* (2016). This method achieved perfect 1 : 1 coalescence of droplets in an isolated environment.

Another strategy to easily produce alginate hydrogel par-

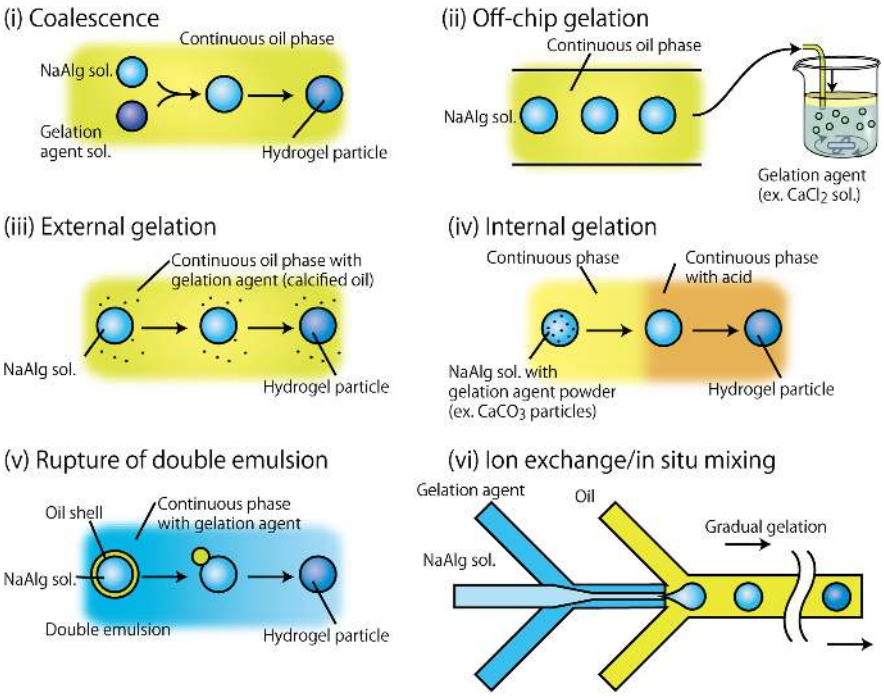

Fig. 3 Microfluidic schemes to produce alginate microparticles from Na-alg droplets. (i) Coalescence of a Na-alg droplet and a droplet with a gelation agent, (ii) off-chip bulk-scale gelation of Na-alg droplets, (iii) external gelation, which utilizes a continuous oil phase containing a gelation agent, (iv) internal gelation, which uses the powder of a poorly soluble salt of a gelation agent suspended in the Na-alg droplets, (v) rupture of water-in-oil-in-water double emulsions, and (vi) ion-exchange or *in-situ* gelation, which initiates slow gelation, in the droplets

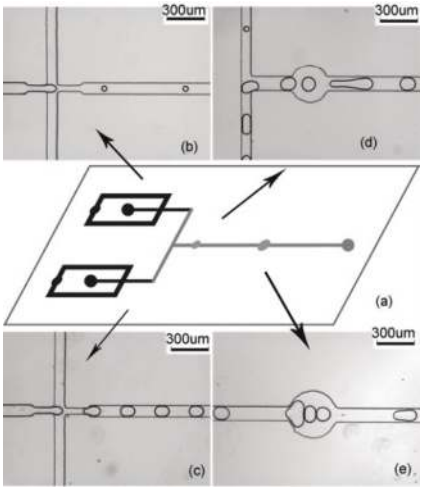

Fig. 4 Coalescence-based production of alginate microparticles. Reprinted with permission from a literature (Liu *et al.*, 2006). Copyright 2006 American Chemical Society

ticles is scheme (ii), i.e., off-chip gelation of Na-alg droplets. Droplets of aqueous Na-alg solution were formed in microchannels, which were subsequently transported to an outer bath containing a gelation agent solution (e.g., aqueous solution of $CaCl_2$). This process is easy to perform, but the interaction of the Na-alg droplets with the water–oil interface often causes droplet deformation, resulting in the formation of non-spherical particles. Capretto *et al.* (2008) reported the production of teardrop-like particles, which were formed by introducing the droplets into a gelation agent solution through an oil phase. Hu *et al.* (2012) further controlled the particle shape from sphere to concaved capsule, by using the aqueous solution of gelation agent with different viscosities. Chuah *et al.* (2009) demonstrated the formation of alginate microparticles with sizes as small as ~10 μm, by using small Na-alg droplets produced using microchannel array devices with a channel width of 8 μm, which were transformed into hydrogel microparticles.

In the aforementioned schemes, there remain some problems to be solved. In scheme (i), a 1:1 pair of two types of droplets should be coalesced to form particles; otherwise, the distribution of the particle sizes becomes large compared to the initial droplets. The double-emulsion-based droplet coalescence scheme (Lee *et al.*, 2016) was perfect in terms of pairing efficiency, but was difficult to perform. In scheme (ii), gelation occurs in a non-controlled manner, thus forming deformed particles and compromising the size uniformity of the particles. As a process of directly supplying multivalent cations through the oil phase, scheme (iii), i.e., the external gelation method, has been reported. Zhang *et al.* (2006, 2007) reported a strategy to produce alginate hydrogel particles using an undecanol solution of CaI_2 as the continuous phase. The gelation speed of this process was not high; it required up to ~100 s to form hydrogel particles. Therefore, hydrogel was not formed at the junction, preventing the microchannel from being clogged with the unintentionally formed hydrogel. By utilizing this external gelation scheme, not only the homogeneous particles, but also hollow hydrogel capsules could be formed when the gelation was incomplete by controlling the time period of gelation (Zhang *et al.*, 2007). This strategy was subsequently modified and further improved to efficiently produce alginate particles. Lian *et al.* (2012) proposed a three-dimensionally expanding microchannel to produce Na-alg droplets based on the rapid change of droplet morphology and interfacial area. Kim *et al.* (2014) reported a microfluidic system that can handle multiple solutions even with a single syringe pump, and applied this system to the external-gelation-based production of alginate microparticles. Lee *et al.* (2011) used a microchannel system that can additionally introduce a flow of calcified oleic acid to the droplet flow in an oil phase, and used this system to produce alginate microparticles. The authors further proposed a technique to effectively remove the continuous phase of oil by using a hydrophobic filter paper (Lee *et al.*, 2014). Furthermore, core-shell microparticles were obtained using a three-dimensional focusing device; droplets incorporating an internal core solution were generated to produce core-shell capsules (Kim *et al.* 2011). Most of these techniques produced living-cell-encapsulating particles, showing the biocompatibility of the particle production process.

As another method to transform Na-alg droplets into hydrogel microparticles, scheme (iv), i.e., the internal gelation method, using multivalent cations incorporated in the Na-alg solution in advance, was developed. This method utilizes the dissolution of a poorly soluble salt of multivalent cations incorporated in the droplets, with response to the change of characteristics of the environment surrounding the droplets. In many cases, $CaCO_3$ powders are used, which are suspended in the dispersed phase (Reis *et al.*, 2006). After generating droplets, a pH changer (e.g., acetic acid) is added to the continuous phase. Acetic acid is rapidly partitioned to the dispersed aqueous phase of the Na-alg droplets, causing the pH decrease of the solution and the dissolution of $CaCO_3$ particles. Consequently, droplets are transformed into hydrogel particles because the released Ca^{2+} ions react with the alginate polymer. This method is advantageous because the time period of gelation can be controlled, and the gelation speed is high. This internal gelation approach was first adopted in a microfluidic process by Tan and Takeuchi (2007) (**Figure 5(a)**). Droplets with a size of ~100 μm were generated in the continuous phase of oleic acid, and the addition of acetic-acid-containing continuous phase triggered the pH change of the droplet, dissolving $CaCO_3$ powders and forming hydrogel particles. The encapsulation of mammalian cells was demonstrated with cell viability higher than 70%. A similar microfluidic process was reported by Zhang *et al.* (2007), and a $BaCO_3$-based internal gelation method was reported by Capretto *et al.* (2008). Furthermore, an internal-gelation-based production of alginate particles using a three-dimensional, axisymmetric flow-focusing device was reported, in which the droplet formation behavior was not significantly affected by the wettability of the microfluidic channel (Morimoto *et al.*, 2009). Janus alginate micropar-

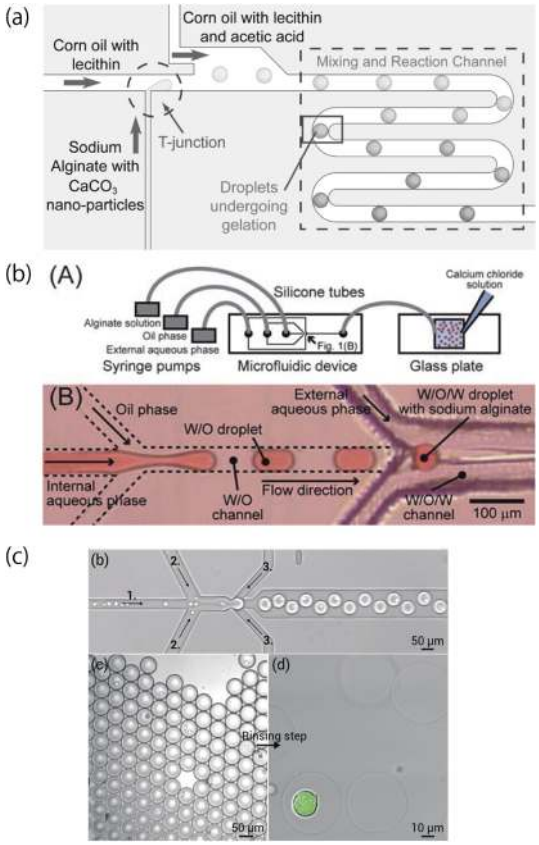

Fig. 5 (a) Production of Ca-alg microparticles by internal gelation. Reproduced from a literature (Tan and Takeuchi, 2007) with permission from Wiley. (b) Rupture of W/O/W double emulsion droplets to form Ca-alg microparticles. Reproduced from a literature (Saeki *et al.*, 2010) with permission from The Royal Society of Chemistry. (c) Alginate particle production by competitive ligand exchange crosslinking. Reproduced from a literature (Hati *et al.*, 2016) with permission from The Royal Society of Chemistry

ticles were also produced by introducing two types of Na-alg solutions with different compositions in microchannels (Marquis *et al.*, 2012). Another salt-based approach was presented by Utech *et al.* (2015), in which Ca-EDTA complex was used. The authors further produced capsules with an aqueous core and an alginate shell that encapsulated living cells in the core (Chen *et al.*, 2016). Akbari and Pirbodaghi (2014) reported a method to minimize the damage to cells caused by the interaction with solid particles. Cells and calcium particles were individually suspended in different Na-alg solutions, which were united at the microchannel confluence, immediately followed by droplet generation, mixing, and subsequent internal gelation. Approximately 85% of the cells were viable after encapsulation, demonstrating the good cytocompatibility of the process.

In scheme (iv) i.e., the internal gelation process, usually, the pH of the aqueous droplets is decreased by the supply of acid, which possibly causes damage to the encapsulated living cells. A method to solve this problem, scheme (v) i.e., the double emulsion rupture scheme, was proposed by Saeki *et al.* (2010). Microfluidic devices were used to produce water-in-oil-in-water (W/O/W) emulsion droplets with a thin oil layer (**Figure 5(b)**). The core was the aqueous solution of Na-alg, and the outer aqueous phase contained a gelation agent. When the thickness of the oil layer was sufficiently small, the double emulsion droplets were easily ruptured, and at this moment, the inner droplets were rapidly gelled. A similar technique was reported by Chan *et al.* (2013), where cells were encapsulated and cultured in the particles produced by this rupture technique.

Furthermore, *in-situ* gelation approaches in the formed droplets have been reported (scheme (vi)). The flows of several types of aqueous solutions were joined in a microchannel immediately before the formation of droplets, and the crosslinking reaction progressed in the droplets. One of the first studies was reported by Zhang *et al.* (2006), wherein the flows of Na-alg solution, $CaCl_2$ solution, and a spacer flow (deionized water) were united, and droplets were subsequently formed. A similar process was reported by Mazutis *et al.* (2015), in which the obtained particles were used to encapsulate/release antibodies. In these techniques, the introduction of the spacer flow was necessary, as it modulates the high gelation speed of alginate. Another interesting approach was recently reported by Hati *et al.* (2016), wherein an *in-situ* gelation method based on ion-exchange was reported, which was called competitive ligand exchange crosslinking. Two types of metal complexes—Ca-EDTA and Zn-EDDA—were used, which were individually introduced into aqueous solutions of Na-alg. Upon mixing these solutions in the formed droplets, the gelling ion (Ca^{2+}) was released in the presence of the exchange ion (Zn^{2+}), and was used to crosslink the alginate and form hydrogel (**Figure 5(c)**). This concept was used not only for particle production but also for preparing alginate microfibers.

3.2 Particle production using miscible water–oil two phase flows

The particle production methodologies presented in Section 3.1 were based on the formation of droplets using immiscible oil and water phases. The typical diameter of the microparticles was in the range of 50–200 μm, and this value was sufficiently large to encapsulate living mammalian cells (of size 10–20 μm) and bacteria (several micrometers) in the hydrogel matrices. However, it was not easy to produce microparticles smaller than ~10 μm using microchannels because the relatively high viscosity of the precursor solution of Na-alg renders it difficult to form smaller-sized droplets. Narrow microchannels possibly solve this limitation, but the pressure required to introduce a highly viscous fluid sample into a much narrower microchannel (e.g., microchannel with a width or depth less than 10 μm) becomes very high, as clearly deduced from the Darcy–Weisbach equation, which potentially compromises the operability of the system.

As an efficient strategy to produce general microparticles with small sizes, a solvent extraction method was developed and utilized to produce various types of particles (Freitas

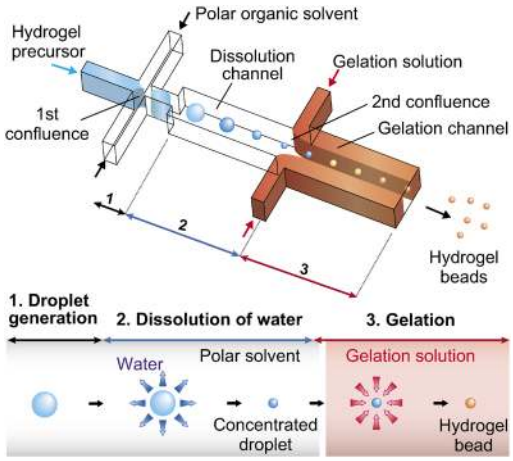

Fig. 6 Production of single micrometer-sized alginate microparticles via the droplet dissolution process in a continuous phase of a polar organic solvent. Reproduced from a literature (Sugaya *et al.*, 2013) with permission from American Institute of Physics

et al., 2005). Several studies have been reported on the microfluidic production of alginate particles by employing this concept (Rondeau and Cooper-White, 2008; Sugaya *et al.*, 2013), wherein a polar organic solvent (e.g., dimethyl carbonate and methyl acetate) that is miscible with water was used as the continuous phase. Sugaya *et al.* (2013) prepared single micrometer-sized alginate microparticles via the rapid dissolution of microdroplets in a non-equilibrium state (**Figure 6**). Droplets of a diluted solution of Na-alg were generated in the continuous phase of methyl acetate. As methyl acetate can dissolve water at 8%, the formed droplets were rapidly shrunk in the microchannel, typically within 1 s, and consequently, alginate molecules were concentrated. After completing the droplet dissolution, the condensed alginate was introduced into the flow of $CaCl_2$ solution, resulting in the formation of single micrometer-sized Ca-alginate microparticles. Owing to the drastic shrinkage of the droplets, particles with initial sizes significantly smaller than the original droplet size could be obtained. This process is advantageous because with the usage of a diluted solution of the precursor (~0.1% Na-alg), the fluid can be manipulated at relatively low pressure. A similar technique to produce small alginate particles was also reported by Pittermannova *et al.* (2016), wherein 1-undecanol with a surfactant was used as the continuous phase. The usage of this non-equilibrium droplet system has been applied to the production of other types of microparticles, including polymeric (Ono *et al.*, 2014), lipid (Mizuno *et al.*, 2015), protein (Yamada *et al.*, 2015; Yajima *et al.*, 2017), and carbon nanotube particles (Tomii *et al.*, 2017), demonstrating its versatility and usefulness.

3.3 Particle production using aqueous–aqueous dispersions

In the aforementioned particle production techniques, the water–oil interface possibly affects the cell viability and function, owing to the relatively high interfacial tension. Furthermore, the continuous oil phase should be finally removed to purify the particles. As a procedure that does not require an oil phase, aqueous–aqueous two phase dispersion has been utilized to produce particles. It is known that an aqueous solution of multiple types of polymers (e.g., PEG and dextran) is spontaneously separated into two immiscible phases: upper PEG-rich phase and lower dextran-rich phase (Soares *et al.*, 2016). Usually, it is not easy to produce an aqueous–aqueous dispersion with controlled droplet size, owing to the significantly low interfacial tension between these two phases (Atefi *et al.*, 2014). However, microfluidic systems have been recently applied to the formation of aqueous droplets in a continuous phase of an aqueous solution (Moon *et al.*, 2015; Moon *et al.*, 2016; Dang and Kim 2017), either by pulsating the inlet pressures or by passive droplet breakup. A microfluidic process to produce alginate-based particles using the aqueous–aqueous two-phase dispersion system was recently reported by Liu *et al.* (2017). The authors produced droplets of a dextran-rich phase containing phenol-modified alginate polymer in a continuous PEG-rich phase. After forming droplets, the alginate polymer was enzymatically crosslinked. By using this method, the oil removal process was dispensed with, and the possible damage to the encapsulated substances (e.g., living cells) caused by the interface could be minimized. The presented system can potentially be extended to the production of various types of biomaterial microparticles without using an oil phase.

3.4 Particle formation via droplets jetting in air

Millimeter-sized alginate hydrogel particles are easily produced simply by dropping Na-alg solution into a gelation agent, and have been utilized for biological encapsulation and immobilization in the past few decades. In this process, droplets are first formed in air and subsequently introduced into the aqueous phase where the gelation is initiated. Attempts have been made to downsize the droplet size using, for example, spraying or atomizing (Herrero *et al.*, 2006). Recently, inkjet techniques have been employed for this purpose, and the formation of alginate microparticles with sizes as small as 20 μm was reported by Kojima *et al.* (2014). Microfluidic technologies have also been employed and combined with the jetting principle, wherein water droplets are formed in air and subsequently dropped into a solution of gelation agent. One of the first studies on air-jet-based microfluidic devices was reported by Sugiura *et al.* (2007), who utilized the air flow to produce small microdroplets. The authors successfully obtained alginate microparticles with sizes of ~150 μm by dropping the formed droplets into a gelation agent. Maeda *et al.* (2012) reported centrifugation-based droplet formation using microcapillary-combined small tubes to produce alginate microparticles (**Figure 7**). Particles with a diameter of ~100 μm were formed, together with satellite particles with a diameter of ~20 μm. Furthermore, the authors prepared multicompartment particles using the combined multiple capillaries. This centrifugal-force-driven jetting scheme was further applied to minimize the dead volume of samples (Onoe *et al.*, 2014); the authors

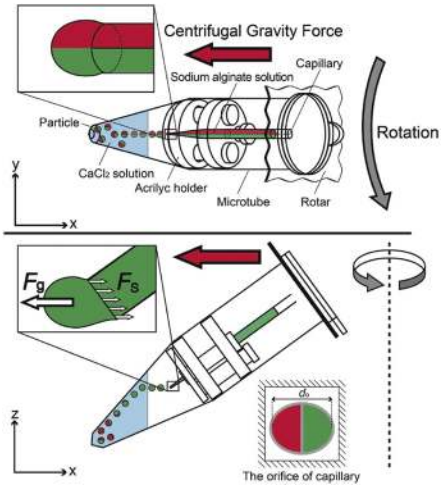

Fig. 7 An example of air-jet-based processes to produce alginate microparticles: centrifugation-based jetting system incorporated in a plastic tube to produce composite alginate microparticles. Reproduced from a literature (Maeda *et al.*, 2012) with permission from Wiley

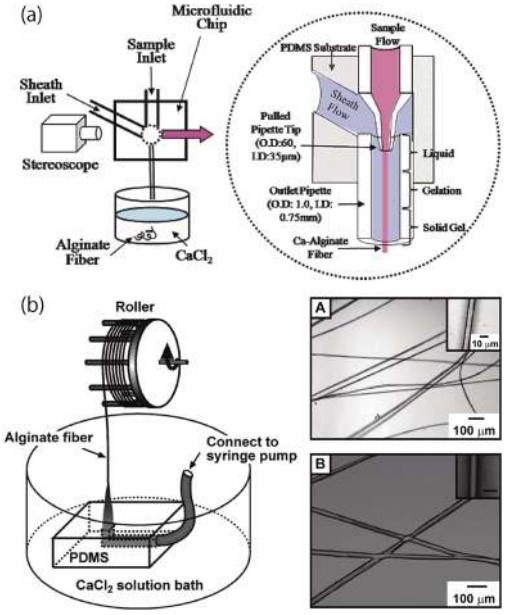

Fig. 8 Microfluidic processes to produce homogeneous alginate hydrogel microfibers. (a) Double-capillary-based flow system to produce alginate hydrogel fibers. Reprinted with permission from a literature (Shin *et al.*, 2007). Copyright 2007 American Chemical Society. (b) Pulling-up process to produce alginate hydrogel fibers using a roller. Reproduced from a literature (Su *et al.*, 2009) with permission from The Royal Society of Chemistry

demonstrated the production of cell-encapsulating alginate beads with an average diameter of ~70 μm, from only a small volume of Na-alg solution (less than 1 μL).

One of the disadvantages of using microfluidic devices is the relatively low throughput of microparticle production. This problem limits the application range of the micrometer-sized materials. Recently, various types of scaling-up approaches have been developed to produce general microparticles, by employing a numbering-up strategy of unit microchannels (Jeong *et al.*, 2016; Han *et al.*, 2017) and a parallel droplet formation scheme using micropillar array structures (Akbari *et al.*, 2017). By utilizing these techniques, the throughput of alginate-based microparticles will be increased.

4. Parallel Microfluidic Flows for Producing Fibers/Sheets

4.1 Continuous production of homogeneous fibers

In addition to microparticle production, microfluidic systems have been recently recognized as a practical tool to produce various types of fibrous materials. There have already been several good review articles reported on the microfluidic production of microfibers, which were mainly utilized for biomedical applications, including cell encapsulation, tissue engineering, and controlled drug delivery (Jun *et al.*, 2014; Sharifi *et al.*, 2016; Cheng *et al.*, 2017). In this article, we focused on the production of alginate-based hydrogel fibers mainly using parallel flows in microfluidic devices.

As mentioned in the above sections, alginate-based microparticles or capsules are advantageous especially for encapsulating small biological substances, including cells, bacteria, and biomacromolecules, because the high surface-to-volume ratio of the small particles significantly enhances the molecular transport through the permeable hydrogel matrices. Recently, microfibers made of alginate-based hydrogels have been produced by using microfluidic devices. For example, simply by extruding a precursor solution through a micronozzle into a gelation agent, the flow of the precursor solution is rapidly gelled and a hydrogel fiber can be continuously generated. One of the first studies of this concept was reported by Takei *et al.* (2006), wherein co-flowing capillaries were used to produce fibers with a width of several hundred micrometers. Shin *et al.* (2007) reported a double capillary system to produce narrower fibers with a diameter of ~20 μm (**Figure 8(a)**). Sugiura *et al.* (2008) applied the micronozzle array device to produce alginate fibers and showed that encapsulated cells proliferated in the hydrogel matrix. Most of these studies reported the encapsulation of living cells in the hydrogel matrices of the fiber. Furthermore, Cuadros *et al.* (2012) characterized the mechanical properties of the alginate fibers produced using microfluidic devices in detail. Apart from the micronozzle-based extrusion process, Su *et al.* (2009) proposed a roller-based pulling up process, combined with a microfluidic device, to produce microfibers with a diameter as narrow as ~10 μm (**Figure 8(b)**). Fibers formed using microfluidic devices have been used to encapsulate various types of cells, including islet cells for immunoprotection reported by Jun *et al.* (2013). Liu *et al.* (2012b) reported a process to generate homogeneous fibers of alginate, modified with the phenolic hydroxyl group, which were obtained by enzymatically crosslinking the phenolic moieties.

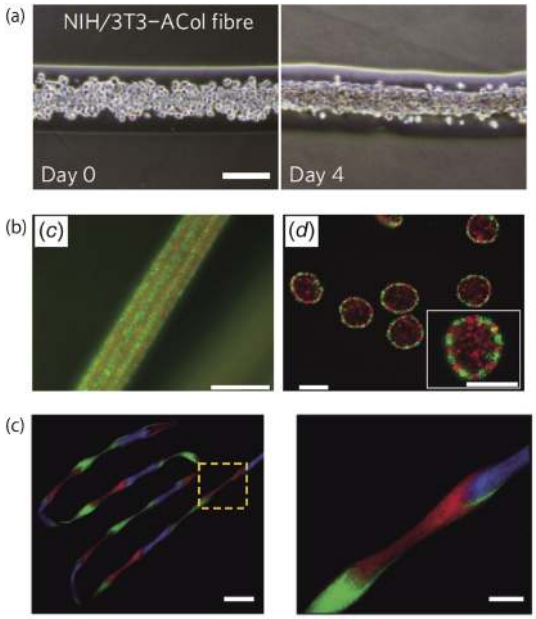

Fig. 9 Complex alginate fibers produced using microfluidic devices. (a) Core-shall fibers, in which the core, containing mammalian cells, was composed of collagen gel. Reproduced from a litearture (Onoe *et al.*, 2013) with permission from Nature Publishing Group. (b) Complex fibers containing different colored particles in the distinct regions in the cross-section. Reproduced from a literature (Kitagawa *et al.*, 2014) with permission from IOP Science. (c) Alginate fibers composed of different-colored regions in the length direction. Reproduced from a literature (Kang *et al.*, 2011) with permission from Nature Publishing Group

4.2 Production of anisotropic and complex fibers

One of the most remarkable advantages of the microfluidic process used to produce complex fibers is the ability to control the cross-sectional morphology and composition. For example, when Na-alg solution and another solution were introduced into the outer and inner capillaries of a double capillary device, respectively, and subsequently, these solutions were extruded into a gelation agent solution, core-shell fibers with an alginate hydrogel shell could be obtained (Hu *et al.*, 2010; Takei *et al.*, 2010; Meng *et al.*, 2016). Most of these studies used a thickener for the core solution, such as dextran and carboxymethyl cellulose. Onoe *et al.* (2013) reported a core-shell microfiber containing collagen gel as the core, and encapsulated various types of mammalian cells in the core and cultured them to form linear microtissues (**Figure 9(a)**). The authors produced pancreatic islet cell-encapsulating fibers, which were transplanted into the kidney capsule of diabetes model mice, and were effective at rescuing the mice from the disease. Hirayama *et al.* (2013) encapsulated and cultured bacterial cells, which produce cellulose, in the core of the core-shell fiber. The formed linear structures made of cellulose were utilized as scaffolds for the mammalian cell culture. Sakai *et al.* (2013) prepared modified alginate-based core-shell fibers utilizing an enzymatic reaction, and cultured different types of cells in the core and on the outer surface of the core-shell fibers. After removing the alginate hydrogel shell, linear microorganoids composed of two types of cells were formed. Zuo *et al.* (2016) prepared composite, double-layered hollow microfibers made of alginate and methacrylated gelatin, which were used to simulate complex tissues. One of the advantages of these systems is the ability to physically separate the inner and outer environments using the thin alginate hydrogel wall to retain specific substances or cells in the restricted linear core regions.

In addition to the relatively simple core-shell structures, various types of significantly more complex hydrogel fibers have been produced by using microfluidic devices. Yamada *et al.* (2012a) reported a microfluidic device with multiple inlet channels for Na-alg solutions with different compositions, and used this device to produce anisotropic fibers, e.g., sandwich-type fibers, composed of hydrogels with different physical stiffness. The authors further applied the system to the formation of hepatic microorganoids composed of two types of cells, by culturing these cells in the matrices of the sandwich-type fibers (Yamada *et al.*, 2012b). The fabrication of more complicated fibers was demonstrated by Kitagawa *et al.* (2014) (**Figure 9(b)**). A multilayered microfluidic device incorporating a micronozzle-array structures was used. The flows of Na-alg solutions with different compositions were introduced into the gelation channel through the micronozzle array to form alginate hydrogel fibers with the cross-sectional pattern corresponding to the arrangement of the nozzles. The authors prepared solid-soft hybrid fibers, in which the direction of cell proliferation and cell-cell network formation of the encapsulated neural cells was controlled in the fiber-length direction. Cheng *et al.* (2016) utilized a multiple capillary-combined microfluidic device, for creating multi-component hydrogel fibers. In addition to the cross-sectional anisotropy, fibers with controlled patterns in the length direction were reported. Kang *et al.* (2011) used a microchannel combined with multiple valve systems to produce coded microfibers (**Figure 9(c)**). Leng *et al.* (2012) produced fibers with mosaic patterns that can be programmed by multiple fluidic controls using valves-integrated microfluidic devices. Furthermore, stripe-patterned hydrogel sheets (Kobayashi *et al.*, 2013) and microfibers/sheets with grooved surfaces (Kang *et al.*, 2012) were produced, which were produced by extruding the precursor solution into a gelation solution through a flat or grooved nozzle structure, respectively.

4.3 Production of functional fibers with complex 3D morphology

In the microfluidic processes to produce hydrogel fibers, not only the straight fibers, but also fibers with highly unique 3D morphologies, including coiled fibers and multiphase fibers, could be obtained. For example, when anisotropic fibers were produced with different compositions in the cross-section, the obtained fibers showed a coiled morphology (Yamada *et al.*, 2012a). In this study, propylene glycol alginate was added to one of the two precursor solutions, which altered the local physical properties of the fiber. Furthermore, unique approaches for changing fiber mor-

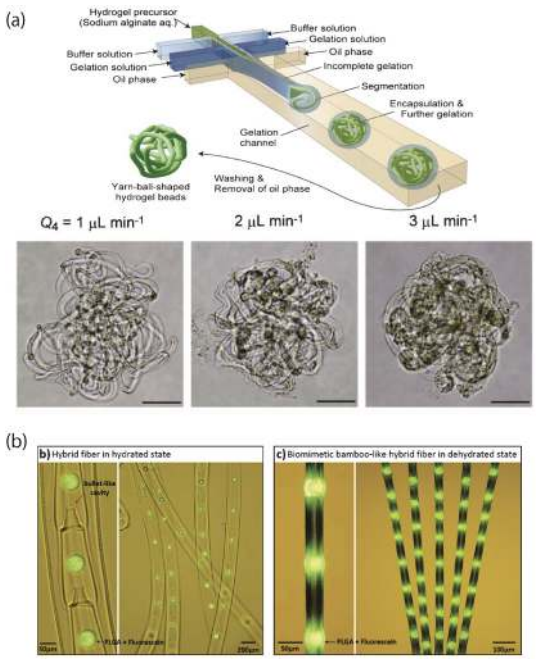

Fig. 10 Creation of unique alginate hydrogel materials by combining a fiber generation system with droplet formation technique. (a) Microfluidic system to produce yarn-ball-shaped alginate hydrogel beads. Reproduced from a literature (Miyama *et al.*, 2013) with permission from The Royal Society of Chemistry. (b) Oil-droplet-encapsulating alginate hydrogel fibers. Reproduced from a literature (Yu *et al.*, 2014) with permission from Wiley

phologies and producing coiled alginate fibers have been developed by controlling the operation conditions such as the fluid viscosities and flow rates (Tottori and Takeuchi, 2015) or by using a nozzle with a diagonally cut edge (Yoshida and Onoe, 2017). These studies demonstrated the ability of microfluidic systems to dynamically tune the 3D morphology and physicochemical properties of the micrometer-sized hydrogel materials.

In addition, microfluidic alginate fiber-producing processes combined with a droplet generation process have been reported to produce unique hydrogel materials. Miyama *et al.* (2013) reported a method to cut alginate fibers undergoing formation into fragments and fold the fiber fragments into yarn-ball shapes (**Figure 10(a)**). Alginate fibers were produced in a microchannel by introducing a Na-alg solution and a gelation agent, and simultaneously, an oil phase was introduced from the outer inlets. At the time of formation of aqueous droplets in the oil phase, the alginate fibers undergoing solidification were fragmented and folded in the droplets. The formed yarn-ball-shaped particles were suitable for cell encapsulation applications because the high surface-to-volume ratio of this morphology enabled the efficient transport of oxygen and nutrition to the cells encapsulated in the hydrogel matrices. A similar droplet-based fragmentation process of alginate fibers was reported by Martino *et al.* (2016). In contrast, by generating oil droplets inside the flow of the precursor solution, oil-droplet-encapsulating hydrogel fibers were fabricated (**Figure 10(b)**) (Yu *et al.*, 2014). These reports are good examples demonstrating the versatility of multiphase microfluidic processes for the production of highly functional materials that are difficult to obtain using conventional operations, but would be useful in various research/industrial fields.

Conclusions and Perspectives

In this article, we have briefly reviewed the fundamentals, recent progresses, and biological applications of alginate-based micromaterials produced in microfluidic devices. Although alginate has a long history of use in food and biomedical industries and biological laboratories, microengineering processes for this material are now accelerating. Further progresses in this research field would be made in the near future, based on the chemical functionalization of the polymer, optimization of the process and microchannel design, deeper understanding of the multiphase mechanics, findings of new non-equilibrium phenomena, and demand for the controlled microenvironments for various types of biological substances.

Acknowledgement

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