Particuology 24 (2016) 44–60

Contents lists available at ScienceDirect

Particuology

journal homepage: www.elsevier.com/locate/partic

Review

Advances in fabricating spherical alginate hydrogels with controlled particle designs by ionotropic gelation as encapsulation systems

Jun-Yee Leong[a], Weng-Hoong Lam[a], Kiang-Wei Ho[a], Wan-Ping Voo[a], Micky Fu-Xiang Lee[a], Hui-Peng Lim[a], Swee-Lu Lim[a], Beng-Ti Tey[a,b], Denis Poncelet[c], Eng-Seng Chan[a,b,*]

[a] Chemical Engineering Discipline, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, Selangor 46150, Malaysia
[b] Advanced Engineering Platform, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, Selangor 46150, Malaysia
[c] Oniris, UMR CNRS 6144 GEPEA, rue de la Géraudière, BP82225, Nantes 44322, France

ARTICLE INFO

Article history:
Received 25 March 2015
Received in revised form 23 August 2015
Accepted 6 September 2015
Available online 3 December 2015

Keywords:
Alginate
Ionotropic gelation
Immobilization
Encapsulation
Hydrogel

ABSTRACT

Alginate is a biopolymer that has exceptional gelling properties, which allow easy gel formation under safe and mild conditions. Consequently, it is often used to encapsulate a variety of cargos, such as cells, enzymes, and lipids, and is typically employed as a model to study hydrogel-based encapsulation systems. Since the first use of alginate in the encapsulation field in the 1970s, many methods have been developed to produce alginate hydrogel particles of different sizes, structures, and morphologies. This review provides an overview of the current progress in the fabrication of alginate hydrogels with various particle designs, including a discussion of dispersion techniques to pre-template alginate particles, gelation mechanisms, considerations in selecting suitable fabrication methods, and future directions.

Contents

Introduction 45
Particle size and morphology 45
Fabrication methods of alginate particles 46
Dispersion methods 46
Liquid–air methods 46
Liquid–liquid methods 48
Self-assembly methods 48
Methods to reduce particle size 49
Gelation mechanisms 49
External gelation 50
Internal gelation 52
Inverse gelation 52
Interfacial gelation 53
Multi-step interrupted gelation 53
Considerations in method selection 55
Particle size and size distribution 55
Economics of production 57
Productivity 58
Technical constraints 58

* Corresponding author at: Monash University Malaysia, Chemical Engineering Discipline, Jalan Lagoon Selatan, Bandar Sunway, Selangor 46150, Malaysia. Tel.: +60 3 55145821; fax: +60 3 55146207.
E-mail address: chan.eng.seng@monash.edu (E.-S. Chan).

http://dx.doi.org/10.1016/j.partic.2015.09.004

Future perspectives and conclusions 58
Acknowledgements 58
References 58

Introduction

Gel-forming polymers from natural sources represent an important class of biomaterials for encapsulation applications because of their renewability, biodegradability, biocompatibility, and non-toxicity. Among this class of polymers, alginate has received much attention in the literature (see Fig. 1), likely because it can form hydrogels primarily by ionotropic gelation with divalent ions (e.g., Ca^{2+}) at room temperature, thereby allowing immobilization to be performed under mild and safe conditions. Furthermore, the most basic particle design of alginate hydrogels (i.e., beads) can be produced using simple equipments, e.g., beakers and syringes, and the encapsulation process can be performed in virtually any laboratory.

Aside from their advantages in the production method, alginate hydrogels possess outstanding physical properties. They are thermally stable and can form gels very rapidly, even in the presence of high solid concentrations (e.g., up to 30% w/v). The mechanical, mass transport, and disintegration properties of alginate hydrogels are also tunable. Furthermore, alginate hydrogels have small-sized pores (5–200 nm) (Martinsen, Storrø, & Skjåk-Bræk, 1992) and are hydrophilic, making them suitable for encapsulating large molecules or hydrophobic materials at high efficiencies, generally higher than 90% (Chan, 2011; Lemoine, Wauters, Bouchend'homme, & Préat, 1998). Depending on their solubility in water, some small hydrophilic molecules, such as melatonin, aspirin, cimetidine, sodium salicylate, and glucose oxidase, have also been successfully encapsulated, though at lower encapsulation efficiencies (Lee, Min, & Cui, 1999; Blandino, Macías, & Cantero, 2001).

To date, alginate has been used for encapsulating various cargos such as living cells, protein drugs, enzymes, food ingredients, volatile compounds, and catalysts. The immobilized systems have been employed in diverse applications including tissue engineering, controlled drug delivery, biocatalysis for chemicals production, stabilization of food ingredients, adsorption of pollutants, and energy storage. Academic interests largely motivate many of these applications, but many industrial companies have begun to offer encapsulation solutions and services over the last decade.

Alginate is a hydrophilic biopolymer derived from brown seaweeds and is composed of (1–4)-linked β-D-mannuronic (M) and α-L-guluronic acid (G) residues (Martinsen, Skjåk-Bræk, & Smidsrød, 1989). This review, however, does not discuss the chemistry or properties of alginate or its derivatives because these topics have been covered in detail elsewhere such as in the recent review paper by Lee and Mooney (2012). In contrast, we provide an overview of alginate hydrogel particle designs formed by various ionotropic gelation process routes. Many methods have been developed to produce alginate hydrogel particles of different sizes and morphologies to suit the needs of various applications. The synthesis routes to form alginate hydrogel particles generally consist of two processing steps i.e., dispersion of the alginate sol, followed by gelation of the sol with cations. Advances in dispersion and gelation methods over the past 10 years have enabled the production of particles that are both small and uniform or complex in their particle morphology. We systematically categorize and review these methods and compare them with respect to particle size, size distribution, and economics and efficiency of production. The shortcomings of the existing methods are discussed, where possible, with opportunities for further research in the field.

Particle size and morphology

The term 'particle' is used broadly throughout this paper without referring to any specific size or morphology. The specific terms used to describe the particle size and morphology of alginate hydrogels are described in Fig. 2. The classic morphologies are 'beads' and 'capsules'. Beads are defined as spheres that have diameters larger than 1000 μm; the immobilized cargo, either hydrophilic or lipophilic, is typically dispersed throughout the polymer matrix within the beads. Capsules, conversely, are spheres that comprise a distinct membrane that engulfs a liquid core containing the cargo.

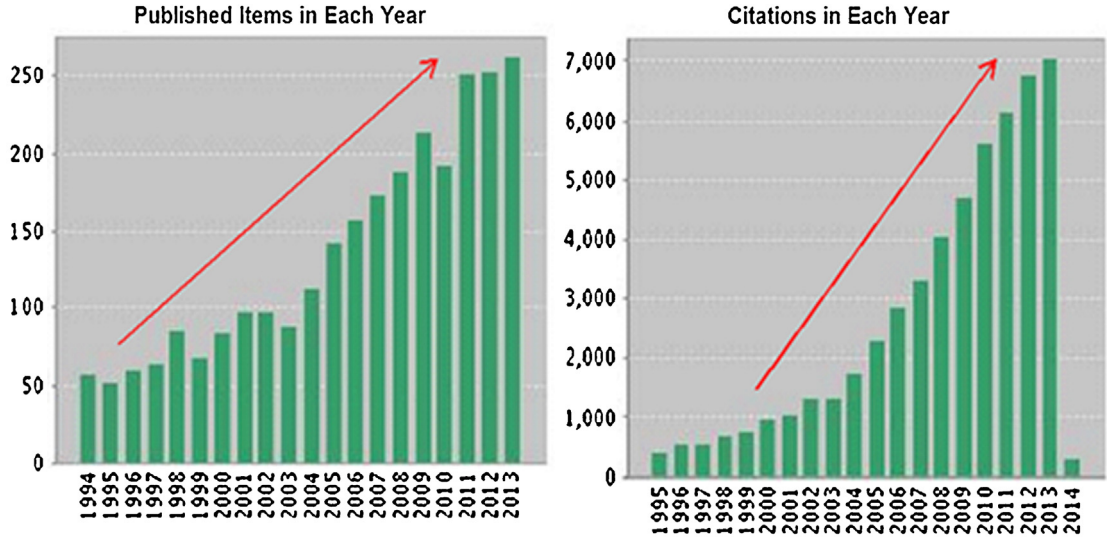

Fig. 1. Publication and citation data on alginate over the past 20 years (*source:* Web of Knowledge). Note, the data were obtained using keywords in the topic and search criteria as follows: {(Alginate) AND (Encapsulation OR Immobilization) AND (Bead OR Capsule OR Particle OR Sphere)}.

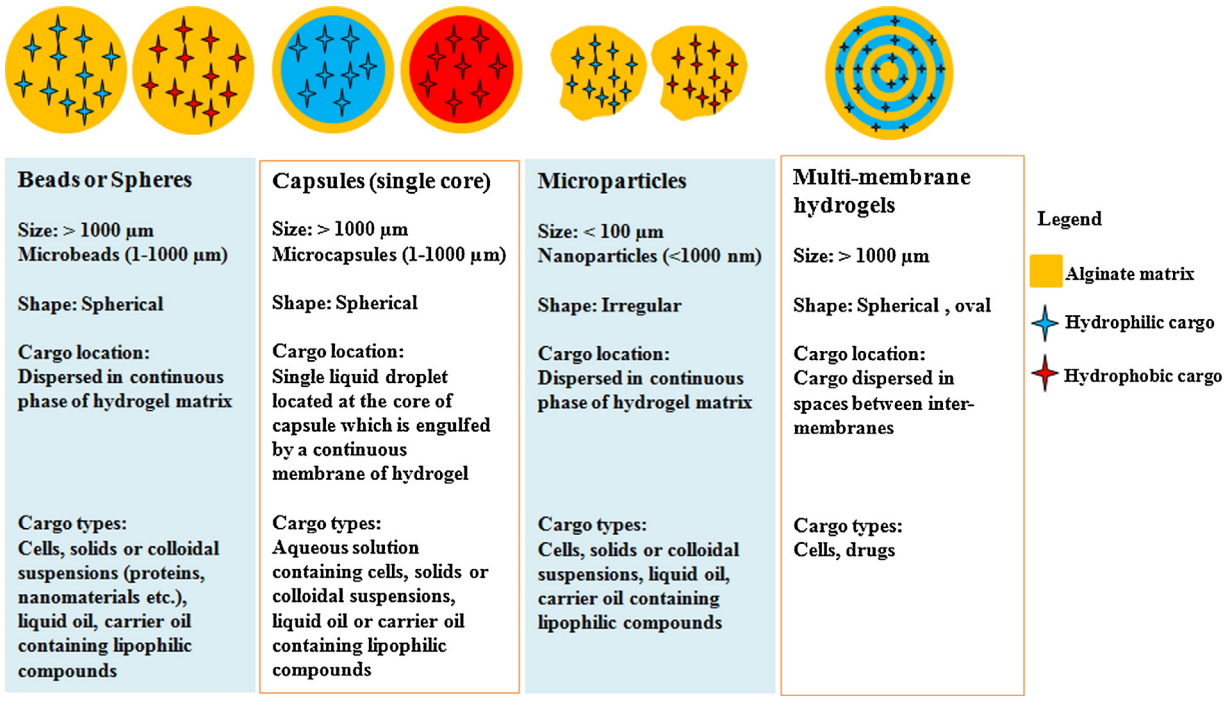

Fig. 2. Particulate designs of alginate hydrogels and their characteristics.

The liquid core can be either an aqueous or oil phase. The term 'microparticles' has also been used in the literature and generally refers to hydrogels that are smaller than 100 μm and irregular in shape. In 2008, another hydrogel design i.e., multi-membrane hydrogels was proposed by Ladet, David, and Domard (2008) and resembles an onion with multiple membranes separated by inter-membrane voids.

The choice of particle size and morphology depends on several factors including the immobilization objectives to fulfill the requirements of a target application (see Table 1). For example, when living cells are immobilized as biocatalysts, the capsule design may be preferred over the bead design to completely isolate the cells from the bulk medium (Yoo, Seong, Chang, & Park, 1996).

Table 1
Purpose of immobilization.

Purpose of immobilization	Common examples
Isolation or separation	Living cells or enzymes are immobilized for isolation from the bulk medium to ease separation for reuse or to mimic the confined environmental of their natural environment Incompatible food ingredients are separated to avoid degradation Chemical catalysts (e.g., metal oxide nanoparticles) are immobilized for ease of separation and reuse
Stabilization or protection	Living cells, enzymes, or food ingredients are protected from external environmental factors (e.g., shear, antibody, oxygen, moisture) to improve longevity and stability
Controlled release	Drugs, food ingredients, or pesticides are released in a controlled manner to improve product efficacy and safety
Improvement of product features	Active ingredients (e.g., fish oil) in the liquid state are converted into solid state for ease of handling The unpleasant taste or smell of ingredients is masked. The aesthetics of products are improved.

For the controlled release of drugs, the bead design may be preferred to manipulate drug diffusion through the hydrogel matrices (Østberg, Lund, & Graffner, 1994). Multi-membrane hydrogels can be considered if a pulsed release of drugs is desired (Dai et al., 2009). Microparticles smaller than 100 μm are preferred in food applications because large beads would otherwise affect the mouthfeel of the food product (Jin et al., 2007). Other important factors that may influence the choice of particle size and morphology include the technical complexity of the fabrication process, economics of production, and process scalability. These factors are discussed in the following sections.

Fabrication methods of alginate particles

The process used to fabricate alginate particles generally involves two basic steps: dispersion of a solution containing alginate or calcium ions into droplets and gelation to solidify the droplets. Herein, we categorize the dispersion methods into three main groups: liquid–air, liquid–liquid, and self-assembly methods (see Fig. 3). The gelation methods are divided into five basic mechanisms: external, internal, inverse, interfacial, and multi-step interrupted gelation. The details of these methods are described in the following sub-sections.

Dispersion methods

Liquid–air methods

Liquid–air methods generally involve the dispersion of an alginate solution into liquid droplets in the air phase using a nozzle system. The liquid–air methods can be divided into three main categories i.e., dripping, jetting, and atomization depending on the volumetric flow rate at which alginate is extruded from the nozzle. At low volumetric flow rates, the pendant drop of the alginate sol accumulated at the nozzle tip detaches as a discrete droplet once the accumulated gravitational force exceeds the surface tension. At higher volumetric flow rates, the extruded alginate sol merges into a continuous stream and experiences a propagation of sinusoidal perturbation waves along the way. Once the amplitude equals the

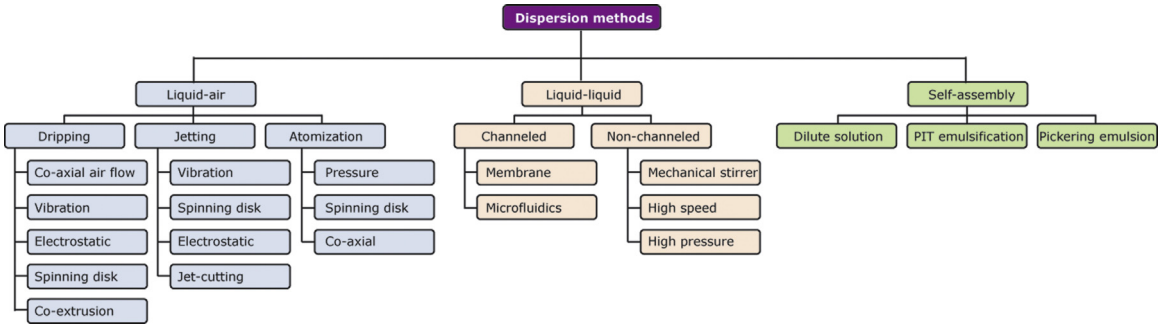

Fig. 3. Methods for dispersion of alginate sol prior to ionotropic gelation.

radius of the jet, the liquid jet breaks into cylindrical segments and eventually forms spherical droplets. When the volumetric flow rate is increased further, the liquid jet starts experiencing strong aerodynamics effects from the quiescent surrounding. The liquid jet atomizes into large droplets that, in turn, break down into smaller droplets.

Dripping method. The dripping method has been widely used to disperse alginate sol because of the simple apparatus required for bead production. Alginate liquid droplets are extruded through a nozzle into the air phase and fall dropwise into a gelling bath containing divalent cations (i.e., Ca^{2+}) to form cross-linked alginate beads. Detachment of the droplet from the nozzle tip is fundamentally governed by Tate's Law (Tate, 1864); however, a number of correction factors have been introduced to improve the accuracy of the model in predicting droplet size (Harkins & Brown, 1919; Chan, Lee, Ravindra, & Poncelet, 2009; Moradian & Mostaghimi, 2011). Droplets formed by the dripping method are typically highly uniform in size. However, the beads produced tend to be large (i.e., >1 mm) because of the accumulation of drop volume at the nozzle tip before droplet breakup (Chan et al., 2009; Lee, Ravindra, & Chan, 2013). This is not favorable in certain applications (e.g., encapsulation of living cells), whereby smaller beads are preferred to allow more efficient mass transfer across the bead boundary. Moreover, the process productivity in terms of bead number per unit time is low.

The bead size can be reduced or the bead productivity can be improved by promoting droplet breakup by applying external forces such as air-driven shearing (Smidsrød & Skja, 1990; De Vos, De Haan, & Van Schilfgaarde, 1997; Kontturi et al., 2011; Sugiura et al., 2007), forced vibration (Lee, Park, Park, & Yang, 1996; Seifert & Phillips, 1997; Brandenberger, Nüssli, Piech, & Widmer, 1999; Del Gaudio, Colombo, Colombo, Russo, & Sonvico, 2005), electrostatic potential (Lewinska, Rosinski, & Werynski, 2004; Klokk & Melvik, 2002; Nedović et al., 2001; Poncelet, Babak, Neufeld, Goosen, & Burgarski, 1999; Goosen et al., 1997; Bugarski et al., 1994), and centrifugal force (Senuma, Lowe, Zweifel, Hilborn, & Marison, 2000; Maeda, Onoe, Takinoue, & Takeuchi, 2012). These methods are used to produce monodisperse alginate beads with sizes in the submillimeter range (i.e., 100–1000 μm), and they can achieve higher bead production rates when compared with the simple dripping method.

The dripping method has also been used to produce liquid-core capsules with either aqueous- or oil-phase cargo. To produce aqueous-core capsules, the cargo is pre-mixed with divalent cations before its dropwise addition to an alginate solution. Upon contact with the alginate solution, divalent cations at the droplet surface cross-link with the alginate polymer to form a continuous shell that encases the aqueous core. This gelation mechanism is known as inverse gelation and is further detailed in Section 3.2.3. In another example, both the alginate solution and cargo (either aqueous or oil phase) can be pre-templated into compound droplets using a concentric nozzle (with alginate as the outer flow and cargo as the inner flow). The compound droplets with cargo as the inner phase are then collected in a gelling bath to solidify the shell. This method is known as co-extrusion and has been used extensively to produce large capsules. Several strategies have also been developed to reduce the capsule size including introducing vibration or electrostatic force to induce detachment of the compound droplets from the nozzle tip (Wang, Waterhouse, & Sun-Waterhouse, 2013; Sun-Waterhouse, Zhou, Miskelly, Wibisono, & Wadhwa, 2011).

The primary drawback of using the dripping method to form alginate beads or capsules is the low production rate in terms of bead number per unit time. The formation of droplets is on a drop-by-drop basis, and the maximum working flow rate is limited by the critical velocity before the extruded alginate liquid merges into a jet. The only way to increase the process productivity to an industrial scale is by using multiple nozzles. The maximum theoretical volumetric productivity for forming alginate droplets with a diameter of 2 mm is approximately 2.5 L/h/nozzle (Poncelet & Neufeld, 1996).

Jetting method. The productivity of alginate beads fabrication can be improved by increasing the volumetric flow rate of the alginate sol to form a laminar liquid jet. This liquid jet is allowed to breakup naturally to form alginate beads. Using this method, alginate droplets of diameters of 2 mm can be produced at approximately 16 L/h/nozzle (Poncelet & Neufeld, 1996). The size of the detached droplets in jetting regimes is primarily influenced by the inner diameter of the nozzle and the viscosity of the alginate sol (Zhang, Li, Zhang, & Xiu, 2007). Producing alginate particles with diameters of less than 1 mm is technically challenging. Several approaches have been attempted to reduce the alginate particle size by introducing external forces to induce droplet detachment including vibration (Heinzen, Marison, Berger, & von Stockar, 2002), electrostatic force (Serp, Cantana, Heinzen, von Stockar, & Marison, 2000), co-axial air flow (Wolters, Fritschy, Gerrits, & Van Schilfgaarde, 1992), and rotating wires (Prüße et al., 1998). By applying these external forces, alginate particles of diameters of 0.08–1 mm were successfully produced. Prüsse et al. (2008) compared the above-mentioned jetting methods and concluded that the use of rotating wires (also known as jet-cutting) was more effective in terms of workable viscosity, and achieving smaller particles formation, narrow size distributions, and higher productivity. However, during the jet-cutting process, a small portion of the liquid jet is wasted by the rotating filaments. This loss is unavoidable but can be minimized by synchronizing the linear velocity of the liquid jet and the rotating wires.

Atomization method. The atomization method typically involves the dispersion of an alginate sol into aerosols that can be gelled to form microparticles. There are three mechanisms by which the

alginate sol can be atomized: (i) pressure nozzle, (ii) co-axial nozzle, and (iii) rotary atomization. In pressure nozzle atomization, the alginate sol is extruded at a high velocity into quiescent air and fragmented into droplets by the drag force between the fluids. In co-axial atomization, the alginate sol is extruded by a high-velocity, co-flowing stream of air. The co-axial airflow exerts drag on the alginate sol liquid surface, which then disintegrates into tiny droplets. The breakup of the alginate liquid thread and droplet occurs when the dynamic pressure of the co-axial gas exceeds the pressure inside the liquid by a fixed extent. In rotary atomization, the alginate sol is fed onto a disk or wheel that rotates at a high speed. The resulting centrifugal force spreads the alginate sol onto the disk into a thin sheet. The alginate sol eventually discharges at the peripheral edge of the disk as tiny droplets.

The atomization method is straightforward and well established, and it uses spray nozzles that are commonly employed in industry for spray drying. The mean size of the particles formed can be altered over a wide range, typically from 10 to 100 μm (Cui, Goh, Park, Kim, & Lee, 2001; Herrero, Del Valle, & Galán, 2006; Hariyadi et al., 2010; Chan, Lim, Ravindra, Mansa, & Islam, 2012). In addition to conventional atomization, Barba, d'Amore, Cascone, Lamberti, and Titomanlio (2009) used ultrasonic atomization to produce alginate microparticles with mean sizes of 50–110 μm. Generally, the size distribution of particles formed by the atomization method is broad because of the chaotic disruption of the liquid thread or droplet under turbulent conditions. Nevertheless, this method is industrially attractive because of its high productivity and is therefore useful in applications that do not require stringent control over the size distribution (e.g., bio-sorbents for water treatment).

Liquid–liquid methods

Liquid–liquid methods typically involve the dispersion of an alginate sol in a continuous phase of immiscible liquid, which forms a template of water-in-oil (W/O) emulsion prior to gelling. These methods are also known as emulsification methods. The oil phase is typically vegetable or mineral oil. The emulsification method is commonly used to produce small alginate particles, generally with a mean size ranging from 1 to 1000 μm. The common factors influencing the droplet size profile in the emulsification method are the alginate concentration (or viscosity of the alginate sol), gelling conditions, and surfactant formulation.

Channeled emulsification can disperse the droplets. In such an approach, the droplets are formed one drop at a time from one channel, and the diameter of the resulting droplets is limited by the length of the channel. Monodisperse droplets are formed using uniform channel openings, as used in microfluidics and membrane emulsification. Alternatively, droplets can be dispersed by non-channeled emulsification, which involves the chaotic mixing of immiscible liquids through mechanical stirring by a rotor/stator or high-pressure homogenizers. The resulting droplets have a broader size distribution than those formed via channel emulsification because a liquid–liquid interface is created through flow turbulence. W/O emulsions can form microbeads immobilizing hydrophilic or large cargos, whereas O/W/O emulsion templates can form microbeads loaded with lipophilic cargos.

Non-channeled emulsification. The classic emulsification method is the non-channeled method, which uses mechanical stirring, typically at stirring speeds below 1000 rpm (Poncelet et al., 1992, 1999). The size of the microbeads formed is inversely proportional to the energy input during agitation. This method can produce microbeads with a large range of mean sizes between 20 and 1000 μm, but the particle size distribution is generally broad and polydisperse. Other non-channel methods, such as high-speed (Ding & Shah, 2009; Lupo, Maestro, Porras, Gutiérrez, & González, 2014) and high-pressure homogenization (Ding & Shah, 2009; Kidane et al., 2002), are capable of producing microbeads with smaller mean sizes and size distributions that are narrower and unimodal compared with those obtained by mechanical stirring. Paques, van der Linden, van Rijn, and Sagis (2013) used rotor speeds between 5000 and 10,000 rpm to prepare alginate O/W emulsions and subsequently cross-linked the alginate droplets using calcium chloride nanoparticles to produce alginate microbeads with mean sizes of approximately 1 μm; some sub-micrometer-sized beads were also produced.

Channeled emulsification. Over the last decade, controlled emulsification in micro-channels using microfluidic devices has generated research interest because this method allows for precise control over the size, morphology, and properties of the droplets formed. For microbeads production, the alginate sol is typically emulsified into a co-flowing stream of immiscible liquid in a micro-channel in which the flow rates of both liquids are precisely controlled. The alginate droplets formed can be gelled inside or outside the channel. The mean size of the microbeads formed can be varied over a wide range from 40 to 2000 μm (Sugiura et al., 2005). Owing to the micrometer-sized capillary, liquid flows in microfluidic devices are completely laminar, and the resulting droplets or microbeads have very narrow size distributions with coefficients of variance of less than 5% (Zhang et al., 2006; Capretto, Mazzitelli, Balestra, Tosi, & Nastruzzi, 2008; Huang, Lai, & Lin, 2006; Choi et al., 2007; Tan & Takeuchi, 2007). Ren, Ju, Xie, and Chu (2010) and Liu et al. (2013) recently fabricated oil-core microcapsules in a microcapillary microfluidic device using an O/W/O emulsion as a template. Monodisperse microcapsules were formed with sizes of 200–500 μm. Furthermore, the inner oil phase of the microcapsules could be removed by extraction with propanol to prepare hollow microcapsules or aqueous-core microcapsules (Liu et al., 2013). Another channeled emulsification method worth highlighting is membrane emulsification, which can produce small microbeads with very narrow size distributions on an industrial scale (Liu et al., 2003; You et al., 2001).

Self-assembly methods

As deduced from the previous sections, the formation of alginate droplets typically involves external forces to break up the bulk alginate sol into smaller pieces. To produce smaller droplets, additional energy is required to disrupt the bulk alginate sol. Bottlenecks in reducing droplet size to the nano-range involve geometric limitations of the dispersing device, the physical properties of the alginate sol, and insufficient energy supplied to the dispersing device. Recent advances in interfacial phenomena in colloidal systems have facilitated the formation of alginate particles via self-assembly without large inputs of energy or sophisticated device requirements. Nano-sized alginate particles can also be produced using this method. The key to these approaches is the formation of alginate colloidal particles through chemical processes before these building blocks are allowed to self-assemble into alginate nanoparticles. To our knowledge, three types of self-assembly processes have been developed to date to produce alginate nanoparticles from (i) dilute solutions, (ii) microemulsions, and (iii) Pickering emulsion templates.

Dilute solutions. In the dilute solution method, alginate nanoparticles are formed by dropping a dilute solution of calcium chloride (e.g., 0.008% w/v) into a dilute solution of alginate polymer (e.g., 0.05% w/v) under vigorous stirring. The ionic interactions between the components lower the apparent viscosity of the system by forming micro-domains with high local concentrations of alginate. The calcium divalent cations are involved in the rearrangement of the alginate molecules to a pre-gel state. As a result, alginate nanoparticles with mean sizes of 70 nm and a polydispersity

index (PDI) of 0.46 were successfully produced (Sarei, Dounighi, Zolfagharian, Khaki, & Bidhendi, 2013). The alginate nanoparticles were successfully used to encapsulate doxorubicin and diphtheria toxoid with a loading capacity of greater than 50 mg of drug per 100 mg of alginate (Rajaonarivony, Vauthier, Couarraze, Puisieux, & Couvreur, 1993), displaying a prolonged *in vitro* release profile.

Microemulsions. Calcium alginate nanoparticles can also be produced from microemulsions without large inputs of mechanical energy (Machado et al., 2012). Unlike other emulsification techniques that involve shear force, droplet formation in microemulsions is spontaneous through self-assembly. For example, decane was emulsified into an aqueous solution of sodium alginate with tetraethylene glycol monododecyl ether ($C_{12}E_4$) as the nonionic surfactant. The temperature of the primary O/W emulsion was then increased from 14 to 40 °C to increase the solubility of $C_{12}E_4$ in decane, which led to phase inversion of the primary emulsion into W/O microemulsions that consisted of nanodroplets of alginate sol. Gelation of the alginate nanodroplets could be achieved by injecting a calcium chloride solution into the nanoemulsion. Alginate nanoparticles 100–200 nm in diameter with a PDI of less than 0.25 were successfully obtained and used to encapsulate DNA at an efficiency of up to 99% (Machado et al., 2013).

Pickering emulsions. Alginate capsules containing oil cores have been successfully prepared from Pickering emulsion templates. In this method, pre-gelled Ca-alginate nanoparticles were first formed and used as a solid emulsifier to deposit onto the oil droplets to stabilize the O/W emulsion. The solvent was then removed to shrink the oil droplets and interlock the Ca-alginate nanoparticles present at the interface to form a continuous shell. This technique successfully produced alginate oil-core capsules of 35–820 nm in diameter with a PDI of less than 0.25. These nanocapsules were successfully used to encapsulate testosterone (Bhowmik, Sa, & Mukherjee, 2006), turmeric oil (Lertsutthiwong, Noomun, Jongaroonngamsang, Rojsitthisak, & Nimmannit, 2008), and an ethanolic extract of *Phyllanthus amarus* (Deepa, Sridhar, Goparaju, Reddy, & Murthy, 2012). Recently, our group developed a method to produce alginate oil-core capsules from O/W Pickering emulsion stabilized by $CaCO_3$ nanoparticles. In this method, oil was emulsified into an aqueous dispersion of $CaCO_3$ nanoparticles to allow self-assembly of the $CaCO_3$ nanoparticles at O/W interface. The $CaCO_3$ nanoparticles were subsequently dissolved and reacted with the alginate polyanions to form a continuous shell surrounding the oil core (Leong, Tey, Tan, & Chan, 2015). This technique can be used to produce alginate microcapsules with diameters of a few to several hundred micrometers depending on the emulsification tool used.

Methods to reduce particle size

The size of the particles formed by liquid–liquid methods can be reduced by using a solvent diffusion method. In a typical example, the alginate sol is mixed with a solvent before dispersing the sol into droplets in an immiscible phase. Upon droplet formation, the solvent is removed, thereby causing the alginate droplets to shrink. The addition of divalent cations causes the alginate droplets to gel. Rondeau and Cooper-White (2008) coupled this technique with microfluidics to fabricate alginate nanoparticles with a narrow size distribution and sizes ranging from 10 to 300 nm. Dilute concentrations of alginate sol were dispersed in a continuous phase of dimethyl carbonate, which was partially miscible; this caused shrinkage of the alginate droplets and condensation of the polymer. The nanodroplets then gelled to form nanoparticles. In another study, Reis, Ribeiro, Houng, Veiga, and Neufeld (2007) produced alginate nanoparticles using a rotor speed of 1600 rpm and then shrunk the gelled particles with dehydrating solvent. The resulting particles featured diameters ranging from 270 nm to 2.7 μm and a unimodal size distribution. The nanoparticles successfully encapsulated insulin with an encapsulation efficiency of 82.5%. Sugaya, Yamada, Hori, and Seki (2013) used a polar organic solvent to shrink alginate droplets produced by microfluidics. Following gelation, the resulting alginate beads were less than 10 μm in diameter.

Gelation mechanisms

After the dispersion of the alginate sol into droplets, the next step is to gel the droplets. This is typically achieved by cross-linking the alginate polymer chains with divalent cations. Divalent cations are believed to bind to the guluronate blocks of the alginate polymer chains, forming an 'egg-box' structure. Alginate displays varying affinities toward different cations (Haug, 1961; Haug & Smidsrod, 1970); the degree of affinity of alginate toward the following cations decreases as Pb > Cu > Cd > Ba > Sr > Ca > Co, Ni, Zn > Mn (Mørch, Donati, Strand, & Skjåk-Bræk, 2006). However, calcium (Ca^{2+}) is the most commonly used cation for ionotropic gelation of alginate because of its non-toxicity compared with other cations.

Among the available calcium sources, calcium chloride ($CaCl_2$) is the most commonly used salt for gelation. $CaCl_2$ is readily soluble in water, and thus Ca^{2+} ions in solution can cross-link with alginate droplets instantaneously to form hydrogel particles. Insoluble calcium salts, e.g., calcium carbonate ($CaCO_3$), can also be used when gradual or controlled cross-linking is desired. The cross-linking process can be initiated by reducing the pH to dissociate the insoluble calcium salt.

Various types of calcium salts can be used to gel alginate particles and can be divided into three main categories: readily soluble, partially soluble, and insoluble. A list of calcium salts and their solubilities are given in Table 2. Readily soluble calcium salts, e.g., $CaCl_2$, can cause spontaneous gelation and are therefore used to prepare alginate particles via external, inverse, or multi-step interrupted gelation mechanisms. The use of partially soluble calcium salts, e.g., calcium sulfate ($CaSO_4$), allows for slow dissociation of Ca^{2+}; however, controlling the gelation kinetics is difficult (Kuo & Ma, 2001).

In contrast, insoluble calcium salts (e.g., $CaCO_3$) are commonly used to prepare alginate particles through internal gelation mechanism, whereby the salt is first dispersed in the alginate sol before emulsification. Upon emulsification, the gelation process can be initiated by solubilizing the salt when required, thus liberating Ca^{2+} for cross-linking with the local alginate polymer chains. Liberation of the salt can be initiated by reducing the pH using a gelling initiator such as the introduction of an acid or UV irradiation in the case of a photo-acid generator. Generally, the pH is reduced by adding acid, such as glacial acetic acid, to an emulsion. A more gradual gelation can be achieved by pre-adding the glucono deltalactone to the alginate sol containing the insoluble salt, where it slowly dissociates the salt (Amici, Tetradis-Meris, de Torres, & Jousse, 2008; Morimoto, Tan, Tsuda, & Takeuchi, 2009). Recently, a photo-acid generator, diphenyliodonium nitrate, was used to initiate the gelation of alginate beads (Liu et al., 2013). The photo-acid generator dissociated and liberated hydrogen ions (H^+) upon UV irradiation. This type of gelling initiator allows for the in situ gelling of the alginate droplets without migration of the acidic solution from the oil phase across the boundary of the alginate droplets, which can deform the beads if the conditions are not optimum. Various gelation mechanisms have been demonstrated in previous research. In addition to solidifying the alginate sol, the gelation mechanism controls the homogeneity of the gel formed and/or creates more complex particle morphologies that cannot be templated by a dispersion method. The five known gelation mechanisms to

Table 2
Solubility of different calcium salts in water at room temperature.

Calcium salt	Chemical formula	Solubility		Reference
		Solubility in water (g/100 mL) at room temperature	Classification	
Calcium acetate	$Ca(CH_3OO)_2{\cdot}H_2O$	34.7	Soluble	Miyazaki, Ohtsuki, and Tanihara (2003)
Calcium chloride	$CaCl_2{\cdot}6H_2O$	74.5	Soluble	Miyazaki et al. (2003)
Calcium citrate	$Ca_3(C_6H_5O_7){\cdot}4H_2O$	0.096	Partially soluble	Perry (2011)
Calcium carbonate	$CaCO_3$	0.00066	Insoluble	Haynes (2013)
Calcium gluconate	$Ca(C_6H_{11}O_7)_2$	3.3	Partially soluble	Igoe (2011)
Calcium lactate	$Ca(C_3H_5O_3)_2$	3.4	Soluble	Igoe (2011)
Calcium phosphate	$Ca_3(PO_4)_2$	0.00012	Insoluble	Haynes (2013)
Calcium sulfate	$CaSO_4$	0.205	Partially Soluble	Haynes (2013)

date include external, internal, inverse, interfacial, and multi-step interrupted gelation.

External gelation

External gelation is a classical and perhaps the most widely used mechanism of ionotropic gelation to form alginate hydrogels. In this process, Ca^{2+} ions are introduced externally into the discrete alginate droplets, which can be formed through liquid–air or liquid–liquid methods. Ca^{2+} ions diffuse inward into the interstitial spaces between the alginate polymer chains to initiate cross-linking. In a typical example, an alginate sol containing cargo is extruded and dropped into a gelling bath containing Ca^{2+} ions (see Fig. 4). Upon contact, the Ca^{2+} ions begin to cross-link with the alginate polymer chains at the periphery of the alginate droplet. This results in the initial formation of a semi-solid membrane encasing the droplet with a liquid core (Zhang et al., 2006). Extended immersion of the droplets in the hardening bath allows for further diffusion of Ca^{2+} across the membrane via a concentration gradient, subsequently leading to the solidification of the droplet core. As a result, an alginate bead is formed in which the cargo is entangled randomly within the cross-linked matrices. For capsule formation, the cargo (aqueous or oil core) is co-extruded with the alginate sol and dropped into a gelling bath. The alginate sol engulfing the liquid core is subsequently gelled, forming a capsule with an insoluble shell. A typical example of alginate capsules produced via external gelation (Stark et al., 2003) is shown in Fig. 5(c).

Because cross-linking is initiated at the periphery of the alginate droplet, the alginate polymer chains are drawn toward

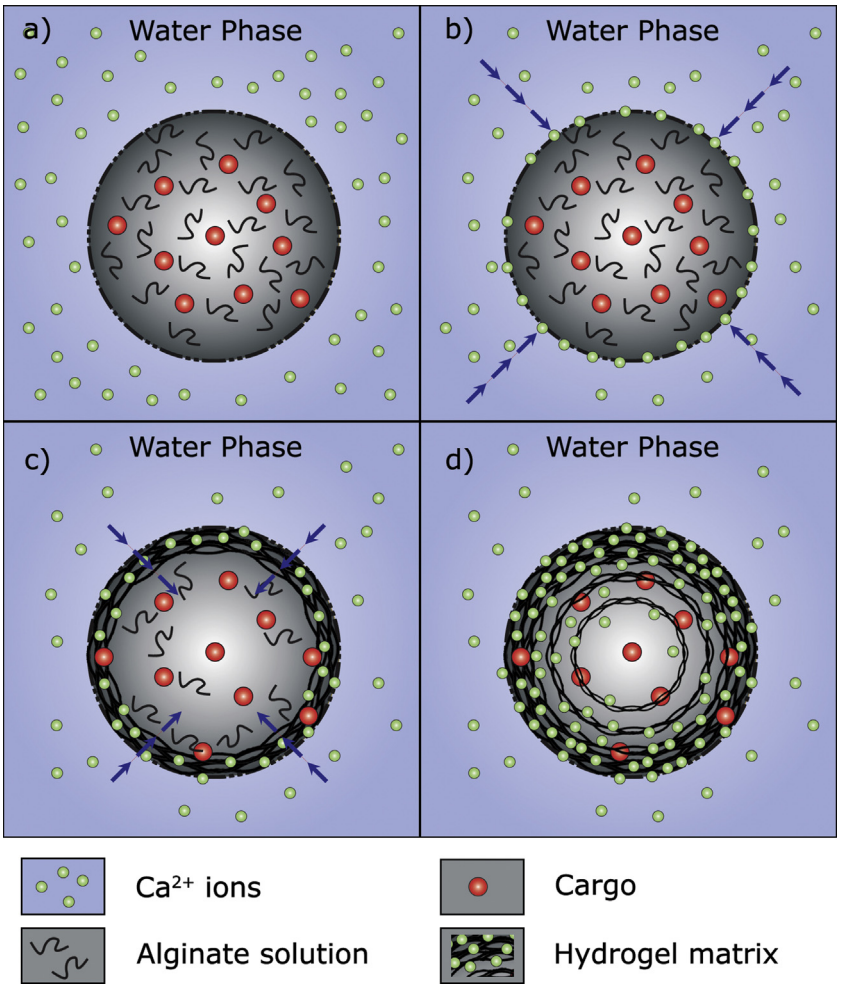

Fig. 4. Mechanism of external gelation for bead formation: (a) alginate droplet in contact with calcium solution, (b) inward diffusion of calcium ions, (c) inward gelation of droplet, and (d) completed gelation.

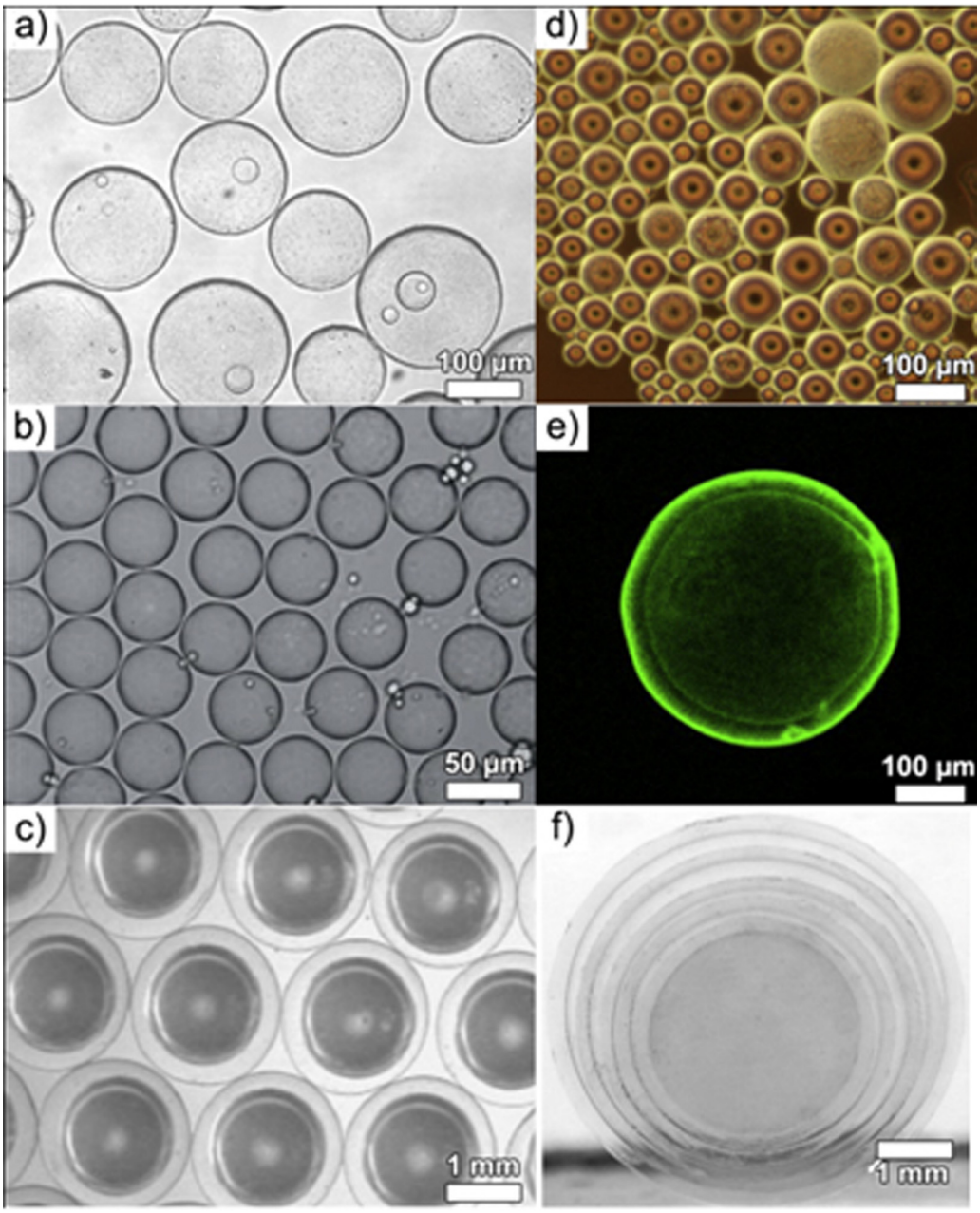

Fig. 5. Alginate particles formed via various gelation mechanisms. Alginate beads produced via (a) emulsification coupled with internal gelation (Song et al., 2013) and (b) microfluidics coupled with internal gelation (Reproduced from Liu et al. (2015) with permission from The Royal Society of Chemistry). Capsule structures of aqueous- and oil-core alginate particles formed via (c) co-extrusion coupled with external gelation (Stark et al., 2003), (d) interfacial gelation performed on an O/W Pickering emulsion and (e) internal gelation performed on an O/W/O emulsion generated in microfluidics (Liu et al., 2013). Multi-membrane alginate capsules fabricated using (f) multi-step interrupted gelation (Reproduced from Dai et al. (2009) with permission from The Royal Society of Chemistry).

the periphery to enable cross-linking (Skjåk-Bræk, Grasdalen, & Smidsrød, 1989). As cross-linking proceeds, the pool of alginate chains within the droplet core becomes diluted over time. When cross-linking is complete, more Ca-alginate networks will have formed at the periphery compared with the center of the bead. The inhomogeneity of the gel formed imparts a higher elastic modulus to the bead and inhibits access to small hydrophilic cargo molecules, thereby improving the encapsulation efficiency (Chan, Lee, & Heng, 2006).

However, the inhomogeneous gel may limit the application of external gelation in encapsulating living cells. A denser peripheral matrix inherently inhibits solute exchange between the bead core and the external environment (Radovich, 1985). Essential life-supporting elements, such as oxygen and nutrients, cannot be effectively transported to the center of the bead because of an increased diffusion barrier. In addition, toxic metabolic wastes are difficult to secrete from the bead. As a result, living cells entrapped at the center of the bead may eventually die.

Several approaches have been adopted to overcome these shortcomings. In a simple approach, living cells were encapsulated in small beads to reduce the diffusion pathway (Annesini et al., 2000). Another method involved the addition of low concentrations of Na^+ or Mg^{2+} ions during gelation to competitively bind with Ca^{2+} ions in alginate (Skjåk-Bræk et al., 1989), resulting in a homogenous Ca-alginate matrix. A homogenous hydrogel matrix can also be formed using an internal gelation mechanism, which is discussed in the next section. Despite the issue of inhomogeneity, external gelation remains a favorable method because of its simple one-step process

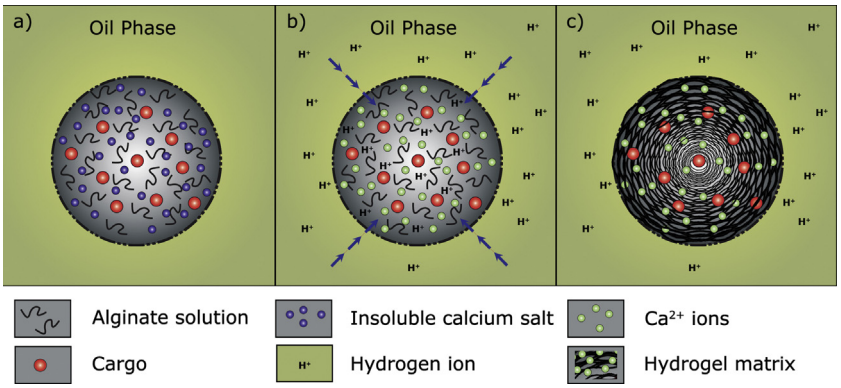

Fig. 6. Mechanism of internal gelation for bead formation: (a) dispersion of alginate droplet in oil, (b) addition of acid to dissolve the calcium salt, and (c) localized gelation of droplet.

to fabricate different particle morphologies (i.e., beads or capsules) without the need for a gelling initiator.

Internal gelation

In the late 1980s, research was channeled toward reducing the bead size and increasing the process throughput for industrial applications. The emulsification method was examined, whereby an alginate sol is dispersed into micro-droplets that were subsequently gelled via external gelation by adding a calcium solution into the emulsion. The bead productivity increased, but the beads tended to coagulate into large masses because of difficulties in controlling the gelling conditions in the emulsion. The gelation mechanism was then modified by controlling the liberation of Ca^{2+} ions for gelation (Lencki, Neufeld, & Spinney, 1989). In this technique, an alginate sol containing a dispersion of insoluble calcium carbonate particles was first emulsified into an oil phase (see Fig. 6). Subsequently, acetic acid was added to the emulsion to lower the pH, thereby inducing the dissolution of calcium carbonate into Ca^{2+}, carbon dioxide, and water. Subsequently, the released Ca^{2+} ions cross-linked with the alginate polymer chains internally. Because cross-linking and gelation were initiated and occurred inside the alginate droplet, this gelling mechanism was named internal gelation. This gelling mechanism is only used when the alginate sol is dispersed using an immiscible liquid. A typical example of alginate beads produced via internal gelation (Song, Yu, Gao, Liu, & Ma, 2013) is shown in Fig. 5(a).

The dissolution of insoluble salts can be precisely controlled and altered by manipulating the pH (Poncelet et al., 1995). Control over the gelation process has enabled the design of particle morphologies and matrix density. Microbeads with sizes of 20–1000 μm (see Fig. 5(b)) have successfully been produced under various process conditions (Poncelet et al., 1995; Ribeiro, Silva, Ferreira, & Veiga, 2005; Liu et al., 2015). Recent work by Liu et al. (2013) revealed that microcapsules can also be formed through internal gelation of a double-emulsion template (i.e., O/W/O) that is generated in a microfluidic device (see Fig. 5(e)).

In contrast to particles formed via external gelation, the gel structure produced by internal gelation is more homogenous (Poncelet et al., 1995). The insoluble calcium salt is dispersed homogenously within the alginate droplet, and dissolution of the insoluble calcium salt within the alginate droplet results in a more uniform polymer distribution across the dispersed droplet when acid is introduced. Therefore, cross-linking between the alginate polymer chains and Ca^{2+} ions proceeds homogenously within the dispersed droplets. Moreover, the liberation of carbon dioxide facilitates the formation of a more porous and looser gel matrix compared with those obtained via external gelation. These features are advantageous in the encapsulation of cargo that requires efficient solute exchange with the external environment such as living cells and enzymes for tissue engineering or biocatalytic applications.

Inverse gelation

In another gelation mechanism, an aqueous solution or liquid oil containing Ca^{2+} ions is extruded dropwise into an alginate solution bath. Upon contact, the Ca^{2+} ions diffuse to the outer periphery of the droplet and ionotropically cross-link with the alginate polymer chains at the droplet interface (see Fig. 7). The ionotropic gelation process continues until the free Ca^{2+} ions are depleted. At the end of the process, the initial liquid droplet is engulfed by a continuous semi-permeable Ca-alginate membrane. In contrast to external gelation, the Ca^{2+} ions diffuse outward from the discrete core into

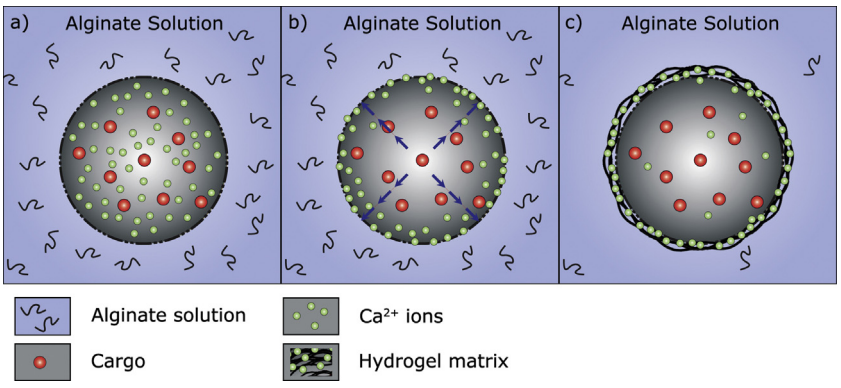

Fig. 7. Mechanism of inverse gelation for liquid-core capsule formation: (a) droplet containing calcium ions in contact with alginate solution, (b) outward diffusion of calcium ions, and (c) gelation at droplet interface.

an external pool of alginate sol; this process is therefore known as inverse gelation. This gelling mechanism is only used when the droplets of liquid core (aqueous or oil) are generated by liquid–air methods. The most attractive feature of this gelation mechanism is the simple apparatus used, in which capsules can be produced without using concentric nozzle.

Inverse gelation was first used to encapsulate monkey kidney cells in 1988 (Nigam, Tsao, Sakoda, & Wang, 1988). Because of the simplicity of the method, its application has widened to encapsulate plant cells, bacteria, whey protein, enzymes, and lipophilic substances such as linalool, carvone, and vegetable oils (López, Maudhuit, Pascual-Villalobos, & Poncelet, 2012; Pascual-Villalobos & López, 2013; Abang, Chan, & Poncelet, 2012). Liquid-core capsules with diameters ranging from 0.4 to 7.8 mm have been successfully fabricated using different dispersion techniques such as dropping (Sakoda, Nigam, & Wang, 1990; Nyende, Schittenhelm, Mix-Wagner, & Greef, 2003), co-axial flow (Dembczynski & Jankowski, 2000), and atomization (Sasaki, Kurayama, Ida, Matsuyama, & Yamamoto, 2008). The thickness of the resulting Ca-alginate membrane is controllable between 0.01 and 1.85 mm by varying the concentrations of Ca^{2+} ions and the alginate solution to suit the target application (Tomida, Nakamura, Yoshitomi, & Kiryu, 1993; Blandino et al., 2001).

However, the aqueous-core capsules produced from inverse gelation are not usually spherical because the shape of the discrete droplets can easily be deformed upon impact with the surface of the alginate bath, which typically has a higher viscosity than the aqueous droplets. The ionotropic gelation occurs faster than the liquid droplets' recovery to a spherical shape. Two approaches can be used to overcome this problem: increasing the viscosity of the dispersed droplets and/or reducing the surface tension of the alginate solution. Thickeners, such as carboxymethyl cellulose, hydroxypropyl methylcellulose, polyethylene glycol, collagen, maltodextrin, starch, xanthan gum, or sucrose, can be added to the aqueous solution to increase its viscosity prior to dispersion (Patel, Pusch, Mix-Wagner, & Vorlop, 2000; Koyama & Seki, 2004a, 2004b; Kim, Lee, & Park, 1998; Torre et al., 2000; Bezbaruah, Shanbhogue, Simsek, & Khan, 2011; Nussinovitch, Gershon, & Nussinovitch, 1996). Surfactants, such as Tween, Span, or Nonoxynol NP95, can be added to the alginate solution to reduce its surface tension (Pascual-Villalobos & López, 2013; Oh & Park, 1998). A low concentration of alginate solution (typically lower than 1% w/v) is usually used to minimize droplet distortion.

A recent study by Martins, Renard, Davy, Marquis, and Poncelet (2015) demonstrated that deformation of oil-core capsules could be inhibited by introducing O/W/O emulsions into the alginate solution, in which the water phase of the multiple emulsions contained Ca^{2+}. The primary W/O emulsion was passively transferred into the alginate solution with the aid of a surfactant. Upon contact with the alginate solution, Ca^{2+} from the water phase of the primary emulsion rapidly formed a continuous Ca-alginate membrane engulfing the oil core. This technique helps avoid the direct impact of droplets onto the alginate solution and thus eliminates deformation. In this work, capsules as small as 340–700 μm were successfully produced. Oil-core capsules in this size range cannot be produced using existing liquid–air techniques because of the weak kinetic force that prevents the dispersed drops from penetrating the alginate solution.

However, several difficulties are inherent to this method. The complex formulation of the core and alginate solutions often requires some trial and error and careful optimization. The weak Ca-alginate membrane formed by low alginate concentrations may cause the capsules to break during post-production handling or during application. Finally, the constant diffusion of free Ca^{2+} ions from the liquid droplets makes control of the gelation process difficult and may cause agglomeration between capsules or gelling of the entire alginate bath.

Interfacial gelation

A novel gelation mechanism that occurs at the interface of immiscible liquids was recently reported (Leong et al., 2015), as shown in Fig. 8. An oil cargo was first emulsified into an aqueous dispersion of insoluble $CaCO_3$ nanoparticles. The $CaCO_3$ nanoparticles were allowed to self-assemble at the interface to form an O/W Pickering emulsion. The Pickering emulsion was allowed to phase separate before the top layer (which was rich in oil droplets) was harvested and re-dispersed into an alginate sol. Then, acetic acid was added to the emulsion system to induce the dissolution of the $CaCO_3$ nanoparticles at the O/W interface. Upon dissolution, Ca^{2+} ions were released and cross-linked with alginate polymers at the O/W interface to form a continuous membrane that engulfed the oil core. Unlike previous gelation mechanisms, the Ca^{2+} ions were released and cross-linked in situ with alginate at the O/W interface. Therefore, this type of gelation mechanism was termed 'interfacial gelation'.

This interfacial gelation is particularly advantageous for forming oil-core alginate capsules when compared with other gelation mechanisms. The process does not require a physical nozzle to pre-template the alginate sol into compound drops to form the capsule morphology. Instead, the capsule morphology is formed through the self-assembly of $CaCO_3$ nanoparticles at the oil–water interface. Any emulsification tool can be used to form the O/W Pickering emulsion template. The size of the alginate capsule is no longer dependent on the nozzle diameter (unlike in the co-extrusion method), but the size of the capsule is inversely proportional to the energy input during the emulsification step. Alginate capsules with diameters of a few to several hundred micrometers can be produced depending on the emulsification method used. Also, the productivity of the process is not limited by the physical nozzle because oil droplets are not formed on a 'drop-by-drop' basis. Large amounts of oil droplets can be produced spontaneously using available industrial-scale emulsification tools (e.g., mechanical stirrer, Shirasu porous glass membrane, or high-pressure homogenizer). The size and size distribution of the capsules formed depend on the choice of the emulsification tool. Fig. 5(d) shows oil-core microcapsules produced using a high-speed homogenizer as the emulsification tool (Leong et al., 2015).

Multi-step interrupted gelation

Recently, Ladet et al. (2008) proposed an onion-like multi-membrane hydrogel structure made of Ca-alginate via a multi-step interrupted gelation. This hydrogel structure was prepared by interrupting and repeating an external gelation mechanism in which the membrane layers were sequentially formed from the outer toward the inner core of an alcogel template (see Fig. 9). Briefly, an alginate alcogel template was alternately immersed in a calcium chloride solution and water bath. The full mechanism of the formation of the alginate multi-membrane hydrogel via external gelation was not explained in the study, but it is likely that the Ca^{2+} ions in the external calcium chloride solution first diffuse through the periphery of the alginate alcogel template before cross-linking and forming a gel membrane layer. The gelation process is then interrupted or stopped by removing the gel template from the calcium solution. The template is then immersed in water to allow the formation of a void between the alginate gel and alginate solution. To form a second layer of gel membrane, the alcogel template is re-immersed in the calcium solution. By repeating this process several times, a multi-membrane hydrogel with inter-membrane voids can be generated. The kinetics of Ca^{2+} diffusion into the alcogel template is crucial because the membranes can only be formed with low concentrations of calcium chloride. This process is more

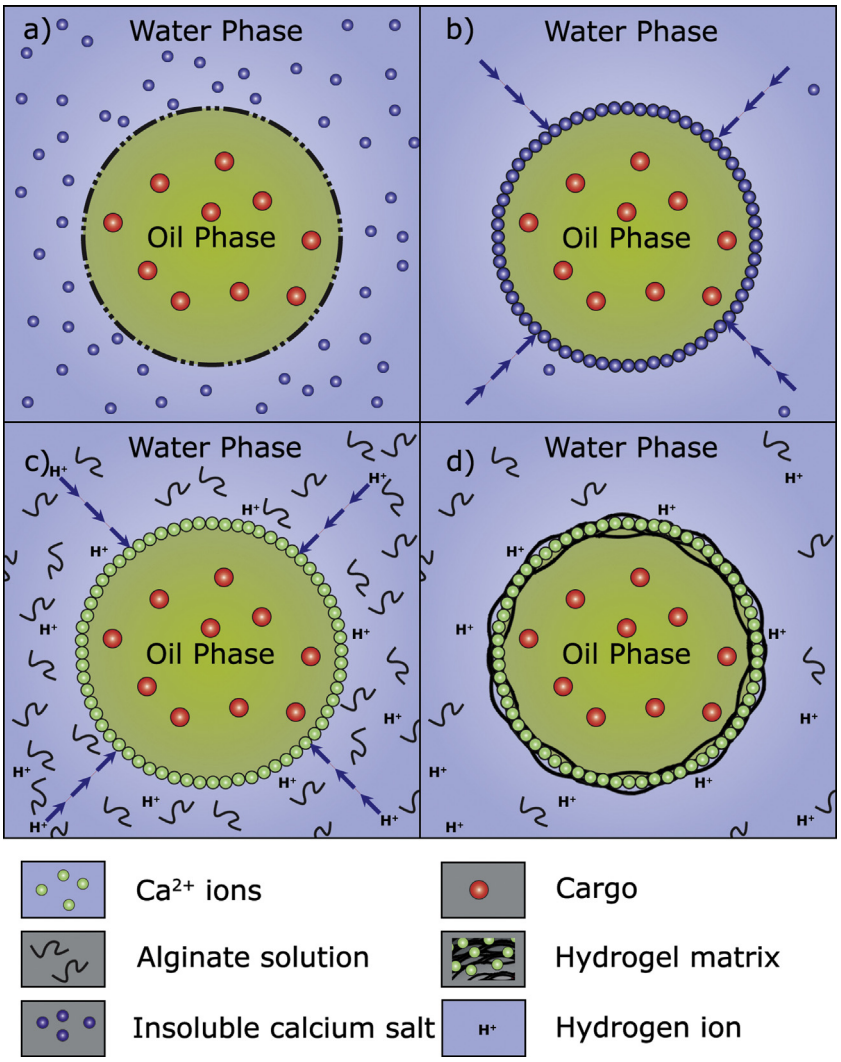

Fig. 8. Mechanism of interfacial gelation for oil-core capsule formation: (a) oil droplet emulsified in a dispersion of $CaCO_3$, (b) self-assembly of $CaCO_3$ at the interface, (c) addition of the alginate solution followed by acid to dissolve $CaCO_3$, and (d) gelation at the droplet interface.

precisely termed multi-step interrupted external gelation, where 'external' indicates the direction of cross-linking, i.e. from the outer to the inner core of the template.

This complex hydrogel architecture represents a significant deviation from the traditional particle morphologies, which are primarily limited to the bulk hydrogel or core–shell structure. The inter-membrane voids may be well suited for cell or drug introduction (Ladet et al., 2008), thereby widening the potential of such a design for applications in cell culture, advanced drug delivery, and investigation of cell–cell interactions (Dai et al., 2009). The potential of such a design in tissue engineering was demonstrated by Ladet et al. (2008), who used multi-membrane hydrogels as chondrocytic cell bioreactors. The authors found that the immobilized chondrocytes successfully formed aggregates, proliferated, and produced cartilage-type matrix proteins that filled the voids between the membranes. This study, however, was performed using chitosan. The use of alginate to form multi-membrane hydrogels for cell culture has yet to be demonstrated.

However, the assembly of multi-membrane hydrogels from the periphery to the core does not allow for targeted loading of different types of biological materials or drugs within the different hydrogel layers (Dai et al., 2009). The cargo needs to be uniformly pre-mixed in the polymer sol, which acts as a master template before membrane formation. As such, new cargo cannot be introduced during membrane formation. Dai et al. (2009) modified the gelation mechanism by constructing multi-membrane hydrogels in an opposite manner i.e., from the core to the periphery using layer-by-layer assembly.

A preformed gel was first used as a template. For example (see Fig. 10), a Ca-alginate gel core (i.e., bead) was formed and then equilibrated with Ca^{2+}. The Ca^{2+}-loaded gel core was then immersed into an alginate solution. The Ca^{2+} ions instantaneously diffused from the inner core and cross-linked with the alginate polymer chains at the periphery of the gel core, forming a single hydrogel membrane layer surrounding the core. The cross-linking process was interrupted by removing the hydrogel from the alginate solution and subsequently immersing it in calcium chloride for curing. Subsequently, the hydrogel was reloaded with Ca^{2+} and re-immersed in the alginate solution to form a second membrane layer. This process can be repeated many times to form a multi-membrane hydrogel. The term multi-step interrupted inverse gelation considers that the alginate polymer chains are cross-linked in an outward direction from the template core.

Dai et al. (2009) showed that the membrane thickness and the presence (or absence) of inter-membrane void can be controlled by the cross-linking time, calcium concentration, and alginate concentration. The flexibility in loading cargo (i.e., blue dextran) in the different membrane layers was also demonstrated; pulse-like

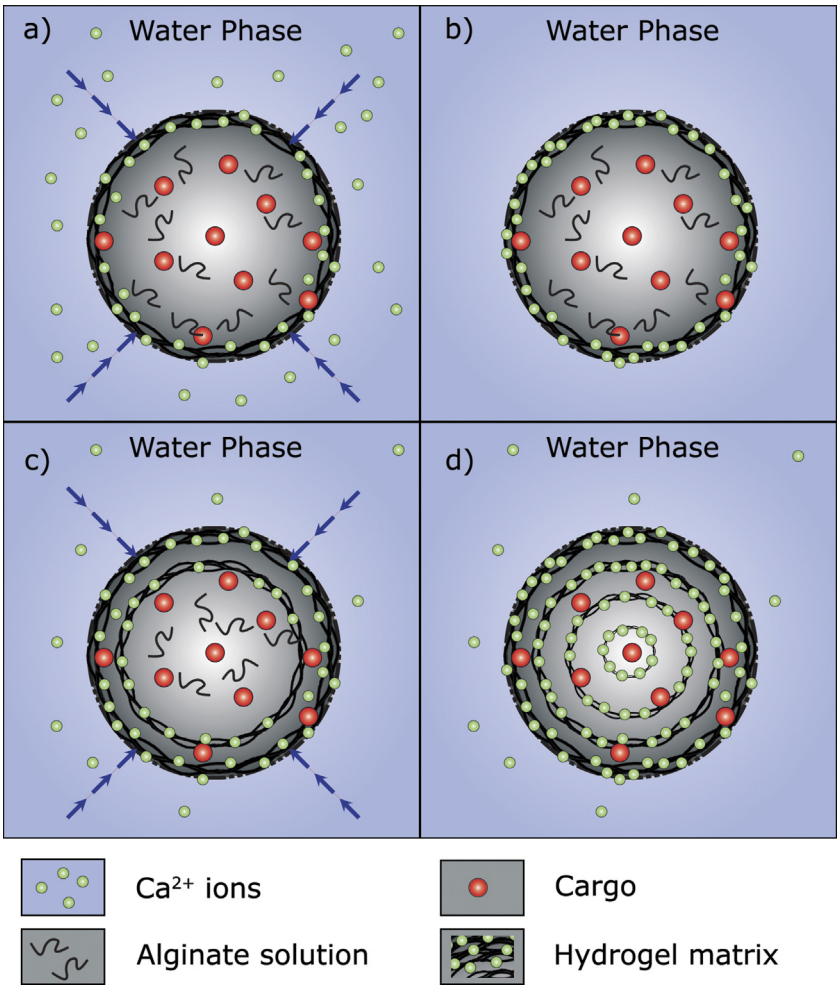

Fig. 9. Mechanism of interrupted external gelation for multi-membrane hydrogel formation: (a) inward gelation of alginate droplets, (b) interruption of gelation by immersing in water, (c) repeated gelation to form the second membrane, and (d) repetition of steps (b) and (c) to form additional membrane layers.

releases of blue dextran were observed when the cargo-loaded membranes were dissolved. Fig. 5(f) shows the onion-like structure of the multi-membrane capsules. Recently, Wei, Zhang, Li, and Lu (2013) showed that alginate multi-membrane hydrogels could control the release of both hydrophobic and hydrophilic model drugs.

Research on multi-membrane hydrogels is still in its infancy. All of the hydrogels reported to date are macroscopic in size, with diameters between 4 and 12 mm. Further research should be directed toward fabricating smaller hydrogels because the resulting increase in the surface-to-volume ratio would promote substrate and product transfer in immobilization systems involving living cells and enzymes and facilitate practical drug delivery by oral, topical, inhalation, or injection routes. However, we foresee many technical challenges when scaling down the size of multi-membrane hydrogels and associated production in large quantities. Because the process involves inverse gelation, the rapid outward diffusion of the pre-loaded Ca^{2+} ions from the gel core toward the bulk solution containing alginate polymers may cause agglomeration between the gel cores. In theory, the extent of agglomeration issue is expected to increase when the size of gel cores becomes smaller because of the faster diffusion of Ca^{2+} ions and the difficulty in dispersing the gel cores away from each other. Addressing this issue may require modifying the existing gelation mechanism.

Considerations in method selection

Many methods have been developed to form droplets of alginate sol, which is a pre-requisite step to forming hydrogel particles via ionotropic gelation. Though both liquid–air and liquid–liquid methods have their advantages and limitations, the selection of a suitable dispersion method typically depends on the target particle size and size distribution, economics of production, productivity, and technical constraints.

Particle size and size distribution

The typical ranges of mean size and size distribution of alginate hydrogel particles that can be formed by various methods are summarized in Fig. 11. Particle size was divided into three classes: nanometer, micrometer, and millimeter. The size distribution was divided into two groups: narrow and broad size dispersion. As observed, liquid–air methods generally dominate the production of large and uniform beads ($\geq$0.1 mm), whereas liquid–liquid methods are more versatile because they can produce uniform particles over a wide range of sizes from 10 nm to 2 mm. Future research should consider expanding the size range obtained by liquid–air methods to produce uniform particles of <100 μm, possibly by adapting microfluidic technologies or coupling the existing

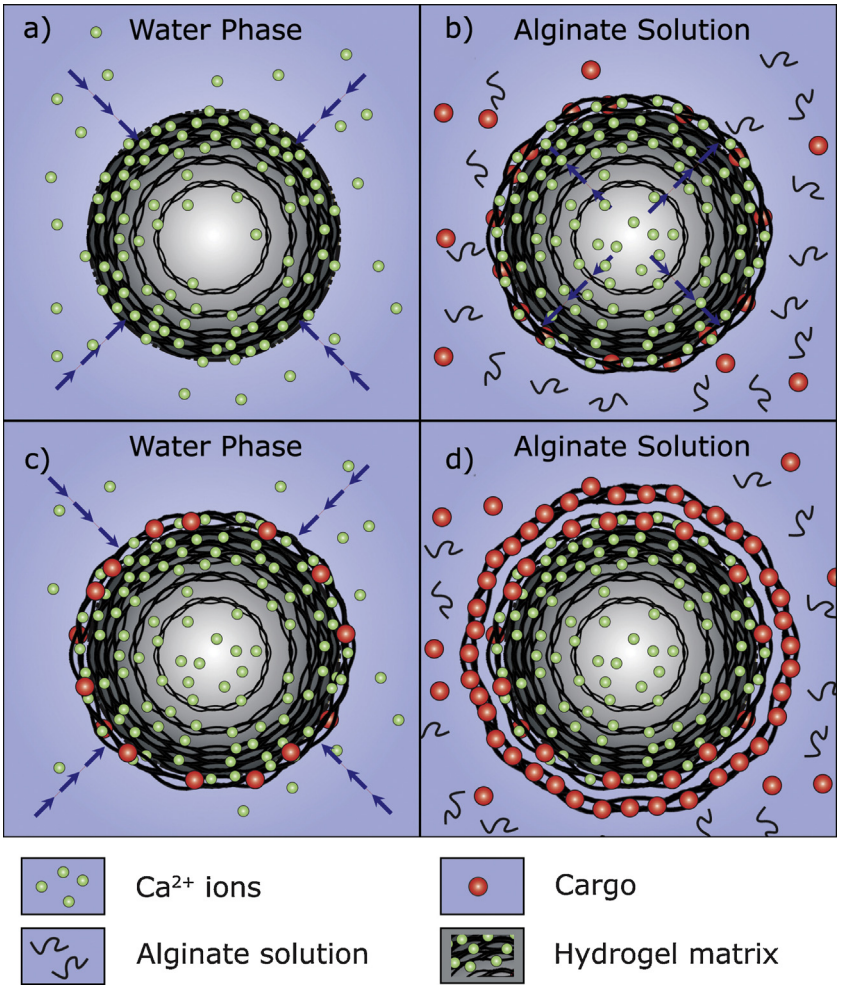

Fig. 10. Mechanism of interrupted inverse gelation for multi-membrane hydrogel formation: (a) equilibration of preformed alginate cores with calcium ions, (b) transfer of alginate cores to the alginate solution to form the first membrane layer at the interface, (c) interruption of gelation by removing the hydrogel from the alginate sol and curing the hydrogel in a calcium chloride solution, and (d) repetition of steps (a)–(c) to form additional membrane layers.

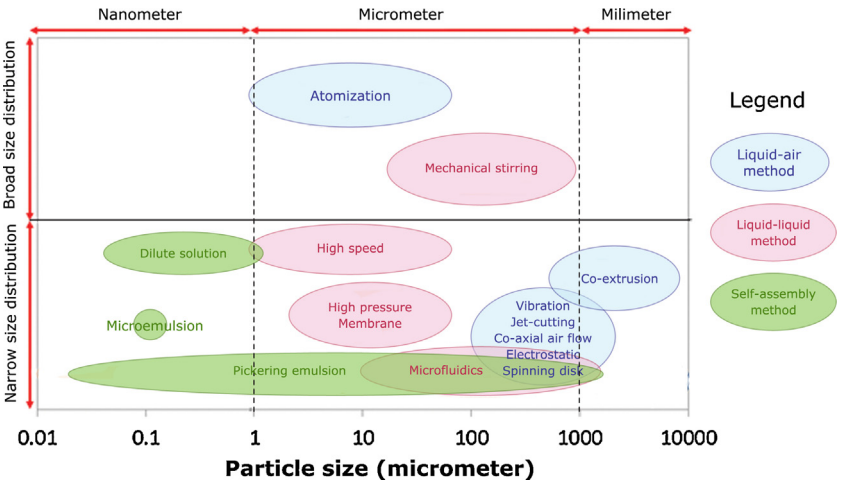

Fig. 11. Comparison of dispersion methods with respect to particle mean size and size distribution of alginate hydrogels.

liquid–air methods with solvent diffusion methods, as used in the liquid–liquid methods.

Economics of production

Another selection consideration is the economics of production. Here, we classify the costs of equipment and consumables used in each method into two categories: high and low cost (see Fig. 12). These costs were estimated based on a laboratory-scale process, allowing for a direct comparison among methods. In general, the equipment cost is correlated to the size and size distribution of the particles that a method can produce. Equipment that can produce small and uniform particles (i.e., <1000 μm) are typically more costly than that capable of producing large beads (>1000 μm) or small particles with a broad size distribution. Special equipment and instrumentation are required to control the size and size distribution of the droplets when compared with those employed in the dripping, atomization, or mechanical stirring method, which all use basic laboratory equipment.

In terms of consumable cost, liquid–liquid methods generally involve higher costs when compared with liquid–air methods because the former methods use immiscible liquids (e.g., vegetable oil or mineral oil) to disperse the alginate sol. Furthermore, the use of oil makes particle harvest more difficult, especially when the particle size is small because multiple steps of washing and rinsing

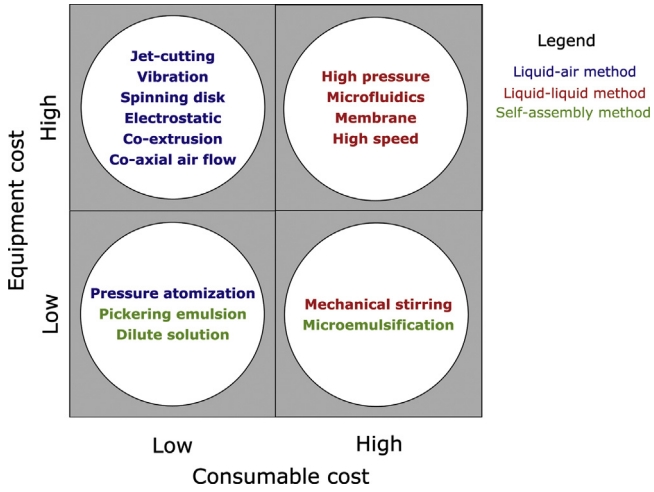

Fig. 12. Cost-effectiveness of different dispersion methods based on the relative cost of equipment and consumables.

are necessary. Typically, the volume of oil used is five times that of the alginate sol. Therefore, a large volume of waste oil and washing liquid is generated. These drawbacks increase not only the operational costs but also the processing time. In this respect, liquid–air

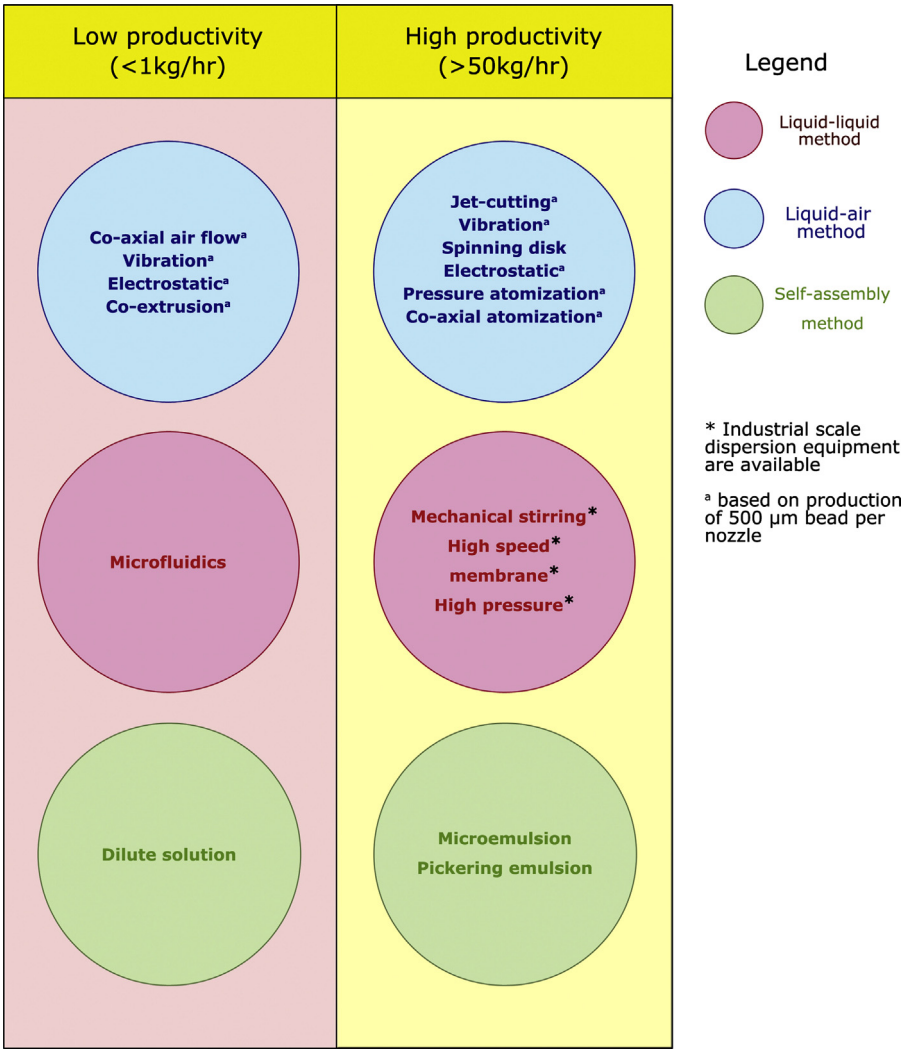

Fig. 13. Productivity of different dispersion methods.

methods are more favorable because particles are formed in an aqueous solution and are easy to separate and clean.

Productivity

Another selection criterion is the productivity of a given method. The productivity required is typically inversely related to the value of a product. For example, high-value products for medical or pharmaceutical use may be needed in small quantities, but low-value products for food, agriculture, or consumer use may require a fabrication process that can be scaled up for mass production.

As shown in Fig. 13, all of the methods developed to date can produce alginate particles in small quantities, e.g., kg/day, but only a few methods can be scalable for mass production from hundreds of kilos to tons per day. For liquid–air methods, the common scale-up strategies involve increasing the number of nozzles employed in production and increasing the liquid feed flow rate. These approaches may sound deceptively simple, but in reality their implementation can be quite challenging because they require specially designed multi-nozzle and instrumentation systems that must precisely control the liquid flow rates and droplet breakup behavior at each nozzle to generate uniformly sized liquid droplets. However, liquid–liquid methods may be easier to scale up because much of the industrial-scale dispersion equipment that can generate emulsions is readily available. However, no studies have been conducted to examine the scale-up potential of these methods for the production of alginate particles. The main obstacle in scaling up liquid–liquid methods may be the tedious post-production process involved, which relates to separating and cleaning the particles formed from the immiscible liquid.

Technical constraints

There are many types of technical constraints that can be placed on a method. For example, some applications may require strict aseptic conditions to immobilize living cells for tissue engineering or bioprocessing. In this case, the equipment involved needs to be sterilized, compact, and airtight to reduce contamination. The production process must also be straightforward and should ideally involve as few post-production treatments as possible. Liquid–air methods are generally preferred for these types of applications. Other technical considerations relate to the formulation of the alginate sol such as the solution viscosity. Solution viscosity has a large influence on the droplet formation mechanism because a higher viscosity gives greater resistance to deformation, which hinders droplet breakup. All of the described methods should be able to operate below 300 mPa s, and many of the discussed methods can operate up to 5000 mPa s. The jet-cutting method has the advantage of operating with high-viscosity liquids (>10,000 mPa s) because droplets are generated by physically cutting the liquid jet using rotating wires.

Future perspectives and conclusions

Since the first paper on alginate beads in 1977, thousands of articles have been published on this material as an encapsulation matrix. Future research should focus on the development of novel particle structures or morphologies that impart new functionalities. The dispersion and gelation methods described in this review provide the basic process tools that can be integrated and combined to develop new or more complex particle designs. Alginate encapsulation has been applied in the industry, with production that could reach 100 tons per year. However, liquid–air methods are primarily used. Therefore, alginate particles of only a certain size range and morphology are being produced, thus limiting the full commercial potential of the alginate-based encapsulation technology. In this respect, future work should involve scaling up existing methods or developing methods that are easy to scale up such as self-assembly. In conclusion, this comprehensive review of the technologies related to encapsulation using alginate and the resulting particle sizes and morphologies should help readers select a suitable method that corresponds to their application and explore future research in this field.

Acknowledgements

The authors thank the Ministry of Science, Technology and Innovation for funding the project under the E-Science grant (06-02-10-SF0157) and Monash University Malaysia for providing postgraduate scholarships for Jun-Yee Leong, Weng-Hoong Lam, Kiang-Wei Ho, Wan-Ping Voo, Micky Fu-Xiang Lee, Hui-Peng Lim, and Swee-Lu Lim.

References

Abang, S., Chan, E. S., & Poncelet, D. (2012). Effects of process variables on the encapsulation of oil in Ca-alginate capsules using an inverse gelation technique. *Journal of Microencapsulation*, *29*(5), 417–428.

Amici, E., Tetradis-Meris, G., de Torres, C. P., & Jousse, F. (2008). Alginate gelation in microfluidic channels. *Food Hydrocolloids*, *22*(1), 97–104.

Annesini, M. C., Castelli, G., Conti, F., De Virgiliis, L. C., Marrelli, L., Miccheli, A., et al. (2000). Transport and consumption rate of O_2 in alginate gel beads entrapping hepatocytes. *Biotechnology Letters*, *22*(10), 865–870.

Barba, A. A., d'Amore, M., Cascone, S., Lamberti, G., & Titomanlio, G. (2009). Intensification of biopolymeric microparticles production by ultrasonic assisted atomization. *Chemical Engineering and Processing: Process Intensification*, *48*(10), 1477–1483.

Bezbaruah, A. N., Shanbhogue, S. S., Simsek, S., & Khan, E. (2011). Encapsulation of iron nanoparticles in alginate biopolymer for trichloroethylene remediation. *Journal of Nanoparticle Research*, *13*(12), 6673–6681.

Bhowmik, B. B., Sa, B., & Mukherjee, A. (2006). Preparation and *in-vitro* characterization of slow release testosterone nanocapsules in alginates. *Acta Pharmaceutica (Zagreb Croatia)*, *56*(4), 417–429.

Blandino, A., Macías, M., & Cantero, D. (2001). Immobilization of glucose oxidase within calcium alginate gel capsules. *Process Biochemistry*, *36*(7), 601–606.

Brandenberger, H., Nüssli, D., Piech, V., & Widmer, F. (1999). Monodisperse particle production: A method to prevent drop coalescence using electrostatic forces. *Journal of Electrostatics*, *45*(3), 227–238.

Bugarski, B., Li, Q., Goosen, M. F., Poncelet, D., Neufeld, R. J., & Vunjak, G. (1994). Electrostatic droplet generation: Mechanism of polymer droplet formation. *AIChE Journal*, *40*(6), 1026–1031.

Capretto, L., Mazzitelli, S., Balestra, C., Tosi, A., & Nastruzzi, C. (2008). Effect of the gelation process on the production of alginate microbeads by microfluidic chip technology. *Lab on a Chip*, *8*(4), 617–621.

Chan, E. S. (2011). Preparation of Ca-alginate beads containing high oil content: Influence of process variables on encapsulation efficiency and bead properties. *Carbohydrate Polymers*, *84*(4), 1267–1275.

Chan, E. S., Lee, B. B., Ravindra, P., & Poncelet, D. (2009). Prediction models for shape and size of Ca-alginate macrobeads produced through extrusion–dripping method. *Journal of Colloid and Interface Science*, *338*(1), 63–72.

Chan, E. S., Lim, T. K., Ravindra, P., Mansa, R. F., & Islam, A. (2012). The effect of low air-to-liquid mass flow rate ratios on the size, size distribution and shape of calcium alginate particles produced using the atomization method. *Journal of Food Engineering*, *108*(2), 297–303.

Chan, L. W., Lee, H. Y., & Heng, P. W. (2006). Mechanisms of external and internal gelation and their impact on the functions of alginate as a coat and delivery system. *Carbohydrate Polymers*, *63*(2), 176–187.

Choi, C. H., Jung, J. H., Rhee, Y. W., Kim, D. P., Shim, S. E., & Lee, C. S. (2007). Generation of monodisperse alginate microbeads and in situ encapsulation of cell in microfluidic device. *Biomedical Microdevices*, *9*(6), 855–862.

Cui, J. H., Goh, J. S., Park, S. Y., Kim, P. H., & Lee, B. J. (2001). Preparation and physical characterization of alginate microparticles using air atomization method. *Drug Development and Industrial Pharmacy*, *27*(4), 309–319.

Dai, H., Li, X., Long, Y., Wu, J., Liang, S., Zhang, X., et al. (2009). Multi-membrane hydrogel fabricated by facile dynamic self-assembly. *Soft Matter*, *5*(10), 1987–1989.

Deepa, V., Sridhar, R., Goparaju, A., Reddy, P. N., & Murthy, P. B. (2012). Nanoemulsified ethanolic extract of *Pyllanthus amarus* Schum & Thonn ameliorates CCl_4 induced hepatotoxicity in Wistar rats. *Indian Journal of Experimental Biology*, *50*, 785–794.

Del Gaudio, P., Colombo, P., Colombo, G., Russo, P., & Sonvico, F. (2005). Mechanisms of formation and disintegration of alginate beads obtained by prilling. *International Journal of Pharmaceutics*, *302*(1), 1–9.

Dembczynski, R., & Jankowski, T. (2000). Characterisation of small molecules diffusion in hydrogel-membrane liquid-core capsules. *Biochemical Engineering Journal*, *6*(1), 41–44.

De Vos, P., De Haan, B. J., & Van Schilfgaarde, R. (1997). Upscaling the production of microencapsulated pancreatic islets. *Biomaterials*, *18*(16), 1085–1090.

Ding, W. K., & Shah, N. P. (2009). Effect of homogenization techniques on reducing the size of microcapsules and the survival of probiotic bacteria therein. *Journal of Food Science*, *74*(6), M231–M236.

Goosen, M. F., Al-Ghafri, A. S., Mardi, O. E., Al-Belushi, M. I., Al-Hajri, H. A., Mahmoud, E. S., et al. (1997). Electrostatic droplet generation for encapsulation of somatic tissue: Assessment of high-voltage power supply. *Biotechnology Progress*, *13*(4), 497–502.

Hariyadi, D. M., Lin, S. C. Y., Wang, Y., Bostrom, T., Turner, M. S., Bhandari, B., et al. (2010). Diffusion loading and drug delivery characteristics of alginate gel microparticles produced by a novel impinging aerosols method. *Journal of Drug Targeting*, *18*(10), 831–841.

Harkins, W. D., & Brown, F. E. (1919). The determination of surface tension (free surface energy), and the weight of falling drops: The surface tension of water and benzene by the capillary height method. *Journal of the American Chemical Society*, *41*(4), 499–524.

Haug, A. (1961). Affinity of some divalent metals to different types of alginates. *Acta Chemica Scandinavica*, *15*(8), 1794–1975.

Haug, A., & Smidsrod, O. (1970). Selectivity of some anionic polymers for divalent metal ions. *Acta Chemica Scandinavica*, *24*(3), 843–854.

Haynes, W. M. (Ed.). (2013). *CRC handbook of chemistry and physics*. New York, NY: CRC press.

Heinzen, C., Marison, I., Berger, A., & von Stockar, U. (2002). Use of vibration technology for jet break-up for encapsulation of cells, microbes and liquids in monodisperse microcapsules. *Landbauforschung Völkenrode*, (SH241), 19–25.

Herrero, E. P., Del Valle, E. M., & Galán, M. A. (2006). Modelling prediction of the microcapsule size of polyelectrolyte complexes produced by atomization. *Chemical Engineering Journal*, *121*(1), 1–8.

Huang, K. S., Lai, T. H., & Lin, Y. C. (2006). Manipulating the generation of Ca-alginate microspheres using microfluidic channels as a carrier of gold nanoparticles. *Lab on a Chip*, *6*(7), 954–957.

Igoe, R. S. (2011). *Dictionary of food ingredients*. Heidelberg: Springer Science & Business Media.

Jin, Y., Perrie, C., Zhang, W., Van Diepen, C., Curtis, J., & Barrow, C. J. (2007). Microencapsulation of marine lipids as a vehicle for functional food delivery. In C. Barrow, & F. Shahidi (Eds.), *Marine nutraceuticals and functional foods* (pp. 115–155). New York, NY: CRC Press.

Kidane, A., Guimond, P., Ju, T. C. R., Sanchez, M., Gibson, J., North, A., et al. (2002). Effects of cellulose derivatives and poly (ethylene oxide)-poly(propylene oxide) tri-block copolymers (Pluronic® surfactants) on the properties of alginate based microspheres and their interactions with phagocytic cells. *Journal of Controlled Release*, *85*(1), 181–189.

Kim, S. P., Lee, D. H., & Park, J. K. (1998). Development of hepatocyte spheroids immobilization technique using alternative encapsulation method. *Biotechnology and Bioprocess Engineering*, *3*(2), 96–102.

Klokk, T. I., & Melvik, J. E. (2002). Controlling the size of alginate gel beads by use of a high electrostatic potential. *Journal of Microencapsulation*, *19*(4), 415–424.

Kontturi, L. S., Yliperttula, M., Toivanen, P., Määttä, A., Määttä, A. M., & Urtti, A. (2011). A laboratory-scale device for the straightforward production of uniform, small sized cell microcapsules with long-term cell viability. *Journal of Controlled Release*, *152*(3), 376–381.

Koyama, K., & Seki, M. (2004a). Cultivation of yeast and plant cells entrapped in the low-viscous liquid-core of an alginate membrane capsule prepared using polyethylene glycol. *Journal of Bioscience and Bioengineering*, *97*(2), 111–118.

Koyama, K., & Seki, M. (2004b). Evaluation of mass-transfer characteristics in alginate-membrane liquid-core capsules prepared using polyethylene glycol. *Journal of Bioscience and Bioengineering*, *98*(2), 114–121.

Kuo, C. K., & Ma, P. X. (2001). Ionically crosslinked alginate hydrogels as scaffolds for tissue engineering: Part 1. Structure, gelation rate and mechanical properties. *Biomaterials*, *22*(6), 511–521.

Ladet, S., David, L., & Domard, A. (2008). Multi-membrane hydrogels. *Nature*, *452*(7183), 76–79.

Lee, B. B., Ravindra, P., & Chan, E. S. (2013). Size and shape of calcium alginate beads produced by extrusion dripping. *Chemical Engineering & Technology*, *36*(10), 1627–1642.

Lee, B. J., Min, G. H., & Cui, J. H. (1999). Correlation of drug solubility with trapping efficiency and release characteristics of alginate beads. *Pharmacy and Pharmacology Communications*, *5*(2), 85–89.

Lee, H. H., Park, O. J., Park, J. M., & Yang, J. W. (1996). Continuous production of uniform calcium alginate beads by sound wave induced vibration. *Journal of Chemical Technology and Biotechnology*, *67*(3), 255–259.

Lee, K. Y., & Mooney, D. J. (2012). Alginate: Properties and biomedical applications. *Progress in Polymer Science*, *37*(1), 106–126.

Lemoine, D., Wauters, F., Bouchend'homme, S., & Préat, V. (1998). Preparation and characterization of alginate microspheres containing a model antigen. *International Journal of Pharmaceutics*, *176*(1), 9–19.

Lencki, R. W., Neufeld, R. J., & Spinney, T. (1989).Method of producing microspheres. *U.S. Patent No. 4,822,534*. Washington, DC: U.S. Patent and Trademark Office.

Leong, J. Y., Tey, B. T., Tan, C. P., & Chan, E. S. (2015). Nozzleless fabrication of oil-core biopolymeric microcapsules by the interfacial gelation of Pickering emulsion templates. *ACS Applied Materials & Interfaces*, *7*(30), 16169–16176.

Lertsutthiwong, P., Noomun, K., Jongaroonngamsang, N., Rojsitthisak, P., & Nimmannit, U. (2008). Preparation of alginate nanocapsules containing turmeric oil. *Carbohydrate Polymers*, *74*(2), 209–214.

Lewinska, D., Rosinski, S., & Werynski, A. (2004). Influence of process conditions during impulsed electrostatic droplet formation on size distribution of hydrogel beads. *Artificial Cells, Blood Substitutes and Biotechnology*, *32*(1), 41–53.

Liu, H., Li, G., Sun, X., He, Y., Sun, S., & Ma, H. (2015). Microfluidic generation of uniform quantum dot-encoded microbeads by gelation of alginate. *RSC Advances*, *5*(77), 62706–62716.

Liu, L., Wu, F., Ju, X. J., Xie, R., Wang, W., Niu, C. H., et al. (2013). Preparation of monodisperse calcium alginate microcapsules via internal gelation in microfluidic-generated double emulsions. *Journal of Colloid and Interface Science*, *404*, 85–90.

Liu, X. D., Bao, D. C., Xue, W. M., Xiong, Y., Yu, W. T., Yu, X. J., et al. (2003). Preparation of uniform calcium alginate gel beads by membrane emulsification coupled with internal gelation. *Journal of Applied Polymer Science*, *87*(5), 848–852.

López, M. D., Maudhuit, A., Pascual-Villalobos, M. J., & Poncelet, D. (2012). Development of formulations to improve the controlled-release of linalool to be applied as an insecticide. *Journal of Agricultural and Food Chemistry*, *60*(5), 1187–1192.

Lupo, B., Maestro, A., Porras, M., Gutiérrez, J. M., & González, C. (2014). Preparation of alginate microspheres by emulsification/internal gelation to encapsulate cocoa polyphenols. *Food Hydrocolloids*, *38*, 56–65.

Machado, A. H., Lundberg, D., Ribeiro, A. J., Veiga, F. J., Lindman, B., Miguel, M. G., et al. (2012). Preparation of calcium alginate nanoparticles using water-in-oil (W/O) nanoemulsions. *Langmuir*, *28*(9), 4131–4141.

Machado, A. H., Lundberg, D., Ribeiro, A. J., Veiga, F. J., Miguel, M. G., Lindman, B., et al. (2013). Encapsulation of DNA in macroscopic and nanosized calcium alginate gel particles. *Langmuir*, *29*(51), 15926–15931.

Maeda, K., Onoe, H., Takinoue, M., & Takeuchi, S. (2012). Controlled synthesis of 3D multi-compartmental particles with centrifuge-based microdroplet formation from a multi-barrelled capillary. *Advanced Materials*, *24*(10), 1340–1346.

Martinsen, A., Storrø, I., & Skjåk-Bræk, G. (1992). Alginate as immobilization material: III. Diffusional properties. *Biotechnology and Bioengineering*, *39*(2), 186–194.

Martinsen, A., Skjåk-Bræk, G., & Smidsrød, O. (1989). Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate gel beads. *Biotechnology and Bioengineering*, *33*(1), 79–89.

Martins, E., Renard, D., Davy, J., Marquis, M., & Poncelet, D. (2015). Oil core microcapsules by inverse gelation technique. *Journal of Microencapsulation*, *32*(1), 86–95.

Miyazaki, T., Ohtsuki, C., & Tanihara, M. (2003). Synthesis of bioactive organic–inorganic nanohybrid for bone repair through sol–gel processing. *Journal of Nanoscience and Nanotechnology*, *3*(6), 511–515.

Moradian, A., & Mostaghimi, J. (2011). Effects of injection angle on the measurement of surface tension coefficient by drop weight method. *Physics and Chemistry of Liquids*, *49*(1), 32–51.

Morimoto, Y., Tan, W. H., Tsuda, Y., & Takeuchi, S. (2009). Monodisperse semipermeable microcapsules for continuous observation of cells. *Lab on a Chip*, *9*(15), 2217–2223.

Mørch, Ý. A., Donati, I., Strand, B. L., & Skjåk-Bræk, G. (2006). Effect of Ca^{2+}, Ba^{2+}, and Sr^{2+} on alginate microbeads. *Biomacromolecules*, *7*(5), 1471–1480.

Nedović, V. A., Obradović, B., Leskošek-Čukalović, I., Trifunović, O., Pešić, R., & Bugarski, B. (2001). Electrostatic generation of alginate microbeads loaded with brewing yeast. *Process Biochemistry*, *37*(1), 17–22.

Nigam, S. C., Tsao, I. F., Sakoda, A., & Wang, H. Y. (1988). Techniques for preparing hydrogel membrane capsules. *Biotechnology Techniques*, *2*(4), 271–276.

Nussinovitch, A., Gershon, Z., & Nussinovitch, M. (1996). Liquid-core hydrocolloid capsules. *Food Hydrocolloids*, *10*(1), 21–26.

Nyende, A. B., Schittenhelm, S., Mix-Wagner, G., & Greef, J. M. (2003). Production, storability, and regeneration of shoot tips of potato (*Solanum tuberosum L.*) encapsulated in calcium alginate hollow beads. *In Vitro Cellular & Developmental Biology-Plant*, *39*(5), 540–544.

Oh, C. Y., & Park, J. K. (1998). The characteristics of encapsulated whole cell β-galactosidase. *Bioprocess Engineering*, *19*(6), 419–425.

Paques, J. P., van der Linden, E., van Rijn, C. J., & Sagis, L. M. (2013). Alginate submicron beads prepared through w/o emulsification and gelation with $CaCl_2$ nanoparticles. *Food Hydrocolloids*, *31*(2), 428–434.

Pascual-Villalobos, M. J., & López, M. D. (2013). New application of guayule resin in controlled release formulations. *Industrial Crops and Products*, *43*, 44–49.

Patel, A. V., Pusch, I., Mix-Wagner, G., & Vorlop, K. D. (2000). A novel encapsulation technique for the production of artificial seeds. *Plant Cell Reports*, *19*(9), 868–874.

Perry, D. L. (2011). *Handbook of inorganic compounds*. New York, NY: CRC Press.

Poncelet, D., Babak, V. G., Neufeld, R. J., Goosen, M. F. A., & Burgarski, B. (1999). Theory of electrostatic dispersion of polymer solutions in the production of microgel beads containing biocatalyst. *Advances in Colloid and Interface Science*, *79*(2), 213–228.

Poncelet, D., De Smet, B. P., Beaulieu, C., Huguet, M. L., Fournier, A., & Neufeld, R. J. (1995). Production of alginate beads by emulsification/internal gelation. II. Physicochemistry. *Applied Microbiology and Biotechnology*, *43*(4), 644–650.

Poncelet, D., Lencki, R., Beaulieu, C., Halle, J. P., Neufeld, R. J., & Fournier, A. (1992). Production of alginate beads by emulsification/internal gelation. I. Methodology. *Applied Microbiology and Biotechnology*, *38*(1), 39–45.

Poncelet, D., & Neufeld, R. J. (1996). Fundamentals of dispersion in encapsulation technology. *Progress in Biotechnology*, *11*, 47–54.

Prüsse, U., Bilancetti, L., Bučko, M., Bugarski, B., Bukowski, J., Gemeiner, P., et al. (2008). Comparison of different technologies for alginate beads production. *Chemical Papers*, *62*(4), 364–374.

Prüβe, U., Fox, B., Kirchhoff, M., Bruske, F., Breford, J., & Vorlop, K. D. (1998). The jet cutting method as a new immobilization technique. *Biotechnology Techniques*, *12*(2), 105–108.

Radovich, J. M. (1985). Mass transfer effects in fermentations using immobilized whole cells. *Enzyme and Microbial Technology*, *7*(1), 2–10.
Rajaonarivony, M., Vauthier, C., Couarraze, G., Puisieux, F., & Couvreur, P. (1993). Development of a new drug carrier made from alginate. *Journal of Pharmaceutical Sciences*, *82*(9), 912–917.
Reis, C. P., Ribeiro, A. J., Houng, S., Veiga, F., & Neufeld, R. J. (2007). Nanoparticulate delivery system for insulin: design, characterization and *in vitro/in vivo* bioactivity. *European Journal of Pharmaceutical Sciences*, *30*(5), 392–397.
Ren, P. W., Ju, X. J., Xie, R., & Chu, L. Y. (2010). Monodisperse alginate microcapsules with oil core generated from a microfluidic device. *Journal of Colloid and Interface Science*, *343*(1), 392–395.
Ribeiro, A. J., Silva, C., Ferreira, D., & Veiga, F. (2005). Chitosan-reinforced alginate microspheres obtained through the emulsification/internal gelation technique. *European Journal of Pharmaceutical Sciences*, *25*(1), 31–40.
Rondeau, E., & Cooper-White, J. J. (2008). Biopolymer microparticle and nanoparticle formation within a microfluidic device. *Langmuir*, *24*(13), 6937–6945.
Sakoda, A., Nigam, S. C., & Wang, H. Y. (1990). Protein separation using membrane-encapsulated soluble ligand conjugates. *Enzyme and Microbial Technology*, *12*(5), 349–354.
Sarei, F., Dounighi, N. M., Zolfagharian, H., Khaki, P., & Bidhendi, S. M. (2013). Alginate nanoparticles as a promising adjuvant and vaccine delivery system. *Indian Journal of Pharmaceutical Sciences*, *75*(4), 442–449.
Sasaki, E., Kurayama, F., Ida, J. I., Matsuyama, T., & Yamamoto, H. (2008). Preparation of microcapsules by electrostatic atomization. *Journal of Electrostatics*, *66*(5), 312–318.
Seifert, D. B., & Phillips, J. A. (1997). Production of small, monodispersed alginate beads for cell immobilization. *Biotechnology Progress*, *13*(5), 562–568.
Skjåk-Bræk, G., Grasdalen, H., & Smidsrød, O. (1989). Inhomogeneous polysaccharide ionic gels. *Carbohydrate Polymers*, *10*(1), 31–54.
Senuma, Y., Lowe, C., Zweifel, Y., Hilborn, J. G., & Marison, I. (2000). Alginate hydrogel microspheres and microcapsules prepared by spinning disk atomization. *Biotechnology and Bioengineering*, *67*(5), 616–622.
Serp, D., Cantana, E., Heinzen, C., von Stockar, U., & Marison, I. W. (2000). Characterization of an encapsulation device for the production of monodisperse alginate beads for cell immobilization. *Biotechnology and Bioengineering*, *70*(1), 41–53.
Smidsrød, O., & Skja, G. (1990). Alginate as immobilization matrix for cells. *Trends in Biotechnology*, *8*, 71–78.
Song, H., Yu, W., Gao, M., Liu, X., & Ma, X. (2013). Microencapsulated probiotics using emulsification technique coupled with internal or external gelation process. *Carbohydrate Polymers*, *96*(1), 181–189.
Stark, D., Kornmann, H., Münch, T., Sonnleitner, B., Marison, I. W., & Von Stockar, U. (2003). Novel type of in situ extraction: Use of solvent containing microcapsules for the bioconversion of 2-phenylethanol from L-phenylalanine by *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering*, *83*(4), 376–385.
Sugaya, S., Yamada, M., Hori, A., & Seki, M. (2013). Microfluidic production of single micrometer-sized hydrogel beads utilizing droplet dissolution in a polar solvent. *Biomicrofluidics*, *7*(5), 054120.
Sugiura, S., Oda, T., Aoyagi, Y., Matsuo, R., Enomoto, T., Matsumoto, K., et al. (2007). Microfabricated airflow nozzle for microencapsulation of living cells into 150 micrometer microcapsules. *Biomedical Microdevices*, *9*(1), 91–99.
Sugiura, S., Oda, T., Izumida, Y., Aoyagi, Y., Satake, M., Ochiai, A., et al. (2005). Size control of calcium alginate beads containing living cells using micro-nozzle array. *Biomaterials*, *26*(16), 3327–3331.
Sun-Waterhouse, D., Zhou, J., Miskelly, G. M., Wibisono, R., & Wadhwa, S. S. (2011). Stability of encapsulated olive oil in the presence of caffeic acid. *Food Chemistry*, *126*(3), 1049–1056.
Tan, W. H., & Takeuchi, S. (2007). Monodisperse alginate hydrogel microbeads for cell encapsulation. *Advanced Materials*, *19*(18), 2696–2701.
Tate, T. (1864). On the magnitude of a drop of liquid formed under different circumstances. *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science*, *27*(181), 176–180.
Tomida, H., Nakamura, C., Yoshitomi, H., & Kiryu, S. (1993). Preparation of theophylline-loaded calcium alginate gel capsules and evaluation of their drug release characteristics. *Chemical and Pharmaceutical Bulletin*, *41*(12), 2161–2165.
Torre, M. L., Maggi, L., Vigo, D., Galli, A., Bornaghi, V., Maffeo, G., et al. (2000). Controlled release of swine semen encapsulated in calcium alginate beads. *Biomaterials*, *21*(14), 1493–1498.
Wang, W., Waterhouse, G. I., & Sun-Waterhouse, D. (2013). Co-extrusion encapsulation of canola oil with alginate: Effect of quercetin addition to oil core and pectin addition to alginate shell on oil stability. *Food Research International*, *54*(1), 837–851.
Wei, S. J., Zhang, M., Li, L., & Lu, L. (2013). Alginate-based multi-membrane hydrogel for dual drug delivery system. *Applied Mechanics and Materials*, *275*(March), 1632–1635.
Wolters, G. H., Fritschy, W. M., Gerrits, D., & Van Schilfgaarde, R. (1992). A versatile alginate droplet generator applicable for microencapsulation of pancreatic islets. *Journal of Applied Biomaterials*, *3*(4), 281–286.
Yoo, I. K., Seong, G. H., Chang, H. N., & Park, J. K. (1996). Encapsulation of *Lactobacillus casei* cells in liquid-core alginate capsules for lactic acid production. *Enzyme and Microbial Technology*, *19*(6), 428–433.
You, J. O., Park, S. B., Park, H. Y., Haam, S., Chung, C. H., & Kim, W. S. (2001). Preparation of regular sized Ca-alginate microspheres using membrane emulsification method. *Journal of Microencapsulation*, *18*(4), 521–532.
Zhang, H., Tumarkin, E., Peerani, R., Nie, Z., Sullan, R. M. A., Walker, G. C., et al. (2006). Microfluidic production of biopolymer microcapsules with controlled morphology. *Journal of the American Chemical Society*, *128*(37), 12205–12210.
Zhang, J., Li, X., Zhang, D., & Xiu, Z. (2007). Theoretical and experimental investigations on the size of alginate microspheres prepared by dropping and spraying. *Journal of Microencapsulation*, *24*(4), 303–322.
Østberg, T., Lund, E. M., & Graffner, C. (1994). Calcium alginate matrices for oral multiple unit administration: IV. Release characteristics in different media. *International Journal of Pharmaceutics*, *112*(3), 241–248.