Preparation of uniformly-sized alginate microspheres using the novel combined methods of microchannel emulsification and external gelation

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ABSTRACT

The purpose of this study was to prepare alginate (ALG) microspheres with narrow size distribution using a combination of microchannel (MC) emulsification technique and external gelation method. ALG solution was dispersed as water-in-oil (W/O) emulsion droplets in iso-octane containing 5 wt% Span 85 as the immiscible continuous phase via MC emulsification technique using hydrophobic MC array. The MC array used in this experiment is a grooved-type MC consisting of 1,070 channels fabricated on a 25 mm x 28 mm silicon microchip. The monodisperse W/O emulsion droplets generated from the MCs were in the mean particle diameter (d_{av}) range of 18 to 22 μm and coefficient of variation (CV) of 5 to 26% at the ALG concentrations of 0.5 to 3.0 wt% and flow rates of 0.05 to 0.4 mL/h. The d_{av} of the emulsion droplets hardly changed below a dispersed phase threshold flow rate of 0.2 mL/h but gradually became smaller when the dispersed phase concentration was increased. The resulting emulsion droplets were then congealed to form rigid ALG gel particles by reacting them with calcium chloride ($CaCl_2$) solution. Gelation of the ALG droplets by calcium ion (Ca^{2+}) resulted in shrinkage of its d_{av}, forming uniformly-sized ALG microspheres with an average diameter of 6.2 μm and a CV of below 10% at the ALG concentration of 3 wt%.

Keywords: Alginate microspheres; Microchannel emulsification; External gelation; Uniformly-sized emulsion; Water-in-oil emulsion

1. Introduction

Hydrocolloid gel particles, for example, alginate (ALG) microspheres have found great potential in the application of encapsulation of materials such as drugs [1], proteins and enzymes [2,3], cells [4,5], DNA [6], probiotics [7], flavors [8] and nutrients [9], owing to their biocompatibility, inert nature, mild encapsulation temperature and high porosity which allows high diffusion rate of macromolecules.

ALG are anionic polysaccharides isolated primarily from brown seaweed. They are linear, unbranched copolymers of β-D-mannuronic acid (M) and α-L-guluronic acid (G) units linked by 1→4 glycosidic bond with the M- and G- residues distributed as homopolymeric blocks of M, blocks of G or heteropolymeric blocks of alternating M and G residues [10]. ALG can form gels in the presence of divalent cations such as calcium due to the interchain binding between G blocks which give rise to a three-dimensional network calcium linked junctions known as "egg box model" [11].

The production of ALG microspheres has traditionally been achieved by extruding an ALG solution from a needle into a divalent cationic solution to induce gelation [12, 13]. This relatively mild gelation process has enabled the retention of full biological activity of proteins, DNA and cells [2]. This method, unfortunately, lacks control over its particle size and size distribution. The resulting particles might simply be too large or polydisperse to be deemed efficacious for some practical applications. Reductions in particle size are of critical importance because the physical properties of the gel particles such as their mechanical strength, dispersion and mass transfer can be improved vastly while minimizing the rupture of beads due to gas formation and accumulation [5, 12]. Also, smaller and uniform microcapsules offer more consistent mechanical properties and the diffusion of oxygen and nutrients into all area of gel can be better facilitated [13].

Recent advances in the state-of-the-art technology of microfabrication have led to the development of an array of techniques such as membrane emulsification [14], microfluidic devices [15, 16] and microchannel emulsification [17, 18] capable of producing monodisperse colloidal particles. In recent years, several groups have attempted to produce monodisperse ALG microspheres using different microfluidic devices [5, 19] and electric field application [20]. Nevertheless, the sizes obtained were relatively large, in the range of 50 μm to a few millimeters in size. Though Rondeau and Cooper-White successfully produced micro and nano sized ALG particles using an axisymmetric flow-focusing microfluidic device [21], the low concentration of ALG solutions used is a limiting factor in producing polymeric particles with enhanced structural and mechanical strength.

Microchannel (MC) emulsification technique is one of the most promising methods to produce monodisperse colloidal droplets to date, having been exploited for the production of a multitude of monodisperse colloidal particles in the size range of 4-100 μm and minimum CV of less than 5% such as oil-in-water (O/W) emulsion [17, 18, 22], water-in-oil (W/O) emulsion [17, 23], solid lipid microspheres [24], gel microbeads [25] and giant vesicles [26] to name a few. Monodisperse emulsion droplets are generated from the MC by permeating a dispersed phase into a continuous phase through a surface-treated silicon MC with well-defined geometries [17]. The driving force of the droplet generation is via the spontaneous transformation of droplets by interfacial tension [27]. This technique is highly energy efficient because energy loss due to viscous dissipation is very much lower than the conventional homogenization method which generally releases its energy as heat [28], thus making it a highly desirable method for the production of emulsions and encapsulation of highly sensitive compounds prone to destruction during processing.

The aim of our study is to use MC emulsification technique to produce uniformly-sized W/O emulsion droplets with ALG solution as the aqueous phase and then using those

emulsion droplets to obtain ALG microspheres via external gelation method with $CaCl_2$ solution. The effects of ALG concentration and its flow rate on the size of the W/O emulsion droplets were also investigated.

2. Materials and methods

2.1 Materials

Sodium ALG (viscosity of 80-120 mPa.s in 10 g/L at 20^{o}C), sorbitan trioleate (Span 85), polyoxyethylene sorbitan trioleate (Tween 85) and $CaCl_2$ were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Iso-octane was obtained from Dojindo Laboratories (Kumamoto, Japan). Hexamethyldisilazane (LS-7150) was supplied by Shin-Etsu Chemical Co. Ltd. (Tokyo, Japan) and was used for surface modification of the silicon MC plate and the glass plate. Milli-Q water was used for the preparation of all aqueous solutions.

2.2 Formation of uniformly-sized ALG microspheres

The preparation of ALG microspheres was based on the modified method of Wan et al. [29]. The formation of uniformly-sized ALG microspheres involved two separate steps: (1) preparation of W/O emulsion droplets by MC emulsification and (2) formation of ALG microspheres by external gelation.

2.2.1 Preparation of W/O emulsion droplets by MC emulsification

Uniformly-sized W/O emulsion droplets were obtained by MC emulsification technique as described previously by Kawakatsu et al. [30]. Fig. 1a shows the experimental setup, which consists of a grooved-type hydrophobized silicon plate (EP Tech. Co. Ltd., Hitachi, Japan, model CMS 6-1) tightly attached to an optically flat hydrophobized glass

plate in the MC module which was initially filled with the continuous phase, two syringe pumps (Model 11, Harvard Apparatus Inc., MA, USA) to supply the dispersed and continuous phases, a heating system set at 40^{o}C to provide temperature controlled water circulation inside the module and a microscope video system to monitor and record the emulsification behavior. Fig. 1b depicts the grooved MC plate, consisting of 1,070 channels fabricated on a 25 mm x 28 mm silicon microchip, in which the channel width is 8 μm, terrace length is 40 μm, MC depth is 4 μm and well depth is 100 μm. Droplet generation occurred when the dispersed phase (ALG solution) was forced through the channels into the continuous phase and was distorted on the terrace, spontaneously transforming them into spherical droplets (Fig. 1c). The droplets formed were then swept away by the flow of the continuous phase which was set at 5 mL/h throughout the experiment. A glass plate was firmly attached to the MC plate to cover the top of the slits on the MC plate and to form channels between them. The glass and silicon MC plates were treated with a silane coupler reagent, hexamethyldisilazane to make their surfaces hydrophobic, so that they will be suitable for the preparation of W/O emulsion according to a modified method by Kawakatsu et al. [30]. Briefly, the glass and silicon MC plates were plasma oxidized and then soaked in 1 M nitric acid overnight. After washing with water and allowing them to dry, both the glass and MC plates were dipped in hexamethyldisilazane (100% concentration for MC plate, 20% in hexane for glass plate) and left for 2 nights at room temperature. Finally, the unreacted materials were washed away.

ALG solutions at varying concentrations (0.1 to 3.0 wt%) were prepared by dissolving ALG powder in Milli-Q water at 60^{o}C for at least 2 hours. After storing the solutions at 4^{o}C overnight to ensure complete hydration, the solutions were then maintained at 40^{o}C (which is the emulsification temperature) before being used as the dispersed phase. The continuous phase consisted of Span 85 at a concentration of 5 wt% dissolved in iso-octane. In order to

improve the stability of the W/O emulsion and to prevent diffusion of the ALG droplets into the organic phase, iso-octane was pre-treated prior to use as follows: Span 85 in iso-octane was saturated with water by allowing it to come into contact with water at a volume ratio of 9:1 (iso-octane:water) for 30 min, after which they were separated by centrifugation at 1200 x g for 15 min using a table centrifuge (KN-70, Kubota, Tokyo, Japan). The iso-octane supernatant part was used as the continuous phase [26].

The MC plate used for producing W/O emulsion in this experiment was cleaned using an ultrasonic bath (VS-100III, As One Co., Osaka, Japan) at a frequency of 45 kHz based on the following sequences: MC plate was cleaned in Milli-Q water for the first 20 min followed by Milli-Q water containing a non-ionic detergent for another 20 min, Milli-Q water containing ethanol (1:1 v/v proportion) for the next 20 min and another round of cleaning with Milli-Q water for the final 20 min. The MC plate was then left to dry in an oven at 60^{o}C before use.

2.2.2 Formation of alginate microspheres by external gelation

Prior to the external gelation process, the W/O emulsion (~3.6 x 10^4 droplets/mL) was diluted with iso-octane solution to a final Span 85 concentration of 2.3 wt% (~1.7 x 10^4 droplets/mL). Estimation of the number of droplets in the emulsion was based on the calculations made according to the amount of solubilized water in the continuous phase measured using the E-684 Karl Fisher Coulometer (Metrohm, Herisau, Switzerland). A solution containing 0.12 g Tween 85 in 0.54 g iso-octane was added to 5 g of the diluted emulsion and mixed gently with a stirrer for 5 min. Subsequently, 1.3 mL of 10 % aq. $CaCl_2$ solution was added into the emulsion to start the gelation process. After gentle mixing for 20 min, the resulting ALG microspheres were rinsed three times with Milli-Q water by

174 successive centrifugation cycles (300 rpm, 10 min) and stored in Milli-Q water at room
175 temperature.
176
177 2.3 Determination of particle diameter
178 The particle diameter of the emulsion droplets and microspheres were determined by
179 measuring the diameter of the captured images of over 200 particles using an image
180 processing software (Winroof, Mitani Co., Fukui, Japan). The coefficient of variation (CV)
181 was calculated based on the following equation:
182 $CV = (\sigma / d_{av}) \times 100$
183 where σ is the standard deviation and d_{av} is the mean particle diameter.
184
185 2.4 Microscopy observation
186 The morphologies of some selected emulsions and ALG microspheres were observed
187 using a light microscope (Leica DM IRM; Leica Microsystems Wetzlar GmbH, Germany). A
188 drop of the sample was placed on a microscope slide and covered with a cover slip.
189 Photomicrography images of the samples were captured using digital image processing
190 software (Canopus Co. Ltd., Japan).
191
192 2.5 Determination of viscosity
193 The viscosities of both the dispersed phase and continuous phase containing 5 wt% Span 85
194 were measured using a vibrational viscometer (SV-10, A&D Company Ltd. Tokyo, Japan) at
195 25^{o}C. Vibrational viscometer measures viscosity by detecting the electromagnetic electric
196 current necessary to resonate the two sensor plates (immersed in the fluid sample whose
197 viscosity is to be determined) at a constant frequency and amplitude. The driving electric
198 current will be detected as the magnitude of viscosity produced between the sensor plates and

the fluid sample. The viscosity measured was then calculated to obtain the absolute viscosity based on the following formula:

$$\text{Absolute viscosity} = \text{viscosity measured} / \text{density}$$

The absolute viscosity value was referred simply as viscosity throughout the manuscript. Each measurement was repeated five times and the calculated mean values were used.

2.6 Determination of interfacial tension

The interfacial tension between an ALG solution (0 to 3 wt%) and the continuous phase was determined by using the pendant drop method. The profile of the drop of ALG solution suspended in 5 wt% Span 85 was measured using a full automatic interfacial tensiometer (PD-W, Kyowa Interface Science Co., Ltd., Saitama, Japan). Each measurement was repeated at least 20 times and the calculated mean values were used.

2.7 Determination of contact angle

The static contact angle in this study was defined as the angle of the ALG solution droplet to the MC plate surface in the continuous phase. These contact angles were measured by analyzing the images captured of the ALG solution droplets after the shape of the ALG solution droplets became stable on the MC plate surface. Each measurement was repeated at least five times and the calculated mean values were used.

3. Results and discussion

3.1 Preparation of W/O emulsion droplets by MC emulsification

W/O emulsion droplets were used as precursors for the formation of uniformly-sized ALG microspheres. Kawakatsu et al. first proposed the production of monodipserse W/O emulsions using hydrophobic grooved MC arrays, which consisted of highly uniform micro-

grooves with a slit-like terrace and a deeply etched well [17]. Fig. 2 shows the successful MC emulsification of uniformly-sized W/O emulsion droplets with 3 wt% ALG solution as the dispersed phase (flow rate of 0.05 mL/h) and 5 wt% Span 85 in iso-octane as the continuous phase (flow rate of 5 mL/h). It has been suggested that a low hydrophilic-lipophilic-balance (HLB) value of the surfactant was necessary to produce stable W/O emulsions by MC [23]. The surfactant used in our experiment, Span 85, has an HLB value of 1.8 and was found to be capable of forming stable W/O emulsions. During droplet generations, the dispersed phase passed through the channels and began to expand in a disk-like shape at the terrace which continued to expand in the well and subsequently detached from the well. The domineering force of the spontaneous transformation of the to-be-dispersed phase can be explained by the interfacial tension effects on the terrace and in the well due to the instability and nonlinearity flow of the to-be dispersed and continuous phases [31]. Microscopic observation of the sample withdrawn from the dispersions before the addition of $CaCl_2$ revealed a morphology that is uniformly-sized, discrete and spherical. The surfaces of the MC plate and glass plate which were initially hydrophilic (due to the presence of silanol groups on their surface) were modified by hexamethyldisilazane to make them hydrophobic. After silanization, the static contact angles of the ALG solutions on the surface of the modified MC plate were measured to be approximately 145^{o}-150^{o} across the various ALG concentration studied (from 0.1 wt% to 3 wt%), indicating that the difference in the ALG concentrations did not have a remarkable effect on the contact angles (Table 1). Sugiura et al. and Kawakatsu et al. reported that when the contact angles were greater than 120^{o}, W/O emulsions were successfully generated from the MCs and these contact angles were found to be dependent on the composition of the oil phase (i.e. the continuous phase of W/O emulsion), the type of surfactant and silane coupler reagent used for the modification of the MC surface [23, 30]. Furthermore, these large contact angles were also an indication of the preferential wetting of the channel surface by

the continuous phase, which is another important prerequisite for the successful generation of uniformly sized droplets [17, 30].

3.2 Effect of ALG concentration in dispersed phase

Other factors have also been identified to affect droplet formation, such as channel geometry, composition of the to-be-dispersed phase and continuous phase and types of surfactants/emulsifiers [32-34]. The viscosity ratio of dispersed phase to continuous phase has also been indicated to be another determining factor influencing the formation time and size of emulsion droplets [30, 35]. In our experiment, the effect of dispersed phase concentration on the d_{av} and CV of the W/O emulsion was investigated (Fig. 3a). The viscosity of the ALG solution, as expected, increased with higher ALG concentration while the interfacial tension at the interface of the ALG solution (concentration of 0.1 wt% to 3.0 wt%) and continuous phase remained almost unchanged (Table 1). The flow rates of the dispersed and continuous phases were controlled at 0.05 mL/h and 5 mL/h respectively. When the ALG concentration was increased, the d_{av} and the CV of the emulsion droplets decreased from ca. 22 μm to 18 μm and 26% to 5% respectively (Fig. 3a). These results correlated well with its particle size distribution which became narrower as the ALG concentration was increased, indicating the formation of a more regular W/O emulsion droplets (Fig. 3b). Kawakatsu et al. [30] demonstrated a decrease in the d_{av} when the ratio of the viscosity of the dispersed phase to the continuous phase increased. As the viscosity of the dispersed phase increased, the rate in which the dispersed phase was transferred to the terrace became slower, causing the amount of the dispersed phase supplied into the well and subsequently the channel exit to decrease [30, 35], hence explained the smaller d_{av} obtained in our experiment. Kobayashi et al. reported that a decrease in the droplet diameter of SDS stabilized O/W emulsion droplets produced via the oblong MCs was influenced by the

increase in the dispersed phase (silicone oil) viscosity at below the threshold level of 100 mPa. On the other hand, they attributed the increase in the droplet diameter above the threshold level to the reduction in the dynamic interfacial tension [36].

A study by Sugiura et al. revealed that a low to-be-dispersed phase viscosity during MC emulsification will lead to an increase in the production rate of droplets from each channel [35]. However, this was apparently not the case in our study as droplet formation from the channels proved to be difficult at low dispersed phase viscosity (i.e. ALG concentration of 0.1 wt%). At the start of the emulsification process, the dispersed phase appeared to have flowed through the channels and reached the channel exits. However, very few droplets were generated as almost no channels were functioning except for a few channels (Fig. 4a). These active channels too soon stopped working shortly after the droplets were formed. Emulsion droplets could only be generated again from the MCs when more force was applied to the dispersed phase flow. Once this force was removed, droplets generation from the channels stopped instantly, concurrently with the withdrawal of the dispersed phase back into the narrow channels.

A close look at these channels showed some small aggregates consisting of tiny droplets accumulating at the channel cleavage and blocking the outflow of the dispersed phase from the channel exits. These aggregates could also be seen to form a layer on the surface of some of the freshly formed droplets under low ALG emulsification condition (Fig. 3c). However, when sodium chloride (~0.5 wt%) enriched ALG solution was used as the dispersed phase, no aggregates were observed and emulsion droplets could be formed easily even without exerting extra pressure to the dispersed phase. Similar observations were also noted by Kobayashi et al. who studied the effect of osmotic pressure of the pure and salted water dispersed phase on the formation behavior of W/O emulsion through an asymmetric straight-through MC array [37]. They reported the formation of highly uniform aqueous

droplets at an osmotic pressure of 0.42 MPa and above but at a low osmotic pressure (i.e. 0.085 MPa and below), droplets formed were polydisperse and were marked by the presence of a rough thin layer surrounding the droplets, in correlation to our observation as mentioned earlier. They suggested this to a phenomenon known as "spontaneous emulsification (SE)", which occurred at the interface between two liquids under a favorable condition.

In order to confirm this phenomenon, the following experiment was carried out to mimic the behavior of the emulsion droplets in the continuous phase. A small drop of the dispersed phase (0.1 wt% ALG concentration) measuring about 1 mm in diameter was carefully dispensed from a syringe needle and allowed to hang in the continuous phase while making sure that the drop did not detach from the tip of the needle during the course of the experiment. Changes to the interface of the drops were observed over time (Fig. 4b(i)). At the start of the experiment, the surface of the drop was clearly smooth (0 min). As time evolved, a whitish layer (which appeared to be aggregates of small droplets) surrounding the bottom portion of the drop started to become visible (15 min). This whitish layer continued to grow over time until it completely covered the whole drop (30 min) and finally formed a rough contour of aggregates around the drop (45 min). The growth of the observed whitish layer is evidently consistent with the behavior of SE. Uricanu et al. in their study on SE behavior at the water-oil interfaces with pure or salted water or gelatin solutions as the aqueous phase and Span 80 in dodecane as the oil phase, discovered the formation of sparse small droplets that increased in number and size over time until the concentration gradients between the oil phase and water-oil interface reached equilibrium [38]. SE can only occur at above a surfactant threshold concentration [38] and in our study, the Span 85 at a concentration of 5 wt% was more than sufficient to induce SE especially at low ALG concentration. Nevertheless, at higher ALG concentration, for example 2.0 wt%, while keeping the concentration of surfactant constant, the process of SE was somewhat delayed or inhibited as

the drop surface appeared to be smooth throughout the duration of the experiment (Fig. 4b(ii)), a result which was similar to the observation during MC emulsification.

The presence of other components (e.g. salt or gelatin) in the aqueous phase has been shown to suppress SE though not likely to arrest the process completely [38]. The presence of sufficiently high ALG concentration in the aqueous phase in our study was believed to have exerted the same effect of protecting the interface against SE. The mechanisms that is responsible for SE is still not very well understood. It has been reported that electrolyte such as potassium chloride, when added to the dispersed phase act as a stabilizer for W/O emulsion droplets [39]. The presence of additives like salt will create an osmotic pressure in the water phase, forcing the surfactant to compete with the salt and interfering in the mass transport of water molecules across the water-oil interface [38]. Therefore, in enriched aqueous phase (with salt or gelatin), a higher surfactant concentration would be needed to induce SE [38]. Also, as a result of preferential hydration of salt, a decrease in the interaction between water molecules and the hydrophilic group of surfactant molecules at the interface of the water-oil could have also strongly suppressed SE [40].

Though the presence of salt in one way will aid in the inhibition of SE, their presence will also result in an ALG gel structure which is physically unstable and weak such as when the anti-gelling cation of sodium and magnesium ions or chelating agents are used [7]. Considering all these factors, the most strategic resolution would be to produce the emulsion using a higher ALG concentration to avoid the incorporation of salt in the aqueous phase. Moreover, ALG particles made from high viscosity ALG solutions will impart the gel structure which is mechanically and physically more superior due to an increase in the polymer chain density and entanglement [4, 13].

3.3 Effect of flow rate of dispersed phase

In order to investigate the effect of flow rate of the dispersed phase on the d_{av} and its CV, the flow rate of the ALG solution was varied from 0.05 mL/h to 0.4 mL/h with the continuous phase flow rate fixed at 5 mL/h and ALG concentration kept constant at 3 wt%. We did not study the effect of the continuous phase flow rate as it has been reported previously that its influence was minimal [41]. Figs. 5a and 5b show changes in the d_{av}, CV and particle size distribution of the O/W emulsion droplets as a function of the dispersed phase flow rate. At a dispersed phase flow rate of 0.2 mL/h and below, the d_{av} of the W/O emulsion droplets was measured to be consistently the same at 18 μm but at a flow rate above 0.2 mL/h, a slight increase in the d_{av} was observed (Fig. 5a). During droplet formation, the formation of larger d_{av} at a higher dispersed phase flow rate could be attributed to the increase in the volume of the dispersed phase that flowed into the well as the dispersed phase flow rate was increase. Nevertheless, the CV remained almost constant and the change in d_{av} was in fact not as pronounced as the one encountered when the ALG concentration was increased. Kobayashi et al. revealed that when soy bean oil or silicone oil was used as the dispersed phase and 1.0 wt% SDS was used as the continuous phase, the size of the droplet diameter of O/W emulsions was independent of the dispersed phase flow rate at below a threshold level but above this critical level, the increase in the droplet size was highly dependent on the dispersed phase velocity inside the channel [42]. Sugiura et al. noted that the increase in the d_{av} as attributed to an increase in the dispersed phase flow rate was especially more obvious when the viscosity of the continuous phase was higher (e.g. in tetradecane or hexadecane) but to a lesser extent when a lower viscosity continuous phase was used (e.g. octane or decane) due to the longer duration taken to form droplets at the former [23].

Fig. 5c shows the droplet productivity at different flow rates of the dispersed phase based on the calculation of the d_{av}. The rate of increment in droplet productivity was especially more conspicuous at the flow rates of 0.2 mL/h and below because the d_{av} obtained

remained relatively unchanged even with an increase in the flow rates of the dispersed phase. In fact, droplet productivity at these flow rates can be expressed by the following simple linear equation:

$$f = 3.2\text{x}10^{8} Q_d$$

where f is the droplet productivity (number of droplets/h) and Q_d is the flow rate of alginate solution (mL/h). In contrast, above these flow rates, the rate of increment in their droplet productivity was less pronounced due to the formation of droplets with larger volume marked by higher flow rates.

3.4 Formation of ALG microspheres by external gelation

Prior to external gelation, Tween 85 was added to the W/O emulsion followed subsequently by the addition of $CaCl_2$ to induce gelation. At the ALG concentration of 3 wt%, shrinkage of the ALG gel particles by approximately 96% (volume basis) from the initial W/O d_{av} of 18.1 μm prior to gelation (Fig. 6a(i)) to ca. 6.2 μm of ALG microspheres after gelation (Fig. 6a(iv)) was observed. The ALG microspheres obtained appeared to be highly spherical and uniform in size with no visible imperfections or irregularities at a CV of below 10%. The decrease in particle size was supported by the particle size distribution which showed a shift of its size distribution from the upper size range to the lower size range (Fig. 6b).

The addition of the right blend of a lipophilic surfactant (i.e Span 85) and a hydrophilic surfactant (i.e Tween 85) has been reported to improve droplet dispersity [43], hence facilitate the formation of a more stable emulsion. In addition, the incorporation of sufficient quantities of the surfactants are essential to ensure that the particles formed are discrete and spherical in shape and not distorted or fused together in large aggregates [44]. Also, the hydrophile-lipophile balance (HLB) of the surfactant mixture has been found to affect the

size distribution, shape and drug release profile of the ALG microspheres [29]. Fig. 6a shows the microscopy images of the W/O emulsion before external gelation and ALG microspheres after external gelation. The ALG microspheres were subjected to different concentration ratios of the surfactants in order to optimize the best condition for the formation of discrete and uniformly-sized ALG microspheres. Both Tween 85 and Span 85 are non-ionic surfactants with HLB values of 11.0 and 1.8 respectively. In the absence of Tween 85 (concentration ratio of Tween 85 to Span 85 of 0), microspheres obtained were highly aggregated (Fig. 6a(ii)). However when Tween 85 was incorporated into the system at a concentration ratio of 0.5, the aggregation appeared to be less pronounced (Fig. 6a(iii)). Nevertheless, in both cases, it was difficult to disperse the aggregated droplets by water bath sonication. At a higher Tween 85 to Span 85 concentration ratio of 1.0 (equivalent to higher HLB value), the microspheres obtained were dispersed as discrete spherical particles (Fig. 6a(iv)). In the event of some slight flocculations, we were able to separate these flocs into discrete particles by subjecting the particles to sonication while maintaining its uniformity. Wan et al. suggested that surfactant mixture with a higher HLB value (equivalent to a higher proportion of the hydrophilic surfactant) has a greater affinity for $CaCl_2$ [44], and hence would be able to form rigid microspheres completely before they could be distorted by the turbulence resulting from introducing $CaCl_2$ solution into the system. Therefore, the right proportion of the two surfactants is critical in order to obtain ALG particles with the desired attributes. Other factors indicated to have pronounced influences on the size and morphology of the ALG microspheres were stirring speed, rate of $CaCl_2$ addition, concentration and composition of encapsulating material [43].

$CaCl_2$ acts as a cross-linking agent, whereby the positively charged Ca^{2+} ions form electrostatic bridges between the negatively charged polysaccharides [45]. These divalent cations participated in the intermolecular binding between the relatively stiff G-blocks on

different ALG molecules by forming junction zones which resulted in a three dimensional network gel. The binding zone between G-blocks is commonly known as the "egg-box model" [11]. $CaCl_2$ would initially react with sodium alginate on the surface of the particles, enveloping the liquid particles with a thin layer of calcium alginate. The gradual diffusion of $CaCl_2$ into the core of the particles would eventually congeal the liquid core into rigid microparticles [43].

It is also well established that calcium–alginate gels shrink during gel formation due to water loss [46]. The kinetics of shrinkage may not solely lie with the gelation process as described above but may also be attributed to the process prior to gelation ie. during the MC emulsification process. Rondeau and Cooper-White attributed the size reduction of the ALG droplets during their transport downstream along the microfluidic channel to loss of water due to diffusion out of the polymeric droplets as a result of its partial miscibility with the continuous phase (which consists of anhydrous dimethyl carbonate) [21]. Presumably, the same phenomena occurred in our system, the diffusion of water at the interface of the two phases ie. the ALG and the Span 85-containing iso-octane phases are expected to proceed till both phases are in equilibrium with respect to each other. There is a possibility that the solvent exchange process could have taken place simultaneously with the gelation process unless the above mentioned equilibrium process is reached before the cross-linking process. Otherwise, the diffusion of the ALG phase would only stopped when it reached a stage in which the gel particles became condensed and finally hardened. Therefore, the relative solvency of the two liquids plays an important role in determining the rate of diffusion. This competing kinetics would have been one of the determining factors affecting its particle size. In addition, an increase in the Ca^{2+} content has also been widely known to cause greater shrinkage due to enhanced crosslinking density [13]. The degree of shrinkage has also been reported to be affected by the initial ALG concentration in the solution with a higher

concentration contributing to a much smaller and slower volume loss [21]. For example, we noticed that the W/O emulsion droplets produced at 0.1 wt% ALG concentration completely disappeared (based on our unaided eye observation) after leaving them overnight. In contrast, the presence of emulsion droplets was still evident after overnight when the concentration of ALG used was greater.

4. Conclusions

Uniformly-sized ALG microspheres were successfully fabricated using the combined MC emulsification technique and external gelation method. The formation of uniformly-sized W/O emulsion by MC emulsification preceded the formation of ALG microspheres. Emulsion droplets formed were between 18 to 22 μm at ALG concentration of 0.5% to 3.0 wt% and flow rate of 0.05 to 0.4 mL/h. Droplet generation appeared to be difficult at low ALG concentration (i.e. 0.1 wt%), probably as a result of the spontaneous emulsification (SE) phenomenon. Nevertheless, the smallest d_{av} and lowest CV of the W/O emulsions were obtained when the dispersed phase viscosity was at its highest while the flow rate of the dispersed phase was at the lowest. The d_{av} and CV of the ALG microspheres prepared at an ALG concentration of 3 wt% upon gelation was ca. 6.2 μm and below 10% respectively.

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References

[1] A.D. Sezer, J. Akbuga, Release characteristics of chitosan treated alginate beads: II. Sustained release of a low molecular drug from chitosan treated alginate beads, J. Microencapsulation 16 (1999) 687-696.

[2] W.R. Gombotz, S. F. Wee, Protein release from alginate matrices. Adv. Drug Delivery Rev. 31 (1998) 267-285.

[3] A.R. Degroot, R.J. Neufeld, Encapsulation of urease in alginate beads and protection from α-chymotrypsin with chitosan membranes, Enzyme Microbial Tech. 29 (2001) 321-327.

[4] O. Smidsrød, G. Skjåk-Bræk, Alginate as immobilization matrix for cells. Trends Biotech. 8 (1990) 71-78.

[5] S. Sugiura, T. Oda, Y. Aoyagi, R. Matsuo, T. Enomoto, K. Matsumoto, T. Nakamura, M. Satake, A. Ochiai, N. Ohkohchi, M. Nakajima, Microfabricated airflow nozzle for microencapsulation of living cells into 150 micrometer microcapsules, Biomed. Microdevices 9 (2007) 91-99.

[6] D. Quong, R.J. Neufeld, DNA protection from extracapsular nucleases, within chitosan- or poly-L-lysine-coated alginate beads, Biotech. Bioeng. 60 (2000) 134-134.

[7] J.S. Lee, D.S. Cha, H.J. Park, Survival of freeze-dried *Lactobacillus bulgaricus* KFRI 673 in chitosan-coated calcium alginate microparticles, J. Agric. Food Chem. 52 (2004) 7300-7305.

[8] A.H. King, Flavour encapsulation with alginates, in: S.J. Risch, G.A. Reineccius (Eds.), Flavour Encapsulation, American Chemical Society, Washington, 1988, pp. 122-125.

[9] K.G.H. Desai, C. Liu, H.J. Park, Characteristics of vitamin C immobilized particles and sodium alginate beads containing immobilized particles, J. Microencapsulation, 22 (2005) 363-376.

[10] K.I. Dragnet, Alginates, in: G.O. Phillips, P.A. Williams (Eds.), Handbook of Hydrocolloids, CRC Press, Boca Raton, FL, 2000, pp. 381-383.

[11] G.T. Grant, E.R. Morris, D.A. Rees, P.J.C. Smith, D. Thom, Biological interactions between polysaccharides and divalent cations: the egg-box model. FEBS Lett. 32 (1973) 195-198.

[12] D. Poncelet, R. Lencki, C. Beaulieu, J.P. Halle, R.J. Neufeld, A. Fournier, Production of alginate beads by emulsification/internal gelation. I. Methodology. Appl. Microbio. Biotechnol. 38 (1992) 39-45.

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

[14] T. Nakashima, M. Shimizu, M. Kukizaki, Particle control of emulsion by membrane emulsification and its application, Adv. Drug Deliver Rev. 45 (2000) 47-56.

[15] T. Thorsen, R.W. Roberts, F.H. Arnold, and S.R. Quake, Dynamic pattern formation in a vesicle-generating microfluidic device, Phys. Rev. Lett. 86 (2001) 4163-4166.

[16] D.R. Link, S.L. Anna, D.A. Weitz, H.A. Stone, Geometrically mediated breakup of drops in microfluidic devices, Phys. Rev. Lett. 92 (2004) 054503.1-054503.4.

[17] T. Kawakatsu, Y. Kikuchi, M. Nakajima, Regular-sized cell creation in microchannel emulsification by visual microprocessing method. J. Am. Oil Chem. Soc. 74 (1997) 317-321.

[18] I. Kobayashi, S. Mukataka, M. Nakajima, Novel asymmetric through-hole array microfabricated on a silicon plate for formulating monodisperse emulsions, Langmuir, 21 (2005) 7629-7632.

519 [19] K.S. Huang, T.H. Lai, Y.C. Lin, Manipulating the generation of Ca-alginate
520 microspheres using microfluidic channels as a carrier of gold nanoparticles, Lab Chip, 6
521 (2006) 954-957.
522 [20] T. Murakata, H. Honma, S. Nakazato, C. Kuroda, S. Sato, Control of particle size of
523 calcium alginate gel bead by application of electric field to interface between aqueous
524 and organic phases, J. Chem. Eng. Jpn. 34 (2001) 299-305.
525 [21] E. Rondeau, J.J. Cooper-White, Biopolymer microparticle and nanoparticle formation
526 within a microfluidic device, Langmuir 24 (2008) 6937-6945.
527 [22] A.M. Chuah, T. Kuroiwa, I. Kobayashi, M. Nakajima, Effect of chitosan on the stability
528 and properties of modified lecithin stabilized oil-in-water monodisperse emulsion
529 prepared by microchannel emulsification, Food Hyd. 23 (2009) 600-610.
530 [23] S. Sugiura, M. Nakajima, H. Ushijima, K. Yamamoto, M. Seki, Preparation
531 characteristics of monodispersed water-in-oil emulsions using microchannel
532 emulsification. J. Chem. Eng. Jpn. 34 (2001) 757-765.
533 [24] S. Sugiura, M. Nakajima, J. Tong, H. Nabetani, M. Seki, Preparation of monodispersed
534 solid lipid microspheres using a microchannel emulsification technique, J. Colloid
535 Interface Sci., 227 (2000) 95-103.
536 [25] S. Iwamoto, K. Nakagawa, S. Sugiura, M. Nakajima, Preparation of gelatin microbeads
537 with a narrow size distribution using microhannel emulsification, AAPS PharmSciTech.
538 3 (2002) 72-76.
539 [26] S. Sugiura, T. Kuroiwa, T. Kagota, M. Nakajima, S. Sato, S. Mukataka, P. Walde, S.
540 Ichikawa, Novel method for obtaining homogenous giant vesicles from a monodisperse
541 water-in-oil emulsion prepared with a microfluidic device, Langmuir 24 (2008) 4581-
542 4588.

543 [27] S. Sugiura, M. Nakajima, S. Iwamoto, M. Seki, Interfacial tension driven monodispersed
544 droplet formation from microfabricated channel array, Langmuir 17 (2001) 5562-5566.
545 [28] G.T. Vladisavljevic, H. Schubert, Preparation of emulsions with a narrow particle size
546 distribution using microporous α–aluminia membranes, J. Dispersion Sci. Tech. 24
547 (2003) 811-819.
548 [29] L.S.C., Wan, P.W.S. Heng, L.W. Chan, Surfactant effects on alginate microspheres, Int.
549 J. Pharm. 103 (1994) 267-275.
550 [30] T. Kawakatsu, G. Tragardh, C. Tragardh, M. Nakajima, N. Oda, T. Yonemoto, The
551 effect of the hydrophobicity of microchannels and components in water and oil phases on
552 droplet formation in microchannel water-in-oil emulsification, Colloids Surf. A:
553 Physicochem. Eng. Aspects, 179 (2001) 29-37.
554 [31] I. Kobayashi, K. Uemura, M. Nakajima, Formulation of monodisperse emulsions using
555 submicron-channel arrays, Colloids Surf. A: Physicochem. Eng. Aspects, 296, (2007)
556 285-289.
557 [32] J. Tong, M. Nakajima, H. Nabetani, Y. Kikuchi, Surfactant effect on production of
558 monodispersed microspehres by microchannel emulsification method, J. Surfactants
559 Detergents 3 (2000) 285-293.
560 [33] I. Kobayashi, M. Nakajima, S. Mukataka, Preparation characteristics of oil-in-water
561 emulsions using differently charged surfactants in straight-through microchannel
562 emulsification .Colloids Surf. A: Physicochem. Eng. Aspects, 229, (2003b) 33-41.
563 [34] M. Saito, L. Yin, I. Kobayashi, M. Nakajima, Preparation characteristics of
564 mondispersed oil-in-water emulsions with large particles stabilized by proteins in
565 straight-through microchannel emulsification, Food Hyd. 9 (2005) 745-751.

566 [35] S. Sugiura, N. Kumazawa, S. Iwamoto, T. Oda, M. Satake, M. Nakajima, Effect of
567 physical properties on droplet formation in microchannel emulsification, Kagaku
568 Kogaku Ronbunsbu, 30 (2004)129-134 (in Japanese).
569 [36] I. Kobayashi, S. Mukataka, M. Nakajima, Effects of type and physical properties of oil
570 phase on oil-in-water emulsion droplet formation in straight-through microchannel
571 emulsification, experimental and CFD studies, Langmuir, 21 (2005) 5722-5730.
572 [37] I. Kobayashi, Y. Murayama, T. Kuroiwa, K. Uemura, M. Nakajima, Production of
573 monodisperse water-in-oil emulsions consisting of highly uniform droplets using
574 asymmetric straight-through microchannel arrays. Microfluid Nanofluid, doi:
575 10.1007/s10404-008-0368-3 (2008) (in press).
576 [38] V.I. Uricanu, M.H.G. Duits, D. Filip, R.M.F. Nelissen, W.G.M. Agterof, Surfactant-
577 mediated water transport at gelatin/oil interfaces, J. Colloid Interface Sci. 298 (2006)
578 920-934.
579 [39] P. Becher (1957), Creaming, inversion and demulsification, In Emulsion: Theory and
580 Practice, edited by Maruzen Asian, Reinhold Publishing Corp., New York, pp. 134-165.
581 [40] F. Opawale, D. Burgess, Influence of interfacial properties of lipophilic surfactants on
582 water-in-oil emulsion stability, J. Colloid Interface Sci. 197 (1998) 142-150.
583 [41] G.T. Vladisavljevic, I. Kobayashi, M. Nakajima, Generation of highly uniform droplets
584 using asymmetric microchannels fabricated on a single crystal silicon plate: Effect of
585 emulsifier and oil types, Powder Tech. 183 (2008) 37-45.
586 [42] I. Kobayashi, S. Mukataka, M. Nakajima, Production of monodisperse oil-in-water
587 emulsions using a large silicon straight-through microchannel plate, Ind. Eng. Chem.
588 Res. 44 (2005) 5852-5856.

[43] L.S.C., Wan, P.W.S. Heng, L.W. Chan, Development of alginate microcapsules by emulsification, Proceedings of the NUS-JSPS Seminar on Recent Developments in Pharmaceutics and Pharmaceutical Technology (1990) 243-255.

[44] L.S.C., Wan, P.W.S. Heng, L.W. Chan, Influence of hydrophile-lipophile balance on alginate microspheres, Int. J. Pharm. 95 (1993) 77-83.

[45] P.A. Williams, G.O. Phillips, The use of hydrocolloids to improve food texture, in: B.M. McKenna (Ed.), Texture in Foods, Volume 1: Semi-Solid Foods, CRC Press, Boca Raton, FL, 2003, pp. 251-274.

[46] A. Martinsen, G. Skjåk-Bræk, O. Smidsrød, Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate beads. Biotech. Bioeng. 33 (1989) 79–89.

FIGURE CAPTIONS

Fig. 1 (a) Microchannel emulsification experimental set-up. (b) Schematic diagram of the surface view and dimensions of CMS 6-1 silicon MC plate. (c) Schematic diagram of the flow in the MC module.

Fig. 2 Microscopy image of droplet formation from the grooved microchannel plate at an alginate concentration of 3 wt%. The flow rates of the dispersed and continuous phases during MC emulsification were 0.05 and 5 mL/h, respectively.

Fig. 3 Effects of alginate concentration in the dispersed phase on the produced water-in-oil emulsions: (a) mean droplet diameters (d_{av}) and coefficient of variations (CV) and (b) droplet size distributions. The flow rates of the continuous and disperse phases during MC emulsion were 5 and 0.05 mL/h, respectively.

Fig. 4 (a) Microscopy image of droplet formation from the grooved microchannel plate at an alginate concentration of 0.1 wt%. The magnified view shows aggregates forming small aqueous droplets surrounding the to-be-dispersed phase prior to detachment as a droplet from the channel. The flow rates of the dispersed and continuous phases during MC emulsification were 0.05 mL/h and 5 mL/h, respectively. (b) Time evolution of spontaneous emulsification for (i) 0.1 wt% (ii) 2.0 wt% alginate concentration/iso-octane system with 5 wt% Span 85.

Fig. 5 Effects of dispersed phase flow rates on the produced water-in-oil emulsions: (a) mean particle diameters (d_{av}) and coefficient of variations (CV), (b) particle size distributions and (c) droplet formation rate. Alginate concentration was prepared at 3 wt%. The flow rate of the continuous phase during MC emulsion was 5 mL/h.

Fig. 6 (a) Effect of surfactant ratio (Tween 85: Span 85) on the microscopy images of alginate microspheres (i) original water-in-oil emulsion (before external gelation) (ii) 0:1 (iii)

637 0.5:1 (iv) 1:1 (b) Particle size distribution of (i) original water-in-oil emulsion (before
638 external gelation) (ii) alginate microspheres after external gelation prepared at alginate
639 concentration of 3 wt%, dispersed phase flow rate of 0.05 mL/h and continuous phase flow
640 rate of 5 mL/h. Alginate microspheres were dispersed in Milli-Q water except for the water-
641 in-oil emulsion droplets which were dispersed in 5 wt% Span 85 in iso-octane.

642

Figure 1a&b

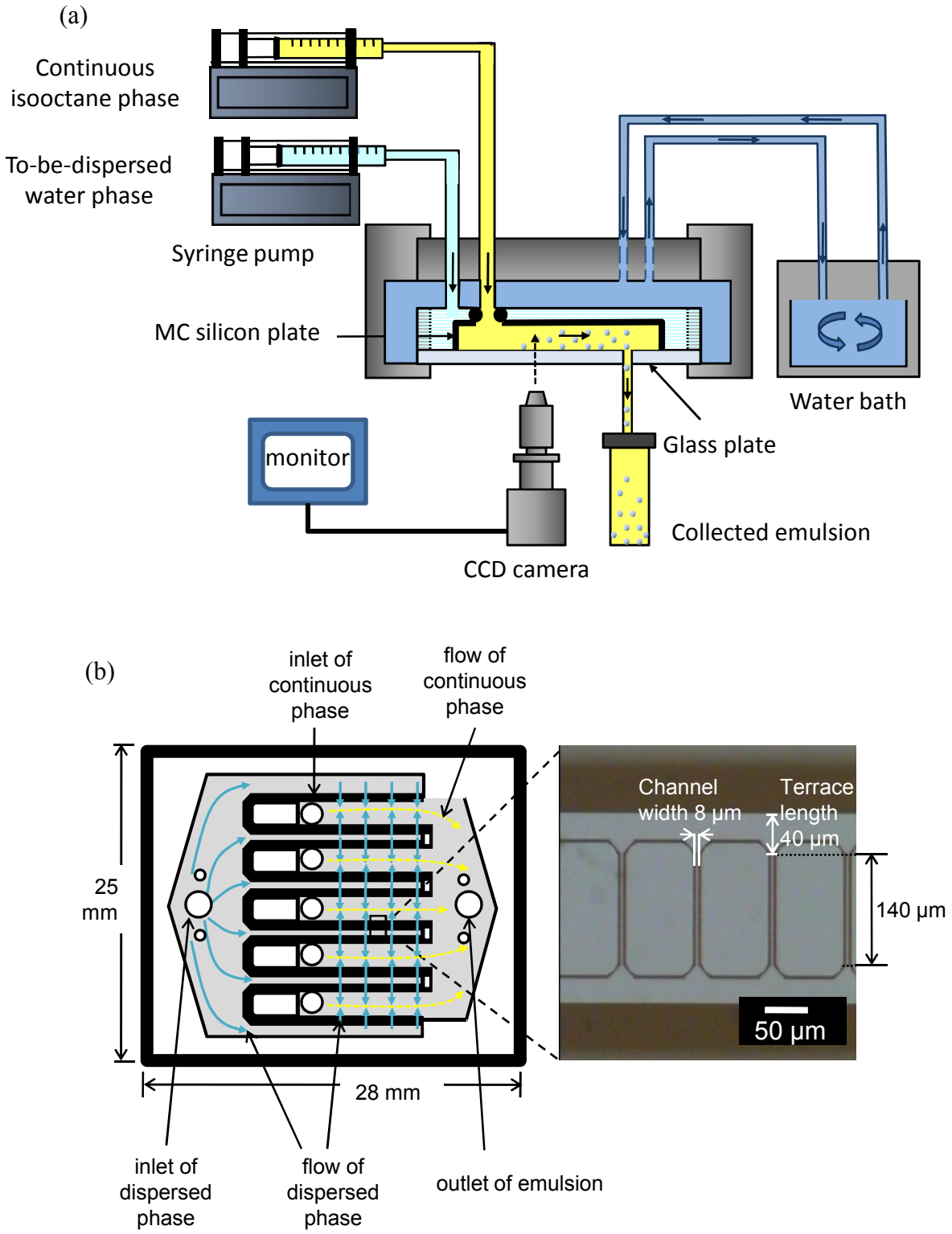


Fig. 1 Chuah et al.

Figure 1c

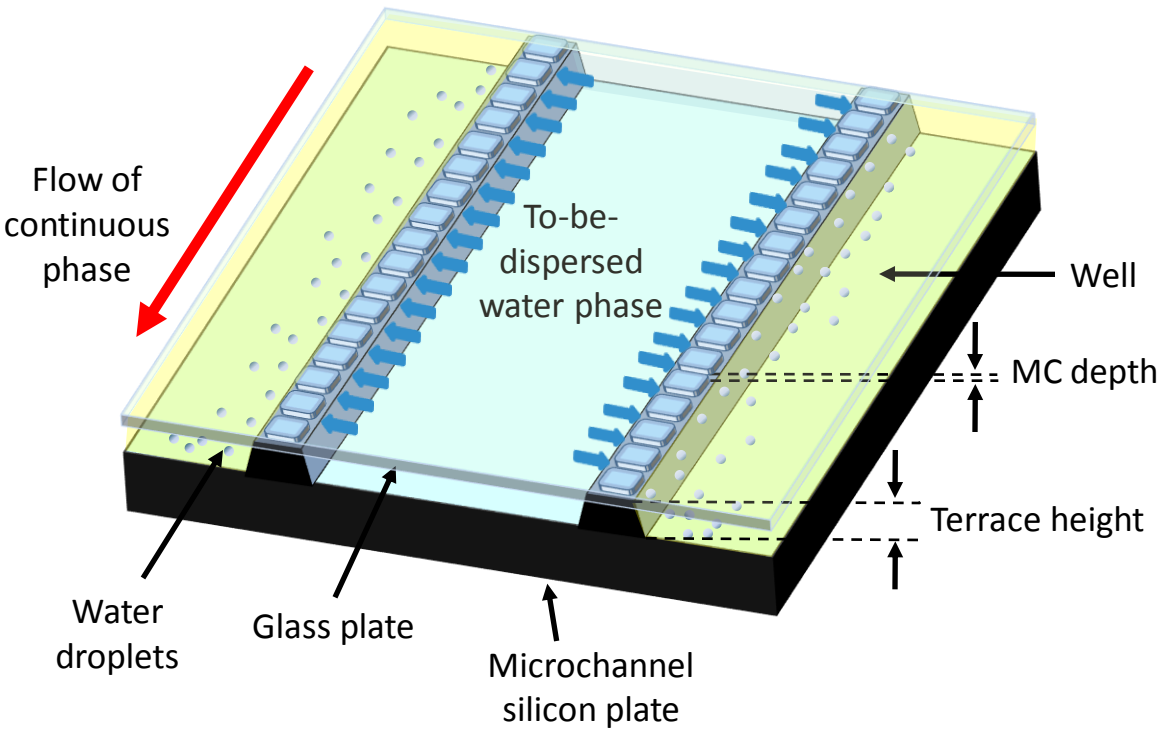


Fig. 1c Chuah et al.

Figure 2

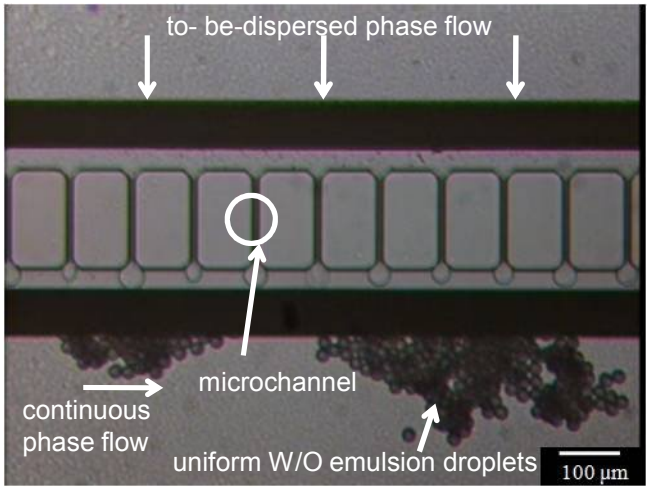


Fig. 2 Chuah et al.

Figure 3a

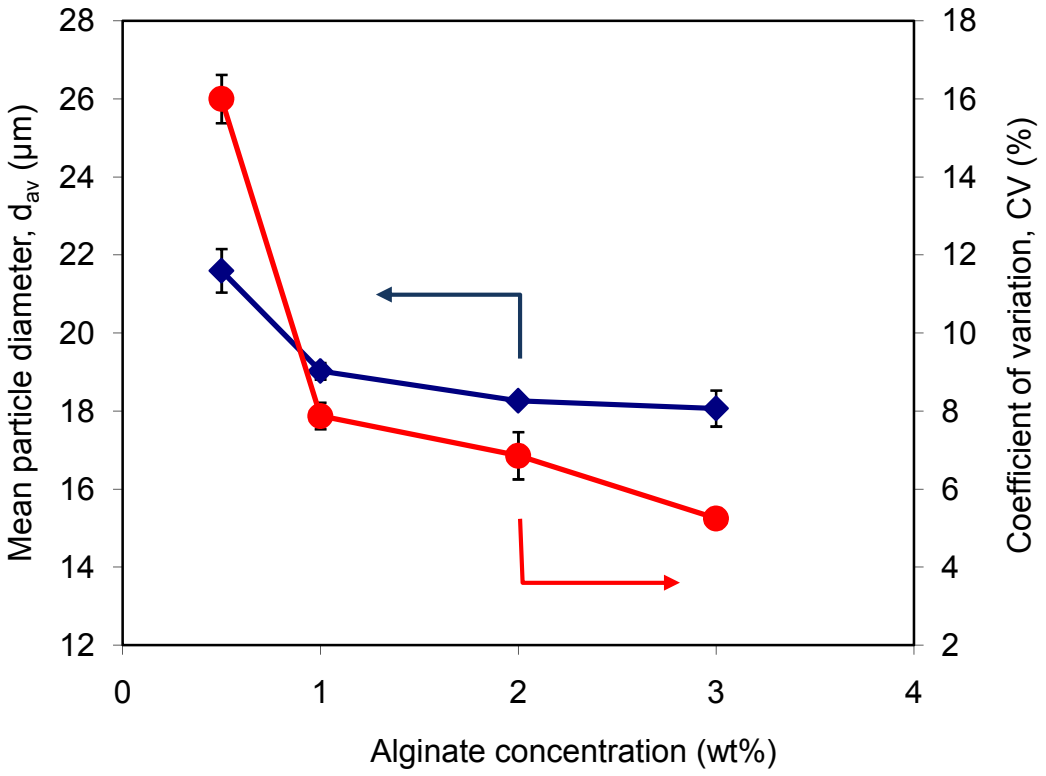


Fig. 3a Chuah et al.

Figure 3b

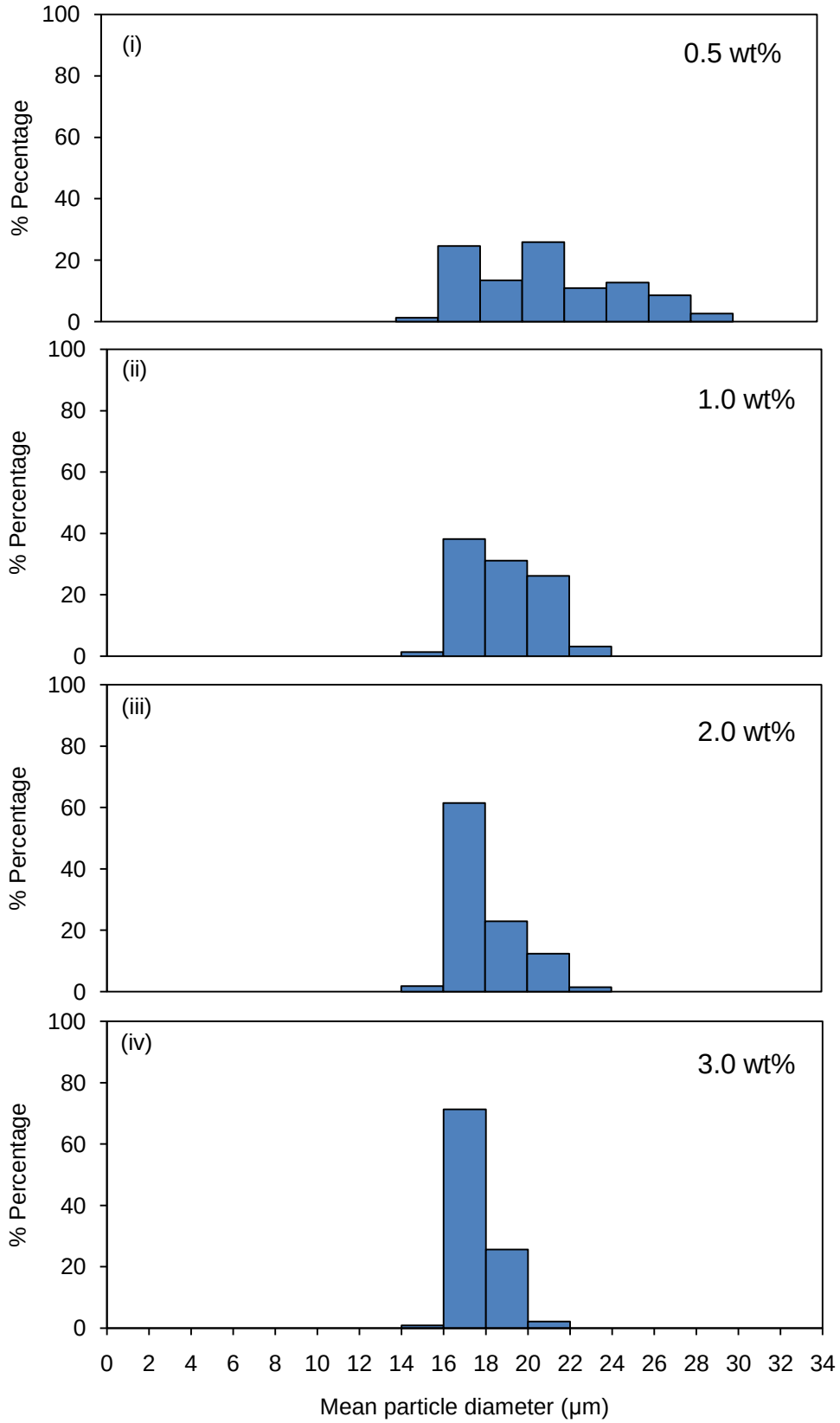


Fig. 3b Chuah et al.

Figure 4a

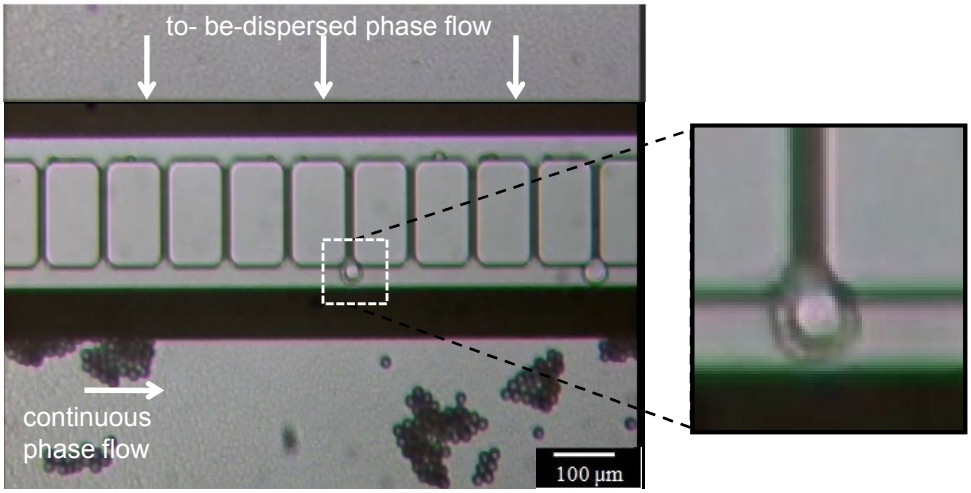
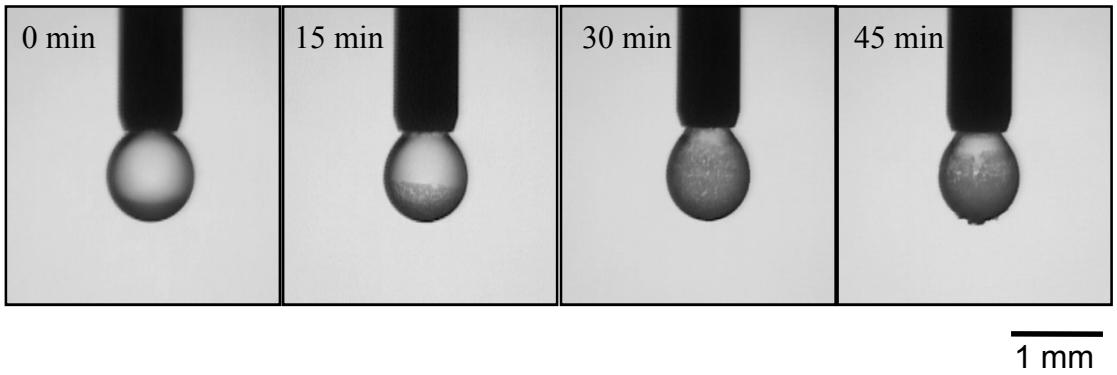


Fig. 4a Chuah et al.

Figure 4b

(i) 0.1 wt% alginate concentration



(ii) 2.0 wt% alginate concentration

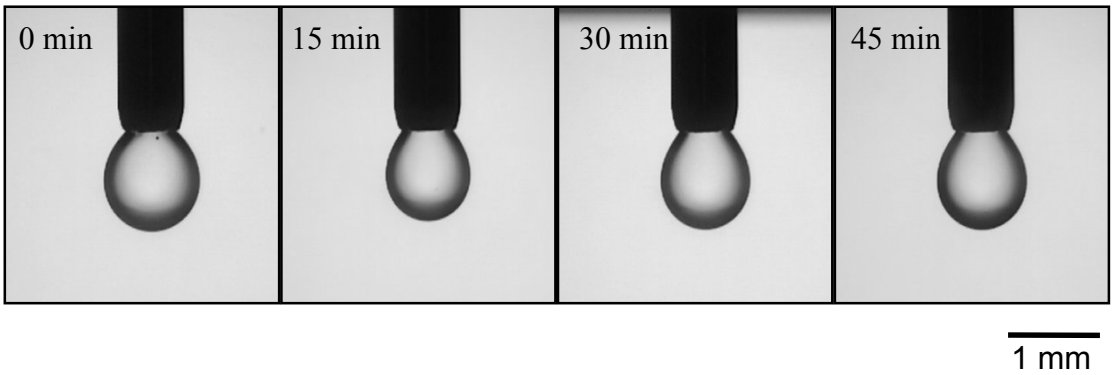


Fig. 4b Chuah et al.

Figure 5a

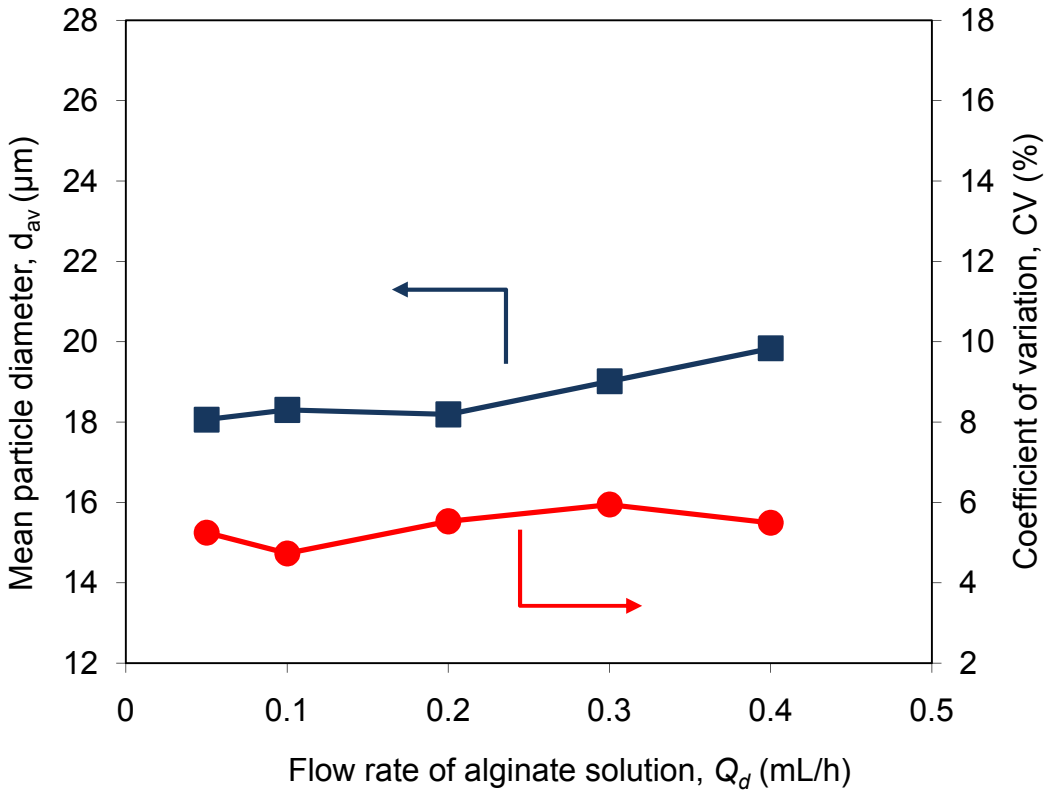


Fig. 5a Chuah et al.

Figure 5b

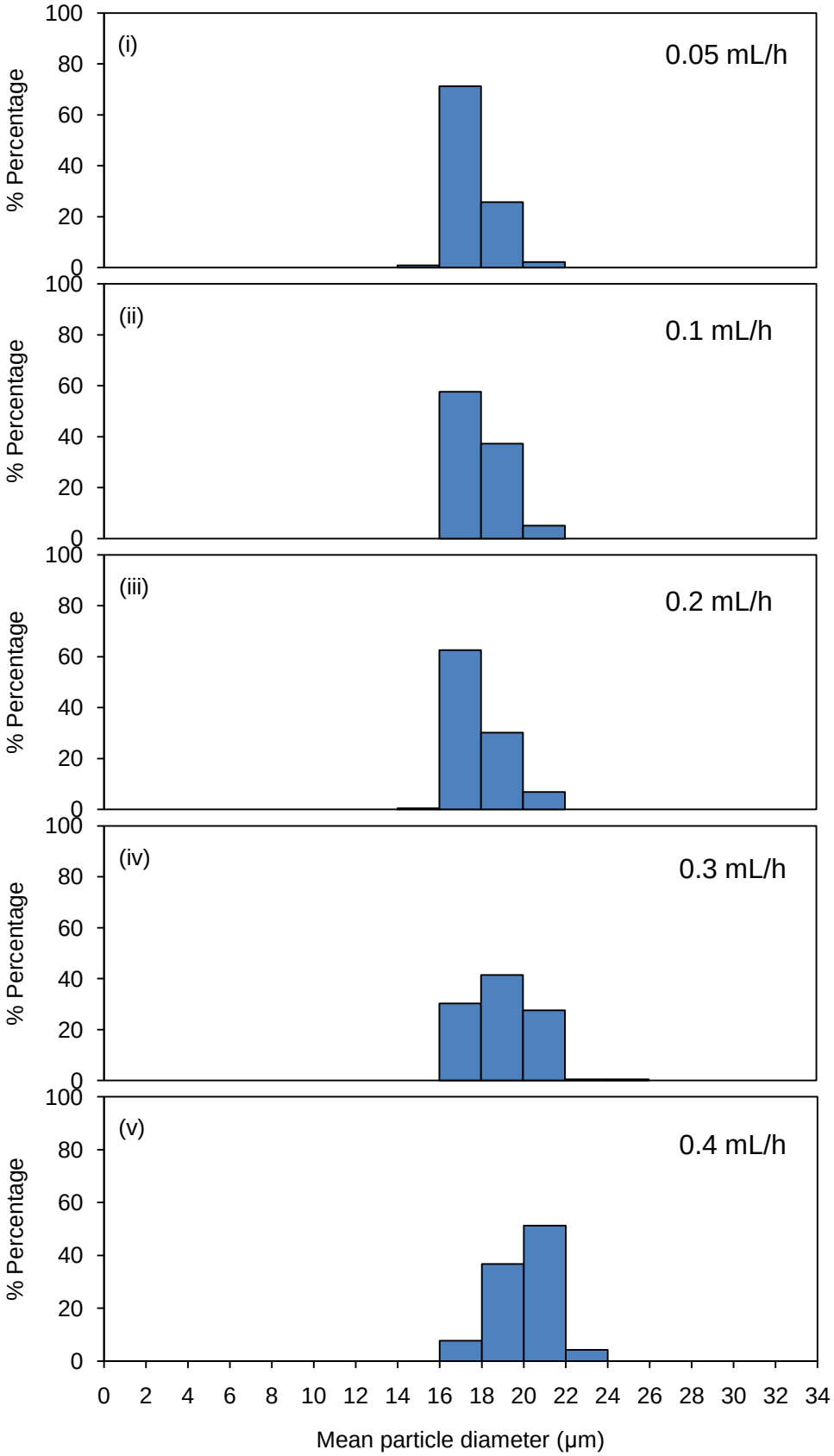


Fig. 5b Chuah et al.

Figure 5c

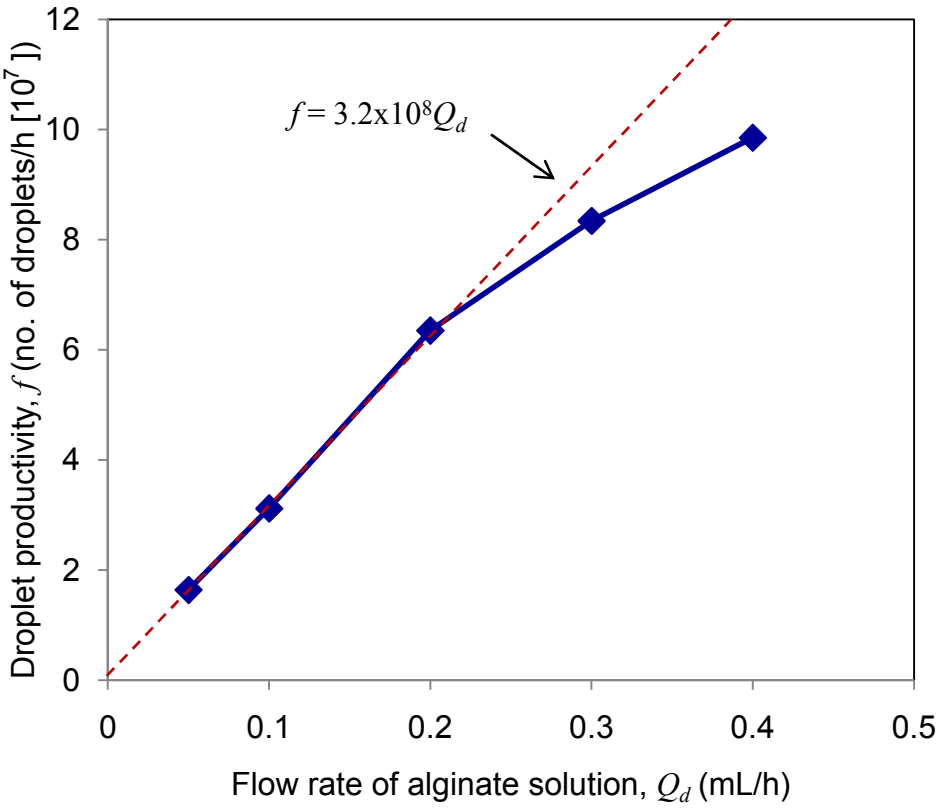


Fig. 5c Chuah et al.

Figure 6a

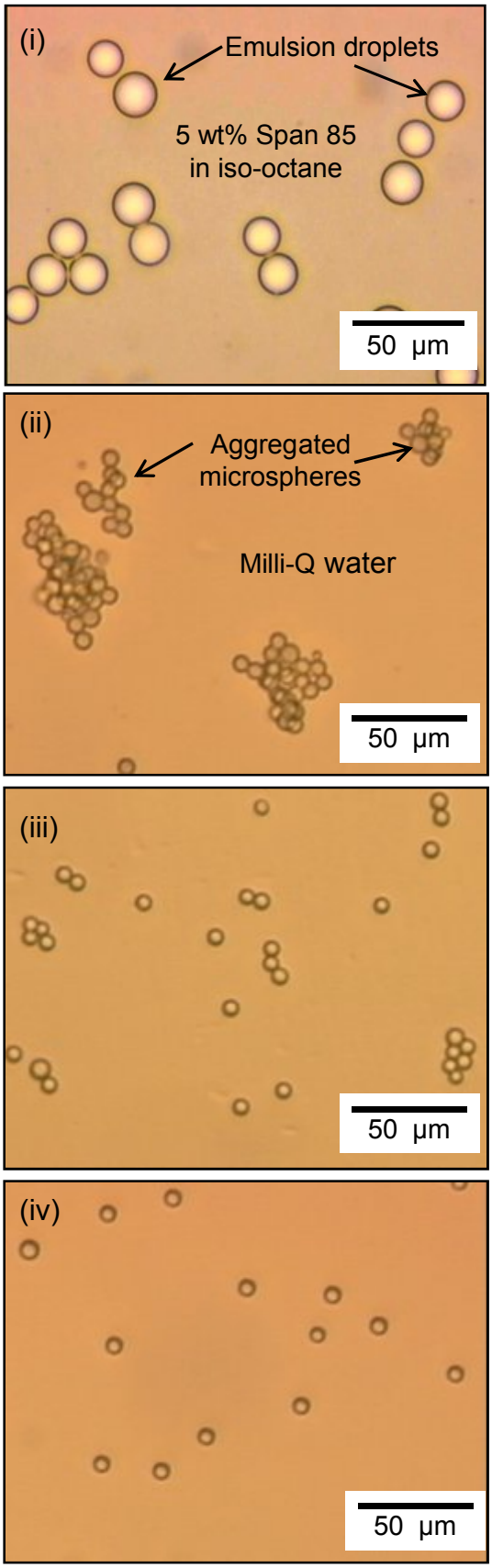


Fig. 6a Chuah et al.

Figure 6b

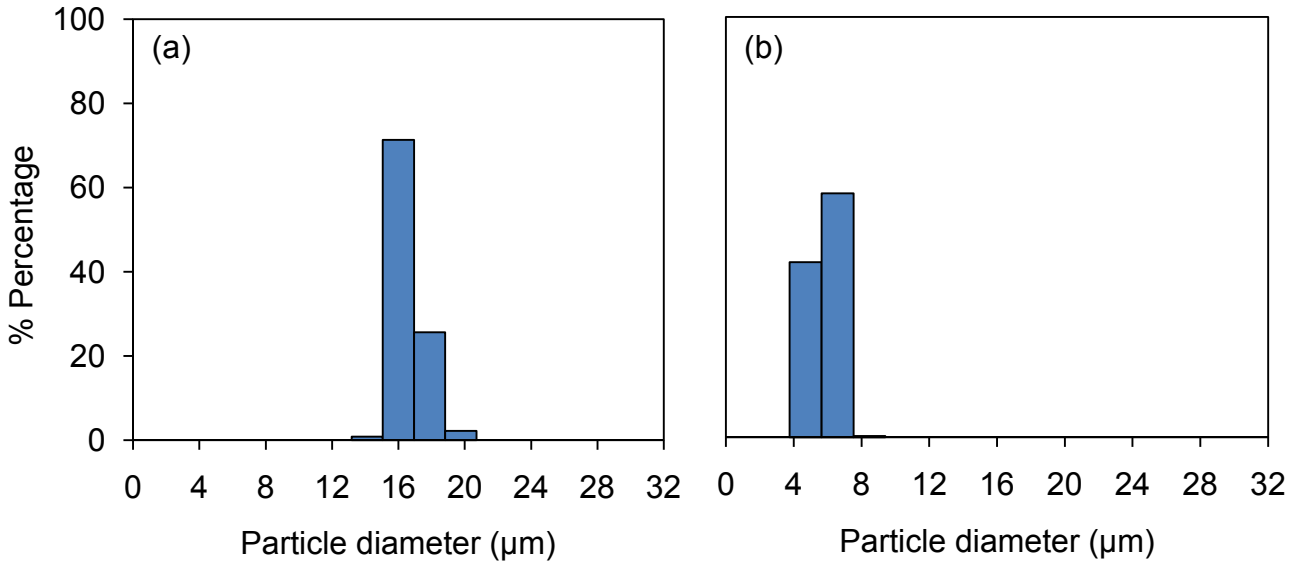


Fig. 6b Chuah et al.

Table 1

Table 1

Viscosity of alginate solutions, interfacial tension and static contact angle of the experimental system used in this study

Concentration (wt%)	Viscosity, η[a] (mPa.s)	Interfacial tension, γ (mN/m)	Static contact angle, θ (^{o})
Dispersed phase (Alginate solution)			
0	0.86	6.7	146.5
0.1	3.49	6.5	149.6
0.5	14.2	6.3	146.2
1.0	47.1	5.6	145.3
2.0	239	5.2	145.1
3.0	658	4.0	148.4
Continuous phase (Span 85 in iso-octane)			
5.0	0.43	-	-

[a] Referred to as absolute viscosity. Measured at 25^{o}C by means of a vibrational viscometer.