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Using a microfluidic chip and internal gelation reaction for monodisperse calcium alginate microparticles generation

Keng-Shiang Huang, Tzung-Heng Lai, Yu-Cheng Lin

Department of Engineering Science, National Cheng Kung University, No.1 University Road, Tainan 701, Taiwan, ROC

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1. ABSTRACT

In this paper, the manipulation of monodisperse Ca-alginate microparticles using a microfluidic chip and a reaction of internal gelation is presented. Our strategy is based on the sheath focusing effect to form uniform water-in-oil (w/o) emulsions in the cross-junction microchannel. These fine emulsions, consisting of 1.5 % w/v Na-alginate and 1.0 % w/v calcium carbonate, are then dripped into an oil solution containing 20 % v/v glacial acetic acid and 1 % v/v Tween 80 to obtain Ca-alginate microspheres in an efficient manner. The mechanism is that acetic acid reacts with the calcium carbonate to release the calcium ions, and these calcium ions, through crosslinking reaction with the alginate, produce Ca-alginate microspheres. We have demonstrated that it is possible to control the size of Ca-alginate microparticles from 80 μm to 800 μm in diameter (with a variation of less than 10 %) by altering the relative sheath/sample flow rate ratio. Experimental data has shown that for a given 0.01 mL/min of the dispersed phase flow (sample flow), the emulsion size decreased as the average velocity of the continuous phase (oil flow) increased. The same tendency was observed in the 0.05 mL/min and 0.10 mL/min of dispersed phase flow. The microfluidic chip is capable of generating relatively uniform micro-droplets and has the advantages of active control of droplet diameter, simple and low cost process, and a high throughput.

2. INTRODUCTION

Ca-alginate is currently gaining a great deal of attention for medical applications as well as for the controlled release of drugs (1, 2). The success of Ca-alginate beads as carriers is due to the following features: (i) they can dissolve poorly soluble drugs and thus increase their bioavailability, (ii) they can stay in the body (in the blood) long enough to provide gradual accumulation in the required area, (iii) their size permits them to accumulate in body regions with leaky vasculature, (iv) they can be tailored to achieve targeting or other desired properties by attachment of a specific ligand to the outer surface, (v) they have low toxicity and high loading capacity, as well as minimize drug degradation and loss, and (vi) they can be easily produced in large quantities (3-6).

To-date, the production of Ca-alginate beads has been accomplished mainly by using external gelation (dripping method). For example, Na-alginate is extruded dropwise through a needle into a solution of divalent cations, which induces cross-linking of the guluronic residues of the alginate polymer (3, 4, 7, 8). The alternative techniques are (i) atomization (spray-drying) (6, 9), (ii) coacervation (10), (iii) emulsification (internal/external gelation, as shown in Figure 1) (11-14), and others. However, the resulting size of the microspheres cannot be

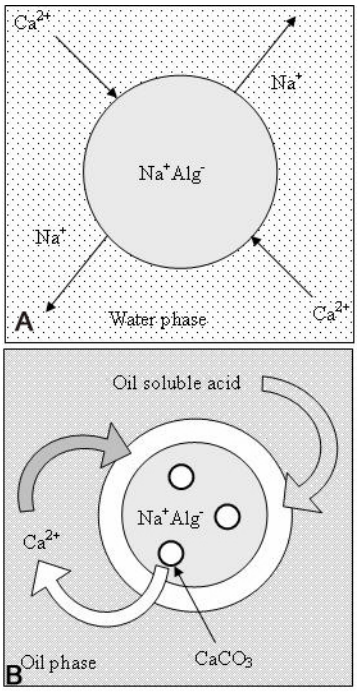

Figure 1. Schematic illustrations of external gelation and internal gelation: (a) external gelation: a Na-alginate droplet is transported to a solution of divalent cations (e.g. $CaCl_2$ solution), which induces cross-linking of the guluronic residues of the alginate polymer. The material to be encapsulated is usually mixed with a Na-alginate solution, and the mixture is dripped into a solution containing calcium ions. Ca^{2+} reacts instantaneous with the carboxylic groups of guluronic acid residues at the droplet surface, then diffuses inward and reacts to form Ca-alginate gel which can entrap drugs within a 3D-lattice. And (b) internal gelation: insoluble calcium salt ($CaCO_3$) is mixed with a Na-alginate solution and is then emulsified in the oil phase containing surfactant and acid. The oil soluble acid diffuses through the oil-water interface and reacts with $CaCO_3$ to release free Ca^{2+}. As a result, Na^+ of alginate is substituted by Ca^{2+} to form Ca-alginate gel (25).

easily controlled by these methods, and the obtained beads tend to coagulate into large masses before hardening properly (12). Control of the particle size and the size distribution is important for controlled-release drug delivery, because they influence the clearance rate from the body and ultimately determine the drug dosage (15). Basically, an ideal particle size could provide the optimal release rate and route of administration.

Many researchers have attempted to make smaller spheres, but less attention has been paid to obtain monodisperse spheres (12, 16, 17). Recently, Nakajima *et al.* developed a novel microfluidic device that utilized a silicon micro-nozzle array and an external gelation reaction to produce 50-200 μm Ca-alginate beads with the variation within 15 % (18). In the present paper a new microfluidic device combined with an internal gelation reaction is developed for size-controlled generation of monodisperse Ca-alginate microparticles. Microfluidic chip (containing cross-junction microchannel) emulsification is a novel technique for preparing water-in-oil (w/o) and oil-in-water (o/w) emulsions (19-23). The mechanism of this type of microfluidic chips in droplet-volume control has been well researched in recent literatures (19, 20). In contrast, no attention has been paid to apply microfluidic chip to control the performance of uniform Ca-alginate microspheres.

The aim of this study is to investigate the size of the Na-alginate micro-emulsions obtained by a different ratio of flow rate in the side inlet channels to that in the center inlet channel. The emulsions containing a suspension of Na-alginate and $CaCO_3$ are transported to an oil solution and then undergo internal gelation reaction which is initiated by the introduction of oil soluble acid to convert insoluble calcium carbonate to free Ca^{2+} resulting in the formation of uniform Ca-alginate microparticles. This proposed platform is easy to fabricate, easy to set-up, and is easily programmed to generate a large set of regular Ca-alginate microspheres.

3. MATERIALS AND METHODS

3.1. Materials

A commercial sample of low viscosity sodium alginate (Na-alginate, viscosity 250 cP in 1.5 % solution at 25°C; brown algae), was purchased from Sigma Chemical Co. (MO, USA). The sunflower seed oil was purchased from Uni-President Enterprises Corp., Taiwan. All other chemicals (10 μm of calcium carbonate, Tween® 80 and glacial acetic acid purchased from Sigma-Aldrich Co.) were of analytical grade and were used without further purification.

3.2. Principle

In this study we reported the use of microfluidics to elicit control over the spontaneous self-assembly of w/o emulsions from a solution of dissolved Na-alginate and $CaCO_3$. These semi-products (emulsions) were then dripped into an oil solution containing acetic acid and Tween 80, resulting in the instantaneous formation of Ca-alginate microspheres (Figure 2). The mechanism of Ca-alginate microspheres synthesis is that acetic acid reacts with the calcium carbonate to release the calcium ions, and these calcium ions then undergo crosslinking with Na-alginate to produce Ca-alginate microparticles (Figures 1b and 2b). The generation of a narrow size distribution of self-assembling emulsions is based on the focusing force in the cross-junction channel. The mechanism of this type of microfluidic chips is that varying the ratio between oil and water flow rates it allows for a finer control of the droplet size (20). Based on the superior performance of the microfluidic chip, we utilize it in this work for pharmaceutics (e.g. Ca-alginate particle generation).

3.3. Experimental procedure

Figure 3 shows an overview of the experimental setup used. The procedure is as follows. First, the fluids of the center and side inlet channels were connected to a pregel solution (the solution containing Na-alginate and $CaCO_3$) and oil, respectively. Generally speaking, the material to be encapsulated is mixed with a pregel solution.

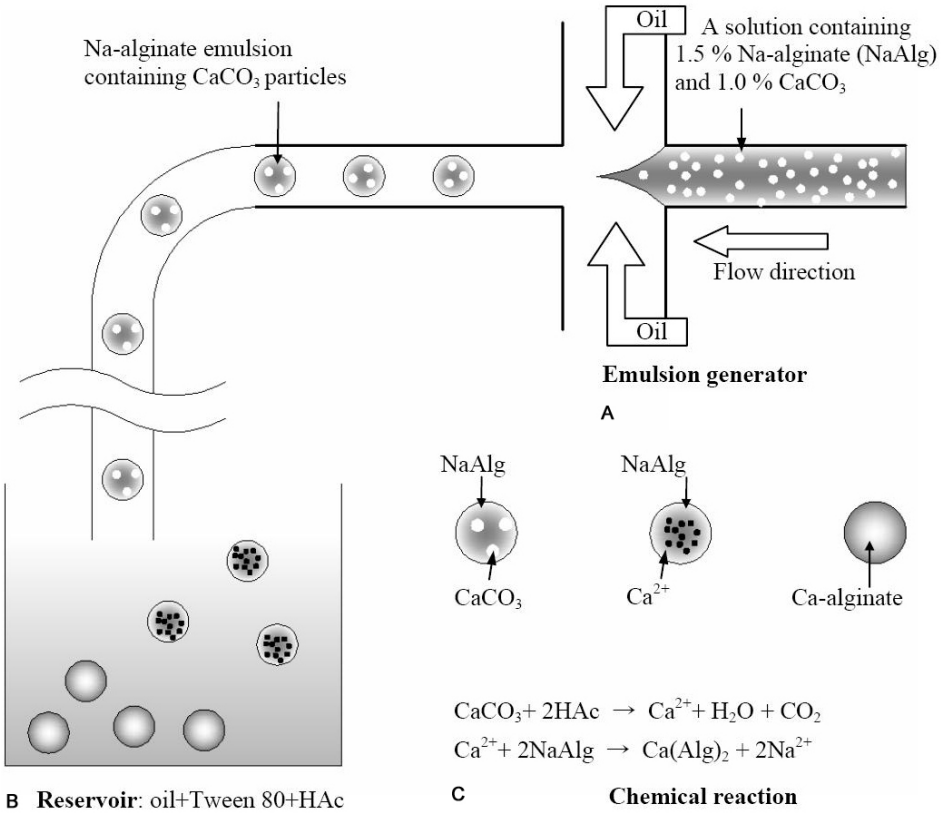

Figure 2. Illustration of microchip emulsification coupled with the internal gelation process (not to scale). (a) Schematic drawing of the formation of Na-alginate (NaAlg) emulsion containing $CaCO_3$ in a cross-junction microchannel. Based on microfluidics to elicit the control focusing force, a large set of uniform self-assembling spheres can be obtained. (b) The emulsions are gelled upon contact with 20 % glacial acetic acid, and the Na-alginate molecules entrapped in the micro container are transformed into Ca-alginate particles in the reservoir. (c) The mechanism of Ca-alginate synthesis.

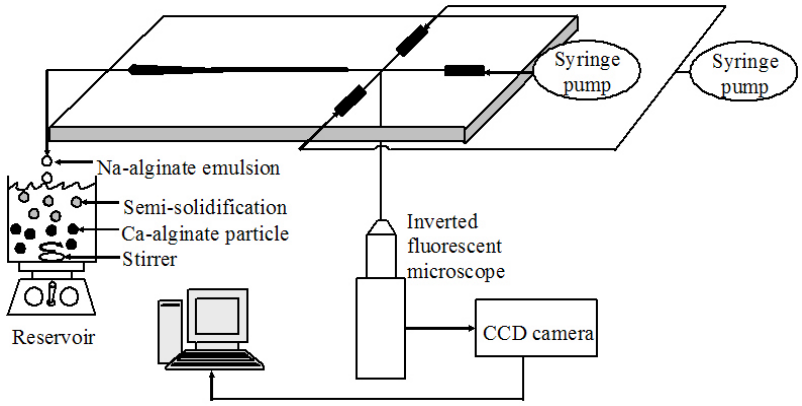

Figure 3. Experimental set up of the microfluidic chip system for Ca-alginate microparticles generation.

Secondly, the fluids were then injected into the microfluidic chip by syringe pumps (Kdscientific KDS230) programmed by a PC. In this work we hydrodynamically focused a stream of aqueous solution (dispersed phase) at a cross-junction microchannel by two oil streams, enabling the construction of water-in-oil (w/o) Na-alginate emulsions containing $CaCO_3$ along the microchannel. Finally, the Na-alginate emulsions were allowed to undergo gelation by dripping them into an oil solution containing Tween 80 and glacial acetic acid. After 20 minutes of hardening, Ca-alginate particles were formed.

3.4. Size measurement of microspheres

A fluorescence microscope is used to observe the experimental results. The image and detection system consist of an optical microscope (IX70, Olympus, Japan)

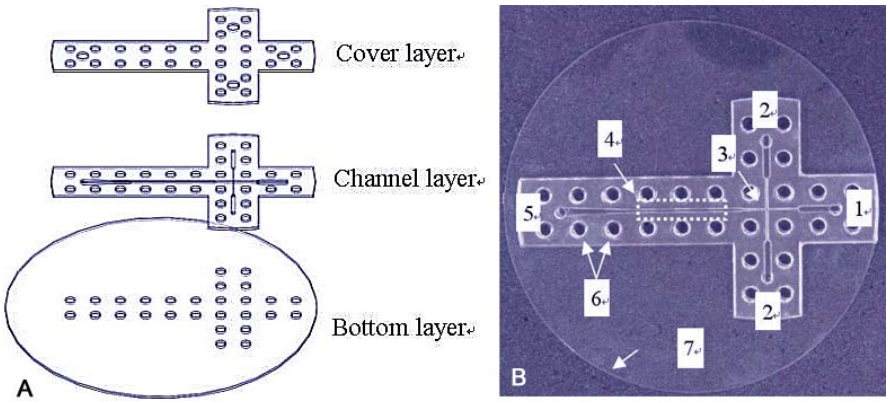

Figure 4. Schematic drawings of a microfluidic chip: (a) the chip in expanded view and (b) the photo image of the chip: 1. sample inlet, 2. oil inlet, 3. cross-junction channel, 4. observation area, 5. outlet, 6. screw holes for binding, 7. bottom layer disk.

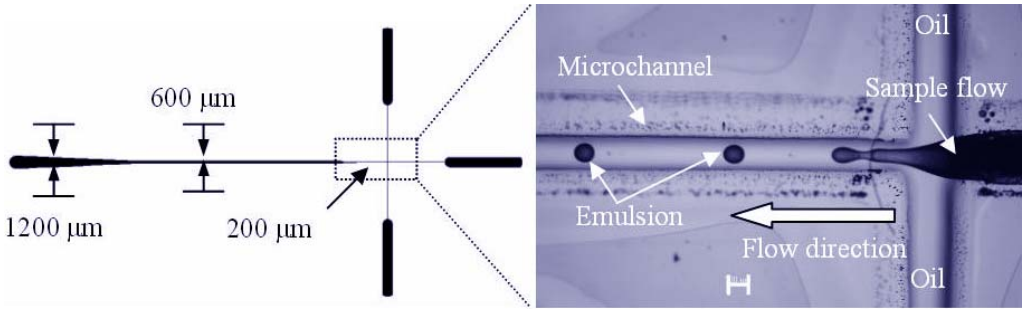

Figure 5. Mono-dispersed Na-alginate microemulsions are generated at the cross-junction with the oil flow at 0.6 mL/min and the sample flow at 0.01 mL/min. The size distribution of the formed emulsions is quite uniform (180 ± 10 μm). The arrow-shaped flow indicates the direction of emulsion generation, and the scale bar is 500 μm.

and a digital camera (DP70, Olympus, Japan). The diameter of each microsphere was measured and averaged. A total of 50 microspheres were measured to provide an average size.

3.5. Design and fabrication of a microfluidic chip

Our microfluidic chip was designed by AutoCAD®, and was constructed on a conventional poly methyl methacrylate (PMMA) substrate (length/width/depth: 270 mm/210 mm/1.5 mm) with a laser micromachining process by a CO_2 laser machine (LaserPro Venus, GCC, Taiwan) (24). The microfluidic chip (length/width/depth: 105 mm /65 mm/4.5 mm) consists of three layers (an expanded view is shown in Figure 4a) which are, from top to bottom: the cover layer (containing one outlet port and three inlet ports), the main layer (cross-junction channel) and the bottom layer (the disk structure, 55 mm in radius, is designed in a modular fashion for placing on an inverted fluorescent microscope). These three layers are integrated by screws (0.5 mm pitch, 4.0 mm in diameter, tightened at 1.0~1.2 Nm), followed by thermal bonding in an oven (OPO-45, CHENG SANG, Taiwan) at 110 ± 5°C for 90 minutes. This device is then naturally annealed to room temperature to produce the microfluidic chip. The microfluidic chip has three inlet ports, one outlet port, one cross-channel, and an observation area, as shown in Figure 4b. The observation area (600 μm channel, near the outlet of the cross channel) is designed for slowing down the flow and enhance the analysis observation. This chip is low cost, easy to fabricate, easy to set up, as well as easy to organize and program.

4. RESULTS AND DISCUSSION

4.1. Formation of monodisperse emulsions

For the uniform w/o Na-alginate emulsions containing $CaCO_3$ generation, the pregel solution (which is prepared by mixing 25 mL of 1.0 % w/v of 10 μm $CaCO_3$ solution and 25 mL of 1.5 % (w/v) Na-alginate solution) and 200 mL sunflower seed oil (125 cP), are employed as the sample-phase fluid and oil-phase fluid, respectively. A water-soluble dye (red ink) is dissolved in the pregel solution for immediate (real-time) observation. This viscous solution is then fluidified by shear forces in the microfluidic chip equipped with a cross-junction channel, and the resulting uniform semi-products (Na-alginate emulsions containing $CaCO_3$) are observed and characterized by an inverted fluorescent microscope.

In the initial experiments, the flow rates of the sample-phase and the oil-phase fluids were set to 0.01 mL/min and 0.6 mL/min, respectively. We found that the sample-phase fluid was compressed to an arrow shape by a shear force (Figure 5) and then separated into emulsions of about 180 μm in diameter. In addition, the diameter distribution of the emulsions formed is quite uniform (180

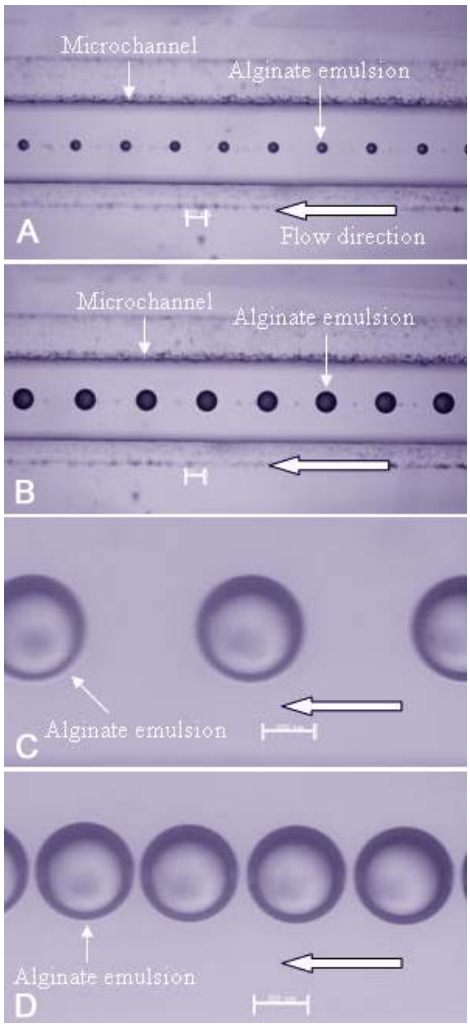

Figure 6. The emulsion formation under (a) oil flow at 0.500 mL/min and sample flow at 0.001 mL/min; (b) oil flow at 0.50 mL/min and sample flow at 0.01 mL/min; (c) oil flow at 0.10 mL/min and sample flow at 0.01 mL/min; (d) oil flow at 0.30 mL/min and sample flow at 0.01 mL/min. (scale bar 200 μm)

± 10 μm), and the gap between each emulsion is stable (1000 ± 30 μm). The flow rates of the oil and the pregel solution were adjusted to control the degree of hydrodynamic focusing and the width of the center stream, resulting in the generation of size-controlled Na-alginate emulsions. Therefore, we conclude that the hydrodynamic focusing can perform the emulsification in a size-controlled manner. In order to remove these emulsions and use them for advanced applications, we solidify the Na-alginate emulsions by gelation.

4.2. Formation of Ca-alginate microparticles

The semi-products (emulsions) are formed in the continuous oil flow. The continuous oil flow prevent the semi-products from fusing together, and transport them to an oil pool containing Tween 80 (1 % v/v) and glacial acetic acid (20 % v/v) through a Teflon tube. The water-soluble Na-alginate emulsions are gelled into solid spheres upon contact with calcium ion (II) by internal-gelation, resulting in water-insoluble Ca-alginate microspheres.

The Ca-alginate microspheres prepared as described above are separated from the oil solution by vacuum filtration. They are then washed twice with 30 mL *n*-hexane/ether, and then cleaned with 10 mL 50 mM Tris–HCl buffer (pH 7.2). All the Ca-alginate microspheres are then subjected to freeze-drying. After being dipped in liquid nitrogen, they are dried at -70°C under vacuum (0.1 mmHg) for 10 hours and then vacuum-dried at room temperature for 1 hour. We found that the shapes of most Ca-alginate microspheres remained spheroid after gelation and freeze-drying.

4.3. Influence of flow rate

To gain a further understanding, the relationship between size and flow speed were studied. The emulsion size is easily varied by changing the flow conditions in the microchannel. Figures 6a and 6b show the images of the emulsions generated at different sample flows under a fixed oil flow rate. The relationship between the average flow speed of the phases and the emulsion size (diameter) are shown in Figure 7. For a given 0.1 mL/min of oil flow rate, the emulsion size increased as the average flow rate of the sample flow rate increased (Figure 7a). The same tendency was observed in the 0.3 mL/min and 0.5 mL/min oil flow rate.

Figures 6c and 6d show the images of the emulsions generated at different oil flows under a fixed sample flow rate. For a given 0.01 mL/min of sample flow, the emulsion size decreased with the average velocity of the oil flow. The same tendency was observed in the 0.05 mL/min and 0.10 mL/min sample flow rate (Figure 7b). Based on Figures 6 and 7, it is evident that the size and gap of the emulsions generated in the cross-junction are controllable and reproducible by using the microfluidic chip. In addition, all of the relative standard deviations shown in Figure 7 are less than 10 %. We propose that the flow condition is not a key parameter for size distribution.

5. CONCLUSIONS

We designed a simple and cost-effective chip for manipulation of Ca-alginate microparticles by the immiscible property of sample and oil solutions in the microchannel and *in situ* internal gelation. In this study we demonstrated a microfluidic device that utilized a cross-junction microchannel, enabling the production of 80–800 μm Ca-alginate beads with a narrow size distribution (<10 %). We found that the oil-phase fluid (the pressure gradient conditions created by the syringe pump) can generate a focusing/shear force on the sample-phase fluid in the microchannel which can be used for the dynamic control of the emulsion size. This method has turned out to be one of the most efficient methods for the production of monodisperse Ca-alginate microparticles. Our platform is very attractive from a practical point of view, since it easily emulsifies and yields extremely uniform micro-emulsions. Our approach for the manipulation of Ca-alginate microspheres will potentially provide many pharmaceutical applications.

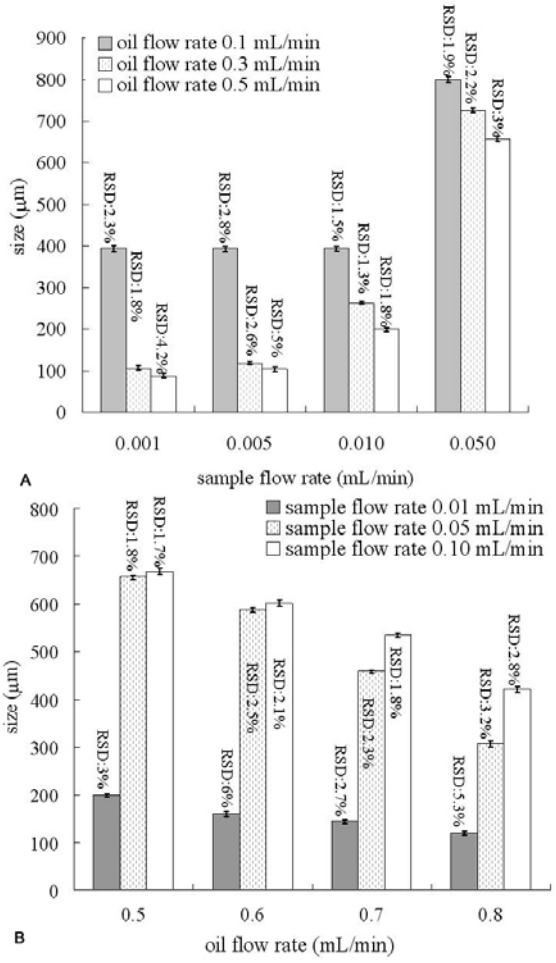

Figure 7. The relationship between particle size and flow rate (sample/oil).

6. ACKNOWLEDGMENTS

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Send correspondence to: Professor Yu-Cheng Lin, Dept. of Engineering Science, National Cheng Kung University, 1 University Road, Tainan 701, Taiwan, R.O.C., Tel: 886-6-276-2395, Fax: 886-6-276-2329, E-mail: yuclin@mail.ncku.edu.tw

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