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Environmental Friendly Flow Focusing Microfluidic Device and Pump System for Microencapsulation of 3D Cells

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Abstract: Microfabrication technique based on microelectronic technology has the capability to produce microfluidic devices in micro or nano-meteric scale but this technique involves with costly process and toxic chemicals. In this study, we proposed the use of patterned vinyl adhesive template to produce a flow focusing microfluidic device on a work bench that was applied to generate microbeads of calcium alginate for microencapsulation of cells. In this researche an infusion pump of high flow rates (1000-5000 μL/min) was developed to be used together with a commercial syringe pump that could pump the fluid to emulsify the continuous and disperse phases leading to the production of microbeads. Microbeads of calcium alginate in the range of 200-800 μm was successfully produced using this simple, environment friendly and efficient technique.

Key words: Microfluidic, vinyl adhesive template, infusion system, calcium alginate, microbeads, 3D cells, microencapsulation

INTRODUCTION

Microencapsulation is the entrapment of particles or materials inside a membrane (Hung and Lee, 2007; Vahabzadeh and Najafi, 2004; Pakzad *et al*., 2013). This technology shows promising potential in biomedicine, agriculture, food, cosmetic and pharmaceutical industries over the last 20 years (Pakzad *et al*., 2013). For Bioengineering application, this technique was employed by Bisceglie in 1933 to transplant tumor cells in polymeric membrane into a pig's abdominal cavity (Sun and Tan, 2013). In the 1960's, Chang proposed the first encapsulation of bioactive materials such as enzyme, proteins and cells in an immuno protective semi-permeable membrane (Pakzad *et al*., 2013; Sun and Tan, 2013). Till the 1980's, Sun and Tan (2013) successfully encapsulated implantable islets cells in alginate-poly-L-lysine. Sodium alginate is a favourable biomaterial for the encapsulation of a variety of cells because cells can maintain viability within the cross linked gel (Wong *et al*., 2016).

Flow focusing microfluidic, microextruder and suspension polymerisation techniques are three different techniques that can be applied to fabricate microbeads (Jiang *et al*., 2012; Moon *et al*., 2016; Winkleman *et al*., 2005; Kim and Pack, 2006). Flow focusing microfluidic is one of the widely used techniques for fabrication of microbeads due to the advantages of producing microbeads in micro or nano-meter size, minimum consumption of materials and safe operation. The size of the microbeads can be varied from 10-200 μm by manipulating the flow rate of the continuous phase (Jiang *et al*., 2012). In flow focusing, a laminar flow regime exists throughout the system and a Newtonian fluid was assumed. For a steady incompressible flow, stokes equation was applied in which the material's density is constant within a fluid parcel:

$$\rho(u\times\nabla)\, u-\mu\nabla^2\, u-\nabla p = 0 \quad (1)$$

Where:

ρ	=	The fluid density (kg/m^3)
u	=	The velocity (m/sec)
μ	=	The viscosity (Pas)
p	=	Pressure (Pa)
$(\nabla\times u)$	=	Divergent
∇p	=	Gradient

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The fabrication of the microbeads continued to grow after year 2005 when Winkleman *et al*. (2005) demonstrated the first fabrication of spherical particles or microbeads on patterned electrodes by applying electric field. Unfortunately, this type of fabrication technique produced flattened microbeads. As the technology advanced, Kim and Pack (2006) reported that the three-dimensional microbeads could be fabricated by using Precision Particle Fabrication (PPF) technology which is also known as microextruding technology. Capillary fluidic device was used by Choi *et al*. (2009) to produce microbeads that are porous. Although, the microbeads were successfully fabricated at narrow distribution of size by using a microextruder and capillary fluidic device but the fabrication method presented was complex and costly due to the precision technology used to produce small diameter of capillary nozzle. More recently, the technology of microencapsulation progressed into the development of microfluidic device using soft lithography technique that was further applied to produce microbeads (Lyu *et al*., 2014; Friend and Yeo, 2010; Johnston *et al*., 2014).

The fabrication of the microfluidic device based on soft lithography gained wide applications but the micropatterning process on a silicon wafer required a clean room facility, masking method and toxic chemical processes. For soft lithography in a clean room, the mask containing the patterns was transferred to a photoresist SU-8, cured by the ultra violet light and patterned template on the silicon wafer is formed via wet etching process. Subsequently, Poly-Dimethylsiloxane (PDMS) would be cured on the micropatterned silicon wafer then the peeled off is the patterned microfluidic device. This technique produced very small microchannels but requires costly facillities and fabrication process. Hence, innovating a fabrication technique which enables production of microfluidic device on a work bench is equivalently plausible and can be more feasible.

An environmental friendly technique was proposed to produce microfluidic device using vinyl adhesive template on a work bench. The development procedure of the microfluidic device based on vinyl adhesive template is simple, economic, highly replicable and inexpensive. Similar work to produce a micromixer using glossy adhesive paper was previously reported. We improved the work by using laser cut vinyl adhesive sheets instead of glossy adhesive paper to make a microfluidic device for microencapsulation.

MATERIALS AND METHODS

Fabrication of microfluidic device: In the flow focusing microfluidic design, a crossed junction channels consisting of two inlets and one outlet was included in order to perform emulsification process (Fig. 1). The design of the template was plotted using Autocad® software (Autodesk®, service pack 1, USA) and the pattern was directly plotted and cut using a vinyl adhesive plotter machine (Roland STIKA SV-12, Japan). Subsequently, the patterned adhesive tape (Fig. 1) was transferred to a petri dish (50 mm in diameter) and the thickness of the adhesive tape was determined using an Alpha-Step IQ surface profiler (ASIQ, USA). The thickness of a layer of the vinyl adhesive tape is approximately 50 μm and hence, six layers of micropatterned vinyl adhesive tapes were stacked together to obtain a total thickness of 300 μm.

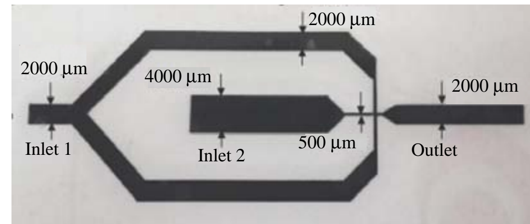

Fig. 1: A layer of vinyl adhesive template designed for the flow focusing microfluidic device with a thickness of 50 μm. White arrows indicate the direction of fluid flow

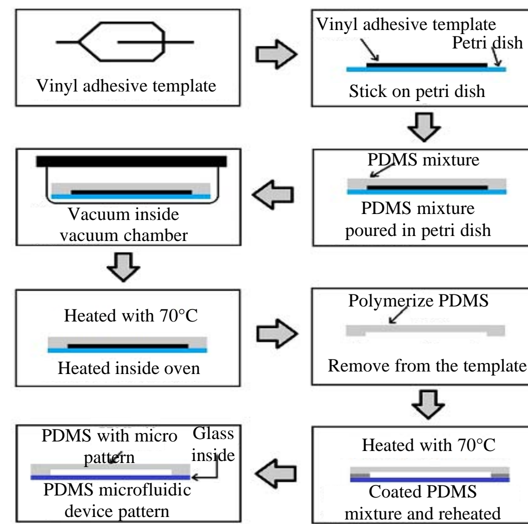

Fig. 2: The process of PDMS microfluidic fabrication based on the vinyl tape templating method

Having the vinyl adhesive template of the microfluidic with a thicknes of 300 μm in a petri dish, the petri dish was filled up with the PDMS mixture as shown in Fig. 2. Subsequently, the PDMS mixture was vacuumed in a 3 gallon vacuum chamber (ProVac, New York, USA)

for 1 h to remove the bubbles formed during stirring (Fig. 2). Then, the PDMS was allowed to be polymerised in an oven (ESCO, Malaysia) at 70°C for 1 h. The PDMS microfluidic was cut into the size of a glass slide (70×20 mm) and covalently bond to the glass slide using PDMS gel. The sandwich of PDMS and glass slide bonded by the PDMS gel were baked in the oven at 70°C for another hour. The holes for the 2 inlets and 1 outlet were made using a puncher and the holes were connected to Tygon® tubing with an outer diameter of 2 mm.

Pump system setup: The electronic infusion system was developed using a lead screw which functions as a linear slider to push or pull the plunger of a 5 mL syringe at a desired flow rate ranging from 1000-5000 µL/min (Winkleman *et al*., 2005). Arduino-uno microcontroller (SparkFun electronics.com, USA) was used as a controller to control a motor driver connected to the stepper motor of the linear slider or infusion system. As shown in Fig. 3, the main component of the infusion system consists of a 9 V Direct-Current (DC) unipolar hybrid stepper motor, Liquid Crystal Display (LCD) and buttons (increase speed, decrease speed, infusion, diffusion and stop). The 12 V, 5 A direct-current power adapter was stepped down to 9 Vdc by using a voltage regulator (LM7809) in order to supply 9 Vdc to operate the arduino-uno microcontroller, LCD and buttons. For a high current demand, the stepper motor was supplied with a switching power supply at 9 V and 4.2 A. Due to the high current consumption, a 12 Vdc ventilation fan was installed to dissipate the heat of the motor driver (L298N). The instruction of the microcontroller was written using C language in an arduino "Integrated Development Environment" (IDE). The infusion system could be controlled to increase or decrease the flow rates; performing the functions of infusion and diffusion and termination. Figure 3 shows the experimental setup of the microfluidic device with the pump system in a biological safety cabinet. The infusion pump and syringe pump were connected to the inlet 1 (continous phase) and inlet 2 (disperse phase) of the flow focusing mirofluidic device, respectively as shown in Fig. 4. The flow rates of the continuous and disperse phases were controllable in the range of 1000-5000 and 50- 300 µL/min, respectively. The intersection of the continous phase and disperse phase at the crossed junction channel is the location where the microbeads of sodium alginate are produced. The downstream flow carries the microbeads out of the channel and fall into a beaker containing the calcium cholride solution to be polymerised as shown in Fig. 5.

Speed measurement of the infusion pump system: The syringe pump functions as an infusion system to infuse

Fig. 3: The experimental setup for fabricating microbeads using the microfluidic infusion system in an ESCO class II biological safety cabinet

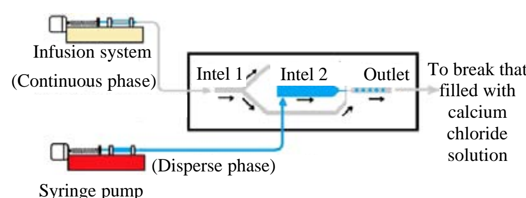

Fig. 4: The experimental setup to produce calcium alginate microbeads

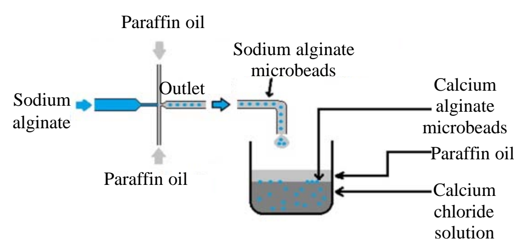

Fig. 5: The concept of producing calcium alginate microbeads via the microfluidic device

the desired flow rate from a 5 mL syringe. Both fluids (distilled water and paraffin oil) were lodged into two independent 5 mL syringes, respectively. Then, one of the syringes was fixed into the system and purged to the microfluidic device at a speed between 0 and 150 rpm with an increment of 10 rpm. The actual speed of the stepper motor was measured by using a digital tachometer (CDT-2000HD, Reliability Direct Inc., League City, Texas, USA) to compare with the programmed speed set in the microcontroller. The flow rate was computed by determining the time (second) required for the infusion system to purge 5 mL of fluid from the syringe.

Cell culture and preparation of cells: The Human keratinocyte cell lines (HaCaTs) were cultured in a 25 cm^2 culture flask with Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, Dorset, UK) supplemented with

L-Glutamine (2 mm, Sigma-Aldrich, Dorset, UK), Penicillin (100 units/mL, Sigma Aldrich, Dorset, UK), Streptomycin (100 mg/mL, Sigma-Aldrich, Dorset, UK), Fungizone (2.5 mg/L, Sigma-Aldrich, Dorset, UK) and 10 % Fetal Calf Serum (Promocell, Heidelberg, Germany). The cells were incubated in an incubator at 37°C. When the cells grew into 80% confluences, the culture media was discarded from the culture flask and the cell suspensions were harvested using standard sub-culture procedure as described by Soon *et al*. (2014). The cells were ready for the microencapsulation experiment using the microfluidic device. The experiment was repeated 3 times and 40 samples of microbeads were taken randomly for size measurement.

Microencapsulation of cells in the microfluidic device: The sodium alginate and calcium chloride were prepared by using a magnetic stirrer (MR Hei-Standard, Heldolph, Germany) which stirred at 1000 prm for 5 min. The solutions were then covered and transferred to an ESCO Class II biological safety cabinet (SC2-4A1, Singapore). The electronic infusion system, commercial syringe pump and microfluidic device were sterilised by using 70% ethanol before used. After the experimental setup, the HaCaT cells at density of 82.8×10^4 cells/mL were mixed with 0.2 mL of sodium alginate and DMEM. At a total volume of 0.4 mL, the HaCaT cells containing in the sodium alginate solution was lodged in a 5 mL syringe to be used for the disperse phase which was purged by the commercial syringe pump using similar setup as shown in Fig. 4 and 5. The paraffin oil was lodged into another 5 mL syringe which was purged using the customised infusion system. The calcium alginate microbeads encapsulated with HaCaT cells were collected in a sterilised petri dish containing the calcium chloride solution. After the 15 min of polymerisation, the calcium chloride solution and paraffin oil were carefully removed from the petri dish by using a micropipette and replenished with 2 mL of DMEM. The petri dish was then incubated at 37°C. The encapsulated cells in calcium alginate microcapsules were observed over a period of 7 days in an inverted phase contrast microscope (TS-100, Nikon, Tokyo, Japan). Images of the microcapsules were captured using a Go-3 camera (QImaging, Surrey, Canada). All experiments were repeated three times.

After 7 days of culture, the cells in encapsulation were stained using 2 μg/mL of 4, 6-diamidino-2 phenylindole (DAPI, Sigma Aldrich, Dorset, UK) to study the distribution of the cells. Then, the alginate encapsulated cells were washed 3 times in HBSS. The microcapsules were transferred to a glass slide and to be observed using a digital camera (DP73, Olympus, Tokyo, Japan) mounted to an fluorescence microscope (BX53, Olympus, Tokyo, Japan). The size of the microcapsules determined using a CellSense Software (Olympus, Tokyo, Japan).

RESULTS AND DISCUSSION

Size distribution of the microbeads: The flow focusing microfluidic device fabricated using vinyl tape templating method is as shown in Fig. 6. Figure 7 shows the microbeads produced using this microfluidic device with a continuous phase of 5000 μL/min and a disperse phase of 300 μL/min. The microbeads were fabricated using continuous flow rates from 1000-5000 μL/min and the disperse flow rate was set constant at 300 μL/min as shown in Fig. 8. The results revealed that the size of the microbeads decreased when the flow rate of the continuous phase increased. At a continuous flow rate of 1000 μL/min, a maximum size of 812 μm±38 was obtained. The minimum size of the microbeads at 460 μm±15 was obtained at a continuous flow rate of 5000 μL/min. When slow continuous phase were induced at 1000 and 2000 μL/min, aggregation of the microbeads were observed at the outlet. However, the problem of aggregation was not found when higher flow rates (3000-5000 μL/min) were applied.

Nonetheless, the size of the microbeads was found increased when the flow rates of the disperse phase increased from 50-300 μL/min at an increment of 50 μL/min in which the continuous phase was fixed at 2000 μL/min (Fig. 9). The maximum size of the microbeads at 799 μm±20 was obtained at a disperse flow rate of 300 μL/min. In addition, the minimum size of the microbeads at 438 μm±38 was obtained at a disperse flow rate of 50 μL/min.

With 20 μL of sodium alginate solution, 50±10 pieces of microbeads were fabricated for each infusion time of 5 sec. The size of the microbeads was distributed between 200-800 μm (Fig. 10). The Coefficient of Variation (CV) obtained was <18% (CV = 79.6 (standard deviation)/454 (mean)×100%) for the experiment. The size of the majority microbeads seemed to be closely associated with the size of the crossed junctions at 500 μm. This size range is suitable to be used for cell encapsulation based on the research reported previously (Wong *et al*., 2016; Santos *et al*., 2015).

Encapsulation of HaCaT cells: The results of the microencapsulated cells in calcium alginate microbeads are as shown in Fig. 11. The phase contrast images indicated that the cells are randomly distributed in a 3D space at different spatial depth in the capsules (Fig. 11a). DAPI staining of the cells clearly showed the each individual cells encapsulated and suggested that higher

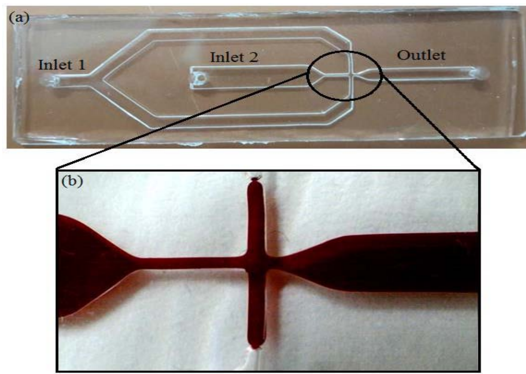

Fig. 6: a) Flow focusing microfluidic device with 2 inlets and 1 outlet and b) The enlarged exert of a crossed junction of the PDMS microfluidic device injected with red food dye

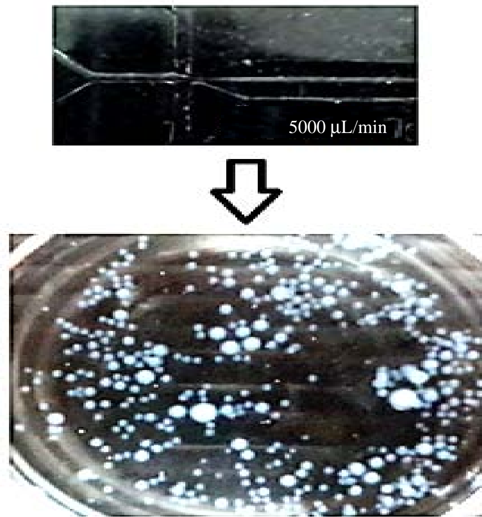

Fig. 7: The micrographs of microbeads at produced at a continous phase of 5000 μL/min and disperse phase of 300 μL/min

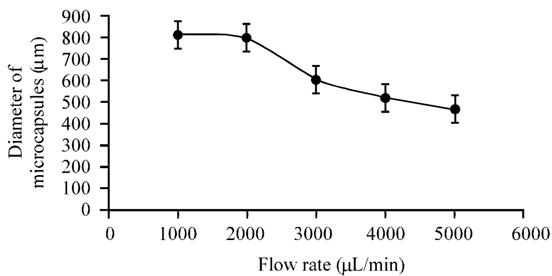

Fig. 8: The diameter of the microbeads at different flow rates of the continuous phase with a disperse phase fixed at 300 μL/min

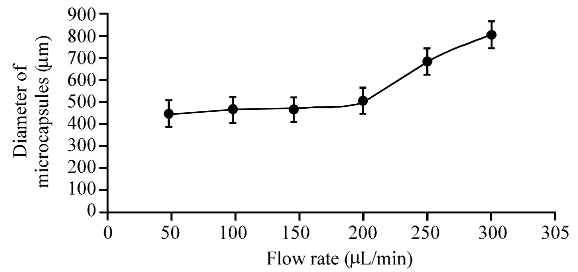

Fig. 9: The diameter of the microbeads at different flow rates of the disperse phase with a continuous phase fixed at 2000 μL/min

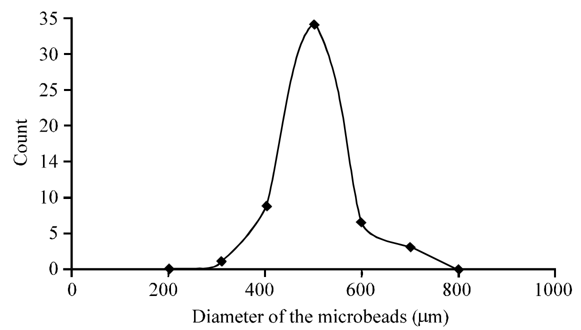

Fig. 10: The size distribution of microcapsules at a continuous phase of 5000 μL/m and a disperse phase of 300 μL/min.

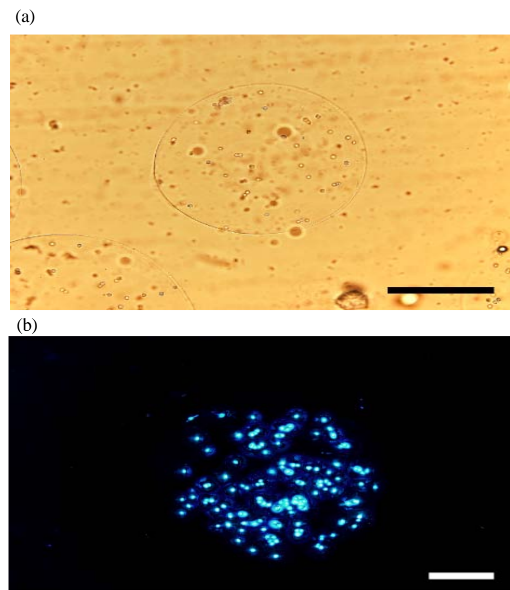

Fig. 11: The phase contrast micrograph of: a) Microencapsulation of cells in microbeads of calcium alginate and fluorescence micrograph of and b) DAPI staining of the HaCaT cells (Scale bar: 100 μm)

density of cells was required to fill up the space (Fig. 11b). The microencapsulation of cells in calcium alginate were successfully demonstrated using the developed system.

CONCLUSION

A flow focusing microfluidic device was designed to perform emulsification of the continuous and disperse phases of two liquids. The PDMS microfluidic device was fabricated using vinyl adhesive templating method without involvement with toxic chemicals during the fabrication. The infusion system was developed to infuse continuous phase at high flow rate. Via the microfluidic device system, the calcium alginate microcapsules were successfully produced. At a continuous phase fixed at 2000 μL/min and a disperse phase set at 50 μL/min, the microcapsules produced were determined at a minimum size of 454 μm±79.6. This study has demonstrated an environmental friendly method to encapsulate 3D HaCaT cells in the calcium alginate microbeads.

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