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Microfabricated airflow nozzle for microencapsulation of living cells into 150 micrometer microcapsules

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Abstract Microencapsulation of genetically engineered cells has attracted much attention as an alternative nonviral strategy to gene therapy. Though smaller microcapsules (i.e. less than 300 μm) theoretically have various advantages, technical limitations made it difficult to prove this notion. We have developed a novel microfabricated device, namely a micro-airflow-nozzle (MAN), to produce 100 to 300 μm alginate microcapsules with a narrow size distribution. The MAN is composed of a nozzle with a 60 μm internal diameter for an alginate solution channel and airflow channels next to the nozzle. An alginate solution extruded through the nozzle was sheared by the airflow. The resulting alginate droplets fell directly into a $CaCl_2$ solution, and calcium alginate beads were formed. The device enabled us to successfully encapsulate living cells into 150 μm microcapsules, as well as control microcapsule size by simply changing the airflow rate. The encapsulated cells had a higher growth rate and greater secretion activity of marker protein in 150 μm microcapsules compared to larger microcapsules prepared by conventional methods because of their high diffusion efficiency and effective scaffold surface area. The advantages of smaller microcapsules offer new prospects for the advancement of microencapsulation technology.

Keywords Material fabrication · Cell encapsulation · Microcapsule · Size control · Microfluidic device

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1 Introduction

Microencapsulation of living cells that have been genetically modified to secrete proteins represents a promising alternative nonviral strategy to gene therapy for a variety of diseases including diabetes (Lim and Sun, 1980; Wang et al., 1997), hormone-deficient diseases (Hortelano et al., 1996), and cancer (Joki et al., 2001; Read et al., 2001; Cirone et al., 2003; Visted et al., 2003). A commonly used system for these microcapsule applications is a polyion complex system based on alginate and poly-L-lysine (PLL), originally developed by Lim and Sun (1980). Generally, calcium alginate beads are formed by dripping an alginate solution into a $CaCl_2$ solution. Alginate-PLL-alginate (APA) microcapsules are formed by coating the formed calcium alginate gel beads with a PLL layer and an outer alginate layer. An alginate-PLL polyion complex

membrane works as the immunoprotection membrane for cell transplantation.

Smaller microcapsules offer many advantages for the use of cell transplantation. Transportation of nutrients and oxygen is theoretically better in smaller microcapsules (Chicheportiche and Reach, 1988; Schrezenmeir et al., 1992). Smaller microcapsules are also characterized by smaller volume, better dispersion and better mechanical strength. These advantages enable injections with a thinner needle or a catheter, allowing potential access to new implantation sites (Leblond et al., 1999). It has also been reported that smaller microcapsules are more biocompatible than larger microcapsules (Robitaille et al., 1999).

Different methods have been developed to prepare small microcapsules, including a concentric airflow method, a vibrating nozzle method and an electrostatic droplet generation (Dulieu et al., 1999). Calcium alginate beads with 300 to 800 μm diameters have been successfully prepared by utilizing these methods. Several research groups have reported the smaller beads by applying a concentric airflow method (Ross and Chang, 2002), a vibrating nozzle method (Nir et al., 1990), and an electrostatic droplet generation method (Bugarski et al., 1994). Although these researches showed successful preparation of smaller beads from 100 to 200 μm, the productivity and production stability for smaller beads would be worse rather than larger beads because of the high pressure loss of thinner needles.

A fabrication of various materials with micrometer scales, including emulsions, particles, gels, and polymers, is one of the interesting applications of microdevices (Nisisako et al., 2002; Albrecht et al., 2005; Liu and Bhatia, 2002). We have been proposed a method for generating monodisperse emulsion droplets from 1μm to 100 μm using a microfabricated channel array (Kawakatsu et al., 1997; Sugiura et al., 2001; Kobayashi et al., 2002). Applying these technologies, we recently proposed a droplet-droplet reaction system in a water-in-oil dispersion using a silicon micro-nozzle array, in which monodisperse alginate droplets and $CaCl_2$ droplets were reacted in soybean oil to form calcium alginate beads (Sugiura et al., 2005). This device enabled us to encapsulate living cells into 162 μm calcium alginate beads. However the oil phase process leaves an adhesive oil layer on the surface of the prepared beads, which disturbs successful PLL coating for preparing an APA microcapsule. Therefore, we prefer the simpler calcium alginate bead formation process without using an oil phase for encapsulating living cells into small microcapsules.

In this study, we have developed a new micro-airflow-nozzle (MAN), which is composed of a nozzle with a 60 μm internal diameter for an alginate solution channel and airflow channels next to the nozzle, in order to effectively produce 100 to 300 μm APA microcapsules. The calcium alginate gel beads were prepared by simply dripping the alginate solution directly into a $CaCl_2$ solution using this device. The living cells were encapsulated in 150 μm APA microcapsules, and the effect of capsule size on the cell growth and secretion activity of marker protein was investigated.

2 Materials and methods

2.1 Silicon MAN and experimental setup

Figure 1 schematically illustrates the MAN fabricated on the silicon plate. The MANs were fabricated by photolithography and the deep reactive ion-etching (RIE) process on a 4-inch silicon wafer with 400 μm thickness. The fabrication process was based on the previously reported method, which was described in previous research (Kobayashi et al., 2002). In order to fabricate thoroughly etched thin holes, the previously reported method was modified. The thoroughly etched holes were etched from both side of the silicon wafer by the RIE. The MAN is composed of a nozzle for an

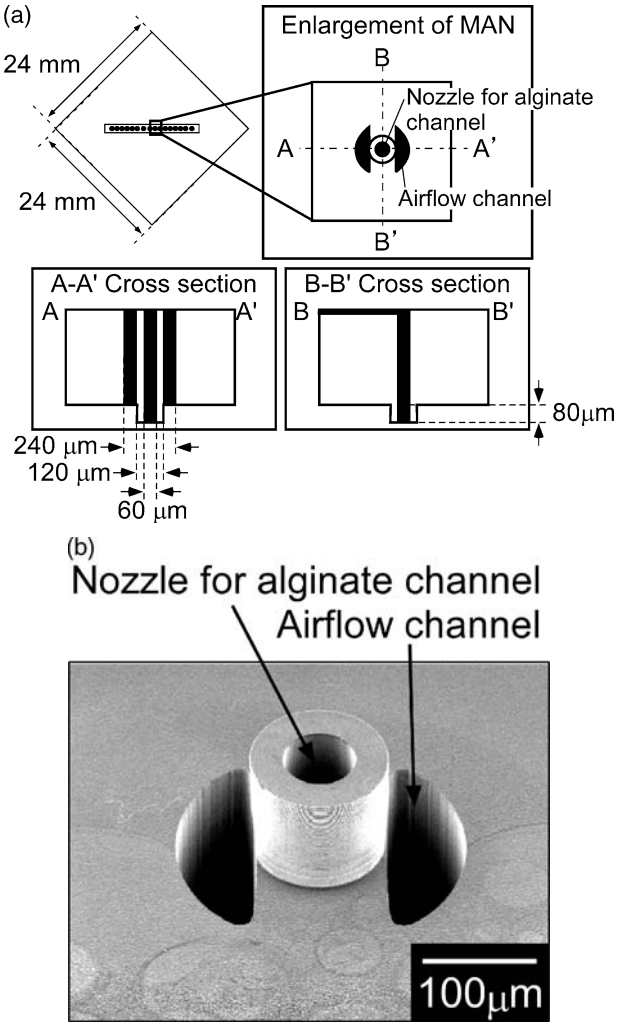

Fig. 1 Schematics (a) and SEM photographs (b) of MAN

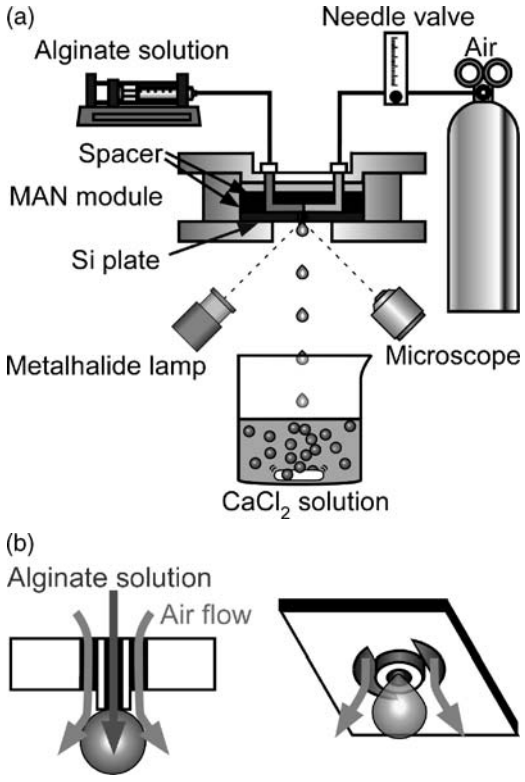

Fig. 2 Experimental setup (a) and schematic flows of alginate solution and airflow (b)

alginate solution channel and airflow channels next to the nozzle. The nozzle has a 60 μm internal diameter, 120 μm external diameter and 80 μm height. The airflow channel is composed of 240 μm cylindrical channels. Fifteen nozzles were fabricated on each silicon plate. The fabricated nozzles had the internal diameters from 59 to 61 μm.

Figure 2(a) depicts the experimental equipment. The silicon plate on which the MANs are fabricated is attached to the MAN module with rubber spacers. Syringe pumps (PHD 2000, Harvard Apparatus Inc., USA) to feed the alginate solution were connected to the MAN module. The airflow rate was controlled with a needle valve. The alginate aqueous solution supplied to the MAN module was sheared by the airflow as shown in Fig. 2(b), and droplets fell into the $CaCl_2$ solution. The droplet formation was observed with a microscope. A high-speed camera (FASTCAM Ultima 1024; Photoron Ltd., Tokyo, Japan) able to capture 16,000 frames/s was attached to the microscope to observe the droplet formation process.

2.2 Cell lines

We constructed cells secreting marker proteins, NK4 (MW 67 kDa) and GFP. NK4 is a known anticancer protein, which is composed of the NH_2-terminal hairpin domain and the subsequent four kringle domains of a-subunit of hepatocyte growth factor (HGF) (Matsumoto and Nakamura, 2003; Kuba et al., 2000; Tomioka et al., 2001; Kushibiki et al., 2004). Chinese hamster ovary (CHO) cell line was obtained from ATCC. For construction of expression plasmid for human NK4, the dihydroforate reductase (DHFR) expression plasmid (pCAGGS-DHFR) was kindly provided by Dr. J. Miyazaki (Osaka University) (Niwa et al., 2004) and cDNA for human NK4 was ligated into Xho I digested pCAGGS-DHFR. Exponentially growing CHO cells were transfected with the expression plasmid for human NK4, using calcium phosphate precipitation method. CHO cell lines secreting higher levels of recombinant human NK4 were selected using ELISA. Biological activities of NK4 secreted from CHO cells were confirmed using in vitro and in vivo experiments (Tomioka et al., 2001; Wen et al., 2004). CHO cell lines secreting NK4 were transfected with pIRES2-EGFP vector (Clontech Laboratories, Inc., Palo Alto, CA, USA) encoding GFP using lipofectamine (Invitrogen Corp., Carlsbad, CA, USA). The transfected cells (CHO/NK4-GFP) were cultured in α-MEM (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% FBS.

2.3 Calcium alginate bead formation and coating with PLL layer

Sodium alginate (Product name IL-2) was provided by Kimica Corp. (Tokyo, Japan). Sodium alginate was dissolved in (*N*-[2-hydroxyethyl]-piperazine-*N*-[2-ethanesulfonic acid])-buffered saline (HBS) and subsequently sterilized with 0.22 μm pore filter (Millex-GP, Millipore, Billerica, MA, USA). Poly-L-lysine hydrobromide (PLL, molecular weight 30,000 to 70,000) was obtained from Sigma-Aldrich Corp. (St Louis, MO, USA).

The calcium alginate bead formation process using MAN is similar to the previously reported concentric airflow method (Van Raamsdonk and Chang, 2001). The sodium alginate (1.5% wt/vol) solution was extruded through MAN with a syringe pump and sheared by the airflow. The resulting alginate droplets fell directly into a 1.1% $CaCl_2$ solution, where they were stirred for 30 min, and calcium alginate beads were formed. To prepare large (290 or 690 μm) microcapsules, calcium alginate beads were prepared by a concentric airflow method using 31G needle (Van Raamsdonk and Chang, 2001). In order to encapsulate the cells into APA microcapsules, the harvested cells were resuspended in a sodium alginate solution at a final concentration of 1.0×10^7 cells/mL, and calcium alginate beads were prepared as described above. The prepared calcium alginate beads were coated with a PLL layer as described previously (Van Raamsdonk and Chang, 2001). Briefly, the outer alginate layer was chemically cross-linked with 0.05% (wt/vol) PLL in saline for 6 min. The capsules were recoated with 0.03% (wt/vol) alginate in saline for 4 min. Finally, the remaining

calcium alginate core was dissolved with sodium citrate for 6 min to yield APA microcapsules, which enables the encapsulated cells to proliferate in the liquid core of the microcapsules.

2.4 Cell proliferation in microcapsules

APA microcapsules containing cells were suspended in α-MEM supplemented with 10% FBS and incubated at 37°C in 5% CO_2. The medium was changed every two or three days for four weeks and assayed for cell proliferation. In order to measure cell proliferation in microcapsules, 5 or 25 μl total volume APA microcapsules were sampled and digested with an enzyme solution composed of 2% Papain W-40 (Amano Enzyme Inc., Nagoya, Japan) and 1% Alginate Lyase (Nagase ChemteX Corp., Osaka, Japan) in PBS at 37°C for 1 h. The digested microcapsules were destroyed by pipetting and the number of cells was counted with a hematocytometer.

2.5 Secretion activity of marker protein from microcapsules

Secretion activity from microcapsules was determined by measuring the secretion of marker protein, NK4. The amount of NK4 released from the unit volume of APA microcapsules was determined. Culture supernatant of 5 or 25 μl total volume of APA microcapsules over a 24 h incubation period was sampled, and NK4 was detected by ELISA for human HGF (Immunis EIA, Institute of Immunology Co., Ltd., Tokyo, Japan), which is known to be capable of detecting NK4 (Kushibiki et al., 2004).

2.6 Microscopy

Scanning-electron microscopy (SEM) photographs of MAN were taken with a JSM-5600LC (JEOL Ltd., Tokyo, Japan). The droplet diameters were determined from pictures taken with the microscope video system. The prepared calcium alginate beads and APA microcapsules were observed with a microscope (DM IRM, Leica Microsystems AG, Wetzlar, Germany) in the bright field and fluorescence modes. The bead and capsule diameters were determined from pictures taken with a camera attached to a microscope. In order to evaluate the homogeneity of the bead size, the coefficient of variation (CV) (%) defined in the following equation was used.

$$CV = (\sigma / D_a) \times 100 \tag{1}$$

where σ is the standard deviation of the diameter (μm) and D_a is the average diameter (μm). Average values of roundness (R) were used to evaluate the shape of the prepared beads, defined as

$$R = \frac{4\pi S}{L^2} \tag{2}$$

where S is the projection area of beads and L is the perimeter of the beads. Nonspherical beads were approximated with an ellipsoid. Winroof (Mitani Corporation, Fukui, Japan) software was used to analyze the captured images.

3 Results

3.1 Calcium alginate bead formation process

Sodium alginate aqueous solution was extruded through the MAN at 5 mL/h flow rate (0.33 mL/h for each nozzle). Airflow was supplied to two side air-channels at 1.2 L/h for each single air-channel. The alginate aqueous solution supplied to the alginate channel was sheared by the airflow, and droplets fell into the $CaCl_2$ solution. The droplet formation was observed with a microscope equipped with a high-speed camera (FASTCAM Ultima 1024; Photoron Ltd., Tokyo, Japan). Figure 3 illustrates the droplet formation process observed with a microscope equipped with a high-speed camera. Identically sized droplets were sequentially prepared every 0.32 s. The measured average droplet diameter was 176 μm. The prepared droplets fell directly into the $CaCl_2$ solution, and calcium alginate gel beads were formed. Figures 4(a) and (b) present microscope photographs of prepared calcium alginate beads at different airflow rates. Airflow rates in each single air channel were 1.2 L/h for Fig. 4(a) and 0.9 L/h for Fig. 4(b). The diameter of the prepared beads decreased as the airflow rate increased, which is similar to the concentric airflow method. Figure 4(c) depicts the diameter distribution of the prepared calcium alginate beads. Number-average diameters and coefficients of variation of prepared calcium alginate beads were 139 μm and 7.3% for a 1.2 L/h airflow rate, and 301 μm and 4.5% for a 0.9 L/h airflow rate. Spherical and monodisperse calcium alginate beads were prepared in both cases. The roundness of the prepared beads was 0.96 for a 1.2 L/h airflow rate and 0.97 for a 0.9 L/h airflow rate.

3.2 Effect of airflow rate on calcium alginate bead formation process

Diameter control of calcium alginate beads is an important factor for practical applications. In order to control the bead diameter, we investigated the effect of the airflow rate on the bead-formation process (Fig. 5). The flow rate of the alginate solution was 5 mL/h (0.33 mL/h for each nozzle).

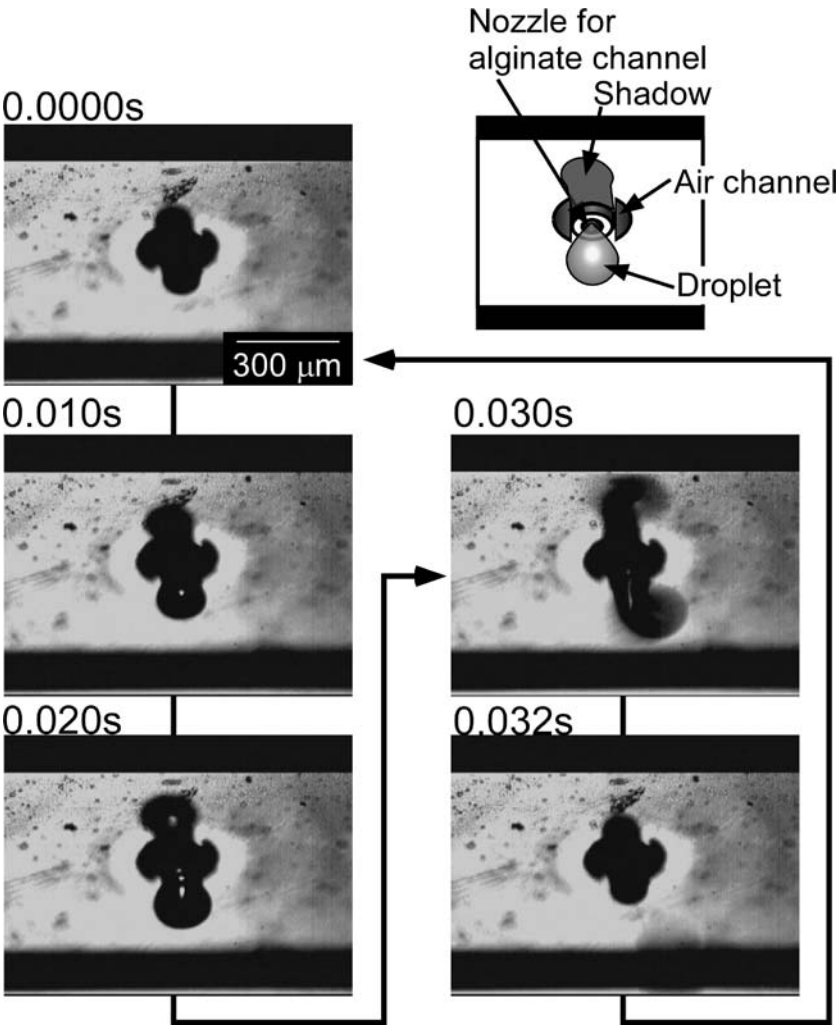

Fig. 3 High-speed camera sequence of droplet formation from MAN. Flow rates of alginate solution and air were 5 mL/hr and 1.2 L/hr for each nozzle and air-channel

Droplet diameter decreased as the airflow rate increased, and resulting bead diameters were controlled between 120 and 300 μm. The coefficient of variation of the prepared calcium alginate beads was less than 7.5% over the entire experimental range and was not affected by the airflow rate. Bead diameter was decreased to 76 to 81% of the original droplet diameter.

3.3 Cell growth and secretion activity in APA microcapsules with different sizes

The bead formation system proposed in this study can be applied for encapsulation of living cells, since the system works under sterile conditions. CHO/NK4-GFP cells were entrapped in the calcium-alginate beads using the MAN. The prepared calcium alginate beads were coated with PLL. APA microcapsules with 150 μm diameter containing CHO/NK4-GFP cells were successfully prepared. We investigated the effect of capsule size on the cell growth and secretion activity. APA microcapsules with 150 μm diameter containing CHO/NK4-GFP cells were prepared using the MAN. APA microcapsules with 290 and 690 μm diameters were prepared using the concentric airflow method using 31G needle. The prepared microcapsules were cultured for four weeks. The morphologies of cells in different sized APA microcapsules were investigated (Fig. 6). The cell proliferation was better in smaller microcapsules. The cells encapsulated in 150 and 290 μm microcapsules can grow in the entire space of the microcapsules. In contrast, the cells encapsulated in 690 μm microcapsules grow only at the bottom, leaving a great deal of dead space in the microcapsules.

We investigated cell proliferation and NK4 secretion for different sized microcapsules. Figure 7(a) shows the time spans of cell concentration in microcapsules with diameters of 150, 290 and 690 μm. The initial cell concentration of 1×10^4 cells/(mm^3-microcapsules volume) increased to 8×10^4 cells/(mm^3-microcapsules volume) at day 14 in a 150 μm microcapsule. In contrast, cells in larger 690 μm microcapsules had increased no more than 2×10^4 cells/(mm^3-microcapsules volume) at day 14. Figure 7(b) shows the

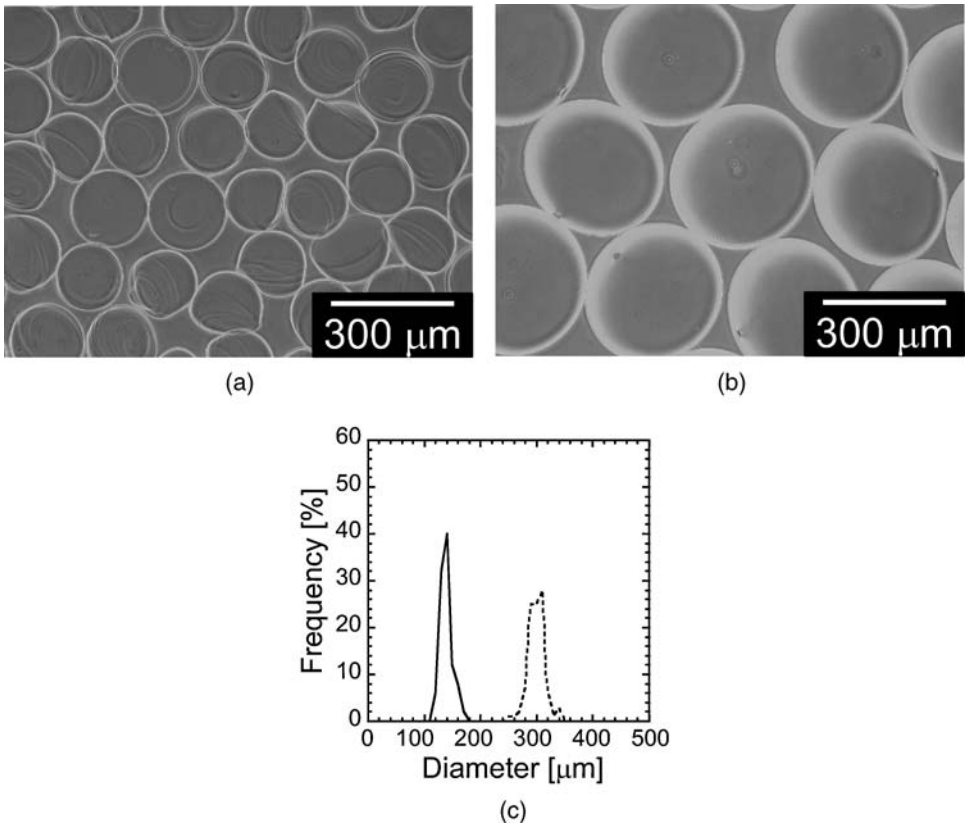

Fig. 4 Microscope photographs (a, b) and diameter distribution (c) of prepared Calcium alginate beads. Airflow rate of each single air-channel was 1.2 L/hr (a, solid line in c), and 0.9 L/hr (b, broken line in c)

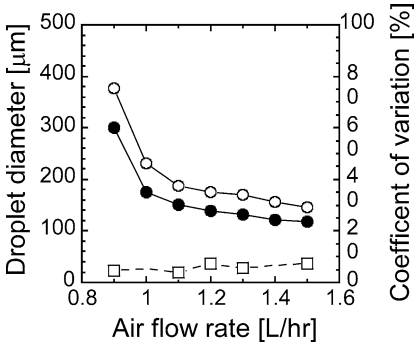

Fig. 5 Effect of airflow rate in each single air-channel on average diameter distribution. Number-average droplet diameter (○), bead diameter (●), and coefficient of variation of the beads (□) are shown

time spans of NK4 secretion activity from a unit volume of microcapsules with diameters of 150, 290 and 690 μm. NK4 secretion activity from 150 μm microcapsule increased to 60 ng/(mm^3-microcapsules volume) at day 28 with increasing cell concentration. In contrast, NK4 secretion from 690 μm microcapsules increased no more than 20 ng/(mm^3-microcapsules volume) throughout the entire experimental period. The cell concentration and secretion rate of marker protein NK4 in 690 μm microcapsules plateaued after one-week incubation. Cell concentrations in 150 and 290 μm microcapsules increased in 14 days and plateaued. The NK4 secretion rate from 150 and 290 μm microcapsules increased in 21 to 28 days. Throughout the entire experimental period, both cell proliferation and NK4 secretion rate were higher in the smaller microcapsules.

4 Discussion

We have presented a novel device for production of small APA microcapsules. It has a microfabricated nozzle with a 60 μm internal diameter. Calcium alginate beads were formed by a simple process, in which an alginate solution was sheared by airflow and the prepared droplets were directly dripped into the $CaCl_2$ solution. The device enables us to prepare 120 to 300 μm APA microcapsules and encapsulate living cells into 150 μm APA microcapsules. Although we previously reported a method for preparing 100 to 300 μm calcium alginate beads, that method adopted a droplet-droplet reaction in a water-in-oil suspension system, which induces an undesirable oil layer covering and is difficult to apply to a coating process with PLL (Sugiura et al., 2005). The present process formed droplets by simple shearing with airflow, which enabled us to perform the coating process used in a conventional APA microcapsule preparation method (Van Raamsdonk and Chang, 2001). The

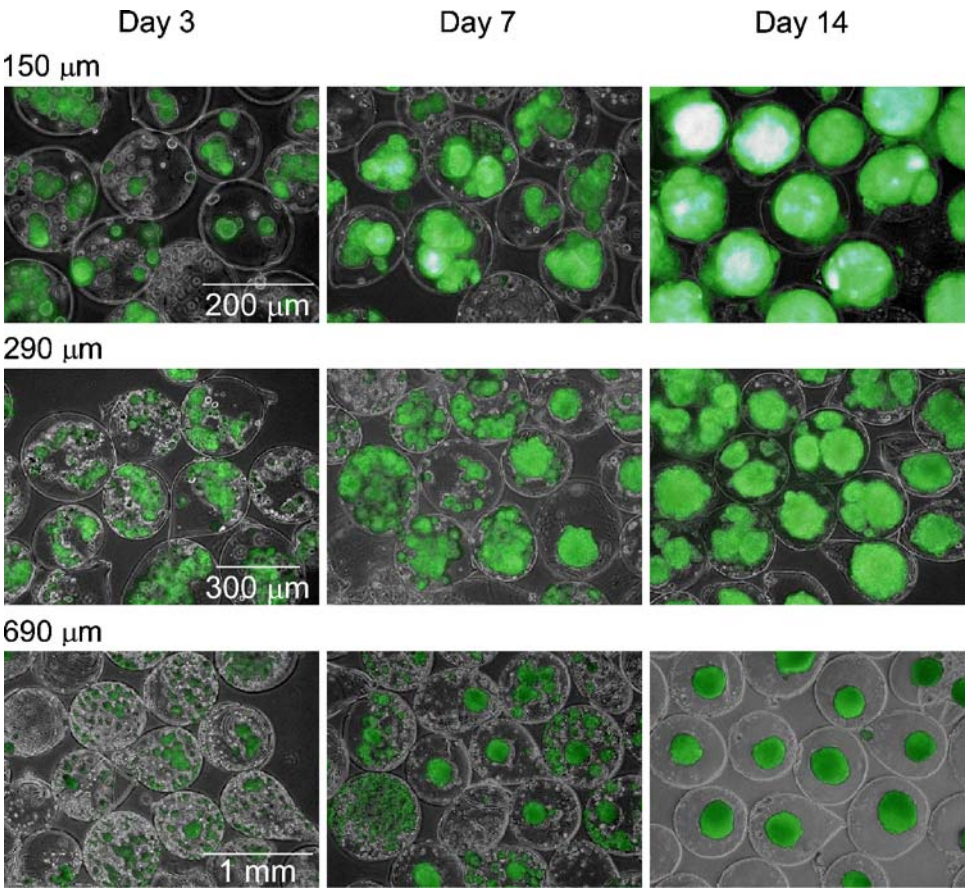

Fig. 6 Microscope photographs of APA microcapsules including CHO/NK4-GFP cells. Green fluorescence images exhibiting GFP were overlaid on to bright field images. Average diameters of microcapsules were 150, 290 and 690 μm

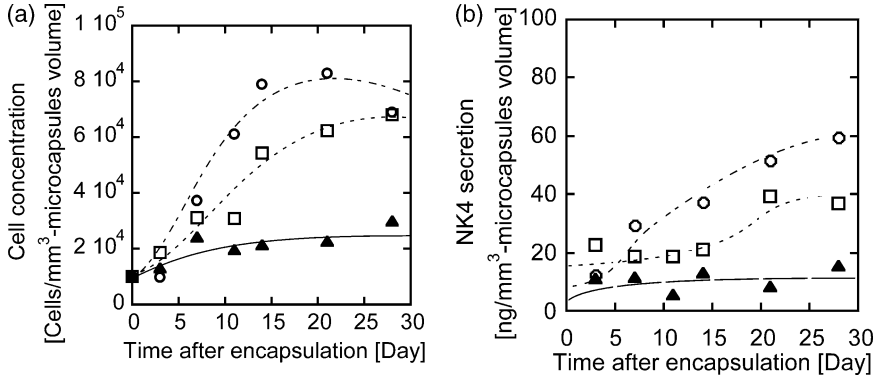

Fig. 7 Cell proliferation in APA microcapsules and NK4 secretion from microcapsules. Average diameters of microcapsules were 150 (○), 290 (□) and 690 μm (▲)

microcapsules were formed under sterile conditions and without chemical irritants, such as surfactant, which enabled successful encapsulation of living cells in the APA microcapsules.

The microfabricated nozzle is advantageous in terms of precision and low pressure loss (short nozzle) compared to the conventional needle-based system. Our device enables to control the size of the calcium alginate beads in the range of 120 to 300 μm by changing the airflow rate. Concerning with the size distribution of the beads, the coefficients of variation was less than 7.5% for the all size range of the prepared beads, which is similar value as the vibrating nozzle method (Nir et al., 1990) and better than the concentric air flow method (Ross and Chang, 2002) and electrostatic droplet generation method (Bugarski et al., 1994) in this size range. Realization of smaller beads is restricted by the diameter of the nozzle. In order to prepare smaller beads, a thinner nozzle should be used. From the point of microfabrication technology, fabrication of a thinner nozzle is possible; however, droplet formation would be unstable if the nozzle size is similar to the cell size. The problem is common for all other conventional methods, including the concentric airflow method (Ross and Chang, 2002), the vibrating nozzle method (Nir et al., 1990), and the electrostatic droplet generation

method (Bugarski et al., 1994), while preparation of smaller beads with the 20 μm diameter have been reported for encapsulation of small microbes (Nir et al., 1990). Concerning with the productivity, the productivity of the present device was 5 mL/hr despite the present small-scale apparatus. The productivity is comparable to both of the airflow and electrostatic droplet generation methods. The droplet formation would be possible at 100 mL/hr in principle. However, the droplet formation was unstable and alginate solution leaked from the air-channel because of insufficient adhesion between the rubber plate and Si plate. Though productivity with the vibrating nozzle method (i.e. 600 (mL/h)) (Brandenberger and Widmer, 1999) is higher than the present system, it may decrease when preparing beads smaller than 300 μm because of the high pressure loss caused by the thin needle. We think modification of the module and application of array-type nozzle arrangement will drastically increase productivity up to 1,000 times.

The encapsulated cells had higher growth rates in smaller microcapsules than in larger microcapsules (Figs. 6 and 7). It leads higher secretion activity of encapsulated cells in smaller microcapsules. Theoretically, smaller microcapsules provide higher diffusion efficiency and large scaffold surface area compared to larger microcapsules. The encapsulated cells formed spheroids 7 to 14 days after encapsulation (Fig. 6). The normal spheroid size seems constant at 200 to 300 μm in diameter (Fig. 6). Larger microcapsules had empty space in the capsule and low cell concentrations. These features resulted in higher cell growth rate in smaller microcapsules than in larger microcapsules. The secretion activity of NK4 was also higher in smaller microcapsules than in larger microcapsules (Fig. 7(b)). The higher cell proliferation rate and diffusion efficiency induced the greater secretion activity of the marker protein.

Smaller capsules had other advantages, such as high mechanical strength and better dispersion. In addition, smaller microcapsules enable injections with a thinner needle and infusions through a selectively placed catheter. This would provide local delivery of recombinant proteins into thinner blood vessels. Smaller microcapsules injected intraportally into the liver caused less portal pressure increase, even though twice the number of the smaller capsules were injected than larger microcapsules (Robitaille et al., 1999). These characteristic physicochemical and biological features of smaller microcapsules prepared by MAN create new prospects for microencapsulation technology due to their various advantages.

5 Conclusions

We have developed a novel microfabricated device that enables us to produce 100 to 300 μm APA microcapsules with a narrow size distribution. The device is composed of a precisely fabricated nozzle (60 μm internal diameter) for an alginate solution channel and airflow channels next to the nozzle. Using this device, we successfully prepared calcium alginate beads with diameters of 120 to 300 μm and coefficients of variation of less than 7.5%. The bead size was controlled by simply changing the airflow rate. The living cells were successfully encapsulated into 150 μm APA microcapsules while maintaining their viability. The encapsulated cells had a higher growth rate and greater secretion activity of marker protein in the smaller microcapsules. Smaller microcapsules provide various advantages including high cell activity, mechanical strength, and potential access to new implantation sites, and will create new prospects for the advancement of microencapsulation technology.

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