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Applications of PLGA Microcarriers Prepared using Geometrically Passive Breakup on Microfluidic Chip

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We present geometrically passive T-junction breakup as a high-throughput preparation method for polymer microcarriers. In passive T-junction breakup, an alternative method that can satisfy requirements for uniform size distribution and high production, a polymer solution droplet is split into numerous smaller droplets as it passes T-junctions, and is then polymerized into particles as solvents evaporate. Microparticles generated from poly(lactic-co-glycolic acid) (PLGA) were used to demonstrate the applicability of this method. The proposed method of droplet fission, controlled by microfluidic flow, allowed for preparation of biopolymer particles at 8000 Hz and size distribution of CV < 5%. Feasibility of the prepared PLGA microparticles was verified as microcarriers for functional materials: lidocaine, carbon nanotubes (CNTs), and 3T3 cells. The prepared microparticles showed a slower and more linear drug release profile compared to those generated using the conventional evaporation method. Highly porous microparticles were also prepared successfully using gelatin as a porogen in the T-junction breakup device.

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NOMENCLATURE

Q_d = dispersed phase flow rate
Q_c = continuous phase flow rate

1. Introduction

Microencapsulation is a widely used method for precise delivery of drugs, vaccines, and various functional materials.[1-5] Microcarriers can be prepared by various methods, but three traditional methods currently exist: solvent evaporation (extraction), phase separation (coacervation), and spray drying.[6] Conventional emulsification techniques have limitation in generating monodisperse droplets, yet many important potential applications require fine control of droplet size and narrow size distribution.[7] Therefore, a new methodology is necessary to produce particles that have homogeneous shapes and narrow size distribution and can be efficiently loaded with functional materials.[8] The droplet-based microfluidic systems have garnered interest as a new methodology that allows for preparation of uniform droplets, fine control of droplet size, low cost, and high loading efficiency for functional materials.[9] Several methods to prepare microparticles in droplet-based microfluidic systems have been developed, such as T-junctions, flow focusing, and sheath flow.[10-12] However, preparation methods based on microfluidic systems are limited in terms of preparation rate because microparticles are generated in a single channel. Although microfluidic systems allow for fine control over the size, shape, and size distribution, scaling up the preparation rate to a level that satisfies the throughput requirements of practical applications is a challenge. Although the frequency of droplet generation rarely exceeds several thousand droplets per second from a single channel in the microfluidic system, there has been trials to increase the throughput. Bardin et al reported the high speed generation of 10^5 droplets per second in a single channel, however the droplet size decreased to 3~6 μm as frequency increased.[13] Nisisako and Torrii reported microfluidic chip with planar 128 droplet formation units (DFUs) with each DFU having ~1.4×10^3 dropets per second, and Conchouso et al stacked four layers of 128 DFUs to increase the number of DFUs.[14,15]

Droplet fission can be another alternative approach in order to increase the throughput, because a large number of droplets can be obtained from one liquid plug as droplets breakup at T-junctions. The

T-junction passive breakup method can generate microdroplets in succession without increasing size distribution. The size of droplets can be controlled by flow rates of disperse and continuous phases. Many studies have been performed to analyze droplet breakup at T-junctions. T-junction breakup has been shown to be a suitable method for droplet generation and the mechanism of droplet breakup at T-junctions has been investigated using numerical analyses.[16,17] Droplet breakup at various junction angles has also been evaluated.[18] Moreover, the breakup of droplets and flow resistance of the sub channels as a function of T-junction size have been examined.[19] However, most studies pertaining to droplet breakup at T-junctions have been focused on the generation of droplets and its behavior as they break up, rather than on extension to production of microparticles or practical applications of the method.

Noting the high throughput of droplet fission method, we employed T-junction passive breakup device for high-throughput preparation of monodisperse microparticles. Microparticles made of poly(lactic-co-glycolic acid) (PLGA) were prepared by using T-junction breakup device. PLGA microcarriers have been explored as potential carriers for cell, drug, and DNA on the micro- and nanoscale.[20-25] Because PLGA is biocompatible, biodegradable and approved by the Food and Drug Administration (FDA), PLGA is widely used as material for surgical string, implant and drug carrier etc. The application of PLGA microcarriers can be extended for medical applications without concerns of cytotoxicity and side effects.[26-29] However, PLGA microparticle generation using conventional methods is limited because of unstable microparticle preparation and wide size distribution. Polydispersed microparticles can give rise to unexpected effects with respect to degradation and in vivo injection of drug.[7] The T-junction breakup method can satisfy requirements for both monodispersed size and improved microparticle preparation throughput.

We propose geometrical T-junction breakup as a preparation method for biodegradable microcarriers. The production rate and size distribution of microparticles generated using this method were evaluated. We used the prepared PLGA microparticles to deliver three functional materials: lidocaine, carbon nanotubes (CNTs), and 3T3 cells.[30-32] Lidocaine, a local anesthetic-type amide used in the treatment of pain, has been used as a model for drug delivery systems. Microparticles prepared using the T-junction breakup method were compared with those generated using the classical evaporation method with respect to size distribution and drug release. The surface of CNT-laden microparticles was inspected using field emission scanning electron microscopy (FE-SEM) and the proliferation of 3T3 cells cultured on microparticles was assessed using a LIVE/DEAD assay kit. Furthermore, we tried to combine the proposed microfluidic chip and W/O/W emulsion generation. Highly porous microparticles were prepared using gelatin by W/O/W emulsion. The morphology of prepared highly porous microparticles was also investigated.

2. Materials and Methods

2.1 Materials

Polydimethylsiloxane (PDMS, Sylgard 184) solution was supplied by Dow Corning. Mineral oil, Span®80, PLGA (MW: 40,000~75,000, 65:35), dimethyl carbonate (DMC, anhydrous, 99%), polyvinyl alcohol (PVA, MW: 31,000~50,000, hydrolyzed), lidocaine (MW: 234.34 Da), gelatin (from pocine skin), dichloromethane (DCM, anhydrous, 99.8%) and collagen fluorescein (FITC) conjugate (Type 1, from bovine skin) were purchased from Sigma-Aldrich. A LIVE/DEAD® Viability/Cytotoxicity kit was purchased from Life TechnologiesTM. Multiwall carbon nanotubes (MWCNTs, over 93% purity, 10~40 nm average diameter, and 1~25 μm long) were purchased from CNT Co., Ltd (South Korea). Reagent grade 60% nitric acid was purchased from Duksan Chemical (South Korea). Dulbecco's modified Eagle's medium, phosphate-buffered saline (PBS), trypsin-EDTA solution (0.25%), and fetal bovine serum were obtained from Thermo Scientific.

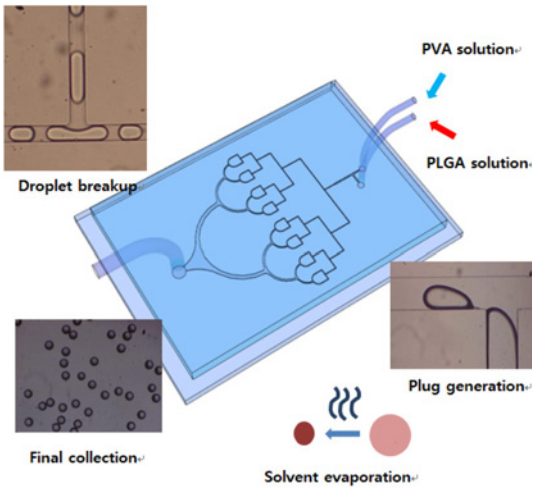

Fig. 1 Schematic outline of poly(lactic-co-glycolic acid) (PLGA) microparticles preparation by using the T-junction passive breakup device

2.2 Methods

2.2.1 Fabrication of the geometrical passive breakup device

Fig. 1 illustrates preparation of PLGA microparticles by using the T-junction breakup method. The T-junction breakup device was designed with fifteen T-junctions to split single droplets into 16 droplets. The cross sectional area of microchannels at T-junctions had dimensions of 100 μm × 140 μm (width × thickness). The T-junction breakup device was fabricated by photolithography by using SU-8 photoresist on a silicon substrate. PDMS prepolymer was mixed with curing agent (10:1). The PDMS solution was poured on the master mold in plastic dish. After curing at 90°C for at least 1 h, the PDMS structure was peeled off the master mold, and bonded to slide glass or a PDMS substrate by using plasma treatment. The microchannels of the T-junction breakup device were modified to be hydrophilic by injecting PVA solution into the microchannels, then heating in oven 3 times for 5 min.

2.2.2 Experimental set-up of the microfluidic system

Syringe pumps (Harvard Apparatus, Pump 11 Elite & Pico Plus) were used to inject liquids of immiscible phases. An inverted optical microscope (CBS-IH5) was used to measure the diameters of prepared microparticles in the microchannels. The droplet generation frequency was measured using a high-speed camera (Motion extra N3, Redlake).

The productivity of the T-junction breakup device was calculated as the droplet generation frequency at T-junctions multiplied by the number of T-junctions along the same branch. The size distributions were analyzed from at least 30 samples from the optical microscopic image. A high-performance liquid chromatography (HPLC) system (Agilent 1100, Agilent Technologies) was used to analyze the drug release profiles of PLGA microparticles used as drug carriers. An inverted fluorescent microscope (Olympus BX51) was used to inspect the surface of collagen-coated and cell-covered PLGA microparticles.

2.2.3 Preparation of lidocaine-loaded drug carriers

PLGA microparticles were prepared using two different methods: the classical solvent evaporation method and T-junction breakup method. The preparation of PLGA microparticles by using the classical solvent evaporation method was as follows: 50 mg of lidocaine was dissolved in 10 mL of PLGA solution (2.5 wt % in DMC) at room temperature. The resulting solution was homogenized in PVA solution at 8000 rpm for 8 min. Synthesis of PLGA microparticles by using the T-junction breakup device required preparation of PLGA solution (2.5 wt % in DMC), with 50 mg of lidocaine as the dispersed phase (Q_d) and PVA solution (1 wt % in DI water) as the continuous phase (Q_c). The two solutions were injected using syringe pumps. PLGA microparticles were generated by using the immiscible phases, with the flow rate of the dispersed and continuous phases being 1.0 mL/h and 10.0 mL/h, respectively. In both methods, the obtained PLGA microparticles were washed three times in DI water to remove PVA and absorbed lidocaine solution on the PLGA microparticle surfaces. The microparticles prepared by both methods were subsequently dried at room temperature to evaporate residual solvent, and were immersed in 40 mL of PBS solution of to analyze lidocaine drug release. We collected 200 μL samples by using a filter syringe every 24 h for 10 days. After 10 days, samples were collected every 5 days for drug release analysis. The amount of lidocaine released was determined at 254 nm by using an HPLC system. The HPLC system was composed by four units: binary pump G1312A, auto sampler G1313A, DAD G1315A, and a RP C18 column (4.6 mm × 150 mm, 5 μm). The sample solutions were filtered using 0.45 μm syringe filters (Acrodisc LC PVDF, PALL life sciences). Acetonitrile : ammonium acetate (70:30, 0.0257 M, pH 4.85) was used as the mobile phase at a flow rate of 0.6 mL/min.

2.2.4 Preparation of CNT-laden PLGA microparticles

The ox-MWCNTs were prepared using the oxidation method described by Rosca et al.[33] ox-CNT was prepared by placing 0.5 g of pristine MWCNTs into 200 mL of 60 wt % nitric acid solution. The MWCNTs in the nitric acid solution were dispersed via sonication for 1 h, using an ultrasonic bath (JAC Ultrasonic 4020P, KODO, South Korea), and were then refluxed at 120°C for 12 h under nitrogen gas-purging conditions. After cooling the dispersed MWCNTs in nitric acid solution, they were washed in DI water several times and passed through a membrane filter (0.22 μm, nitrocelluose, Millipore, USA). The obtained MWCNTs were then dried in an oven at 60°C for 48 h. Prepared ox-CNT was mixed with PLGA solution (1 wt % in DMC) by sonicating for 5 min. Finally, the CNT-PLGA solution was prepared using the T-junction breakup device. The ox-CNT and PLGA solution mixture was used as the disperse phase. The rest of the procedure was performed as previously described.

2.2.5 Cell culture on PLGA microparticle surfaces

To culture 3T3 cells on PLGA microparticles, a collagen layer was coated on PLGA microparticles to improve cell adhesion. Microparticles prepared using the T-junction breakup device were immersed in collagen solution for 1 h. Excess collagen was washed off several times with PBS solution. Coated microparticles were put into petri dish filled with culture medium. Then, 3T3 cells were seeded in the petri dish and incubated in a CO_2 incubator (100% humidity, 37°C, 5% CO_2). The cells on microparticles were inspected for 3 days by inverted microscope. To confirm collagen had coated the PLGA microparticles, the collagen was labeled with FITC and visualized under fluorescent microscopy. The condition of 3T3 cells seeded on PLGA microparticles were evaluated using a LIVE/DEAD assay kit.

2.2.6 Preparation of PLGA porous microparticles

PLGA porous microparticles were prepared in the passive breakup device using the gelatin porogen method described by Chieh-Cheng Huang et al with some minor modifications.[34] Firstly, water-in-oil emulsion of porogen and PLGA solution was prepared using homogenizer (HG-15D, DAIHAN Scientific Co., Ltd., South Korea). Then, the prepared emulsion was introduced as a disperse phase into the passive breakup device.

75 mg of PLGA powder was dissolved in organic solvent (2.0 wt % in DMC) at room temperature. As a porogen material, gelatin solution was prepared by resolving 50 mg of gelatin powder in 1% PVA solution at 37°C (7.5 % w/w). Water-in-oil emulsion of porogen and PLGA solution was prepared by mixing 50 mg of gelatin solution (7.5 wt % in DI water) and 600mg of PVA solution (1 wt %) in PLGA solution (6 g, 2 wt %) using homogenizer for 3 min at 20,000 rpm. The prepared emulsion was introduced as a disperse phase into the passive breakup device, while an aqueous solution with 1wt % PVA was used as the continuous phase (Q_d = 1 mL/h, Q_c = 10 mL/h). Water-in-oil-in-water droplets prepared in the passive breakup device were collected in ice-cold water and were stored overnight at room temperature in order to remove organic solvent by evaporation. The micropaticles were washed three times with DI water, and finally were put into water bath at 37°C for several hours in order to remove gelatin and to obtain the porous structures.

3. Results and Discussion

3.1 Evaluation of the T-junction breakup device

Fig. 2(a) shows the generation frequency of the micro water droplets according to flow rates of Q_c and Q_d. The droplet generation frequency increased with increasing flow rates. The highest generation frequency of the micro water droplets was approximately 8000 Hz (Q_d = 20 mL/h, Q_c = 35 mL/h). At higher flow rate, leakage occurred at inlet tubing. Fig. 2(b) shows the effect of Q_c/Q_d on the diameter of the micro water droplets. When Q_d is constant, the diameter of the micro water droplets decreases with increasing Q_c. The diameter of the micro water droplets was mainly dependent on the flow rate. The generation of a plug at the T-junction is governed by the relation between the interfacial tension of

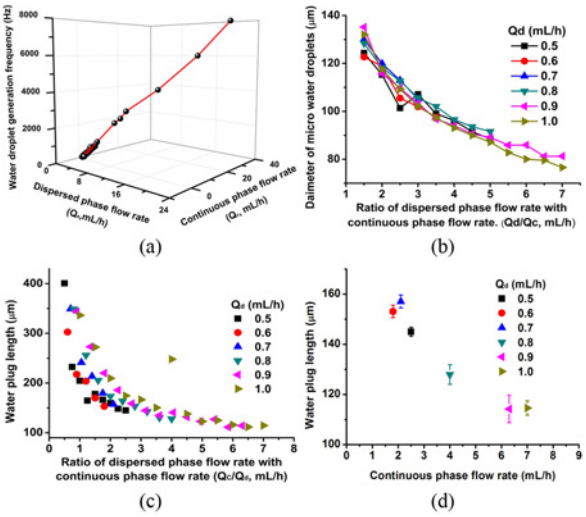

Fig. 2 (a) Productivity and (b) diameter of water droplet as a function of flow rate. (c) The water plug length, (d) Minimum water plug length according to flow rates

the dispersed phase and the shear force of the continuous phase.[35] Because the shear force increased at high Q_c/Q_d, smaller droplets were generated at the initial T-junction. As a result, the size of droplets prepared at the final T-junction decreased. Using the geometrical passive breakup device, the size distribution of the micro water droplets was 98.87 μm in diameter with a standard deviation of 3.20 μm (Q_d = 0.5 mL/h, Q_c = 1.75 mL/h, C.V. < 3.24%). Fig. 2(c) illustrates water plug length measured in the last T-junction according to Q_c/Q_d. The length of the water plug decreased as Q_c/Q_d increased until the length reached the minimum value that the plug did not split. Fig. 2(d) shows minimum sizes of plug necessary for breakup according to each Q_d dependent on Q_c. As Q_c/Q_d increase, smaller water plug could be spitted because the pressure for splitting the droplets increases.

PLGA microparticles were successfully prepared by using the T-junction geometrical passive breakup device. As compared with microdroplets, the size of microparticles decreased drastically as the solvent evaporates. The size of the PLGA microparticles increased slightly with increasing concentration of PLGA. However, the size ratio of microparticles with microdroplets was no more than 40%, meaning that even if there was a difference in the length of the plugs at the T-junctions, this difference can be reduced during the evaporation process. For this reason, the geometrical passive breakup device can be a good choice for the preparation of microparticles using solvent evaporation.

3.2 In vitro lidocaine release assay

Fig. 3(a)~(b) shows images of PLGA microparticles prepared by the classical evaporation method and by using the T-junction breakup device. Microparticles prepared using classical evaporation had a broad size distribution, while those prepared using T-junction breakup had a narrow size distribution. The classical method allowed for the generation of smaller microparticles compared to the T-junction breakup method; the mean diameter of microparticles prepared using the classical evaporation method was 13.04 μm with standard deviation of 4.19 μm (CV < 32.15%), while that of PLGA microparticles prepared using the T-junction breakup device was 34.51 μm with standard deviation of 1.52

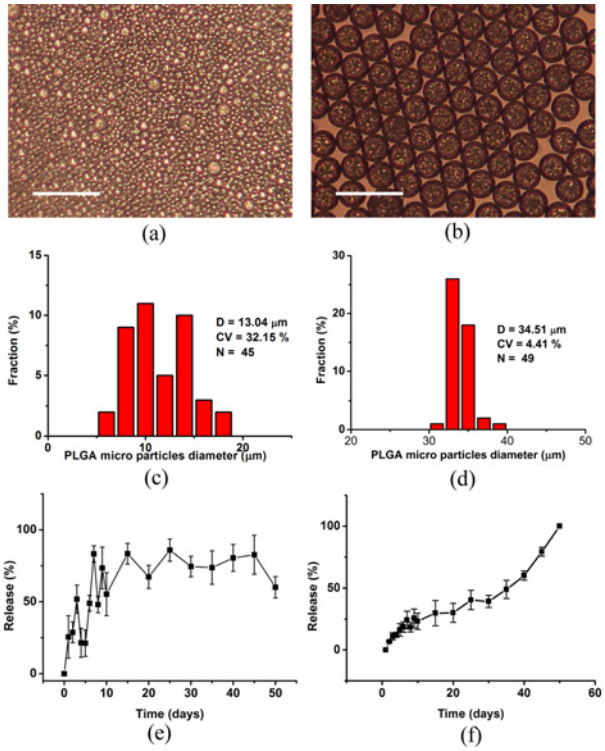

Fig. 3 Optical images of microparticles (scale bar: 100 μm), size distribution and drug release profile of PLGA microparticles prepared using the classical evaporation method ((a), (c), (e)) and T-junction breakup device ((b), (d), (f))

μm (Q_d = 1 mL/h, Q_c = 10 mL/h, CV < 4.41%), as shown as Fig. 3(c)~(d).

The T-junction breakup method generated microparticles that were more monodispersed than those prepared using the classical evaporation method. Notably, the size distribution of microparticles is considered as important factor affecting drug-release profile properties.[36] Fig. 3(e)~(f) shows the lidocaine drug-release profile of PLGA microparticles, according to preparation method, monitored over 2 months by using HPLC. For PLGA microparticles generated using the classical evaporation method, 75% of the drug was released quickly from the microparticles within 10 days, while the remaining 25% of drug was released slowly during the following 40 days. In contrast, PLGA microparticles prepared using the T-junction breakup method showed a slower lidocaine release rate. Approximately 25% of the lidocaine was released within the first 20 days, while the remaining 75% was released during the following 30 days.

3.3 CNT-loaded PLGA microcarrier morphology

Fig. 4(a) shows an image of a CNT-laden PLGA (1% in DMC) microdroplets prepared using the T-junction breakup method. Typically, the size of particles decreased as the solvent evaporated from the droplets. As the size decreased during solvent evaporation, CNT embedded in the droplet emerge from the droplet surfaces to become partially exposed, as shown in SEM images in Fig. 4(b). Dimples are often observed on the surface of PLGA microparticle. It is attributed to evaporation of solvents. The formation of dimples with irregular size shown in Fig. 4(b) is attributed to disturbance of solvent evaporation due to the embedded CNT in mirocarriers. Exposed CNT fibers on

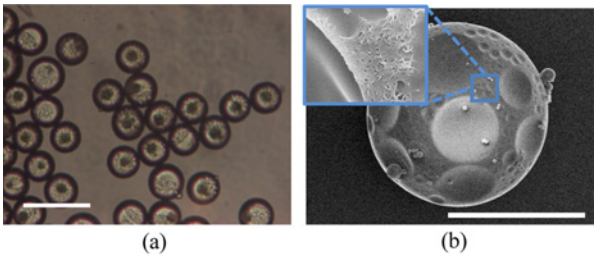

Fig. 4 (a) Images of carbon nanotube (CNT)-laden PLGA microdroplets (scale bar: 100 μm) and (b) scanning electron microscopy images of CNT-laden PLGA microparticle surfaces (scale bar: 30 μm)

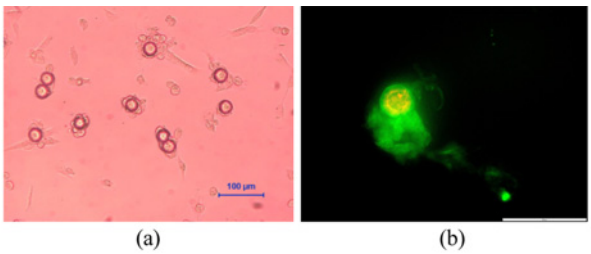

Fig. 5 (a) Optical and (b) fluorescent images of 3T3 cell on PLGA microparticles coated with collagen (scale bar: 100 μm)

microparticles can be used to improve the loading efficiency of functional materials onto carrier and to increase their reaction area.

3.4 3T3 cells cultured on PLGA carriers

The prepared PLGA microparticles were tested to check their feasibility as microcarriers of 3T3 cells. The microparticles were coated with a collagen solution to improve cell adhesion for the cell culture. 3T3 cells were seeded on the microparticles in culture medium, and the cells bound to the PLGA particles were inspected using microscopy. Fig. 5(a) shows images of 3T3 cells on the surface of the collagen coated microparticles. Migration of 3T3 cells to microparticles was confirmed in these results, demonstrating the compatibility between 3T3 cells and collagen-coated PLGA.[37] Proliferation of 3T3 cell cultured on microparticles was confirmed using LIVE/DEAD assay kit after 3 days (Fig. 5(b)). These data showed that PLGA microparticles could be used successfully as carriers to deliver cells.

3.5 PLGA porous microparticles prepared by T-Junction passive breakup

Fig. 6(a)~(b) shows an SEM image of porous PLGA microparticles prepared using W-O-W (water in oil in water) method in the T-junction breakup device. The prepared particles showed highly porous structures which are interconnected. The size of the particles was 55.92±10.11 μm. The pore size of microparticles was 11.31±4.40 μm. The size deviation of porous particles showed larger than that of other cases. It is attributed to low dispersibility of gelatin and PLGA solutions which affected the fission of droplet in the device. Prepared PLGA porous microparticles can be used as cell carrier with high loading efficiency and viability of cells because the highly porous structure and interconnectivity of microparticles can provide improved mass transfer

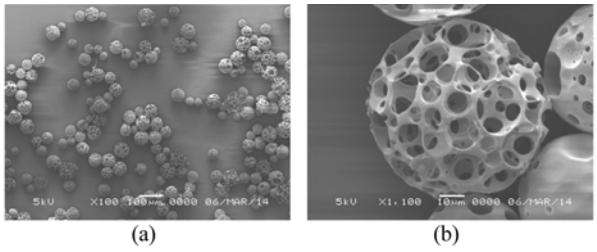

Fig. 6 ((a), (b)) Scanning electron microscopy images of porous PLGA microparticle prepared by T-junction passive breakup device

of oxygen and nutrients that is beneficial to cell viability.[27]

4. Conclusions

We showed that PLGA microparticles preparation using the T-junction breakup device is a suitable alternative methodology to improve microparticle production. The droplet generation was investigated at various flow rate conditions. When Q_c/Q_d increased, diameter of the microdroplets decreased. Monodispersed PLGA microparticles (C.V. < 5%) could be prepared by using the geometrical passive breakup device. Prepared microparticles were tested as microcarriers to deliver lidocaine, CNT, and 3T3 cells. Compared to classical evaporation method, microparticles prepared using the passive breakup had a monodispersed size distribution. In addition, the microparticles showed a slower and more linear drug release profile compared to those generated using the evaporation method. CNT-laden microparticles were also prepared using T-junction breakup. PLGA microparticles with CNTs exposed from their surfaces were successfully prepared using the T-junction breakup device and had increased surface area for loading of functional materials. PLGA microparticles were shown to be useful as cell carriers by attaching cells to microparticle surfaces. Highly porous microparticles were also prepared successfully by T-junction breakup using gelatin as a porogen.

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