Continuous preparation of cytocompatible poly(2-methacryloyloxyethyl phosphorylcholine) microcapsule for cell immobilization using microfluidics

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We have studied the preparation of 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer microcapsules as a carrier for living cells by two microfluidic methods as follows: (i) By photoinduced radical polymerization of MPC, the microcapsules with ca. 85 μm in the diameter were obtained, however, they had low mechanical strength to maintain their shape. (ii) By cross-linking of poly(MPC-*co-n*-butyl methacrylate-*co*-4-vinylphenyl boronic acid) (PMBV) and poly(vinyl alcohol) (PVA), oval-shaped capsules were obtained. Even the capsules were not spherical, the size of the capsules were uniformed. Resultant capsules had mechanical strength enough for maintaining their shape. Sedimentation of the capsules, leading to collapse and coalescence, was prevented by using silicone oil as collection medium. The capsules prepared by the cross-linking method would be suitable candidate for microsized cell carriers and cell culture vessels because the MPC polymers possess highly cytocompatibility.

Key words: Microcapsule, microfluidics, phospholipid polymer, biocompatibility, cell

1. INTRODUCTION

An *in vitro* biologically testing with cultured cells has been required in various fields such as clinical medicine, drug discovery research, and food industry. In these fields, cell analysis is an important and essential process for examining an effectiveness and toxicity of interest materials. However, there have been some problems in a conventional cell analysis. Most of cells within the human body interact with neighboring cells and extracellular matrix to construct three-dimensional (3-D) tissues. Nevertheless, cells form two-dimensional (2-D) tissue in conventional cell culture system such as using microplate. As a result, loss of tissue-specific properties is common in these 2-D cultures [1]. For example, hepatocytes that have been commonly used for activity and toxicity testing of drugs cannot exhibit tissue-specific functions enough until forming 3-D tissues [2]. In order to overcome these problems, many efforts have been devoted to build multi-cellular spheroid (e.g., culturing on nonadhesive surface [3] or hanging drop method [4]). Although these methods have been developed to some extent, uniformity of the cell number or shape of spheroid has not yet been improved. Therefore, we employed microfluidic devices for preparing spheroids under controlling the number of cells (Fig. 1). Most of methods for the preparing capsule encapsulating materials are complicated and time consuming. Furthermore, there have been few methods for synthesis of micro-sized capsules with narrow size distribution. Actually, microfluidic devices can produce monodispersed droplets or particles within microscale [5]. When two immiscible liquids, such as pair of aqueous solution and oil, introduce into a microchannel having Y- or T-shaped junction, monodispersed droplets would be generated at the junction by shearing stress between two immiscible phases. In addition, these devices allow us to encapsulate materials in a single process and control the

amount of encapsulated materials [6]. In case of using cell suspension as aqueous phase, cells could be enclosed in droplets. Moreover, in order to immobilize cells into capsules robustly, curing of liquid droplets should be needed. We employed photoinduced radical polymerization and cross-linking of prepolymers for curing droplets. In both methods, we prepared 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers as cytocompatible materials [7]. The MPC polymers would be suitable for cell encapsulation due to good permeability of solute and hydrophilic nature [8]. In photoinduced polymerization, aqueous solution of MPC, and paraffin oil containing oil soluble surfactant and photoinitiator were introduced into the polydimethylsiloxane (PDMS) microchannel. When preparing aqueous droplets in oil (O/W emulsion) as in this case, it is suitable to use PDMS microchannel due to its hydrophobicity. The hydrophobic surface can inhibit adhesion of the droplets to interior wall of channel. In cross-linking method, we employed pair of poly(MPC-*co-n*-butyl methacrylate-*co*-4-vinylphenyl boronic acid) (PMBV) and poly(vinyl alcohol) (PVA) as prepolymers. Cross-linking between boronic acid moiety in PMBV and hydroxyl groups in PVA occurs spontaneously and quickly after just mixing of each polymer solutions ([7], Fig. 2). Previous study revealed that PMBV/PVA gel prepared in microscaled chamber (thickness: 400 μm , diameter: 5.0 mm) exhibited long term-cell viability [9]. Herein, we expect that cells immobilized in cytocompatible polymer microcapsules could grow up to spheroid *in situ*. The development of this method will enable us to prepare uniform spheroids or engineered tissue. Moreover, such encapsulated cells would be applicable to cell-based transplantation therapy [10]. Here, we investigated suitable condition for preparing cytocompatible polymer capsules.

Fig. 1 Schematic illustration of microfluidic system (a), designed microchannel (b), and magnified view of the junction of microchannel (c).

2. EXPERIMENTAL

2. 1. Fabrication of microchannel.

2. 1. 1. Microchannel mold.

Epoxy resin mold for fabricating PDMS microchannels was prepared by UV lithography. First, microslide glass (S1112, MATSUNAMI GLASS) was washed with ethanol in a sonicator bath, and then treated with oxygen plasma at 300 W for 10 min in Plasma Reactor PR 500 (Yamato Scientific Co., Ltd.) prior to use. Second, a certain amount of epoxy resin prepolymer (SU-8 50,

Table 1 Preparation recipes of MPC polymer microcapsules

	photo-polymerization	cross-linking prepolymers
P _{d1} *	MPC (20 wt%), BIS (2.0 wt%)	PMBV (5.0 wt%)
P _{d2} *	MPC (20 wt%), BIS (2.0 wt%)	PVA (2.0 wt%)
P _c **	span 80 (2.0 wt%), Irgacure OXE01 (4.0 wt%)	span 80 (2.0 wt%)

*aqueous solution. **paraffin oil solution.

Microchem Co.) was coated onto the microslide glass with spin coating at 500 rpm for 5.0 s, and subsequently at 1000 rpm for 30 s. And then heated at 65 °C for 10 min and subsequently at 95 °C for 30 min. After heating, the designed photomask was attached onto the coated prepolymer on the microslide glass and UV light was irradiated to the prepolymer at 340 W/cm² for 40 s. After irradiation, the prepolymer was heated at 95 °C for 10 min and then rinsed in SU-8 developer (Microchem) for 10 min in order to dissolve non-irradiated prepolymer.

2. 1. 2. PDMS microchannel.

A PDMS microchannel was prepared by pouring a 10:1 ratio by weight mixture of PDMS (silpot 184, Dow coming TORAY) and curing agent onto the mold at a depth of ca. 4.0 mm. The mixture were heated at 65 °C for 4 h in order to solidify the mixture. And then solidified PDMS was removed from the mold. Resultant PDMS was bound to another PDMS plate as a bottom by oxygen plasma treatment at 85 W for 30 s. And stored at 85 °C for 2.0 h in an oven.

2.2. Preparation of polymer microcapsules by photoinduced polymerization of MPC in microchannel.

Paraffin oil (Wako Pure Chemical) containing surfactant (span 80, 2.0 wt%, TCI) and photoinitiator (Irgacure OXE01, 4wt%, BASF Japan Ltd.) was injected into P_c inlets (Fig. 1 (b), Table 1). Aqueous mixture of MPC (20wt%) and *N, N'*-methylenebisacrylamide (BIS, 2.0 wt%, Kanto Chemicals) as cross-linker was also introduced into both P_{d1} and P_{d2}. The MPC was synthesized as reported by us elsewhere [8]. The injection was performed with gastight syringe (1001TLL,

Fig. 2 Chemical structure of PMBV (a). Schematic illustration of gelation mechanism of PMBV and PVA (b).

Hamilton Company) equipped with microsyringe pump (BS-MD1001, Bioanalytical Systems Inc.) to control the flow rate. The generation of MPC aqueous droplets was observed with optical microscope (Axioskop 2 plus, Carl Zeiss) equipped with camera (VB-7010, KEYENCE Corp.). The UV irradiation was carried out onto the droplets in order to cure the droplets through objective lens (5x) on the microscope (light source; Hg lump, power; 60-70 mW/cm²). Resultant capsules were collected in paraffin oil.

2.3. Preparation of PMBV

MPC, BMA (Kanto Chemicals Co., Inc.), VPBA (Tokyo Chemical Industry Co., Ltd. (TCI)), and 2,2'-azobisisobutyronitrile (AIBN, Kanto Chemicals) were dissolved in 200 mL ethanol (Kanto Chemicals) and poured into 300 mL four-neck round bottom flask equipped with thermometer, Allihn condenser, argon gas inlet, septum cap and magnetic stirrer bar. Argon gas was flown into the solution for 20 min to remove the oxygen prior to copolymerization. Copolymerization was performed at 60 °C for 12 h in an oil bath. Resulting polymer was purified by reprecipitation against ether/chloroform (70/30, vol%) solution and dialysis against water for a week with dialysis membrane (Spectra/Por 7 (MWCO: 3500), Spectrum Laboratories Inc.). Characterization of the polymer was performed with ¹H-NMR (JNM-NR30, JEOL Ltd.) and gel permeation chromatography (JASCO Corp.) with OHpak SB-804 HQ column (Shodex®). The column was calibrated with poly(ethylene glycol) standards. Molecular weight and polydispersity index of the PMBV were 70k and 2.0, respectively.

2.4. Preparation of polymer microcapsules by cross-linking the prepolymers in microchannel.

Each PMBV and PVA (polymerization degree: ~1000, saponification value: 96.0 mol%, Wako Pure Chemical) were dissolved in Dulbecco's modified eagle medium (DMEM; cat. No. 11885076, Invitrogen Corporation). Both of solutions were injected separately into P_{d1} and P_{d2} inlets (Fig. 1 (b), Table 1). Paraffin oil containing span 80 (2.0 wt%) was injected into P_c inlets. To confirm the production of droplets, we employed the optical microscope. Resultant capsules were collected in paraffin oil (0.86-0.89 g/mL) or silicone oil (1.07 g/cm³, KF-54, Shin-Etsu Chemical Co., Ltd.)

3. RESULTS AND DISCUSSION

3.1. Preparation of MPC polymer microcapsules by photoinduced polymerization of MPC in microchannel.

Our preliminary experiment revealed that more than 10 s of UV irradiation was needed to polymerize the MPC monomer in microchannel. And we also investigated the upper limit of irradiation time. In case of using UV irradiation for curing the MPC aqueous droplets, excess irradiation might damage a cell. When UV light (80 mW/cm²) exposed to cervical carcinoma cells (HeLa cells) in DMEM for more than 60 s, viability of the cells decreased to less than 80 %. Therefore, UV irradiation should be limited between 10 s and 60 s in order to

Fig. 3 Plots of droplet diameter as a function of flow rate of dispersed phase. Error bar indicates standard distribution ($n=50$).

Fig. 4 Microscopic images of MPC aqueous droplets under irradiation of UV light (a) and resulting MPC polymer capsules collected in paraffin oil after UV irradiation (b).

polymerize the MPC and to keep cells alive. In case of using objective lens on the microscope for UV irradiation and keeping the limitation of irradiation time, total flow rate ($= 2 \times (Q_d + Q_c)$) should be set up from 2 to 11 μL/min (Q_d and Q_c stands for flow rate of dispersed phase and continuous phase, respectively). Within this flow rate, monodispersed MPC aqueous droplets could be obtained. And the diameter of droplets increased with increasing Q_d under constant Q_c (Fig. 3). This result indicated that the diameter of the droplets could be controlled by altering the flow rate.

To obtain the MPC polymer capsules, we irradiated UV onto the droplets. However, precipitates were generated in the microchannel after the irradiation. Droplets produced from the junction were collapsed by hitting onto the precipitates. The precipitates might be derived from activated initiator because they could not be generated without initiator and/or UV irradiation. Addition of 2,2-diethoxyacetophenone to the continuous phase could prevent these precipitates from generating due to solubilizing initiator. Although droplets generated from the junction were monodispersed state, resulting

Fig. 5 Microscopic images of PMBV/PVA droplets generated from microchannel (a), PMBV/PVA microcapsules in paraffin oil at 1.0 min after collection (b), and at 10 min after collection (c), and in silicone oil at 10 min after collection (d).

capsules after curing had a wider size distribution and some capsules were collapsed (Fig. 4(a), (b)). This result indicated that coalescence between each capsules was occurred after polymerization due to low mechanical strength of the capsules. To prevent the coalescence, the stiffness of capsules needs to be increasing by using large amount of MPC in feed.

3.2. Preparation of MPC polymer microcapsules by cross-linking the prepolymers in microchannel.

In case of using 5.0 wt% PMBV and 2.0 wt% PVA solution, after these solutions passed through the junction, flux of the solution was elongated (Fig. 5(a)). Pinching off of the elongated flux led to generating MPC polymer capsules continuously. And the generation frequency of the capsules increased with the flow rate ratio. Resulting capsules were oval-shaped and have some pits in the center of the capsules (Fig. 5(b)). When employed ellipsoidal-approximated image on image processing software (imageJ, <http://rsb.info.nih.gov/ij/>), the major axis and the minor axis of the capsule were $141 \pm 6 \mu\text{m}$ and $124 \pm 4 \mu\text{m}$, respectively. And the variation coefficients (Cv) of these axes were 4.5 % and 2.8 %, respectively. The reason why these capsules have pits would be explained as follow. First, PMBV and PVA run into each other at the junction. Each prepolymers might start cross-linkage in mixed region as soon as this encounter. Remained prepolymers that have not cross-linked yet would gelate subsequently. Therefore, such gradual mixing process might lead to uneven cross-linking of the prepolymers. Gelated region at the first contact might remain such pits on the capsules. Applying for winding channel into the microchannel would achieve uniform mixing of prepolymers [10]. When the capsules were collected in paraffin oil, the capsules went down to the bottom of glass reservoir and spreaded on the bottom (Fig. 5(c)). To prevent the

sedimentation of the capsules, we used silicone oil as collection medium. Using silicone oil could keep the capsules suspend in reservoir and inhibit collapse of the capsules (Fig. 5(d)). Since UV irradiation is not necessary for curing liquid droplets in this cross-linking method, encapsulated cell would not be damage. Moreover, the resulting capsules kept their shape stably. Therefore, the cross-linking method might be suitable for preparing a cell carrier rather than the photoinduced method.

4. CONCLUSION

We demonstrated that MPC polymer capsules could be prepared by two types of microfluidic methods. And resulting capsules were uniform-sized in both methods. The capsules prepared by photoinduced polymerization collapsed due to low mechanical strength. On the other hand, the capsules prepared by cross-linking method were expected to become a candidate for cell carrier because of their mechanical strength enough to maintain their shape.

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