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Development of Cell Culture Scaffold Composed of Microcapsule: Determination of Preparation Condition

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A novel cell culture scaffold composed of gelatin microcapsules for suspension culture is proposed: In the process of suspension culture, the cells are separated from the scaffold without centrifugation after the trypsin treatment. To this purpose, gelatin emulsified in organic solvent was irradiated with UV ray to cross-link the gelatin at the interface (microencapsulation). The stability of the microcapsules was examined by raising the temperature and by incubating them at the physiological condition. The optimum condition for preparing the microcapsules has been determined.

Key words: microcapsule, cell culture scaffold, gelatin

1. INTRODUCTION

Recent development in regenerative medicine has stimulated technological studies of large-scale cell culture [1]. Since most animal cells can be cultured only on an appropriate rigid scaffold, the surface area of the scaffold need to be increased to enhance the cell yield in culture [2]. Suspension culture is one of the most efficient methods to satisfy this requirement [3]. Serial sub-cultivation by means of the suspension culture consists of three steps: (1) cell culture on the surface of microparticles in the medium, (2) cytodetachment from the microparticles with peptidase such as trypsin, and (3) separation of cells from microparticles. However, cell yield is often significantly reduced at the third step. It is urgent interest to raise the efficiency and simplify the process. Our strategy to solve this problem is that the microparticles are replaced by trypsin-degradable microcapsules, and by using these microcapsules we can skip the third process. Required characteristics for the microcapsules are (1) diameters of 100-500μm, (2) nontoxicity, (3) high cell adhesion, and (4) autoclavability. In this study we tried to prepare gelatin microcapsules with thin wall membranes. The requirements of (2) and (3) are satisfied by gelatin. Thus, the critical condition is the size and trypsin-degradability of the gelatin particles and the autoclavability. We used the electrostatic atomization and simple stirring to prepare gelatin droplets with appropriate sizes, one of environmentally friendly cross-linking methods of the ultraviolet (UV) irradiation for microencapsulation and ethanol sterilization in place of autoclaving.

2. MATERIALS AND METHODS

2.1 Preparation of gelatin microcapsules

Porcine gelatin (type:APH-250, Nitta Gelatin Inc) was dissolved in MilliQ water at 40℃ at the percentage of weight of 5% or 10% to make gelatin solutions. Aliquot of surface-active agent, tetra glycerin fatty acid ester (SYglyster CR-310, Sakamoto Yakuhin Kogyo Co. Ltd), was added to reagent-grade isooctane (Wako Pure Chemical Industries Ltd) at 5wt% to make a dispersing medium. A 0.5ml of 5wt% or 10wt% gelatin solution was added to 30ml of the dispersing medium. Dispersions of gelatin droplets were prepared by the following two ways. (1) The suspension was simply stirred at 40℃ at the stirring rate of 500 rpm to emulsify the solution (Simple stirring) . (2) The suspension was extruded from a syringe with the bore diameter of 100μm at the rate of 0.082ml/min using a micro feeder (JP-S, Furue Science Inc), and applied a 5.5kV using a DC high voltage generator (SL300, Spellman) to a round ring, the center of which was settled at the exit of the syringe (Electrostatic atomization). The size of the droplets depends on the balance of surface tension and electrostatic force according to Rayleigh's equation [4]. The droplets were dipped on the dispersing solution. The emulsions were then incubated at 15℃ for 10min to turn the droplets into gel particles, which were collected as precipitates. The gelatin gel particles were washed in hexane (Wako Pure Chemical Industries Ltd) for three times. Finally we poured 0.5g of gelatin particles in 10ml of hexane in a beaker with the diameter of 3.5cm. The suspension was then irradiated with UV ray at 3400μW/cm^2 of 254nm (CSL-100C, Cosmo Bio Co. Ltd) for 0-15h. The distance between the light source and the surface of the suspension was 5.5cm. The obtained gelatin particles were washed in 2-propanol for three times, and then in ethanol for three times. The suspension was sterilized in 70v/v% ethanol for one day. Finally, they were

dispersed in MilliQ water or a phosphate acid buffer solution at pH=7.0. All the organic solvents were reagent grade ones purchased from Wako Pure Chemicals. Co. Ltd.

2.2 Measurements

The gelatin particles were observed with an inverted microscope equipped with a water bath, and the digital data were taken with a CCD camera. The temperature of the water bath was raised from 25℃ to 75℃ at the rate of 0.105K/min, and the number of particles that remains insoluble was measured. The melting point of the particles was determined as the temperature when half of the particles were dissolved in water. The stability of the particles was also examined by incubating them in the phosphate buffer at 37℃ for 5 days.

2.3 Cell Culture

The gelatin particles prepared with UV irradiation for 3 hours were used. A 0.5 g of the particles was suspended in 10 ml of Minimum Essential Medium including the fetal bovine serum at 10v/v% in a petri dish. Then Human Fibroblast WI-38 was suspended in the medium at the number concentration of $4x10^4$/ml. The cell culture was performed with a seesaw type incubator at 37℃ for five days. The suspension was stirred for two minutes at the interval of 30 min for initial three hours and then continuously.

2.4 Trypsin Treatment

After removing the supernatant of the suspension, 1.5 ml of 0.25wt% trypsin-EDTA-4Na (Invitrogen Corporation) was added to the suspension. Then the suspension was rinsed with the medium to extract the cells.

3. SUMMARY OF THEORETICAL ARGUMENT OF UV CROSS-LINKING OF GELATIN PARTICLES

Gelatin turns into gel due to hydrogen bondings at low temperatures. The melting temperature T_m is enhanced with increasing the molecular weight. UV irradiation for gelatin induces intermolecular covalent bondings, which results in molecular weight increase. Let's assume that (a) the rate of a binding after the UV irradiation is proportional to the number density of the un-reacted functional groups, and (b) the potential energy resulted from a deviation r of a gelatin molecule from its equilibrium position is given by

$$U(r) = \frac{1}{2}k|r^2| \quad (1).$$

Here, k is the elastic coefficient of the binding, and is proportional to $P^{2/3}$ in which P is the degree of polymerization of the gelatin molecule. From (a) , for the irradiation time t, we have $P = 1 + \beta t$, where β is a constant depending on the gelatin weight fraction. According to the Lindeman's law [5] based on (b), the melting point of the gelatin gel is given by

$$T_m \propto (1+\beta t)^{2/3}, \quad (2)$$

The melting temperature reduced by that for gelatin particles without irradiation T_0 is obtained as

$$T_m / T_0 = (1+\beta t)^{2/3} \quad (3)$$

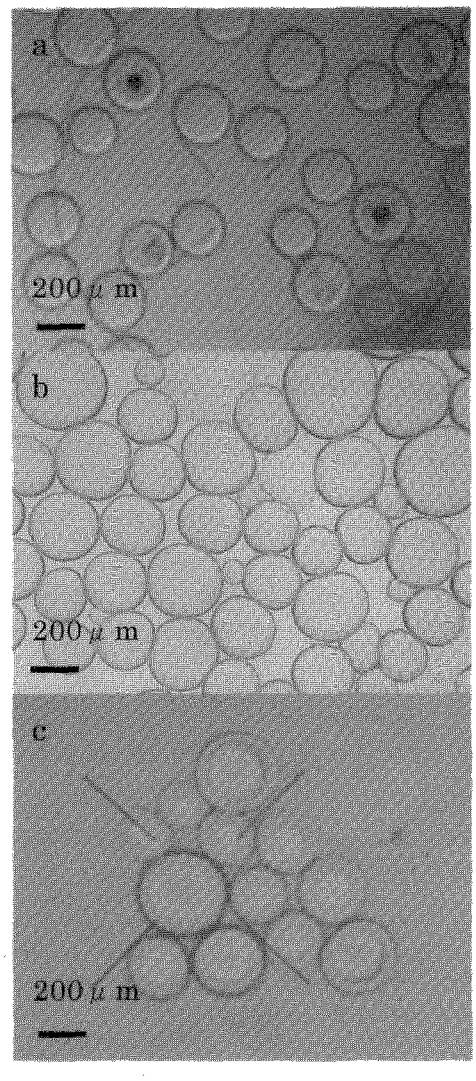

Fig.1 Gelatin emulsions prepared from 5 wt% gelatin solutions with electrostatic atomization (a) and simple stirring (b), and gelatin microcapsules prepared by UV irradiation time of 3h for 10wt% gelatin solution emulsified with simple stirring (c).

4. RESULTS AND DISCUSSION

Figure 1(a) and (b) show the photographs of gelatin particles prepared by electrostatic atomization and simple stirring, respectively. Figure (c) shows the gelatin particles after by UV irradiation for the sample emulsified with simple stirring. From the figures the particles have inner core and thin wall membrane, i.e., microcapsules, as we expected. Figure 2 shows the size distribution of the gelatin emulsions prepared at different conditions. The electrostatic atomization gives much narrower size distribution than simple stirring. The range of the size of the emulsions is satisfactory for both methods of electrostatic atomization and simple stirring. Because the time required for preparing enough amounts of particles for cell culture is much shorter for simple stirring than for electrostatic atomization, we used simple stirring in the following measurements.

Figure 3 shows the reduced melting temperature as a function of UV irradiation time. As expected from Eq. (3), two third power of the reduced temperature is proportional to UV irradiation time. Therefore, we can estimate the irradiation time for preparing particles with desired melting temperature. For cell culture performed at 37°C, more than 30min incubation is necessary.

Figure 4 shows the relative number of microcapsules insoluble after the incubation. The microcapsules prepared with short irradiation less than 1 h and long irradiation more than 10h were dissolved in the medium gradually. The microcapsules prepared with irradiation time in the range between 3h and 8h remained insoluble after 5 days incubation required for cell culture.

Figure 5 shows the fibroblast cells cultured on microcapsules. No significant difference in the growth rate was observed between the present microcapsule scaffolds and Cytodex 1 (Amersham Biosciences) used as a control. Trypsin treatment for the suspension successfully resulted in solubilization of the gelatin scaffolds, and cultured cells were extracted without centrifugation. The time required for dissolving the microcapsules was smaller for smaller UV irradiation time. From these experimental results, the optimum irradiation time was determined as 3h for preparing gelatin microcapsule scaffolds.

In conclusion, trypsin-degradable microcapsules for cell culture scaffolds were successfully prepared. It is hoped to test the present scaffold for various types of cells.

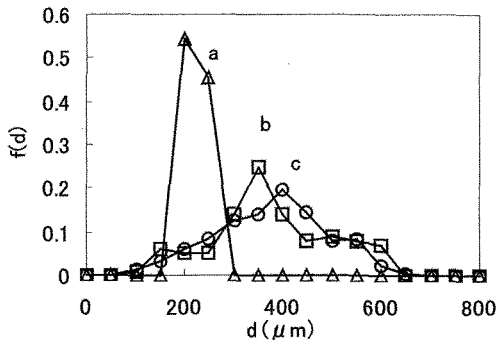

Fig.2 Size distribution of gelatin emulsions prepared with electrostatic atomization for 5wt% gelatin solutions (a), and with simple stirring for 5wt% (b) and 10wt% (c) gelatin solutions in hexane. The average and the standard deviation are 198 ± 18μm, 355 ± 115μm and 364 ± 121μm, respectively, for samples (a), (b) and (c).

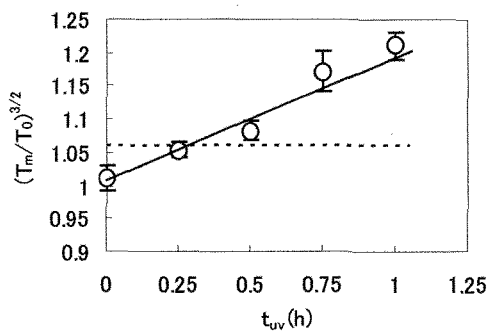

Fig.3 Melting temperature of gelatin microcapsules prepared with different UV irradiation times. The dashed line indicates the incubation temperature for cell culture of 37°C.

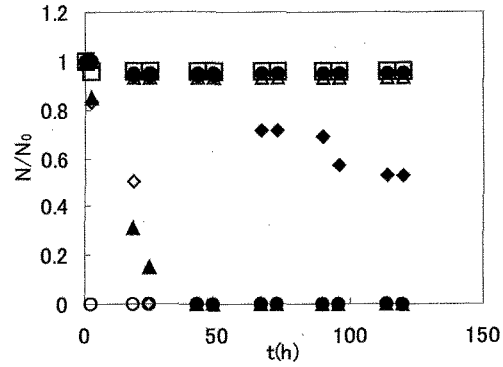

Fig.4 The number N of microcapsules insoluble after the incubation at 37°C divided by the initial one N_0 plotted as a function of incubation time. The symbols indicate UV irradiation time of ○ 0h, ▲ 1h, □ 3h, ● 5h, △ 8h, ◆ 10h, and ◇ 15h.

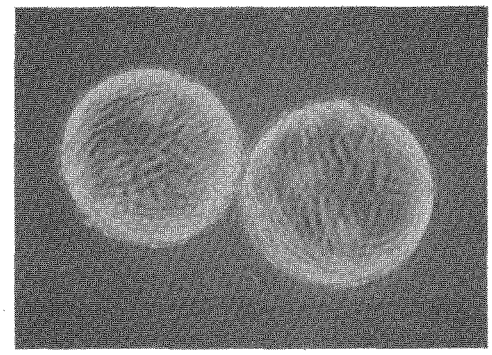

Fig.5 Cultured fibroblasts on gelatin microcapsule scaffolds prepared with UV irradiation to gelatin emulsions for 3h.

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