Tissue-Engineered Bioreactors with Flow Channels Molded by Polypod Particles

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Abstract Good mass transfer and high cell density culture are required for bioreactors using animal cells. These criteria can be met by fabricating a tissue in the bioreactor. In the present study, polypod particles were prepared using agarose, carrageenan, calcium alginate, and hydroxyapatite. The particles were then packed into reactors, and the reactors were filled with enzymatically cross-linked gelatin. Reactors with flow channels were then obtained upon dissolution of the gel particles. Cell adhesion, growth, and expression of organ (liver) function in the reactor were subsequently examined. Experiment using CHO-K1 cells suggested that the cells adhered and grew on the internal surface of the flow channels. HepG2 cells inoculated into the reactor expressed liver-specific functions over the 3-day culture period examined. Thus, the current findings demonstrate that the method developed can be applied to fabricate bioreactors to provide physiologically active substances and medical treatments for tissue engineering. Furthermore, this method was extended to the preparation of a hydroxyapatite-packed reactor by combining calcium alginate gel particles and hydroxyapatite. Therefore, this technique is expected to be applicable to both soft tissue models and hard tissue models such as bone.

Keywords: bioreactor, high cell density culture, tissue engineering, medium circulation culture.


1. Introduction

Bioreactors combined with animal cell culture technology are used in the production of bio-pharmaceuticals or as medical engineering devices [1]. To date, bioreactors can achieve a maximum cultured cell density 1/10–1/100 of the native tissue cell density. However, for application to the production of bio-pharmaceuticals or medical engineering devices, culture of higher cell density is required. Achieving a higher cell density culture is challenging because it is difficult to realize uniform conditions in the reactor. For instance, constructing uniform flow channels in the reactor for the cell culture medium to flow through efficiently is difficult. Accordingly, scaling up the fabrication of reactors that are able to achieve a high cell density culture is challenging [2].

Tissue engineering has been proposed as a technique for constructing tissues [3]. This technique enables the construction of tissues in vitro and/or in vivo by combining the target cells, growth factor, and scaffold. The scaffold provides the surface for cell adhesion and maintains the space required for tissue construction. The growth factor promotes proliferation, migration, and differentiation of cells and expression of cell functions. In our previous studies, we developed a material that can immobilize growth factors [4–8]. Collagen and gelatin, which are biodegradable and biocompatible, were used as base materials. The resulting growth factor-immobilized materials were able to promote cell growth and angiogenesis for cell organization in reconstruct-ed tissues [9].

In the present study, we aim to produce cultures with higher cell density in bioreactors by integrating tissue engineering technology. Specifically, we aim to achieve culture with cell density comparable to that in the native tissue, by constructing a tissue in the bioreactor onto which the seeded cells can assemble. To obtain uniform flow channels in the bioreactor, polypod particles are examined. The polypod particles are first synthesized by a templating method. Second, the polypod particles and a biodegradable solution that contains the growth factor-immobilizable material are packed in the reactor, and gelation of the solution is initiated. Third, the polypod particles are dissolved to generate the flow channels. Cells in suspension are seeded in the gel or in the flow channels in the reactor. Finally, cell culture takes place and cell growth and angiogenesis are promoted in the reactor. A high cell density culture can thus be realized in the reactor using the concept outlined above (Fig. 1).

In this report, we present a protocol for constructing bioreactors consisting of a gel containing flow channels. The flow channels were formed using polypod particles that were fabricated for the purpose of this study. CHO-K1 cells, which are widely used in the production of bio-pharmaceuticals, were used as model cells that were seeded and cultured in the reactor. Additionally, HepG2 cells, which have been used in trials with hybrid artificial liver devices, were seeded in the reactor to demonstrate the applicability of the present bioreactor as a medical engineering device.

2. Methods

2.1 Preparation of the Polypod Particles

The procedure employed to prepare the template of polypod (tetrapod in this study) particles template is shown in Fig. 2A. The template was prepared from a polydimethylsiloxane (PDMS; SYLGARD 184, Dow Corning, Inc., Midland, MI, USA) mold. The PDMS mold was prepared as follows. An acrylic plate was drilled to design the shape of the PDMS mold (effectively the
polypod particles template), as shown in Fig. 2B, using a Computer Numerical Control vertical milling machine (Dyna Myte 2400, Mycom, Inc., Kyoto, Japan). Then, PDMS was introduced into the acrylic mold and stored at room temperature for 48 h to create the first PDMS mold. A flat acrylic board was used to cover the PDMS-filled acrylic plate. The resulting PDMS mold was then soaked in 1% bovine serum albumin (BSA; Wako Pure Chemical Industries, Ltd., Osaka, Japan) aqueous solution for 1 h, then dried at room temperature. (BSA coating of the first PDMS mold was performed for easy detachment of the second PDMS mold from the first mold.) Subsequently, PDMS was poured into a 60-mm dish for immersion of the (first) BSA-coated PDMS mold to generate the second mold. After deaeration, the entire PDMS mold was stored at room temperature for 48 h. Finally, removal of the first PDMS mold generated a PDMS template for the polypod particles.

The tetrapod particles were prepared from four types of aqueous solutions (described below) using the prepared PDMS template. Agarose (Agar), carrageenan (Carr), and calcium alginate (CaA) were selected because these materials are biocompatible and are typically used for cell culture dish coating and as cell-embedding materials. Hydroxyapatite (HAp) was selected because it is a major component of bone and for its high biocompatibility. Therefore, the cytotoxicity of the above materials is not an issue if some of the materials do not dissolve completely in the reactor. The Agar and Carr gels can be dissolved by applying the appropriate temperatures. In contrast, CaA gel and HAp can be dissolved by chelation with calcium ions. Specifically, 1.0, 3.0, and 5.0% Agar (MP Biomedicals, LLC., Santa Ana, CA, USA) solutions, and 2.0, 2.5, and 3.0% Carr (Kanto Chemical Co. Inc., Tokyo, Japan) solutions with microwave heating were poured into the prepared template, then cooled to room temperature, resulting in the formation of the corresponding gel particles. The sol–gel transition temperature was determined by varying the storage...
temperature. Additionally, 1% alginate sodium (Kimica Co. Inc., Tokyo, Japan) solution was poured into the prepared PDMS template, then soaked in 2.84 mM CaCl₂ (Wako). Gelation occurred, thereby generating CaA gel particles. To prepare the HAp particles, first, hemihydrate gypsum and water were mixed at a ratio of 10:9 to obtain gypsum dihydrate. The latter was then dried at 45°C and soaked in 0.5 mol/L (NH₄)₂HPO₄ aqueous solution for 4 days at 85°C (hydrothermal treatment).

2.2 Fabrication of the Reactor and Observation of the Flow Channels

A vinyl chloride tube (inner diameter: 10 mm) and a Nylon 6 rod (diameter: 18 mm) were used to construct the reactor. Ten tetrapod particles, made of CaA, HAp, 3% Agar, or 2.5% Carr, were packed into the reactor. Enzymatically cross-linked 9% gelatin [8] was inoculated from the bottom of the reactor unless otherwise stated. CaA or HAp particle-packed reactors were soaked in 0.2 M ethylenediaminetetraacetic acid (EDTA; Dojindo, Kumamoto, Japan) aqueous solution and shaken attempting to dissolve the particles to form flow channels in the reactors. To dissolve the Agar or Carr gel particles and form flow channels in the reactors, the gel particle-packed reactors were soaked in water at the required temperatures. Additionally, a HAp-based reactor was fabricated by packing Agar tetrapod gel particles in the reactor and inoculating HAp instead of gelatin gel. The Agar particle-packed reactor was then soaked in water at 85°C to dissolve the particles to obtain flow channels in the HAp-based bioreactor.

To observe the formation and connectivity of the flow channels in the reactor, the following procedure was used. Resin (Mercox II, Ladd Research Inc., Williston, VT, USA) was injected into the flow channels and cured. The reactor system was then soaked in 0.2 M EDTA aqueous solution to dissolve the HAp that was used as the surrounding matrix (instead of gelatin). The resin molded the flow channels.

2.3 Cell Adhesion to the Gel

Three types of gels were prepared to analyze cell adhesion in the reactor: (i) 9% gelatin gel (which was the same material as the surrounding gel in the reactor); (ii) 3% Carr gel (which was the same material from which the tetrapod particles were made of); and (iii) Carr-treated gelatin gel. The latter gel was prepared as follows. First, a 9% gelatin gel was prepared. Subsequently, a 3% Carr gel was layered over the gelatin gel. Then, the 3% Carr gel was dissolved to produce Carr-treated gelatin gel. This treatment was employed to mimic the internal surface of the flow channels in the reactor.

The three types of gels (300 μL) were prepared in a 48-well plate. The water in each gel was replaced with Ham’s F-12 medium containing 10% fetal bovine serum (FBS; J R Scientific Inc., Woodland, CA, USA). CHO-K1 cells were then seeded at a density of 1 × 10⁶ cells/cm². Then, the gels were incubated at 37°C in 5% CO₂. To determine the time required for cell adhesion, the culture medium was changed and the gels were observed by microscopy every 30 min after seeding.

2.4 Medium Circulation Culture of Cell-Inoculated Reactor

Gelatin gel with flow channels in the reactor was prepared using tetrapod Carr gel particles and gelatin gel. Water was replaced with the culture medium, and 5 mL of the cell suspension was seeded. After seeding, the device was rotated three times at 90° every 2 h. Then, medium circulation was initiated at 0.55 mL/min. After 1 h, the medium was changed to remove the non-attached cells.

In the first experiment, CHO-K1 cells were seeded at a density of 4.27 × 10⁶ cells/mL and Ham’s F-12 medium containing 10% FBS was used. The medium was sampled (100 μL) every 2 h over a culture period of 30 h. The CHO-K1 cell density in the reactor was calculated from the glucose concentration determined during medium circulation. Glucose in the sampled medium was analyzed using a Glucose CII kit (Wako).

Next, HepG2 cells were seeded at a density of 5 × 10⁶ cells/mL, and Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS was used. The medium was sampled (300 μL) daily. HepG2 cells, a type of liver cell, exhibit gluconeogenesis and albumin synthesis, which are specific to the liver. Therefore, the albumin concentration in the sampled medium was determined using a human albumin ELISA quantitation kit (Bethyl Laboratories, Inc., Montgomery, TX, USA).

3. Results

3.1 Formation of Polypod Particles

To synthesize the polypod particles, a PDMS template was prepared using an acrylic mold and a BSA-coated PDMS mold (Fig. 2C). The Agar, Carr, CaA, and HAp tetrapod particles are shown in Fig. 3.

In this study, 1.0, 3.0, and 5.0% Agar gel particles and 2.0, 2.5, and 3.0% Carr gel particles were prepared. The sol–gel transition temperatures observed for the two types of gel particles are shown in Fig. 4.

The prepared polypod gel particles should be sufficiently strong to support their weight upon packing in the reactor to maintain the final structure after packing. Concurrently, they should be easy to dissolve. The study showed that 3.0 and 5.0% Agar gel particles and 2.5 and 3.0% Carr gel particles maintained their final structures after packing in the reactor device. Thus, 3% Agar and 2.5% Carr were studied further.

3.2 Fabrication of the Reactor and Observation of Flow Channels

The CaA and HAp particles, which were packed in the reactor filled with cross-linked gelatin, were not dissolved by shaking in

**Fig. 3** Tetrapod particles: (A) 3.0% Agar; (B) 2.5% Carr; (C) 1.0% CaA; and (D) HAp. Scale bar is 4 mm.
0.2 M EDTA solution. Therefore, a gelatin gel with flow channels could not be produced using the CaA and HAp particles. In contrast, the Agar and Carr tetrapod gel particles dissolved upon soaking in water at 80°C and 60°C, respectively, resulting in the formation of flow channels within the cross-linked gelatin matrix in the reactor. Because gelatin is prone to damage under high temperature conditions, Carr is a better option to fabricate a gelatin-based reactor. Combining the Agar tetrapod gel particles with HAp (instead of gelatin) also led to the successful fabrication of a reactor with flow channels. The connectivity among the flow channels was determined by infiltrating the reactor with resin. The molded resin indicated that the flow channels were connected from one end of the reactor to the other end (Fig. 5).

3.3 Cell Adhesion on the Gel
Animal cells are typically cultured at 37°C. They cannot survive during dissolution of the tetrapod particles as performed in this study. Therefore, cells were seeded after dissolution of the tetrapod particles by introducing the cell culture medium into the flow channels. The seeded cells are expected to attach to the internal walls of the flow channels. Therefore, adhesion of cells on the gels is important and was analyzed by observing the gels after cell seeding. The results after 1 day of seeding suggested that the CHO-K1 cells did not effectively attach to the Carr gel (data not shown). In contrast, cell adhesion was observed on the Carr-treated gelatin (Fig. 6A–C) and gelatin (data not shown) gels. The number of CHO-K1 cells on the Carr-treated gelatin gel increased after 1 day of seeding (Fig. 6D). This result suggests that the Carr gel, which was initially overlaid on gelatin gel matrix, was effectively removed by dissolution of the gel in hot water. Thus, the surface of the flow channels of the gelatin gel in the reactor, formed by a combination of Carr gel particles and gelation gel, is expected to exhibit cell adhesion properties.

Additionally, cell attachment to the gel could be observed after 0.5 h of seeding (Fig. 6A). And, the cell number appeared to increase slightly after 1 h of seeding (Fig. 6B). This result suggests that a minimum of 1 h is required for cell attachment. Therefore, rotation of the reactor by 90° every 2 h was performed during seeding of the cells in the reactor.

3.4 Medium Circulation Culture in Cell-Inoculated Reactor
The glucose concentrations measured after seeding CHO-K1 cells in the reactor and the albumin concentrations measured after seeding HepG2 cells in the reactor are shown in Fig. 7. The concentration of glucose in the circulation medium decreased as a function of time. The number of cells attached to the surface of the reactor flow channels was estimated as follows: \( r_t = \mu \cdot C_t \), where \( r_t \) is the cell proliferation rate; \( C_t \) is the cell density; \( \mu \) is the specific proliferation rate; \( \mu_{\text{max}} \) is the maximum specific proliferation rate; and \( t \) is the culture time. Integration of the equation gives the following equation:
where $\mu_r$ can be described from the Monod equation [10], $\mu_r = \mu_{\max} \frac{C_s}{K_s + C_s}$, where $C_s$ is the concentration of glucose (1.8 g/L) and $K_s$ is the substrate saturation constant ($10^{-7}$ g/L [11]). Thus, under the assumption that $\frac{C_s}{K_s + C_s} = 1$, parameter $\mu_r$ is simply described as $\mu_r = \mu_{\max}$.

Conversely, \[
\frac{dC_r}{dt} = GCR \cdot C_s,
\]
where GCR refers to the glucose consumption rate.

Combining Eqs. (1) and (2) gives \[
\frac{dC_s}{dt} = GCR \cdot C_s - C_s \frac{dC_r}{dt} = GCR \cdot C_s - \frac{GCR}{\mu_r} C_s \cdot e^{\mu_r t},
\]
which upon integration gives the following equation:

\[
C_s = C_{s0} + \frac{GCR}{\mu_r} C_{s0} \left( e^{\mu_r t} - 1 \right),
\]
where $C_{s0}$ refers to the initial glucose concentration in the medium.

Under the assumption that the cells grow by a factor of two, $\mu_r = \frac{\ln 2}{t_d}$, where $t_d$ refers to the doubling time.

Thus, $C_{s0} = 1.8$ g/L, GCR = 1.33 $\times$ $10^{-10}$ g/(cell-h) (measured value), and $t_d = 15$ h (measured value) [12, 13].

The volume of a tetrapod particle was determined to be 50 $\mu$L. Hence, the volume of the flow channels, formed by ten tetrapod particles, was estimated to be 500 $\mu$L. $C_{s0}$ was then changed to fit the curve of the measured data (Fig. 7A).

The number of cells initially attached to the gel was estimated to be $2.5 \times 10^7$ based on the results of the curve fitting. The seeded cell number was $2.1 \times 10^6$. Therefore, the cell adhesion efficiency was estimated to be ~10%. In contrast, $1.0 \times 10^6$ cells were expected to remain in the reactor after seeding for 30 h, and the number of cells was expected to increase by four-fold in the reactor.

Furthermore, the albumin concentration in the circulation medium increased when HepG2 cells were cultured in the reactor. The albumin concentration, $C_A$, can be expressed as follows: \[
\frac{dC_A}{dt} = APR \cdot C_{s0} e^{\mu_r t},
\]
where APR refers to the albumin proliferation rate. Integration of the equation gives the following equation:

\[
C_A = C_{A0} + \frac{APR \cdot C_{A0}}{\mu_r} \times (e^{\mu_r t} - 1),
\]
where $C_{A0}$ refers to the initial albumin concentration.

A doubling time $t_d = 40$ h (measured value) was used for the HepG2 cells study. Parameter $C_{A0}$ was estimated to be $1/10$ of the seeded cell number from the above result.

Then, APR was changed to fit the curve of the detected data (Fig. 7B). Based on the result, the albumin production rate was calculated as 0.26 pg/(cell-h), which corresponds to 1.3 $\mu$g per day.

4. Discussion

The present study demonstrated the construction of a bioreactor consisting of a biodegradable gel with flow channels (Figs. 3 and 7). Cell growth and expression of organ (liver)-specific function were observed during the medium circulation culture studies performed using the constructed reactor (Fig. 7). Thus, we propose a new method for the construction of bioreactors.

Albumin production was observed as a liver-specific function in the reactor. The albumin production rate [6.26 pg/(cell-days)] was calculated based on the hypothesis that the seeded HepG2 cells grow at the same rate as in a monolayer culture and albumin is produced at a stable rate. This result is consistent with the value [4 pg/(cell-days)] reported by some studies [14, 15]. Thus, the above results indicate that HepG2 cells in the fabricated bioreactor would proliferate effectively similar to a monolayer culture, and demonstrate the potential of the present bioreactor system for application to functional cell culture.

The prepared tetrapod particles allowed easy construction of a reactor featuring flow channels. Gelatin was used as a matrix for filling the reactor and supporting the tetrapod particles. In previous studies, we demonstrated a technique of imparting growth factor immobilization ability to gelatin [4–7]. Thus, by using heparin-immobilized gelatin, instead of gelatin, some growth factors can be immobilized within the gel surrounding the polypod particles. Such a phenomenon is expected to promote angiogenesis inside the gel, expression of cell functions, and further cell growth in the reactor [8, 9]. Although cells were seeded in the flow channels in the present study, cells are expected also to grow in the surrounding gel.

Additionally, carrageenan gel was used to make the dissolvable gel particles for creating the flow channels. In the present study, water at 60°C was used to dissolve the particles. However, if dissolution of the particles can be achieved under milder conditions, for example, using either enzymes or other types of gels such as temperature-responsive polymer gels, the cells could be packed in the gel surrounding the polypod gel particles. Consequently, if hepatocytes are seeded in the surrounding gel and blood vessel cells are seeded in the flow channels, the reactor may function as an organ model that can promote angiogenesis inside the gel for growth of the seeded cells. Then, the reactor may be used not only as an artificial liver, but also for drug evaluation. Moreover, this would consequently allow high cell density culture. This method is expected to be useful for constructing bioreactors to provide physiologically active substances or medical treatments of tissue engineering.

A HAp-packed reactor with flow channels was successfully constructed using the present method (Fig. 5). HAp is a major material used as a scaffold in bone tissue engineering [16]. Therefore, bone remodeling can be studied using this reactor. The present technique for constructing bioreactors can be applied to hard tissue models such as bone and soft tissue models.

5. Conclusion

A method for preparing polypod particles was developed in the present study. The particles were subsequently used for fabricating reactors with flow channels. Cell growth and expression of organ (liver)-specific function were observed in the medium circulation culture studies performed using the reactor.

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Conflict of Interest

We have no conflicts of interest relationship with any companies or commercial organizations based on the definition of Japanese
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