Short communication

Hydroxyapatite-pulp composite fiber sheet bed: A new material for the immobilization of CHO-K1 cells

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Abstract

A new immobilization material for cell culture, a hydroxyapatite-pulp composite fiber (HAPC) sheet bed, was used to grow CHO-K1 cells. The sheet bed for cell culture was prepared from HAPC fiber by paper-making techniques. Scanning electron microscopic analysis revealed that the HAPC sheet bed had a structure consisting of piled fibers with spaces 10–200 μm in diameter and a pore surface area of 0.32 $\text{m}^2 \text{g}^{-1}$. Using a 25 $\times$ 25 mm^2 square HAPC sheet bed 0.41 mm in thickness (85 g m^{-2} basis weight) for cell culture, CHO-K1 cells grew to a cell density of 3.7×10^7 cells cm^{-3} in a 60 mm plastic dish over a 6-day culture period. High-density culture of CHO-K1 cells was successfully performed using the HAPC sheet bed in a 500 ml spinner flask over a 21-day culture period. The HAPC sheet bed, wound around the stirrer paddle, was rotated in the spinner flask in order to supply nutrients and remove waste products efficiently. The HAPC sheet bed has a large surface area to support cell growth and there is large diffusion space inside of the bed. This new autoclavable substrate for anchorage-dependent cells can be easily scaled-up.

Introduction

Because adherent cells have to be cultured on some solid support for cell attachment, they are usually cultured on a plastic dish coated with attachment factors such as fibronectin or collagen. A glass fiber support (Perry and Wang, 1989), a macroporous cellulose support (Ogawa et al., 1992), and polyurethane foam (Ijima et al., 1992) have been developed for large-scale cultivation. Fiber sheet matrixes are especially advantageous because of their high surface-to-volume ratios. However, conventional materials such as glass fiber and polyester fiber (Mitsuda et al., 1990) have poor biocompatibility.

Hydroxyapatite (HAp) has high biocompatibility and has been applied to artificial tooth-bone ceramics sinter materials (Akao, 1985) and cell attachment ceramics sinter beds (Suzuki et al., 1990). Many

studies on the biocompatibility of HAp have been performed using osteoblasts (Labat et al., 1999; Di Silvio et al., 1998; Wilke et al., 1998). HAp-coated hollow microspheres have been used for 3-dimensional bone tissue formation (Oiu et al., 1999).

Recently, hydroxyapatite-pulp composite fiber (HAPC), consisting of pulp coated with HAp, has been developed (Kawakatsu, 1993). HAPC fiber looks like wood pulp fiber and sheets can be easily formed by paper-making techniques. Since the entire surface of the HAPC sheet is coated with HAp, these sheets are expected to be a suitable biocompatible substrate for cell culturing adherent cells. There are many spaces 10–200 μm in diameter in the HAPC sheet bed. These spaces provide a large cell attachment area (0.32 $\text{m}^2 \text{g}^{-1}$), and a suitable environment for cell growth. This paper is the first report demonstrating that CHO-K1 cells can be cultured at a high cell density in a HAPC

Figure 1. Scanning electron microscopic images of the HAPC(85) bed. The magnification is shown at the bottom of each photo. 10 points = 150 or 60 μm .

Figure 2. Comparison of static culture of CHO-K1 cells on a HAPC(85) bed and on a plastic dish. CHO-K1 cells (inoculum: 3×10^5 cells) were cultured on a $25 \times 25 \text{ mm}^2$ HAPC(85) bed in a 60 mm plastic dish containing 10% FBS/F12 medium. For comparison, the same number of cells were cultivated on a 60 mm plastic dish. The number of cells was counted using a Burkert-Turk blood cell counter. ●, HAPC(85) bed; ○, 60 mm dish.

sheet bed in either a plastic dish or a large-scale spinner flask.

Materials and methods

Preparation of the hydroxyapatite-pulp composite fiber sheet bed

HAPC was synthesized by the HAp liquid reaction (Kawakatsu, 1993). Briefly, $(\text{NH}_3)_2\text{HPO}_4$ solution was dropped into $\text{Ca}(\text{NO}_3)_2$ solution containing commercial softwood-bleached kraft pulp (N-BKP) and HAp precipitated onto the surface of the pulp fibers. Because the core of the HAPC is pulp fiber, it is easy to prepare sheets from the HAPC by paper-making techniques. A wet HAPC sheet was made using a $25 \times 25 \text{ cm}^2$ hand-made sheet machine. Then the wet sheet was dried at 120°C in a cylindrical electric heat drier. This HAPC sheet was again broken up to HAPC fibers to remove HAp powder unattached to pulp in the sheet. Then a mixture of the HAPC fibers and 20% binder (synthetic polyethylene fiber) was processed to form a sheet for use as a cell attachment bed using a hand-made sheet machine and

a drying oven (SANYO, MOV-212) at 135°C . Two kinds of HAPC sheet beds differing in basis weight, 85 g m^{-2} and 175 g m^{-2} , were prepared, and these were called the HAPC(85) bed and the HAPC(175) bed, respectively. The pore size, surface area and specific gravity of the sheet bed for cell attachment was measured using a pore size analyzer (MICROMERITICS, Poresizer 9310) equipped with a mercury porosimeter. This method of analysis is based on the capillary law governing mercury penetration into small pores. The volume of mercury penetrating into the pore is measured directly as a function of the pressure applied, providing information on pore structure (volume, diameter, surface area and density).

Cells and methods of cell culture

Experiments were performed using a Chinese Hamster Ovary cell line (CHO-K1, Riken Gene Bank, RGB0285). In some experiments, the cells were cultured in 60 mm plastic dishes (Nalge Nunc, USA) containing Ham's F12 medium (Gibco BRL, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in a humidified atmo-

Table 1. Characteristics of the HAPC(85) bed and the HAPC(175) bed

		HAPC(85) bed	HAPC(175) bed
Basis weight	(g m ⁻²)	85	175
Thickness	(mm)	0.41	0.83
HAp weight %	(%)		15
Bulk specific gravity	(g cm ⁻³)		0.0324
Pore volume	(ml g ⁻¹)		2.86
Pore surface area ^a	(m ² g ⁻¹)		0.32
Surface-to-volume ratio ^a	(cm ⁻¹)		104

^a Pore diameter: 10~200 μ m.

Figure 3. Illustration of a HAPC(175) bed wound around the stirrer paddle in a spinner flask.

sphere of 5% CO₂/95% air. In other experiments, the cells were cultured in 500 ml spinner flasks (BELLCO Glass, USA) containing Ham's F12 medium supplemented with 10% FBS and 20 mM HEPES buffer pH 7.3 (Gibco) at 37 °C in an atmosphere of 100% air.

Immobilization and cultivation of cells in HAPC beds

A HAPC(85) bed 25 × 25 mm² in size was washed with distilled water 3 times and autoclaved in distilled water at 121 °C for 20 min. The bed was then washed

with culture medium 3 times and placed in a 60 mm plastic dish for static culture. After inoculating the HAPC(85) bed with 3×10^5 cells, 7 ml of Ham's F12 medium supplemented with 10% FBS was added to the dish. The cells were cultured in a humidified atmosphere of 5% CO₂/95% air at 37 °C. The culture medium was changed routinely every 2 days by transferring the sheet bed into a new dish containing 7 ml of fresh culture medium.

In the case of the rotating sheet bed culture in a spinner flask, a HAPC(175) bed 35 × 410 mm² in size was wound around the paddle of a 500 ml spinner flask. A HAPC(175) bed thicker than HAPC(85), was used in the rotating culture in the spinner flask, because it retained the wound shape. The HAPC(85) bed was too thin to hold the spiral shape in the rotating culture. The bed was washed with distilled water three times and autoclaved in distilled water at 121 °C for 20 min. Then the bed was washed with culture medium twice. The bed was wound around the paddle was inoculated with 3×10^6 cells and 450 ml of culture medium was added to the spinner flask. The culture was incubated with stirring, with the paddle rotating at 60 rpm, in an atmosphere of 100% air at 37 °C. The culture medium was changed three times during the 21-day cultivation period.

Cell counting

The cultured HAPC(85) bed was removed from the 60 mm plastic dish, and washed with PBS solution (Gibco). The cells were detached by treatment with 0.2% trypsin/0.02% EDTA (Gibco), and suspended in 30 ml of culture medium in a 50 ml plastic tube by shaking vigorously. The viability of the suspended cells was assessed by the 0.4% trypan blue (Gibco) exclusion method. Cell numbers were determined by counting cells under a phase-contrast microscope.

Figure 4. Long-term culture of CHO-K1 cells immobilized on a HAPC(175) bed wound around the stirrer paddle in a spinner flask. CHO-K1 cells (inoculum: 3×10^6 cells) were cultured on a HAPC(175) bed in a spinner flask containing 10% FBS/F12 medium. The concentrations of glucose and lactate were determined every day. The medium was replaced with fresh medium on days 9, 13 and 17. ●, glucose concentration; □, lactate concentration.

Scanning electron microscopic analysis

The sheet bed with the culture cells was removed from the dish or spinner flask, and washed with PBS solution. The cells on the sheet bed were dehydrated by treatment with a series of ethanol solution increasing concentration (70, 80, 90, 95, and 100%), and finally immersed in t-butyl alcohol. The samples were dried with a freeze dryer (HITACHI ES-2030), coated with Pt-Pd using an ion sputter (HITACHI E-1030), and then observed with a scanning electron microscope (SEM) (HITACHI S-4500).

Determination of glucose and lactate concentrations

The concentrations of glucose and lactate in the waste medium were measured by means of a Biotech Analyser (ASAHI CHEMICAL AS-210) equipped with glucose oxidase membranes or lactate oxidase membranes.

Results and discussion

The HAPC sheet bed surface is suitable for attachment of adhesive cells

Table I shows the characteristics of the HAPC(85) bed. The bed has 10–200 μm pores providing a large surface area of $0.32 \text{ m}^2 \text{ g}^{-1}$, with suitable space for cell

attachment. This surface area is about 1/10 of that of a porous microcarrier, $2.8 \text{ m}^2 \text{ g}^{-1}$ (Yanagida et al., 1997). The surface area to volume ratio (S/V) is 104 cm^{-1} . This value is very similar to the S/V of microcarrier, 120 cm^{-1} and bigger than that of hollow fiber, 25 cm^{-1} (Sakuma, 1990). The sheet bed has hydroxyapatite on the surface which has been well studied as a surface-active bioceramic in medical applications, so the HAPC surface seems to be preferable for mammalian cell culture.

The pore area of the HAPC bed is larger than that of N-BKP paper, because the HAPC bed was made by a technique in which the spaces are enlarged by drying the wet HAPC sheet bed under low pressure. The N-BKP paper is dried with a cylindrical electric heat drier under high pressure, resulting in the formation of a high-density sheet. The pores are spatially connected to each other and the medium passes through from the surface of the bed to the reverse side, because HAPC is piled up in the course of preparation of the sheet bed structure. As shown in Figure 1, scanning electron microscopic analysis revealed that HAPC looked like ribbon tape and the shape was different from that of the columnar shape of glass fiber (Perry and Wang, 1989). The HAPC sheet beds have advantageous characteristics such as large surface area to support cell growth, good diffusion inside the bed, and autoclavability.

(A)

(B)

Figure 5. Scanning electron microscopic images of CHO-K1 cells immobilized on a HAPC(85) bed and on a HAPC(175) bed. (A) CHO-K1 cells cultivated for 6 days on a HAPC(85) bed in a 60 mm plastic dish. (B) CHO-K1 cells cultivated for 21 days on a HAPC(175) bed in a spinner flask. 10 points = 60 μm .

Static culture in the HAPC sheet bed

CHO-K1 cells were cultured on a $25 \times 25 \text{ mm}^2$ HPAC(85) bed in a 60 mm plastic dish. As shown in Figure 2, the CHO-K1 cell growing on the HAPC(85) bed showed no signs of cytotoxicity. The stationary phase of growth was reached on day 6 of the culture period. The cell number on day 6 was 9.4×10^6 cells per $25 \times 25 \text{ mm}^2$ piece of HAPC(85) bed, indicating a high cell density, $3.7 \times 10^7 \text{ cells cm}^{-3}$. In contrast, in conventional static culture on a plastic dish, the cells reached confluence at a density of $1.1 \times 10^5 \text{ cells cm}^{-2}$, more than 10 times less than that in the HAPC(85) bed.

Rotating culture on a HAPC sheet bed in a spinner flask

In order to demonstrate the effectiveness of the HAPC bed for large-scale culture, a rectangular HAPC(175) bed ($35 \times 410 \text{ mm}^2$) was wound around the stirrer peddle in a 500 ml spinner flask (Figure 3). After inoculation of the bed with 3×10^6 CHO-K1 cells, the cells were cultivated in 450 ml of medium on the rotating bed at 60 rpm for 21 days. Figure 4 shows the glucose consumption and lactate production during the 21-day culture period. The entire medium was replaced with fresh medium on days 9, 13, 17. The glucose consumption and lactate production increased during the cultivation period, suggesting that the cells were growing in the HAPC bed. After stopping cultivation on day 21, the cells were not counted. It seems likely that frequent medium exchange would have allowed further growth of the cells, since the results of scanning electron microscopic observation (Figure 5) revealed that the cells did not cover the entire surface area of the HAPC(175) bed. However, it was difficult to continue culturing the cells in a spinner flask of this scale beyond day 21 because of the very rapid consumption of glucose.

So far non-woven fabrics have been used as cell culture beds. The fabrics beds are cut into strips and the strips are fixed inside a roller bottle (Mitsuda et al., 1990) or set in a spinner flask in a cylindrical configuration (Robert et al., 1992). In both cases, the fabric bed is fixed and the medium is circulated. However, in the present study, the HAPC(175) bed was rotated with a paddle to supply nutrients and remove waste products efficiently.

Figure 5 shows scanning electron microscopic images of CHO-K1 cells attached to the surface of a HAPC sheet bed in a dish (Figure 5A) or a spinner

flask (Figure 5B). The morphology of the cells on the HAPC(85) bed in the dish, seemed to be normal and the outline of the cells was clear. However, in the case of the HAPC(175) bed in the spinner flask, there were many cells exhibiting irregular shapes and there was a lot of material other than cells which appeared to be extracellular matrix material (Figure 5B). Furthermore, many clumps of cells that filled the inner space of the HAPC(175) bed were observed.

In conclusion, the HAPC sheet bed, a new cell-immobilization material for cell culture, was found to be suitable for high-density culture of CHO-K1 cells. Further investigation to develop new high-density culture systems using the HAPC sheet bed is desirable.

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