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Journal of the European Ceramic Society 23 (2003) 2939–2946
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A new approach to an artificial joint based on bio-cartilage/porous β-tricalcium phosphate system

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Abstract

A new artificial joint model based on the system of bio-cartilage and porous β-tricalcium phosphate (TCP) was fabricated. The porous β-TCP with 100 μm or so of average pore diameter was prepared by hydrothermal treatment and successive ceramic foaming method. The bio-cartilage part consisted of 0.15% collagen gel with chondrogenic ATDC5 cells. ATDC5 cells in the bio-cartilage/porous β-TCP system were cultured to give an artificial joint model. In the artificial joint model, metachromasia was observed with toluidine blue staining. From the FT-IR spectra of the bio-cartilage, formation of proteoglycans was suggested. In addition, alkaline phosphatase activity increased dramatically. ATDC5 cells grew to produce cartilage matrices in this system.

Keywords: Artificial joint; ATDC5 cells; Metachromasia; Porous β-TCP

1. Introduction

There has been considerable interest in the artificial joint replacements. Especially, for regeneration of cartilage by tissue engineering [1] because articular cartilage lacks the capability of recovery. Wakitani et al. reported cultivation of chondrocytes embedded in collagen gel (bio-cartilage) and the introduction of the bio-cartilage into osseocartilaginous loss part.[2,3] Chen et al. reported that chondrocytes were cultured in the PLGA (polylactic glycolic acid)-collagen gel hybrid sponge[4] and that the cell proliferation and the formation of hyaline cartilage of chondrocytes were confirmed. In the replacements with cultured cartilage, the following points should be considered; (1) the introduced bio-cartilage often didn't adhere to cartilage tissue and living bone in vivo,[5–8] (2) the cell differentiation of chondrocytes in the bio-cartilage must be controlled.[9]

As a new artificial joint model for the improvement of the adhesion between bio-cartilage and living bone, the system of bio-cartilage (collagen gel with chondrocytes;ATDC5 cells[10]) and porous β-TCP was fabricated as shown in Fig. 1. As β-TCP has been used as a biodegradable artificial bone, the porous β-TCP could adhere to a living bone. ATDC5 cells are known as the cell line whose differentiation process from the early stage to the late stage could be tracked.[11,12] Conceptually, our system may be satisfied with the requirement mentioned above. We present herein fabrication of bio-cartilage/porous β-TCP system and the cultivation of ATDC5 in our model system.

2. Experimental procedure

2.1. Materials and methods

α-Tricalcium phosphate with 5 μm of the average particle size was supplied from Taihei Chemical Co. (Nara, Japan). A 50 wt.% solution of polyethylenimine in water was obtained from Aldrich Co.. Detergent (MORE; Kao, Japan) was used as a foaming agent. Glycerol diglycidyl ether as a cross-linking agent was obtained from Sigma (St. Louis, MO, USA). Chondrogenic ATDC5 cells were obtained from the Riken Cell Bank (Tsukuba, Japan). Dulbecco's modified Eagle's medium and Ham's F-12 medium (D-MEM/F-12), Fetal bovine serum (FBS) and 0.25% trypsin were obtained from GIBCO Laboratories (Grand Island, NY, USA). A 0.3% type I collagen (Cellmatrix type I-A; 0.3% acid-soluble type I collagen obtained from porcine tendon) and 1% collagenase solution were

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doi:10.1016/S0955-2219(03)00305-4

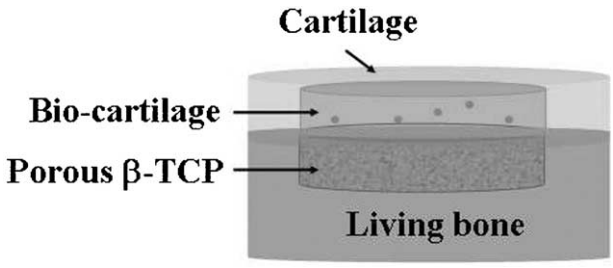

Fig. 1. Schematic view of Bio-cartilage/porous β-TCP system.

obtained from Nitta Gelatin Inc. (Osaka, Japan). Ten percent buffered formalin, 0.05% toluidine blue, 1 × tris-buffered solution (1 × TBS), and "Alkali Phospha K-Test Wako" Kit for the measuring of Alkaline phosphatase (ALPase) activity were purchased from Wako Pure Chemical Industry (Osaka, Japan).

Crystalline phases of the fabricated porous material were identified by X-ray powder diffractometry (XRD) (Rigaku Rint-1200) at 50 kV and 150 mA with Cu$K\alpha$. The microstructure of products was observed after the sputtered coating with Au/Pt by scanning electron microscopy (SEM, Hitachi S-2150). The specific surface area of porous materials were determined by the BET method at 77 K using N_2 as adsorptive agent. FT-IR spectra were recorded on a Fourier transform infrared (FT-IR) spectroscopy (WINSPEC-100; Jeol). Twenty spectral scans were performed over a wavenumber range of 4000–400 cm^{-1} with 2 cm^{-1} resolution. Electronic spectra were recorded on a spectrophotometer (BioSpec-mini Shimadzu) in a range of 190–1100 nm. Ultrasound homogenizer (Heidolph DIAX100) was used for the preparation of the measuring samples of ALPase activity. Phase-contrast microscope (Olympus BX40) was used for the observation of the growth process of ATDC5 cells.

2.2. Preparation and characterization of porous β-tricalcium phosphate (β-TCP)

The porous material was prepared by the combination of hydrothermal reaction and forming ceramics method.[13]

Eight grams of α-TCP and 12 g of 15 wt.% polyethylenimine aquous solution were put into a 100-ml round bottom flask with a stirring bar. The mixture was stirred for 30 min and dispersed with an ultrasonic homogenizer for 30 min. To the slurry was added 1 g of detergent and 0.968 g of glycerol diglycidyl ether. After stirring for 3 min, the slurry was cast into 5-ml plastic cylinder and polymerized at 60 °C for 30 min in a dry oven. After removal of plastic cylinder, the obtained sample was cut into the dimensions ∅ 12 × 1 mm with a cutter blade. After the green bodies were put into a steel vessel with 50 ml of distilled water, its vessel was autoclaved at 121 °C for 24 h. The samples were dried at 60 °C for 24 h in a dry oven and sintered at 1150 °C for 3 h in air.

2.3. Fabrication of bio-cartilage/porous β-TCP system

2.3.1. Cultivation of ATDC5 cells

ATDC5 cells were cultured for 7 days in a 1:1 mixture of D-MEM/F-12 medium containing 5 vol.% (i.e. 5% of total volume) FBS at 37 °C in a humidified atmosphere of 5% CO_2 in air in 60 mm dish. Every other day 5 ml of the medium was replaced. The monolayer cultured ATDC5 cells were treated with 1 ml of 0.25% trypsin. To the dish was added 5 ml of D-MEM/F-12 medium containig 5 vol.% FBS. The suspension was transferred into a 50 ml centrifuging tube and centrifuged at 1000 rpm for 3 min. The supernatant was aspirated to give ATDC5 cell pellets.

2.3.2. Preparation of collagen solution

Collagen solution was prepared by the mixing of the solution A, B, and C in a volume ratio of solution A:solution B:solution C/8:1:1 according to the reported method.[14] Solution A is 0.15% type I collagen, which was diluted with 0.1 M HCl of 0.3% type I collagen, solution B is 10 × D-MEM/F-12 medium, and solution C is the buffer solution for reconstruction of collagen gel.

2.3.3. Fabrication of bio-cartilage/porous β-TCP system

The ATDC5 cell pellet was dispersed into the collagen solution. The autoclave sterilized porous β-TCP sintered compact was placed into a 35-mm dish and the prepared collagen solution-cell mixture (final concentration: 7×10^6 cells/ml) was introduced into the dish, and then the mixture solution was gelled in a CO_2 incubator at 37 °C for 30 min. The system was cultured in 2 ml of D-MEM/F-12 medium with 5 vol.% FBS for 21 days. The medium was changed every other day.

2.4. Characterization of bio-cartilage/porous β-TCP system

2.4.1. Formalin fixing and lyophilization

After cultivation for certain days (3, 7 and 21 days), a sample was well rinsed with a phosphate-buffered saline (PBS) and fixed with 5 ml of 10% buffered formalin for 1 day. The fixed sample was used for the toluidine blue staining and collagenase treatment.

Samples cultured for certain days were well rinsed with PBS and lyophilized. The lyophilized samples were used for SEM observation, FT-IR measurement, and measurement of ALPase activity. A sample for SEM observation was cut into the dimensions 2 mm × 2 mm ×1 mm with a cutter blade, which was sputter coated with Au/Pt.

2.4.2. Collagenase treatment

The cut sample (the dimensions: 2 mm × 2 mm ×1 mm) was treated with 2 ml of 1% collagenase solution in a 15 ml centrifuging tube at 37 °C for 3 h, and was dried at the room temperature for 1 day.

2.4.3. Toluidine blue staining

After cultivation, the system fixed with 10% buffered formalin solution was washed with 5 ml of distilled water for three times and were stained with 5 ml of 0.05% toluidine blue for 30 min. The stained system was rinsed with 95% ethanol three times for 5 s and absolute ethanol once for 10 s. The stained bio-cartilage part in the system was observed with a phase-transfer microscope.

The stained bio-cartilage was sandwiched between two quartz plates. Electronic spectra between 400 and 800 nm were measured with a spectrophotometer.

2.4.4. Alkaline phosphatase (ALPase) activity

The lyophilized system was (approximately 14.5 mg) put into a 15-ml centrifuging tube with 0.5 ml of 1 × TBS and then were homogenized with an ultrasound homogenizer for 1 min. After the suspension was centrifuged for 15 min at 3000*g*, the supernatant liquid was obtained.[15] ALPase activity in an aliquot of the supernatant was measured by the modified Kind-King method[16] using phenyl phosphate as a substrate by measurement of the absorbency at 500 nm with a spectrophotometer.

3. Results and discussion

3.1. Characterization of porous β-TCP

Fig. 2 shows X-ray diffraction patterns of the samples before and after sintering. All peaks of the green body after hydrothermal treatment were attributed to those of calcium-deficient hydroxyapatite (Ca-dHAp). After sintering at 800 or 1150 C, all the peaks of the porous materials were attributed to those of β-TCP. As Ca-dHAp powders with higher Ca/P ratio (> 1.50) are sintered at 1200 °C to give Hap–β-TCP system,[17] the green body after hydrothermal treatment should have the lower Ca/P ratio (<1.50).

Fig. 3 shows SEM photographs of the fracture surface of the prepared porous materials. The average pore diameter was over 100 μm. This porous β-TCP sintered at 1150 °C had coral reef-like microstructure.

The specific surface area of the green body after hydrothermal treatment was 24.46 m^2/g and those of sintered samples at 800 and 1150 °C were 1.61 and 0.46 m^2/g, respectively.

Whisker-like crystals were formed on the surface by hydrolysis of α-TCP, which led to increase of specific surface area. Although the specific surface area decreased after sintering, the surface roughness was maintained. By the combination of the hydrothermal treatment and the ceramic foaming technique, enough pore size and surface roughness[18] for cell cultivation were achieved.

The porous β-TCP obtained by sintering at 1150 °C was used for further three-dimesional cultivation of ATDC5 cells because the porous β-TCP sintered at 800 °C was brittle.

3.2. Characterization of the bio-cartilage/porous β-TCP system

3.2.1. Phase-contrast micrographs

Fig. 4 shows the phase-contrast micrographs of ATDC5 cells in bio-cartilage (inside and outside of porous β-TCP). ATDC5 cells were dispersed in collagen matrix evenly. The shapes of ATDC5 cells were spherical type immediately after cultivation. After cultivation over 3 days, the spherical type cells were not observed because of the adhesion to the collagen fiber. ATDC5 cells grew inside and outside of pores in this system with the increase of cultivation time. In porous β-TCP part, ATDC5 cells adhered inside of porous

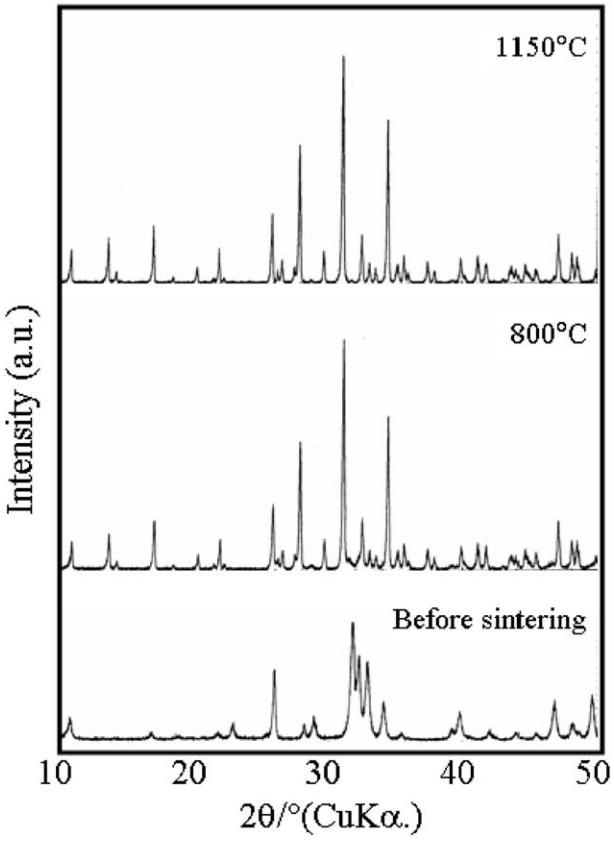

Fig. 2. X-ray diffraction patterns of porous products before and after sintering.

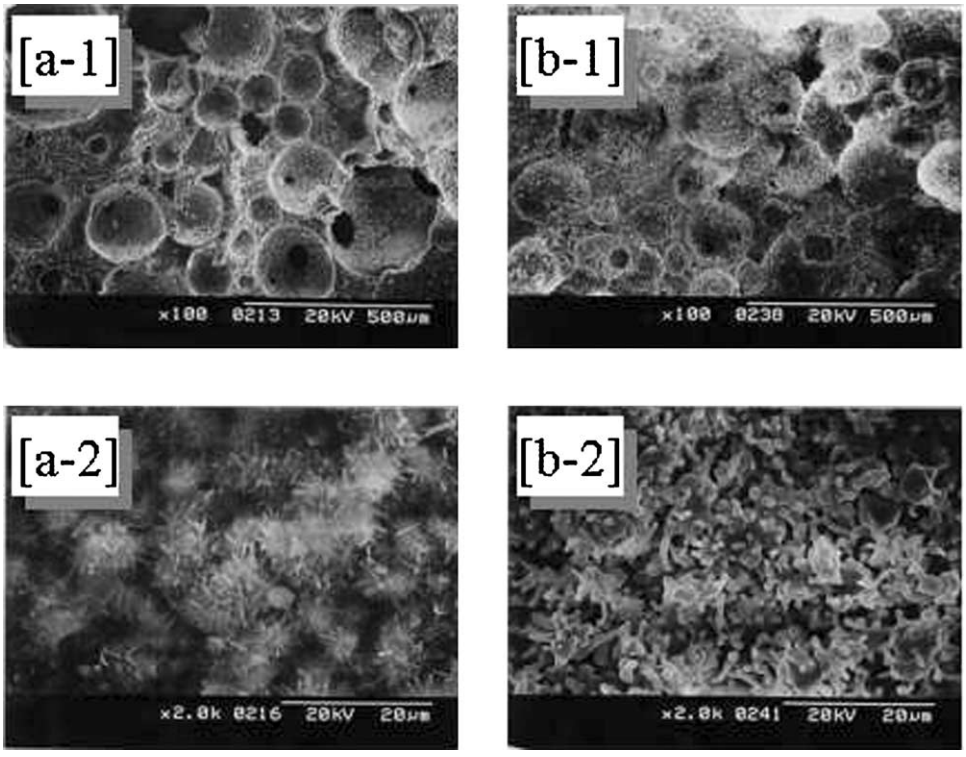

Fig. 3. SEM photographs of porous calcium phosphates with hydrothermal before and after sintering. *Sintering temperature: 1150°C. Sintering time: 3 h. [a–1] Before sintering (×100); [a–2] before sintering (×2000; [b–1] After sintering (×100); [b–2] after sintering (×2000).

β-TCP. Porous β-TCP didn't inhibit growth of ATDC5 cells.

3.2.2. SEM observation

From SEM photographs shown in Fig. 5, the fracture surface of the system after cultivation for 21 days was covered by fibrous substances. When the cultured samples were treated with 1% collagenase, the fibrous substances were not observed. Therefore, the observed fibrous substances were fibrous collagen produced by ATDC5 cells in the course of the cultivation.

3.2.3. FT-IR measurement

From FT-IR spectra of porous material of the system after cultivation for 21 days, no change of the absorption bands between 1200 and 800 cm^{-1} corresponded to phosphate of β-TCP was observed.

Fig. 6 shows the FT-IR spectra of collagen gel (0.15%) and the bio-cartilage part of the system after cultivation for 21 days in the range 1800–800 cm^{-1}. The absorption band at 1550 and 1250 cm^{-1} of the bio-cartilage part of the system was increased, compared with those of collagen gel (0.15%). The absorption band at 1550 cm^{-1} was assigned to C–N stretch, N–H bend combination (Amide II) of proteoglycans.[19–22] The increase of the shoulder at 1250 cm^{-1} was assigned to the antisymmetrical stretching mode, $\nu_{as}(SO_3^-)$ derived from sulfate of proteoglycans.[23]

3.2.4. Toluidine blue staining

The change of color of toluidine blue from blue to magenta was observed at the cultured sample for 21 days with a phase-contrast microscope apparently. The color change is called metachromasia and derived from the reaction of toluidine blue and cartilage matrices produced by chondrocytes. Fig. 7. shows the electronic spectra of collagen gel, bio-cartilage and collagen gel with 1.0 mg/ml sodium chondroitin sulfate C (NaChS) as a positive control. The absorption of collagen gel with ATDC5 cells after cultivation for 3 days increased at 593 nm, compared with that of collagen gel. The absorption peak increased and shifted to low wavelength (from 593 to 530 nm) with the increase of the cultivation time. In the collagen gel with ATDC5 cells after cultivation for 21 days, the shape of the absorption peak is similar to that of collagen gel with 1.0 mg/ml NaChS. Although the metachromasia was not observed with a phase-contrast microscope after 7 days, the metachromasia was observed in the electronic spectra after 3 days.

3.2.5. Alkaline phosphatase (ALPase) activity

The increase of the ALPase activity for ATDC5 cells indicates that the growth (proliferation and differentiation) of ATDC5 cells.[24] Fig. 8 shows the ALPase activity of the system after cultivation and porous β-TCP as a control. The ALPase activity increased with the

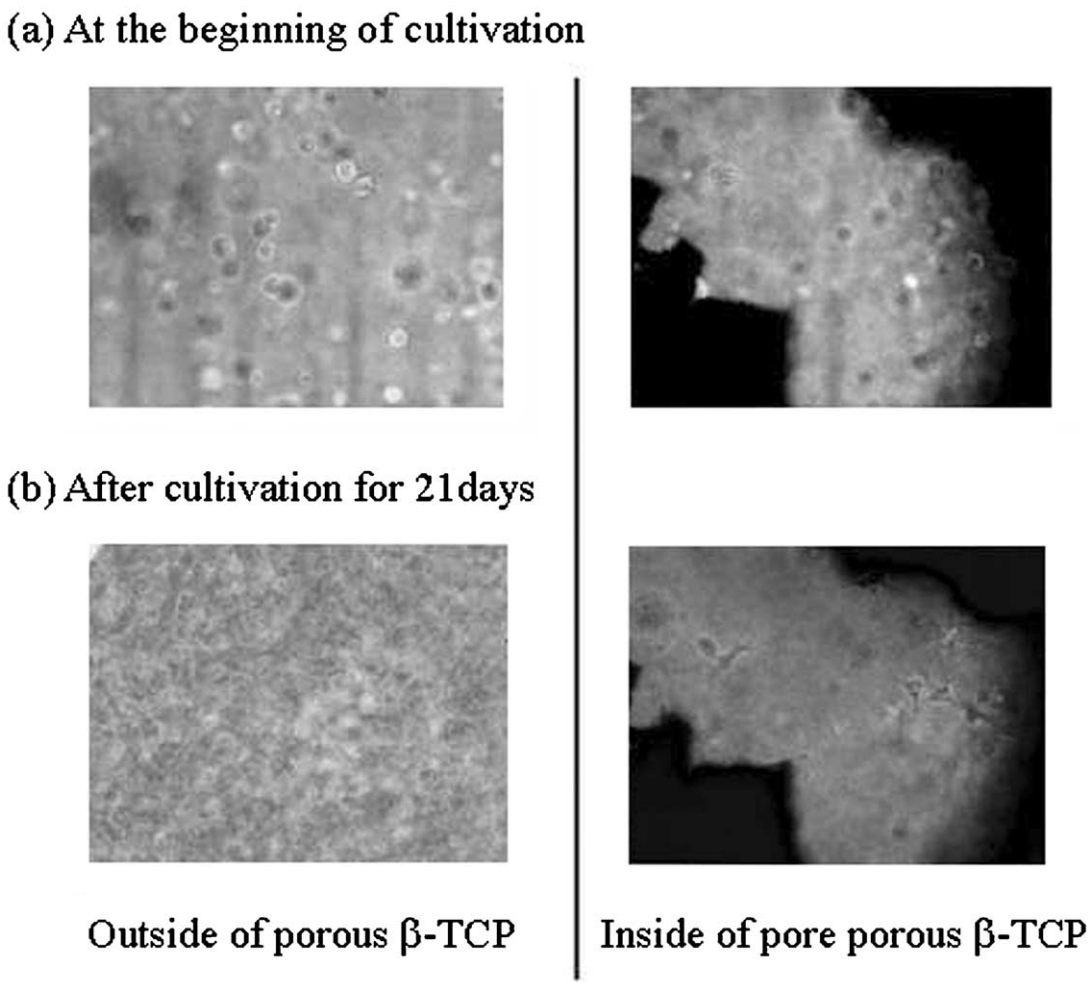

Fig. 4. Phase-transfer micrographs of ATDC5 cells in collagen gel inside and outside of porous β-TCP. (a) At the beginning of cultivation. (b) After cultivation for 21 days.

increase of cultivation time. The ALPase activity after 21 days increased 15-fold after 3 days.

3.2.6. *Growth of ATDC5 in the bio-cartilage/porous β-TCP system*

From ALP activity, electronic spectra, and FT-IR spectra, the cell growth both in collagen gel outside and inside of porous β-TCP was suggested after cultivation for 3 days. After 21 days the differentiation of ATDC5 cells reached the maturation stage from the shapes of ATDC5 cells, and ATDC5 cells produced considerable amount of proteoglycans.

In addition fibrous collagen produced by the grown ATDC5 cells was observed in the system. This fibrous collagen adhered inside of porous β-TCP with rough surface. The adhesion between fibrous collagen and

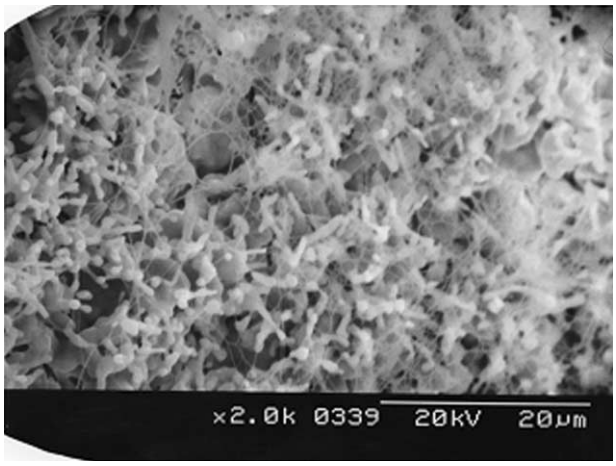

Fig. 5. SEM photographs of the fracture surface of Bio-cartilage/porous β-TCP system for 21 days.

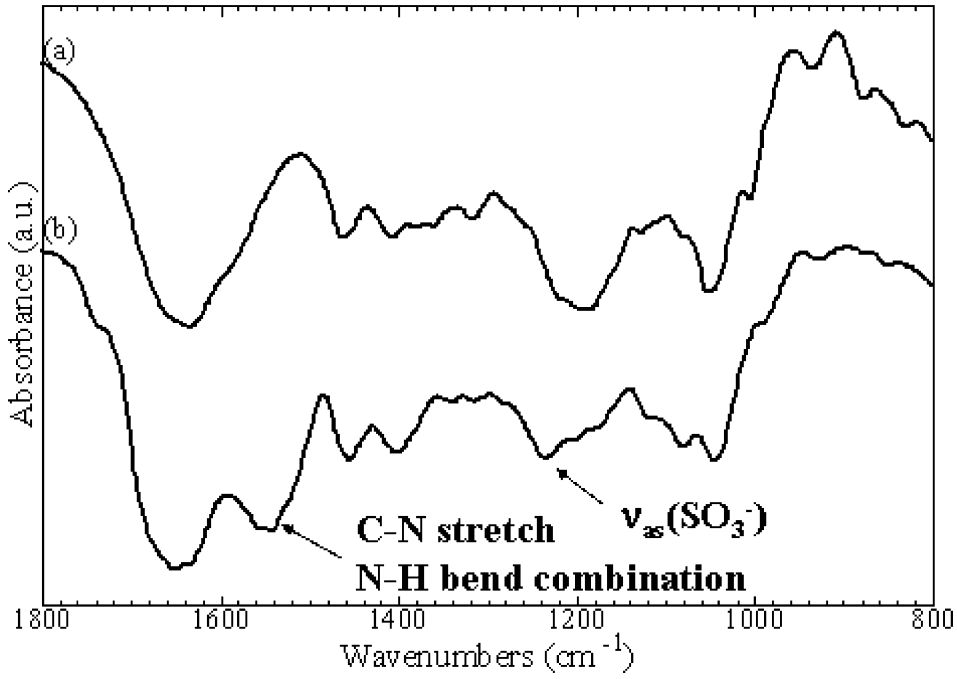

Fig. 6. FT-IR spectra of the lyophilized gel part with Bio-cartilage/porous β-TCP system. (a) Collagen gel (0.15%) as reference; (b) The gel part with Bio-cartilage/porous β-TCP system after 21 days cultivation.

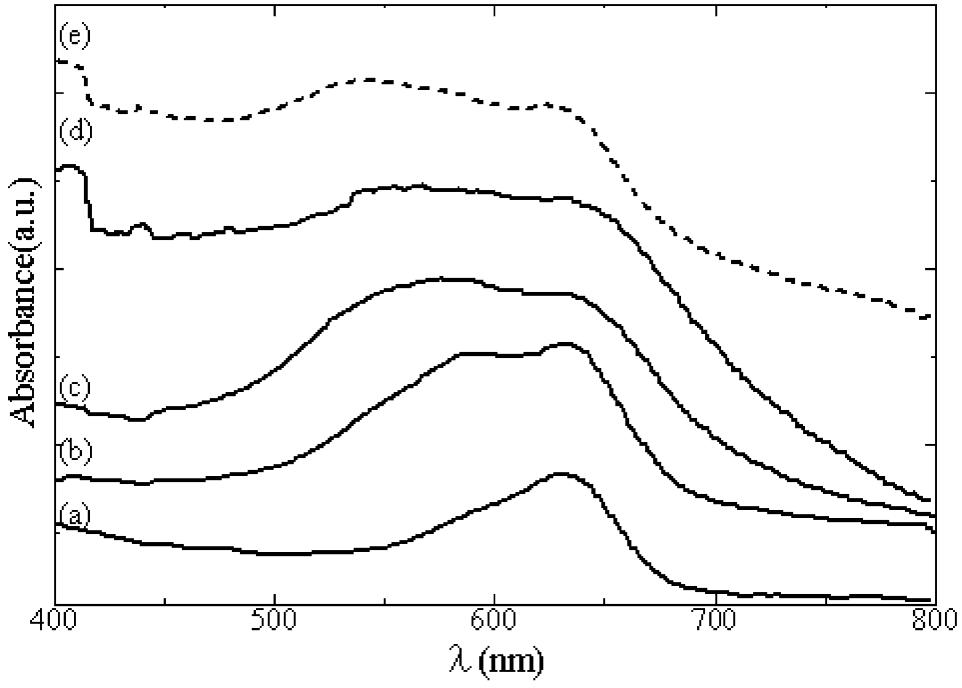

Fig. 7. Electronic spectra of the bio-cartilage part outside of porous β-TCP with toluidine blue staining: (a) 0.15% collagen gel; (b) bio-cartilage (3 days); (c) bio-cartilage (7 days); (d) bio-cartilage (21 days); (e) Collagen gel with 1.0 mg/ml sodium chondroitin sulfate C.

porous β-TCP may improve the adhesion between bio-cartilage and porous β-TCP. Inside of porous β-TCP, β-TCP didn't inhibit the growth of ATDC5 cells because ATDC5 cells adhered inside of porous β-TCP. By production of cartilage-like matrices porous β-TCP seems to be fixed with bio-cartilage.

3.2.7. Scope of our artificial joint model

For the replacements with cultured cartilage, improvement of adhesion between cartilage tissue and living bone and the control of the cell differentiation of chondrocytes are required. In our new artificial joint model the tight adhesion between porous β-TCP and

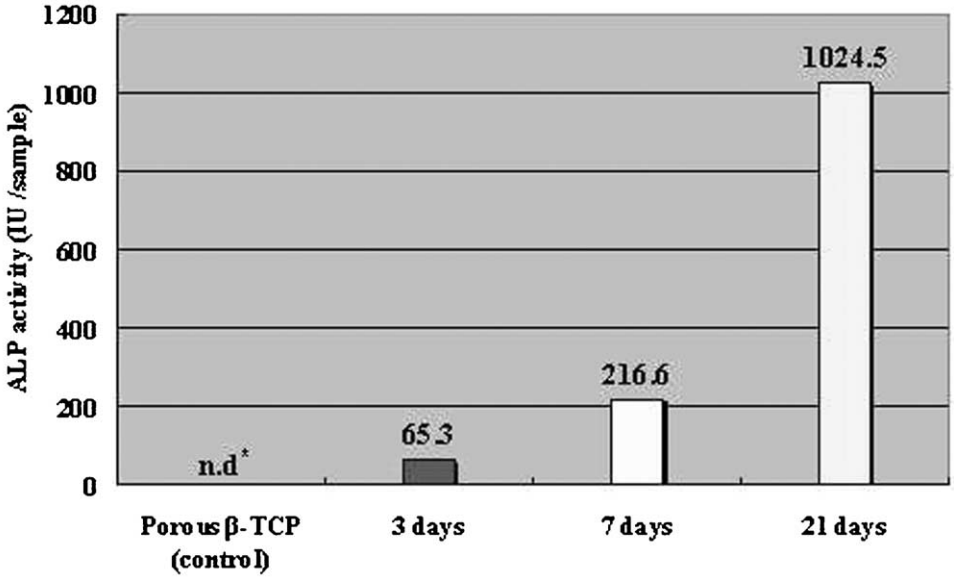

Fig. 8. ALPase activity of Bio-cartilage/porous β-TCP system. *n.d. = not detected.

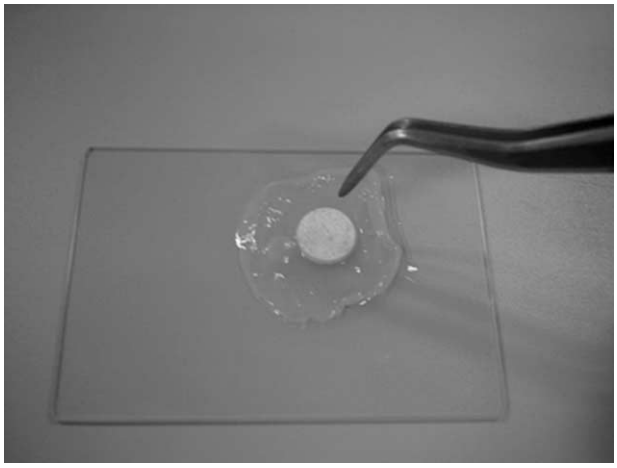

Fig. 9. Photograph of Bio-cartilage/porous β-TCP system.

living bone are expected, as β-TCP is a bioabsorbable material and is replaced by new bone.

ATDC5 cell is a chondrogenic clonal cell line and its differentiation process from the early stage to the late stage is demonstrated. In addition, the effects of the addition of the growth factor on the differentiation process were reported.[24] Especially, the calcification of ATDC5 cells is promoted by BMP-6.[25] By, applying the calcification to the bio-cartilage/porous β-TCP system, not only tight adhesion between bio-cartilage and porous β-TCP but also the bonding boundary with gradient structure like a growth plate[26–30] may be achieved.

4. Conclusions

The bio-cartilage/porous β-TCP system was fabricated in vitro as a new artificial joint model (Fig. 9). Porous β-TCP with enough pore size and specific surface area was obtained by the combination of the hydrothermal treatment and the ceramic foaming technique. ATDC5 cells in our system were cultured to produce the cartilage matrices. After the cultivation, the bio-cartilage was fixed on porous β-TCP, apparently. Further studies on adhesion strength between the bio-cartilage and porous β-TCP are in progress. As the production of cartilage matrices and the resulting fixation were achieved, our model is satisfied with fundamental requirements for the cultured cartilage in our bio-cartilage/porous β-TCP system.

Acknowledgements

This work has performed under the Center of Excellence (COE) program supported by the Ministry of Education, Science, Sports, and Culture, Japan.

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