

- cyte monolayers: cell viability and cytochrome P450 content in post-thaw cultures. *Toxicol In Vitro* **16**, 71, 2002.
19. Ikeguchi, R., Kakinoki, R., Matsumoto, T., Yamakawa, T., Nakayama, K., Morimoto, Y., Nakamura, T. Successful storage of peripheral nerves using University of Wisconsin solution with polyphenol. *J Neurosci Methods* **159**, 57, 2007.
 20. Hyon, S.H., Kim, D.H. Long-term preservation of rat pancreatic islet under physiological conditions. *J Biotechnol* **85**, 241, 2001.
 21. Tipoe, G.L., Leung, T.M., Hung, M.W., Fung, M.L. Green tea polyphenols as an anti-oxidant and anti-inflammatory agent for cardiovascular protection. *Cardiovasc Hematol Disord Drug Targets* **7**, 135, 2007.
 22. Neuhaus, T., Voit, S., Lill, G., Vetter, H., Schor, K., Weber, A.A. Platelet aggregation induced by the C-terminal peptide of thrombospondin-1 (4N1-1) is inhibited by epigallocatechin gallate but not by prostaglandin E1. *Platelets* **15**, 455, 2004.
 23. Ivanov, V., Roomi, M.W., Kalinovsky, T., Niedzwiecki, A., Rath, M. Anti-atherogenic effects of a mixture of ascorbic acid, lysine, proline, arginine, cysteine, and green tea phenolics in human aortic smooth muscle cells. *J Cardiovasc Pharmacol* **49**, 140, 2007.
 24. Omasa, M., Fukuse, T., Matsuoka, K., Hyon, S.H., Wada, H. Effect of green tea extracted polyphenol on ischemia/reperfusion injury after cold preservation of rat lung. *Transplant Proc* **35**, 138, 2003.
 25. Lee, S.R., Suh, S.L., Kim, S.P. Protective effects of the green tea polyphenol (-)-epigallocatechin gallate against hippocampal neuronal damage after transient global ischemia in gerbils. *Neurosci Lett* **287**, 191, 2000.
 26. Varilek, G.W., Yang, F., Lee, E.Y., Villiers, W.J.S.D., Zhong, J., Oz, H.S., Westberry, K.F., McClain, C.J. Green tea polyphenol extract attenuates inflammation in interleukin-2-deficient mice, a model of autoimmunity. *J Nutr* **131**, 2034, 2001.
 27. Ahmad, N., Gupta, S., Mukhtar, H. Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor κ B in cancer cells versus normal cells. *Arch Biochem Biophys* **376**, 2, 338, 2000.
 28. Hayek, T., Fuhrman, B., Vaya, J., Rosenblat, M., Belinky, P., Coleman, R., Elis, A., Aviram, M. Reduced progression of atherosclerosis in apolipoprotein e-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. *Arterioscler Thromb Vasc Biol* **17**, 2744, 1997.
 29. Jankun, J., Selman, S.H., Swiercz, R., Jankun, E.S. Why drinking green tea could prevent cancer. *Nature* **387**, 561, 1997.
 30. Seglen, P.O. Preparation of isolated rat liver cells. *Methods Cell Biol* **13**, 29, 1976.
 31. Moldeus, P., Hogberg, J., Orrenius, S. Isolation and use of liver cells. *Methods Enzymol* **52**, 60, 1978.
 32. Schwerer, B., Bach, M., Bernheimer, H. ELISA for determination of albumin in the nanogram range: assay in cerebrospinal fluid and comparison with radial immunodiffusion. *Clin Chim Acta* **163**, 237, 1987.
 33. Landry, J., Bernier, D., Ouellet, C., Goyette, R., Marceau, N. Spheroidal aggregate culture of rat liver cells: histotypic reorganization, biomatrix deposition, and maintenance of functional activities. *J Cell Biol* **101**, 914, 1985.
 34. Matsuhita, T., Ijima, H., Koide, N., Funatsu, K. High albumin production by multicellular spheroids of adult rat hepatocytes formed in the pores of polyurethane foam. *Appl Microbiol Biotechnol* **36**, 324, 1991.
 35. Robert, R.A., Soames, A.R. Hepatocyte spheroids: prolonged hepatocyte viability for *in vitro* modeling of nongenotoxic carcinogenesis. *Fundam Appl Toxicol* **21**, 149, 1993.
 36. Cho, H.R., Choi, D.H., Ko, B.K., Nam, C.W., Park, K.M., Lee, Y.J., Lee, S.G., Lee, J.S., Lee, K.A., Lee, E.A., Ju, S.A., Kim, B.S. Cold preservation of rat cultured hepatocytes: the scoparone effect. *Transplant Proc* **32**, 2325, 2000.
 37. Antonenas, V., Garvin, F., Webb, M., Sartor, M., Bradstock, K.F., Gottlieb, D. Fresh PBSC harvests, but not BM, show temperature-related loss of CD34 viability during storage and transport. *Cytherapy* **8**, 158, 2006.
 38. Hyon, S.H. A non-frozen living tissue bank for allotransplantation using green tea polyphenols. *Yonsei Med J* **45**, 1025, 2004.

Address reprint requests to:

Tetsuji Yamaoka
 Department of Biomedical Engineering
 Advanced Medical Engineering Center
 National Cardiovascular Center Research Institute
 5-7-1 Fujishirodai, Suita
 Osaka 565-8565, Japan.

E-mail: yamtet@ri.ncvc.go.jp

Received: August 14, 2008

Accepted: November 20, 2008

Online Publication Date:

8

PRESERVATION OF PORCINE HEPATOCYTES ◀ AU1

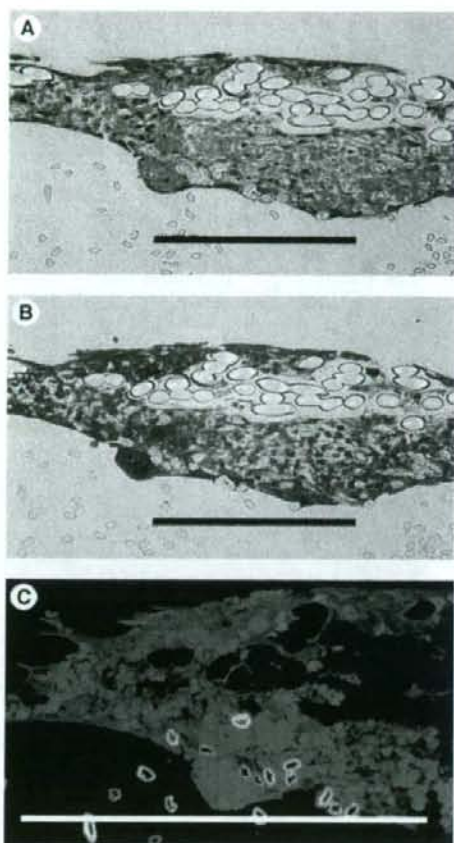


FIG. 7. (A) Hematoxylin and eosin staining, (B) Azan staining, and (C) albumin immunostaining of cultured hepatocytes in bioreactor. Cells were preserved in the presence of 0.25-mg/mL epigallocatechin-3-gallate for 4 days and cultured in normal medium for another 5 days. Bars represent 300 μ m. Color images available online at www.liebertonline.com/ten

Disclosure Statement

AU25 ▶ No competing financial interests exist.

References

- Demetriou, A.A., Brown, R.S., Busuttill, R.W., Fair, J., McGuire, B.M., Rosenthal, P., Am Esch, J.S. 2nd, Lerut, J., Nyberg, S.L., Salizzoni, M., Fagan, E.A., de Hemptinne, B., Broelsch, C.E., Muraca, M., Salmemon, J.M., Rabkin, J.M., Metselaar, H.J., Pratt, D., De La Mata, M., McChesney, L.P., Everson, G.T., Lavin, P.T., Stevens, A.C., Pitkin, Z., Solomon, B.A. Prospective, randomized, multicenter, controlled trial of a bioartificial liver in treating acute liver failure. *Ann Surg* 239, 660, 2004.
- Bilir, B.M., Guinette, D., Karrer, F., Kumpe, D.A., Krysl, J., Stephens, J., McGavran, L., Ostrowska, A., Durham, J. Hepatocyte transplantation in acute liver failure. *Liver Transpl* 6, 32, 2000.
- Baccarani, U., Sanna, A., Cariani, A., Barriga, M.S., Adani, G.L., Zambito, A.M., Piccolo, G., Risaliti, A., Nanri-Costa, A., Ridolfi, L., Scalomogna, M., Bresadola, F., Donini, A. Isolation of human hepatocytes from livers rejected for liver transplantation on a national basis: results of a 2-year experience. *Liver Transpl* 9, 506, 2003.
- Opolon, P. High permeability membrane hemodialysis and hemofiltration in acute hepatic coma. Experimental and clinical results. *Artif Organs* 3, 354, 1979.
- Millis, J.M., Cronin, D.C., Johnson, R., Conjeevaram, H., Conlin, C., Trevino, S., Maguire, P. Initial experience with the modified extracorporeal liver-assist device for patients with fulminate hepatic failure: system modifications and clinical impact. *Transplantation* 74, 1735, 2002.
- Terry, C., Dhawan, A., Mitry, R.R., Hughes, R.D. Cryopreservation of isolated human hepatocytes for transplantation: State of the art. *Cryobiology* 53, 149, 2006.
- Azizi, M., Noriko, S., Tetsuji, Y., Hiroshi, U., Makoto, K. Radial flow type bioreactor for bioartificial liver assist system using PTFE non-woven fabric coated with poly-amino acid urethane copolymer. *Macromol Symp* 249-250, 151, 2007.
- Innes, G.K., Fuller, B.J., Hobbs, K.E. Functional testing of hepatocytes following their recovery from cryopreservation. *Cryobiology* 25, 23, 1988.
- Pahemik, S.A., Thasler, W.E., Mueller-Hoecker, J., Schildberg, F.W., Koebe, H.G. Hypothermic storage of pig hepatocytes: Influence of different storage solution and cell density. *Cryobiology* 33, 552, 1996.
- Guillouzo, A., Rialland, L., Fautrel, A., Guyomard, C. Survival and function of isolated hepatocytes after cryopreservation. *Chem Biol Interact* 121, 7, 1999.
- Darr, T.B., Hubel, A. Postthaw viability of precultured hepatocytes. *Cryobiology* 42, 11, 2001.
- Yagi, T., Hardin, J.A., Valenzuela, Y.M., Miyoshi H., Gores, G.J., Nyberg, S.L. Caspase inhibition reduces apoptotic death of cryopreserved porcine hepatocytes. *Hepatology* 33, 1432, 2001.
- Rauen, U., Kerkweg, U., Weisheit, D., Petrat, F., Sustmann, R., Herbet, D.G. Cold-induced apoptosis of hepatocytes: Mitochondrial permeability transition triggered by non-mitochondrial chelatable iron. *Free Radic Biol Med* 35, 12, 1664, 2003.
- Muller, P., Aurich, H., Wenkel, R., Schaffner, I., Wolff, I., Walldorf, J., Fleig, W.E., Christ, B. Serum-free cryopreservation of porcine hepatocytes. *Cell Tissue Res* 317, 45, 2004.
- Li, L., Li, C.M., Zhang, B.Y., Hu, M.D., Li, X.Y., Ran, J.H., Huang, M. Apoptosis of rat liver in cold preservation with custom-designed KYL solution. *Hepatobiliary Pancreat Dis Int* 6, 497, 2007.
- Lloyd, T.D.R., Orr, S., Skett, P., Berry, D.P., Dennison A.R. Cryopreservation of hepatocytes: a review of current methods for banking. *Cell Tissue Bank* 4, 3, 2003.
- Watts, P., Grant, M.H. Cryopreservation of rat hepatocytes monolayer cultures. *Hum Exp Toxicol* 15, 30, 1996.
- McKay, G.C., Henderson, C., Goldie, E., Connel, G., Westmoreland, C., Grant, M.H. Cryopreservation of rat hepatocytes

MISKON ET AL.

7

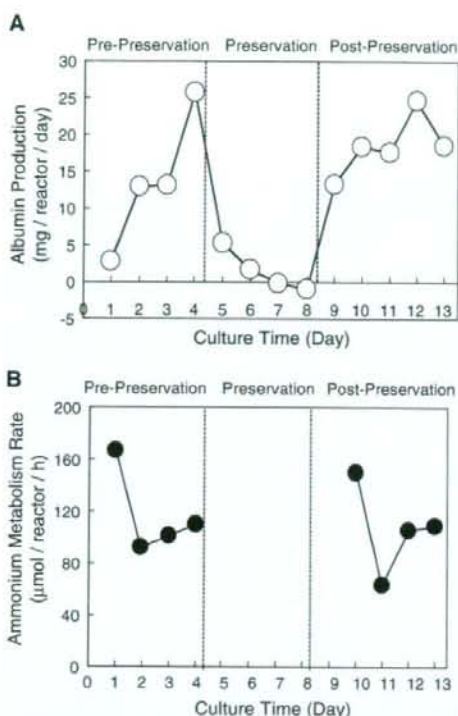


FIG. 5. Albumin production (A) and ammonium metabolism rate (B) of hepatocytes in three-dimensional bioreactor during pre-preservation, preservation in 0.25-mg/mL epigallocatechin-3-gallate-containing medium, and post-preservation.

confirmed in monolayer culture. Then, the concentration of EGCG in the 3D system was set to 0.25 mg/mL. The results of this study imply that 0.25-mg/mL EGCG-containing medium could preserve hepatocyte function for 4 days at room temperature.

In monolayer culture, only the albumin production rate was fully recovered after preservation when EGCG concentration was high (0.25 mg/mL). In contrast, albumin production and ammonium metabolism in a 3D bioreactor under the same conditions recovered to $72 \pm 16\%$ and $98 \pm 32\%$, respectively, of their levels before preservation. The reason for the better result in ammonium metabolism in the 3D system is unclear but is considered to relate not only to the effect of EGCG, but also to the spheroid-like structure, which is known to be preferable condition for hepatocytes.³³⁻³⁵ Medium flow may also play an important role for the recovery of function in the 3D bioreactor. The continuously perfused medium might have effectively removed the adsorbed EGCG, whose desorption from the cells was suggested to be slow.²⁰ Then hepatocyte function might have recovered more effectively in a 3D system than in static culture.

Conclusion

In conclusion, EGCG-containing medium were found to be able to maintain the cultured hepatocyte morphology and functions. This system is useful for BAL preparation. Nevertheless, further studies are required to characterize the full effects of EGCG on hepatocytes as well as on how to preserve the cultured hepatocytes for longer than 4 days.

Acknowledgments

This work was partly supported by Grant-in-Aid for Scientific Research (B) in the Ministry of Education, Culture, Sports, Science, and Technology. We thank Ms. Sachiyo Fujinobu for her outstanding technical assistance. The author would like to thank Ministry of Higher Education Malaysia and Tun Hussein Onn University of Malaysia for funding his study under the Academic Training Scheme.

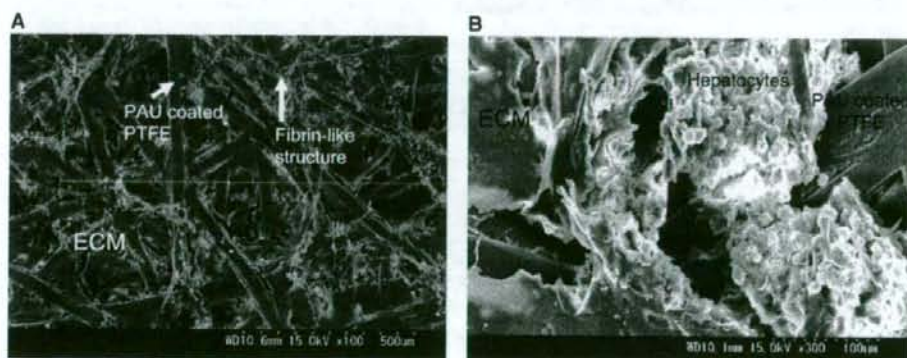
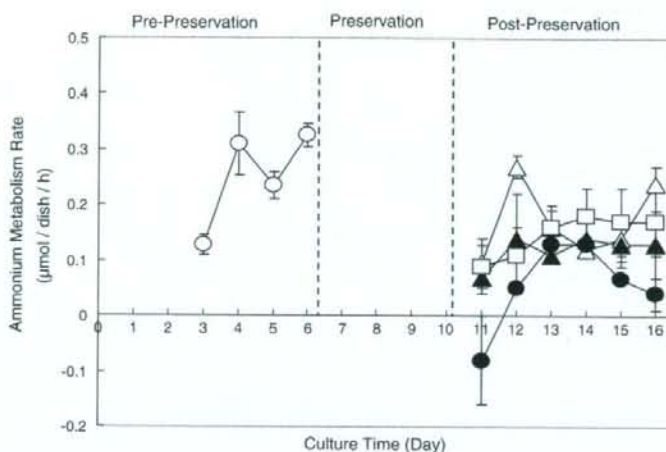


FIG. 6. Scanning electron microscopy photographs of hepatocytes preserved in the presence of 0.25-mg/mL epigallocatechin-3-gallate and cultured for 5 days in a bioartificial liver. (A) Extracellular matrix (ECM) and fibrin-like structures covering the non-woven fabric. (B) The aggregations of hepatocytes were observed under the ECM.

FIG. 4. Ammonium metabolism rate of monolayer-cultured hepatocytes during pre-preservation (○); preservation in epigallocatechin-3-gallate (EGCG)-free medium (●) and in 0.0025- (△), 0.025- (▲), and 0.25-mg/mL EGCG-containing medium (□).



The maximum rates of ammonium metabolism before preservation and after preservation were 167 $\mu\text{mol}/\text{reactor}$ per hour and 149 $\mu\text{mol}/\text{reactor}$ per hour, respectively, whereas the minimum rates before preservation and after preservation were 92 $\mu\text{mol}/\text{reactor}$ per hour and 64 $\mu\text{mol}/\text{reactor}$ per hour, respectively. The 3D bioreactor system is much more effective at maintaining the ammonium metabolic activity of the hepatocytes than the monolayer culture (Fig. 4), which is an almost 100% ($98 \pm 32\%$) recovery relative to the rate observed before preservation.

SEM and histological observation for perfusion culture

The hepatocytes preserved in 0.25-mg/mL EGCG-containing medium and post-cultured for 5 days produced extracellular matrix (ECM) proteins and were covered by them (Fig. 6A). Hepatocytes form aggregates which has spheroid-like structure were surrounded by the ECM structure rather than adhering onto PAU-coated PTFE, as shown in Figure 6B. This result was similar to our previous report in which the hepatocytes were cultured continuously for 1 week (supplementary data 2).⁷ In addition, fibrin-like fibers were observed on the ECM layer (Fig. 6A).

The difference between the hepatocytes adhering onto substrates in 2D culture and forming the spheroid-like structure would related to the higher functions of hepato-

cytes in a 3D system.³³⁻³⁵ This was also proven using H&E staining, Azan staining, and albumin immunostaining, as shown in Figures 7A, B, and C. These images indicate that hepatocytes producing albumin were located in the ECM layer composed of collagen.

Discussion

Cho *et al.* reported that the viability of hepatocytes decreased lower than 60% when preserved at low temperature in University of Wisconsin (UW) and Euro-Collins (EC) solutions for 48 h.³⁶ Not only the hepatocytes, but also the other valuable cells generally lost their viability in preservation. For example, peripheral blood stem cells and hemopoietic progenitor cells lost more than 50% of their viability after 3 days preserved at room temperature.³⁷ When hepatocytes were preserved at room temperature in monolayer culture for 4 days, more than half of the hepatocytes died and detached from the culture dish (Fig. 2A). In contrast, cell detachment was almost completely prevented, and the functions of the hepatocytes could be preserved in 0.25-mg/mL EGCG-containing medium for 4 days at room temperature. Albumin production in static culture increased after preservation at room temperature with increasing EGCG concentration.

A mechanism of cell or tissue preservation using EGCG has not been clarified, but it has been considered to be related to the intrinsic characteristics of polyphenol, that is, its amphipathic property.^{19,20,38} The amphipathic property of EGCG might be feasible to pass through the plasma membrane and the polyphenol adsorbs to the cellular receptors because of the high affinity that polyphenol and protein have for one another.³⁸ They hypothetically proposed a hibernation-like phenomenon of the cells or tissues. As the polyphenol reversibly leaves the cell membrane with time, and the cell cycle resumes.^{20,38}

Our final aim is to develop a 3D BAL system for clinical use. Because a bioreactor system needs a large amount of hepatocytes, the effect of EGCG on hepatocyte function was

TABLE 2. RELATIVE AMMONIUM METABOLISM OF MONOLAYER-CULTURED HEPATOCYTES

Culture	EGCG Concentration (mg/mL)	Relative Ammonium Metabolism (%)
Before-Preservation	—	100 \pm 6.4
Post-Preservation	0	17 \pm 23.8
	0.0025	53 \pm 21.1
	0.025	40 \pm 8.3
	0.25	52 \pm 11.5

- AU14 ▶ microscopy (DMR, Leica Co., Japan). For albumin immunostaining, the transverse section was permeabilized with 0.3% Triton X-100 in PBS for 10 min. Cells were incubated with 3 mL/L of H₂O₂ in methanol for 10 min to block endogenous peroxidase activity and then washed twice in PBS for 5 min. Nonspecific binding sites were blocked with 3 mL/L normal goat serum. Cells were incubated overnight at 4°C with primary antibody (goat anti-pig albumin antibody, Bethyl Lab, 1:200). After being washed for 15 min in PBS twice, slides were incubated at 37°C for 30 min with secondary antibody (anti-goat immunoglobulin G rhodamine-conjugated donkey antibody, Chemicon International), with streptavidin marked using horseradish peroxidase. The positive cells presented as brownish yellow.
- AU15 ▶
- AU16 ▶

Results

Isolation of hepatocytes

More than 2.5×10^9 hepatocytes were routinely obtained from a lobe. Viability determined according to the trypan blue exclusion method was higher than 90%.

Morphology of the monolayer-cultured hepatocytes

- Three days after preservation in 0.25- or 0.025-mg/mL EGCG-containing medium, cells kept their morphologies and adhered to the culture dish, as shown in Figures 2B and C, whereas cells preserved in 0.0025 mg/mL and EGCG-free medium started to detach from the culture dish, as shown in Figures 2A and D. The number of detached cells in 0.0025-mg/mL EGCG-containing medium was much smaller than in EGCG-free medium. These results indicate that cellular damage in normal medium at room temperature leading to cell detachment can be prevented by adding EGCG to the medium.
- F2 ▶

Function of monolayer hepatocytes

- The albumin production of the hepatocytes started to increase on the third day after isolation and reached the highest value on the fourth day, as shown in Figure 3. Albumin production rates slowly decreased over the culture period. During the preservation period, albumin production rates decreased drastically, to 0 µg/dish per day. After preservation in EGCG-containing medium, the cells were washed four times with culture medium to remove EGCG and incubated in a 5% CO₂/air atmosphere at 37°C for another 6 days. The highest albumin production was observed when the cells were preserved in the presence of 0.25-mg/mL EGCG.
- F3 ▶
- AU17 ▶

Albumin production had increased to 14.5, 8.0, 1.1, and 8.9 µg/dish per day on the first day of preservation in 0.25-, 0.025-, and 0.0025-mg/mL EGCG-containing media and EGCG-free medium, respectively, as shown in Figure 3. Albumin production on the first day of preservation in EGCG-free medium was high, probably because of albumin released from the dead hepatocytes and not because of the albumin secretion process. The albumin production of hepatocytes preserved in 0.25-mg/mL EGCG-containing medium had drastically increased, up to a maximum rate of 30.9 µg/dish per day, on the third day of the post-preservation period, which was much higher than in the other conditions. The

effect of EGCG concentration on the relative albumin production rate during the post-preservation period was compared with that before preservation (Table 1). Relative albumin production rate was calculated as follows:

◀ T1

$$\text{Relative of albumin production rate} = \frac{\text{Average value of albumin production rate during post-preservation}}{\text{Albumin production rate before preservation}} \times 100$$

From Table 1, albumin production of hepatocytes after preservation increased with increasing EGCG concentration. Production after preservation in 0.25-mg/mL EGCG-containing medium completely recovered and reached $111 \pm 25.1\%$, but the albumin production rates of hepatocytes preserved in 0.025- and 0.0025-mg/mL EGCG-containing medium and EGCG-free medium recovered only to 74 ± 32.1 , 58 ± 21.9 , and $36 \pm 23.7\%$ of their pre-preservation levels, respectively.

The ammonium metabolism rate for hepatocytes preserved in EGCG-containing medium also recovered and reached higher levels than that of the hepatocytes preserved in EGCG-free medium (Fig. 4). Relative ammonium metabolism rate was calculated as follows:

◀ F4

$$\text{Relative of ammonium metabolism rate} = \frac{\text{Average value of ammonium metabolism rate during post-preservation}}{\text{Ammonium metabolism rate before preservation}} \times 100$$

The relative ammonium metabolism rates in hepatocytes preserved in 0.25-, 0.025-, and 0.0025-mg/mL EGCG-containing medium and EGCG-free medium after preservation were $52 \pm 11.5\%$, $40 \pm 8.3\%$, $53 \pm 21.1\%$, and $17 \pm 23.8\%$, respectively, who the rate before preservation, as shown in Table 2. These results indicate that maintaining the ammonium metabolism functions of hepatocytes in the monolayer culture is difficult even in the presence of EGCG.

◀ T2

Function of hepatocytes in 3D bioreactor

As shown in Figure 5A, albumin production of hepatocytes in the bioreactor reached a maximum rate (25.8 mg/reactor per day) at 4 days of pre-preservation culture. During the preservation period, albumin production decreased almost to 0 mg/reactor per day on the eighth day of culture. Albumin production increased during post-preservation culture and reached a maximum rate of 24.6 mg/reactor per day in 5 days, which is $72 \pm 16\%$ recovery relative to the rate observed before preservation. The ammonium metabolism rate after preservation was almost the same as that for pre-preservation, as shown in Figure 5B.

◀ F5

TABLE 1. RELATIVE ALBUMIN PRODUCTION OF MONOLAYER-CULTURED HEPATOCYTES

Culture	EGCG Concentration (mg/mL)	Relative Albumin Production (%)
Before-Preservation	—	100 ± 37.8
Post-Preservation	0	36 ± 23.7
	0.0025	58 ± 21.9
	0.025	74 ± 32.1
	0.25	111 ± 25.1

concentration of EGCG fixed in the monolayer culture system was applied to the 3D culture system and preserved at room temperature for 4 days. The medium perfusion and oxygen, air, and CO₂ supplies were stopped during the preservation period. The preserved cells were properly washed with 3 L of culture medium and cultured for 5 days in the normal medium. Medium exchange was performed every 24 h throughout the culture period.

Hepatocyte functions

Albumin production rate. One mL of medium was taken before and after the medium change in static and perfusion culture for measuring albumin production using enzyme-linked immunosorbent assay.³² For the perfusion culture, the albumin concentration in the conditioning vessel and the plasma bottle were measured. Briefly, a 96-well plate was coated overnight with 1000-times-diluted goat anti-pig albumin antibody (Bethyl Lab, Inc.) at 4°C. The plate was washed four times with 1% (v/v) Tween 20 in phosphate buffered saline (PBS (-)), incubated with 0.01 g/mL block ace (Dainippon Sumitomo Pharma, Japan) for 3 h at 37°C to block unspecific binding, and washed four times. Samples were added to the plate, incubated for 3 h at 37°C, and washed with 1% (v/v) Tween 20 in PBS (-) five times. The peroxidase-conjugated goat anti-pig albumin (Bethyl Lab, Inc., 15,000 times diluted) was added to each well and incubated for 2 h at 37°C. Afterward, the plate was washed, and the o-phenylenediamine (Wako, Ltd.) solution in 0.01% hydrogen peroxide (H₂O₂, 3 mg/mL) was added. The color reaction was stopped by adding 50 μ L of 1.0 M sulfuric acid, and the ultraviolet absorbance was measured using a microplate reader at 490 nm. The albumin secretion is indicated as the amount in a dish and in a reactor per day (μ g/dish per day, mg/reactor per day).

Ammonium metabolism rate

Static culture. To assess the ammonium metabolism of the hepatocytes, 1 mM ammonium chloride (NH₄Cl) was added to the medium, and the ammonium concentration was measured after 0, 3, and 6 h using the Amicheck meter (Arkray Factory Inc., Japan). Ammonium metabolism rate defined as amount of decreased ammonium in a dish per hour (μ mol/dish per h).

Perfusion culture. The plasma bottle (Fig. 1A(14)) of the 3D bioreactor system was filled with 300 mL of 1 mM NH₄Cl-containing medium, and the hepatocytes grown on the 3D scaffold (Fig. 1B) were perfused with this medium. The ammonium concentrations in the conditioning vessel and plasma bottle were measured after 0, 3, and 6 h using the Amicheck meter (Arkray Factory Inc.). The ammonium metabolism rate was expressed as μ mol/reactor per hour.

Scanning electron microscopy

Hepatocytes attached to the scaffold in the bioreactor were fixed with 2.5% glutaraldehyde, dehydrated with graded ethanol (50%, 60%, 70%, 80%, 90%, 95%, and 99.5%), and dried with tert-butyl alcohol (t-BuOH) freeze-drying (t-BuOH freeze dryer VFD-21; Vacuum Devices Co., Ltd., Japan). The specimens were coated with palladium by sputtering (E-1030, Hitachi Co., Japan) and then observed using scanning electron microscopy (SEM; S-3000N, Hitachi Co.).

Histological study

The transverse section (2 μ m thick) was taken from the construct in the bioreactor. The section was stained with hematoxylin and eosin (H&E) and azan to demonstrate hepatocytes and collagen and was examined using light

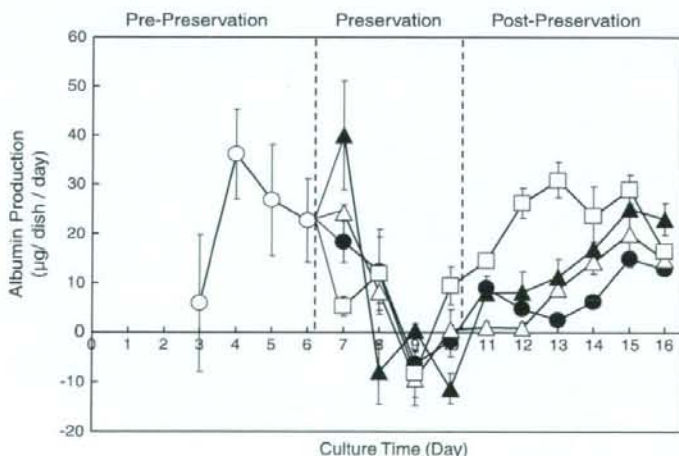


FIG. 3. Albumin production of monolayer-cultured hepatocytes during pre-preservation (○); preservation in epigallocatechin-3-gallate (EGCG)-free medium (●) and in 0.0025- (△), 0.025- (▲), and 0.25-mg/mL EGCG-containing medium (□).

AU6 ▶ Glass Co., LTD, Japan) and incubated in a 5% carbon dioxide (CO₂)/air atmosphere at 37°C for 6 days. We started monitoring the hepatocyte functions on day 3 and continued for 4 more days (pre-preservation). After that, to preserve the hepatocytes, the given concentrations of EGCG (0, 0.25, 0.025, and 0.0025 mg/mL) were added to the culture medium and kept at room temperature for 4 days. CO₂ was not supplied during the preservation period. The preserved cells were properly washed with culture medium four times and cultured for 6 days in the normal medium (post-preservation). The medium was changed every 24 h.

The cell morphologies were observed at 3 days pre-preservation, on day 3 of the preservation period, and on 6 days in post-preservation using a Nikon TMS phase-contrast light microscope (Nikon, Japan). Images were taken using a Nikon CoolPix 990 digital camera (Nikon). The hepatocytes adhered in 2 days after seeding. Because of the high density of the seeded cells, the cells were in the confluent stage, and then the cells do not proliferate even for the pre-preservation.

Perfusion culture. Perfusion culture was performed in the radial flow bioreactor system that we reported previ-

ously.⁷ In brief, hepatocyte suspension (2.0×10^9 cells in 50 mL) was inoculated into a conditioning vessel (Fig. 1(7)) and perfused to a bioreactor module composed of a 3D scaffold made of PAU-coated PTFE non-woven fabric at the flow rate of 17 mL/min for 10 min. The suspension in the conditioning vessel was turbid because of the high density of inoculated cells before use (Supplementary data 1A). After 10 min of perfusion at 17 mL/min and 2 h of perfusion at 84.2 mL/min, the suspension became clear (Supplementary data 1B), and no cells were detected in the medium, indicating that the cell seeding efficiency was high and that almost all inoculated cells were adhered onto or entrapped in the non-woven scaffold.

The pH and oxygen concentration of the medium were fixed at 7.3 and 313 μ M, respectively, using the pH and oxygen sensors of the conditioning vessel (Fig. 1(7)). The gas controller equipment (Fig. 1(4)) controlled air flow and carbon dioxide flow at 10 mL/min to control the pH. Meanwhile, the controller controlled oxygen at 50 mL/min to maintain the diluted oxygen rate.

We started monitoring the hepatocyte functions 24 h after inoculating the cells and continued for 4 days. The best

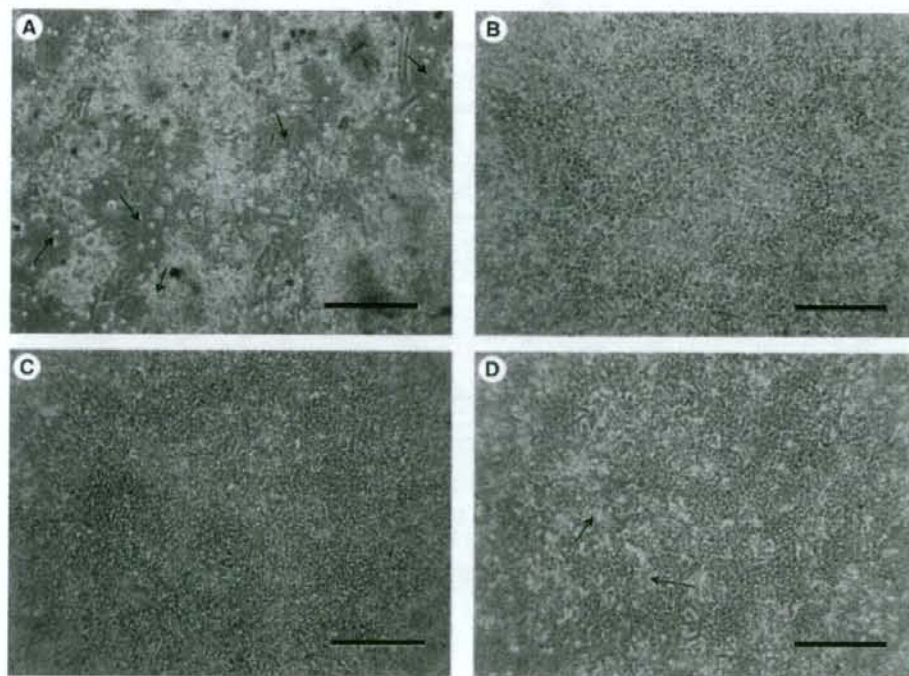


FIG. 2. Light microscopic images of porcine hepatocytes preserved for 3 days in (A) epigallocatechin-3-gallate (EGCG)-free medium or in EGCG-containing medium at the concentration of (B) 0.25-, (C) 0.025-, and (D) 0.0025-mg/mL EGCG. The arrows show the cells detaching from the dish. Bars represent 200 μ m. Color images available online at www.liebertonline.com/ten.

2

PRESERVATION OF PORCINE HEPATOCYTES ◀AU1

original BAL, which consisted of a perfusion-type bioreactor and a polytetrafluoroethylene (PTFE) non-woven fabric coated with the copolymer of poly (γ -methyl-L-glutamate) and poly urethane (PAU).⁷

Materials and Methods

Animals and hepatocytes

Mini pigs (male, 4–16 weeks) were purchased from Japan Farm LTD. (Kagoshima, Japan). Hepatocytes were isolated from the liver lobes (70–90 g) and used for a 2D monolayer culture experiment at the National Cardiovascular Center Research Institute (Osaka, Japan). The Animal Subjects Committee of the National Cardiovascular Center (Osaka, Japan) had reviewed and approved the experimental protocol.

A slaughtered pig (male, 7 weeks) was purchased from Kitayushu Prefecture Edible Meat Center (Fukuoka, Japan). Hepatocytes were isolated from a liver lobe (103.4 g) and used for 3D BAL experiments at the Kyushu Institute of Technology (Fukuoka, Japan). The animal experiment was conducted in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Science, approved by the Physiological Society of Japan.

Hepatocytes were isolated using the collagenase perfusion technique with modifications.^{30,31} The liver was perfused with 1000 mL of calcium-free ethylenediaminetetraacetic acid (EDTA) buffer solution (0.15 M sodium chloride (NaCl), 5.63 mM calcium chloride (KCl), 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.02 M sodium bicarbonate (NaHCO₃), and 0.99 mM EDTA disodium salt (EDTA-2Na; Wako, Ltd., Japan), pH 7.4) at 80 mL/min and 37°C to remove the blood and then perfused with 500 mL of

calcium-free buffer solution to rinse the EDTA-2Na, because EDTA-2Na reduces collagenase activity. After that, 500 mL of dispase solution (0.15 M NaCl, 5.63 mM KCl, 0.02 M HEPES, and 6.0×10^8 U Dispase; Gibco Co.) and then 500 mL of collagenase solution (0.15 M NaCl, 5.63 mM KCl, 0.02 M HEPES, 0.02 M NaHCO₃, 3.74 mM calcium chloride dihydrate (CaCl₂·2H₂O), and 6.5×10^4 U collagenase (Wako, Ltd., Lot no. 06032W)) were perfused. The perfused liver was carefully minced with a scalpel blade, and the resulting crude homogenate was shaken in the presence of the last perfusate. The resulting cell suspension was filtered through nylon mesh with a 200- μ m pore size and centrifuged at 600 rpm at 4°C for 2 min. The hepatocytes were washed with washing buffer with DNase (0.12 M NaCl, 6.2 mM KCl, 1.26 mM CaCl₂·2H₂O, 0.49 mM magnesium chloride·6H₂O, 0.41 mM magnesium sulfate heptahydrate, 10 mM HEPES, 1.0 g/L of bovine serum albumin (Wako, Ltd.), and 10 mg/L of DNase (Roche Diagnostics GmbH, Germany, Lot 13738000)) and with washing buffer (0.12 M NaCl, 6.2 mM KCl, 1.77 mM CaCl₂·2H₂O, 0.02 M HEPES, and 1.0 g/L of bovine serum albumin (Wako, Ltd.)) twice.

Isolated hepatocytes were cultured in William's E medium with L-glutamate (Sigma-Aldrich Inc.) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich Inc., Lot no. 043K83032), 100 IU/L penicillin-streptomycin, 1.0×10^{-7} M insulin (Wako, Ltd.), 1.0×10^{-6} M dexamethasone (Wako, Ltd.), 25 μ g/L epidermal growth factor (Sigma-Aldrich Inc.), and 1.5 mM L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako, Ltd.).

Cell culture

Static culture. Hepatocytes (1.0×10^6) were initially seeded on a 60-mm collagen type I-coated dish (Iwaki, Asahi

AU2 ▶

◀AU3

◀AU4

◀AU5

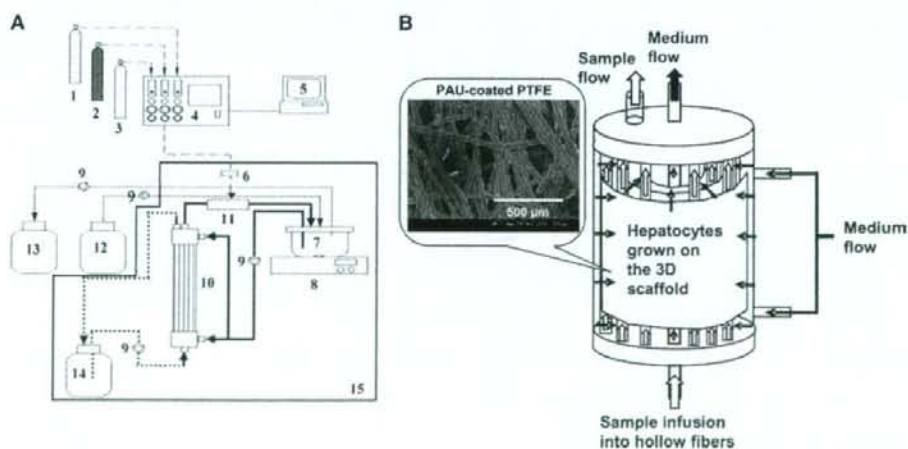


FIG. 1. (A) A schematic diagram of a bioartificial liver (BAL) (1) carbon dioxide cylinder, (2) oxygen cylinder, (3) air cylinder, (4) gas control equipment, (5) computer, (6) membrane filter, (7) conditioning vessel, (8) stirrer, (9) peristaltic pump, (10) radial flow bioreactor module, (11) silicone oxygenator module, (12) fresh medium tank, (13) spent medium tank, (14) plasma bottle, and (15) incubator. →: medium flow for hepatocytes culture. ←: Sample flow for ammonium metabolism evaluation. (B) Radial flow bioreactor module.

Preservation of Porcine Hepatocytes in Three-Dimensional Bioreactor at Room Temperature Using Epigallocatechin-3-Gallate

Azizi Miskon, M.Eng.,^{1,2,3} Tetsuji Yamaoka, Ph.D.,¹ Suong-Hyu Hyon, Ph.D.,⁴
Makoto Kodama, Ph.D.,³ and Hiroshi Uyama, Ph.D.²

A bioartificial liver (BAL) assist system employing a three-dimensional (3D) bioreactor has been studied as a temporary support in liver failure. In the present study, a novel preservation method of primary cultured porcine hepatocytes in monolayer and 3D culture systems was studied. Epigallocatechin-3-gallate (EGCG), which has recently been found to have various bioactivities, was selected as a key compound for hepatocyte preservation. Hepatocytes isolated from porcine liver using the collagenase perfusion method were pre-cultured for 6 days, preserved at room temperature in the presence of EGCG at various concentrations for 4 days, and post-cultured in normal medium for another 6 days. In the monolayer culture, only albumin production rate was fully recovered after preservation when EGCG concentration was high (0.25 mg/mL). In contrast, albumin production and ammonium metabolism in the 3D bioreactor under the same condition recovered to $72 \pm 16\%$ and $98 \pm 32\%$, respectively, of levels before preservation. These results indicate that hepatocytes can be preserved in the presence of 0.25 mg/mL of EGCG at room temperature, especially in a 3D culture system, which is promising technology for BAL preparation.

Introduction

VARIOUS CELL-BASED THERAPIES have been proposed and widely studied recently. Among them, hepatocyte-based treatments for fulminant or chronic hepatitis have been attracting great attention. Injection of hepatocytes isolated from unused donor livers and bioartificial liver (BAL) assist systems is used for treating acute liver failure and liver-based metabolic defects.¹⁻⁵ A large number of hepatocytes must be prepared for emergency care and for repeated treatment,⁶ but because the isolation and reconstruction into a three-dimensional (3D) structure of the hepatocytes would take a long time and requires complex steps, metabolic activity rapidly decreases during the preparation process. We previously reported that the BAL system, using a radial flow bioreactor, needs at least 1.0×10^9 hepatocytes to maintain function.⁷ Therefore, it is essential to preserve cells that maintain their functions in BALs. However, maintaining the specific functions of the hepatocytes using conventional cryopreservation is difficult.⁸⁻¹⁶

Various strategies to maintain cell functions have been proposed. Watts *et al.* and McKay *et al.* reported that mono-

layer culture is superior to suspension culture in preserving hepatocytes.^{17,18} Kakinoki *et al.* have succeeded in storing peripheral nerves for longer than 1 month using green tea polyphenol,¹⁹ and Hyon *et al.* reported that green tea polyphenol also preserved the rat pancreatic islet for longer than 2 months.²⁰

The green tea polyphenol is composed of epigallocatechin gallate, epicatechin gallate, epicatechin, and epigallocatechin, but the effect of each component on cell preservation is unknown. We focused on epigallocatechin-3-gallate (EGCG) because it is the main component of the green tea polyphenol and is well known to have a wide range of pharmacological activities, including antioxidant, anti-inflammatory, anti-atherogenic, and anti-thrombotic.²¹⁻²³ Recently, the effects of polyphenol on various diseases such as tissue ischemia,^{24,25} neoplasm inflammation,^{26,27} and arteriosclerosis^{28,29} have been reported.

In the present study, we attempted to preserve primary cultured hepatocytes for a 3D BAL system. The effect of EGCG on hepatocyte preservation at room temperature was evaluated using monolayer culture. The preservation experiments in 3D BAL systems were also conducted in our

¹Department of Biomedical Engineering, Advanced Medical Engineering Center, National Cardiovascular Center Research Institute, Osaka, Japan.

²Department of Chemical Engineering, Osaka University, Osaka, Japan.

³Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, Kitakyushu City, Fukuoka, Japan.

⁴Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan.

- [4] Anderson AJ, Dawes EA. Occurrence, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol Rev* 1990;54:450–72.
- [5] Doi Y. *Microbial polyesters*. New York: VCH; 1990.
- [6] Kato M, Bao HJ, Kang CK, Fukui T, Doi Y. Production of a novel copolyester of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids by *Pseudomonas* sp. 61-3 from sugars. *Appl Microbiol Biotechnol* 1996;45:363–70.
- [7] Hocking PJ, Marchessault RH. Biopolyesters. In: Griffin GJL, editor. *Chemistry and technology of biodegradable polymers*. London: Chapman & Hall; 1994. p. 48–96.
- [8] Nelson T, Kaufman E, Kline E, Sokoloff L. The extraneural distribution of gamma-hydroxybutyrate. *J Neurochem* 1981;37:1345–88.
- [9] Taylor MS, Daniels AU, Andriano KP, Heller J. Six absorbable polymers: *in vitro* acute toxicity of accumulated degradation products. *J Appl Biomater* 1994;5:151–7.
- [10] Iwata T, Tsunoda K, Aoyagi Y, Kusaka S, Yonezawa N, Doi Y. Mechanical properties of uniaxially cold-drawn films of poly[(R)-3-hydroxybutyrate]. *Polym Degrad Stab* 2003;217–24.
- [11] Sudesh K, Abe H, Doi Y. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog Polym Sci* 2000;25:1503–55.
- [12] Griffith LG. Emerging design principles in biomaterials and scaffolds for tissue engineering. *Ann N Y Acad Sci* 2000;961:83–95.
- [13] Zong X, Bien H, Chung CY, Yin L, Fang D, Hsiao BS, et al. Electrospun fine-textured scaffolds for heart tissue constructs. *Biomaterials* 2005;26:5330–8.
- [14] Li WJ, Laurencin CT, Catterton EJ, Tuan RS, Ko FK. Electrospun nanofibrous structure: a novel scaffold for tissue engineering. *J Biomed Res* 2002;60:613–21.
- [15] Kim J, Reneker DH. Mechanical properties of composites using ultrafine electrospun fibers. *Polym Composites* 1999;20:124–31.
- [16] Lee WH, Azizan MNM, Sudesh K. Effects of culture conditions on the composition of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) synthesized by *Comamonas acidovorans*. *Polym Degrad Stab* 2004;84:129–34.
- [17] Siew EL, Rajab NF, Osman A, Sudesh K, Inayat-Hussain SH. *In vitro* biocompatibility evaluation of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) copolymer in fibroblast cells. *J Biomed Res A* 2007;81A:317–25.
- [18] Dong H, Nyame V, Macdiarmid Jr AG, Jones WE. Polyaniline/poly(methyl methacrylate) coaxial fibers: the fabrication and effects of the solution properties on the morphology of electrospun core fibers. *J Polym Sci Part B: Polym Phys* 2004;42:3934–42.
- [19] Lyons J, Li C, Ko F. Melt-electrospinning part I: processing parameters and geometric properties. *Polymer* 2004;45:7597–603.
- [20] Fong H, Chun I, Reneker DH. Beaded nanofibers formed during electrospinning. *Polymer* 1999;40:4585–92.
- [21] Zuo W, Zhu M, Yang W, Yu H, Chen Y, Zhang Y. Experimental study on relationship between jet stability and formation of beaded fibers during electrospinning. *Polym Eng Sci* 2005;45:704–9.
- [22] Zong X, Ran S, Kim KS, Fang D, Hsiao BS, Chu B. Structure and morphology changes during *in vitro* degradation of electrospun poly(glycolide-co-lactide) nanofiber membrane. *Biomacromolecules* 2003;4:416–23.
- [23] Li WJ, Cooper Jr JA, Mauck RL, Tuan RS. Fabrication and characterization of six electrospun poly(α -hydroxyester)-based fibrous scaffolds for tissue engineering applications. *Acta Biomaterialia* 2006;2:377–85.
- [24] Jeong EH, Im SS, Youk JH. Electrospinning and structural characterization of ultrafine poly(butylene succinate) fibers. *Polymer* 2005;46:9538–43.
- [25] Yokouchi M, Chatani Y, Tadokoro H, Teranishi K, Tani H. Structural studies of polyesters: 5. Molecular and crystal structures of optically active and racemic poly(β -hydroxybutyrate). *Polymer* 1973;14:267–72.
- [26] Su F, Iwata T, Tanaka F, Doi Y. Crystal structure and enzymatic degradation of poly(4-hydroxybutyrate). *Macromolecules* 2003;36:6401–9.
- [27] Doi Y, Kanesawa Y, Kawaguchi Y, Kunioka M. Biodegradation of microbial polyesters in the marine environment. *Polym Degrad Stab* 1992;32:173–7.
- [28] Mitomo H, Hsieh WC, Nishiwaki K, Kasuya K, Doi Y. Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) produced by *Comamonas acidovorans*. *Polymer* 2001;42:3455–61.
- [29] Di Lorenzo ML, Raimo M, Cascone E, Martuscelli E. Poly(3-hydroxybutyrate)-based copolymers and blends: influence of a second component on crystallization and thermal behavior. *J Macromol Sci Phys* 2001;40:639–67.
- [30] Gogolewski S. Resorbable polymers for internal fixation. *Clin Mater* 1992;10:13–20.
- [31] Marois Y, Zhang Z, Vert M, Deng X, Lenz R, Guidoin R. Mechanism and rate of degradation of polyhydroxyoctanoate films in aqueous media: a long term *in vitro* study. *J Biomed Mater Res* 2000;49:216–24.
- [32] Lo CM, Wang HB, Dembo M, Wang YL. Cell movement is guided by the rigidity of the substrate. *Biophys J* 2000;79:144–52.
- [33] Pelham RJ, Wang YL. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc Natl Acad Sci U S A* 1997;94:13661–5.
- [34] Rappolee DA, Mark D, Banda MJ, Werb Z. Wound macrophages express TGF- α and other growth factors *in vivo*: analysis by mRNA phenotyping. *Science* 1988;241:708.
- [35] Martin DP, Skraly FA, Williams SF. Polyhydroxyalkanoate compositions having controlled degradation rates. PCT Patent Application No. WO 99/32536; 1999.
- [36] Qu XH, Wu Q, Zhang KY, Chen GQ. *In vivo* studies of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) based polymers: biodegradation and tissue reactions. *Biomaterials* 2006;27:3540–8.
- [37] Ali SAM, Doherty PJ, Williams DF. Molecular biointeractions of biomedical polymers with extracellular exudates and inflammatory cells and their effects on the biocompatibility *in vivo*. *Biomaterials* 1994;15:779–85.
- [38] Saad B, Neuenschwander P, Uhlenschmid GK, Suter UW. New versatile, elastomeric, degradable polymeric materials for medicine. *Intern J Biol Macromol* 1999;25:293–301.
- [39] Tracy MA, Ward KL, Firouzabadian L, Wang Y, Dong N, Qian R, et al. Factors affecting the degradation rate of poly(lactide-co-glycolide) microspheres *in vivo* and *in vitro*. *Biomaterials* 1999;20:1057–62.
- [40] Mukai K, Doi Y, Sema Y, Tomita K. Substrate specificities in hydrolysis of polyhydroxyalkanoates by microbial esterases. *Biotechnol Lett* 1993;15:601–4.
- [41] Maim T, Bowald S, Bylock A, Busch C, Saldeen T. Enlargement of the right ventricular outflow tract and the pulmonary artery with a new biodegradable patch in transannular position. *Eur Surg Res* 1994;26:298–308.

the main scaffold (Fig. 7D4). More macrophages were found to be present along the interface connected to this copolymer in comparison to the electrospun P(3HB-co-7mol%-4HB) and P(3HB-co-5mol%-3HHx) (Fig. 7B4 and C4). This phenomenon is desirable during wound healing because the presence of macrophages is necessary for the regeneration of many cell types [34]. The presence of thin connective tissue surrounding the electrospun P(3HB-co-97mol%-4HB) was also observed.

The most promising finding was the tissue response after 12 weeks of implantation for the electrospun P(3HB-co-97mol%-4HB). No fibrous encapsulation was observed around the degraded copolymer and there was also a substantial drop in the number of inflammatory cells (Fig. 7D12). This observation is similar to a study done on the biocompatibility of P(4HB) implanted subcutaneously in rats by Martin et al. [35], that reported minimal inflammatory responses. In this study, the number of inflammatory cells surrounding the electrospun P(3HB-co-7mol%-4HB) and P(3HB-co-5mol%-3HHx) did not appear to have lessened. The muscle cells surrounding these two scaffolds appeared compact as a result of inflammatory reaction (Fig. 7B and C). After 12 weeks of implantation, the number of macrophages bordering the electrospun P(3HB) increased. Inflammation was obvious due to the compacted muscle cells surrounding the scaffold. The difference in tissue response to the electrospun P(3HB-co-97mol%-4HB) and the electrospun scaffolds with higher molar fraction of 3HB reflected their distinct physical properties. It has been reported that rigid polymer, such as P(3HB), elicit acute inflammatory reaction because it exerts a continuous mechanical stimulus to the surrounding tissues of the implants [36]. Although the tissue response to the electrospun P(3HB-co-7mol%-4HB) and electrospun P(3HB-co-5mol%-3HHx) was slightly more pronounced than that of the electrospun P(3HB-co-97mol%-4HB), the overall local tissue response to all three copolymers was found to be mild. The results have confirmed the biocompatibility of all three types of electrospun PHA copolymers.

3.6. Bioabsorption mechanism

The results from various analyses clearly demonstrated that the bioabsorption rate of the electrospun P(3HB-co-97mol%-4HB) was the fastest relative to the other two PHA copolymers. Three possible reasons for this observation are as follows: Firstly, the P(3HB-co-97mol%-4HB) with low crystallinity is more susceptible to bioabsorption as water and enzymes penetrate easier into the amorphous regions. Secondly, previous studies have established that macrophages are able to phagocytize PHA *in vitro* [37,38] and free radicals, acidic products or enzymes produced by these cells may also accelerate the degradation [39]. As seen in Fig. 7, the number of inflammatory cells was the most concentrated at the interface of electrospun P(3HB-co-97mol%-4HB) suggesting their active part in the bioabsorption process. Thirdly, possibly the enzymatic degradation by lipase also contributed to the rapid bioabsorption of the electrospun P(3HB-co-97mol%-4HB). PHA can be enzymatically degraded by PHA depolymerases, but there is no

evidence to date that these are present *in vivo* [1]. P(4HB) was found to be also highly susceptible to lipase hydrolysis as opposed to P(3HB) [40]. Besides having good mechanical properties and biocompatibility, it is desirable for a medical implant to show good bioabsorption after its primary function has been fulfilled. The persistence of polymer at a wound healing site may lead to chronic inflammation as shown by the slowly degrading P(3HB) patches that elicited a long-term (greater than 2 years) macrophage response [41]. Hence the fast bioabsorption rates of the electrospun PHA containing 4HB have confirmed their potential in the application for medical implants.

4. Conclusion

In this study, electrospinning proved to be a simple and adaptable fabrication technique in producing constructs with dimensions approaching the native profile of ECM. Sterilization did not cause discoloration and damage to the PHA scaffolds. SEM revealed that both the *in vivo* and *in vitro* surface erosion of the electrospun PHA scaffolds progressed dependently on the individual fiber dimensions and monomeric contents. The mechanical properties demonstrated by all samples were comparable to those of human skin thus suggesting that their structures are able to provide sufficient biomechanical support. The electrospun scaffolds consisting of high 3HB content had higher degree of crystallinity and thus, they showed slower bioabsorption rate. GPC revealed that the *in vitro* degradation of the electrospun PHA scaffolds proceeded at a much slower rate in comparison to the *in vivo* bioabsorption. Histological evaluation showed that subcutaneous implantations of the electrospun PHA scaffolds were well tolerated *in vivo* as the tissue response continued to be very mild throughout the course of the study. Our results revealed that by changing the molar fraction of monomers in the PHA copolymers, it is possible to create tissue-engineering scaffolds that are tailor-made to meet the various needs in regenerating different cell type. The electrospun PHA copolymers proved to be promising biomaterials for scaffolds because of their biodegradability, flexible mechanical properties and excellent biocompatibility.

Acknowledgement

This work was supported by a Grant-in Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (No.19350075) (to T. Iwata) and by a grant for Ecomolecular Science Research II provided by RIKEN Institute.

References

- [1] Williams SF, Martin DP. Applications of PHAs in medicine and pharmacy. In: Steinbüchel A, editor. Series of biopolymers in 10 volumes, vol. 4. Wiley/VCH/Verlag; 2002. p. 91–121.
- [2] Chen GQ, Wu Q. The application of polyhydroxyalkanoates as tissue engineering materials. *Biomaterials* 2005;26:6565–78.
- [3] FDA clears first of its kind suture made using DNA technology. Available from: FDA News <http://www.fda.gov/fbs/topics/NEWS/2007/NEW01560.html>; 2007 February 12 [accessed 25.04.07].

indicates that the P(3HB-co-97mol%-4HB) fibers are more amorphous than the other scaffolds, and this is consistent with the WAXD results. The distinct mechanical properties of the PHA could find different use as scaffolds for tissue engineering. For example, the 3HB-rich scaffolds which are more rigid could serve as preferential substrates for directional cell migration [32] while the compliant 4HB-rich scaffolds could be used to promote cell motility [33]. The EtO sterilization and the immersion in PBS buffer little affected the mechanical properties of all the scaffolds.

3.5. Histological observation

The histological sections of the electrospun PHA scaffolds at different period of subcutaneous implantation are shown in Fig. 7. Histological observations indicate that all the three electrospun copolymers elicited fairly mild tissue response relative to that of the electrospun P(3HB) throughout the course of study. After 4 weeks of implantation, some parts of the electrospun P(3HB-co-97mol%-4HB) bordering the interface were degraded as evidenced by the small fragments broken off from

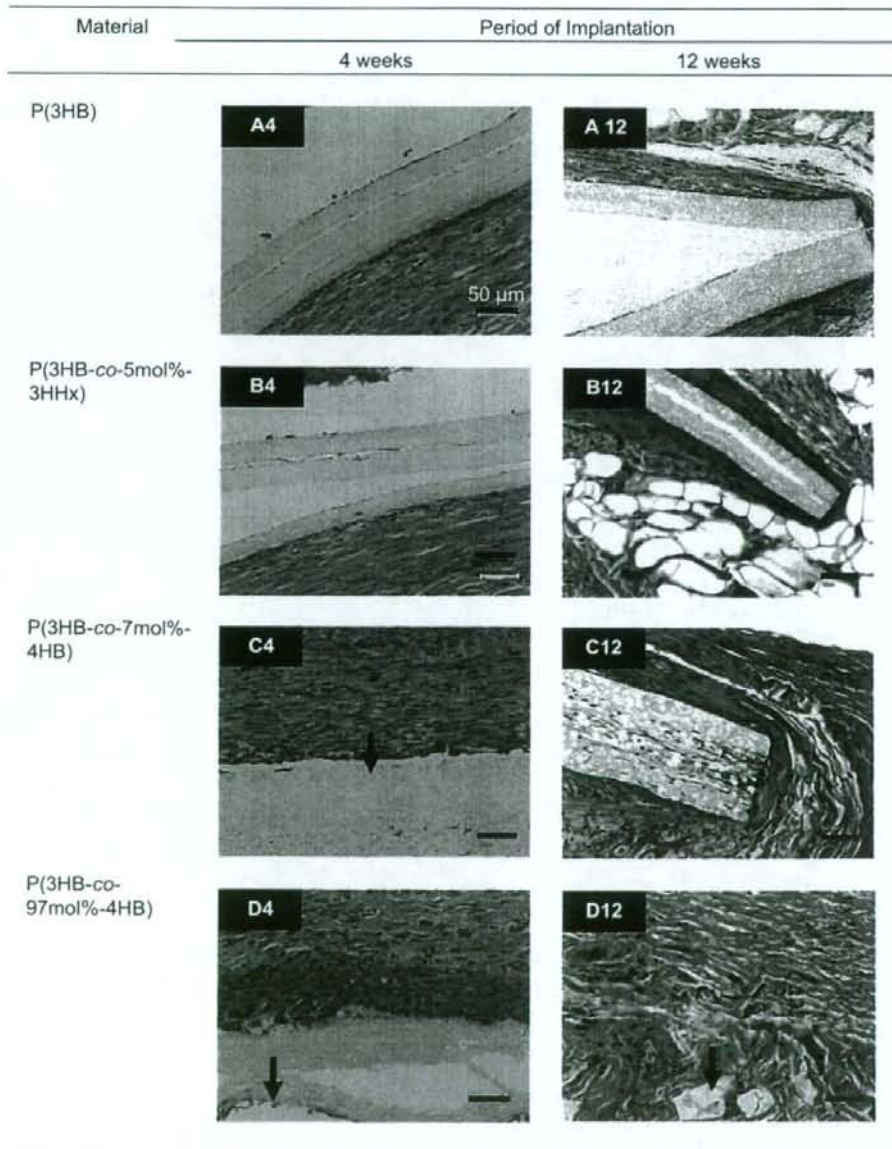


Fig. 7. Histological sections of the electrospun PHA scaffolds at different period of subcutaneous implantation. Arrows indicate the polymer surface.

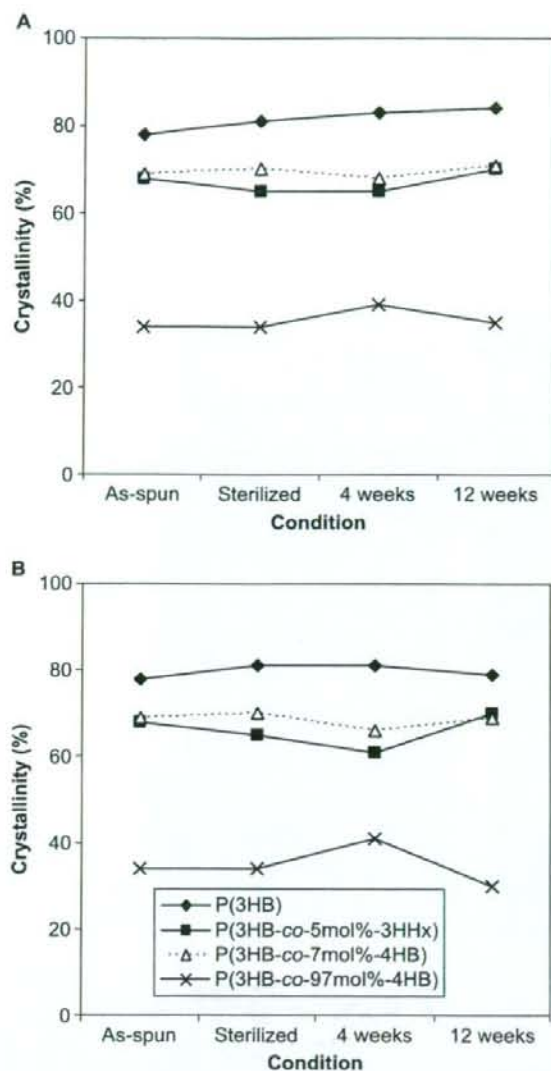


Fig. 6. Crystallinities of the electrospun PHA scaffolds in different conditions: (A) *in vivo* and (B) *in vitro*.

3.3. Molecular weight changes of electrospun PHA scaffolds

Table 1 summarizes the change in M_w and polydispersity index (M_w/M_n) for the as-spun scaffolds and scaffolds following sterilization, 4 and 12 weeks of *in vivo* and *in vitro* studies. After sterilization, all of the electrospun PHA scaffolds showed no significant differences in their molecular weight. Despite the large surface area of the fibers, PHA scaffolds remained intact in the *in vitro* study because they have higher resistance to hydrolysis in non-biological environment where specific enzymes are absent [27,31]. Furthermore, the immersion in PBS (pH 7.4) under sterile conditions up to only 12 weeks is short and

the temperature is low for any significant hydrolysis to occur. The subcutaneous implantation, however, seems to cause decrease in the M_w of PHA copolymers with 4HB unit. At 4 weeks, bioabsorption was the most pronounced for the electrospun P(3HB-co-97mol%-4HB) with 47% loss M_w , while the M_w of P(3HB-co-7mol%-4HB) showed no decrease. Following longer implantation period, the electrospun P(3HB-co-7mol%-4HB) lost 43% of M_w . Unexpectedly, the electrospun P(3HB-co-97mol%-4HB) recorded only 37% of M_w loss after 12 weeks. It was confirmed that the number of main-chain carbon atom strongly influences the rate of hydrolysis.

3.4. Mechanical properties of electrospun PHA scaffolds

Table 2 summarizes the mechanical properties of electrospun PHA scaffolds obtained. The mechanical properties of all the as-spun scaffolds were comparable to those of human skin, and hence suggest they are mechanically stable in supporting regenerated tissues. The Young's modulus of the as-spun scaffolds increased in the order of P(3HB-co-97mol%-4HB) \ll P(3HB-co-7mol%-4HB) < P(3HB) < P(3HB-co-5mol%-3HHx). Low Young's modulus, that is, high elasticity is a characteristic property in rubber-state amorphous polymers. Accordingly, this

Table 2
Mechanical properties of the PHA scaffolds

Material	Condition	Mechanical properties	
		Tensile strength (MPa)	Young's modulus (MPa)
P(3HB)	As-spun	17	223
	Sterilized	15	234
	4 weeks <i>in vivo</i>	12	182
	4 weeks <i>in vitro</i>	14	220
	12 weeks <i>in vivo</i>	15	152
	12 weeks <i>in vitro</i>	13	194
P(3HB-co-5mol%-3HHx)	As-spun	15	277
	Sterilized	12	272
	4 weeks <i>in vivo</i>	12	268
	4 weeks <i>in vitro</i>	13	208
	12 weeks <i>in vivo</i>	ND ^b	ND ^b
	12 weeks <i>in vitro</i>	15	230
P(3HB-co-7mol%-4HB)	As-spun	8	184
	Sterilized	8	139
	4 weeks <i>in vivo</i>	ND ^b	ND ^b
	4 weeks <i>in vitro</i>	8	163
	12 weeks <i>in vivo</i>	ND ^b	ND ^b
	12 weeks <i>in vitro</i>	9	110
P(3HB-co-97mol%-4HB)	As-spun	13	9
	Sterilized	15	16
	4 weeks <i>in vivo</i>	4	12
	4 weeks <i>in vitro</i>	11	14
	12 weeks <i>in vivo</i>	ND ^b	ND ^b
	12 weeks <i>in vitro</i>	14	16
Skin ^a		5–30	15–150

^a Data obtained from Ref. [13].

^b Not determined as the retrieved scaffolds from rat had cracks on the surface that prevented tensile test.

Table 1
Molecular weight properties of the electrospun PHA scaffolds

Material	Condition	$M_w \times 10^5$	M_w/M_n
P(3HB)	As-spun	11	3.1
	Sterilized	9.1	3.3
	4 weeks <i>in vivo</i>	12	2.6
	4 weeks <i>in vitro</i>	11	2.6
	12 weeks <i>in vivo</i>	8.5	2.9
	12 weeks <i>in vitro</i>	17	3.3
P(3HB-co-5mol%-3HHx)	As-spun	13	3.6
	Sterilized	12	3.6
	4 weeks <i>in vivo</i>	12	3.1
	4 weeks <i>in vitro</i>	11	4.3
	12 weeks <i>in vivo</i>	11	3.3
	12 weeks <i>in vitro</i>	13	4.3
P(3HB-co-7mol%-4HB)	As-spun	7.0	3.0
	Sterilized	6.8	3.0
	4 weeks <i>in vivo</i>	6.4	2.6
	4 weeks <i>in vitro</i>	6.1	2.6
	12 weeks <i>in vivo</i>	3.9	2.7
	12 weeks <i>in vitro</i>	5.0	2.6
P(3HB-co-97mol%-4HB)	As-spun	1.7	1.5
	Sterilized	1.9	1.8
	4 weeks <i>in vivo</i>	1.0	1.6
	4 weeks <i>in vitro</i>	2.5	2.0
	12 weeks <i>in vivo</i>	1.2	2.0
	12 weeks <i>in vitro</i>	2.2	1.8

The *in vivo* and *in vitro* observations using SEM revealed that fibers with smaller diameter were more prone to fragmentation because of increased water contact due to large surface area. Thus, it can be concluded that surface erosion of the electrospun PHA scaffolds depends upon individual fiber dimensions and monomeric content.

3.2. Crystallinity

The WAXD profiles of the as-spun PHA scaffolds are displayed in Fig. 5. The profiles are the ones after subtraction of background. As shown in this figure, the crystalline reflections for the P(3HB) and the 3HB-rich copolymers could be indexed on the basis of P(3HB) α -form structure [25] while the crystalline phase of P(3HB-co-97mol%-4HB) fibers adopted the P(4HB) crystal structure [26]. From the 1D profiles, the crystallinity of as-spun, sterilized and scaffolds after *in vivo* and *in vitro* studies is estimated, according to the method described above. As shown in Fig. 6, the crystallinity of the as-spun PHA scaffolds increased in the order of P(3HB-co-97mol%-4HB) < P(3HB-co-5mol%-3HHx) \approx P(3HB-co-7mol%-4HB) < P(3HB). This tendency is the same as the case of those bulk materials [27]. It has been reported that the crystallinity of P(4HB) homopolymer is much lower than that of P(3HB) homopolymer [28]. The slight lowering of the crystallinities of the 3HB-rich copolymers is due to the exclusion of second monomer unit from the crystalline lattice [29]. It is evident that the degradability of the scaffolds, as shown in Figs. 2 and 3, strongly depends on the crystallinity.

We confirmed that the EtO sterilization did not appear to have any effect on the crystallinities of all the electrospun

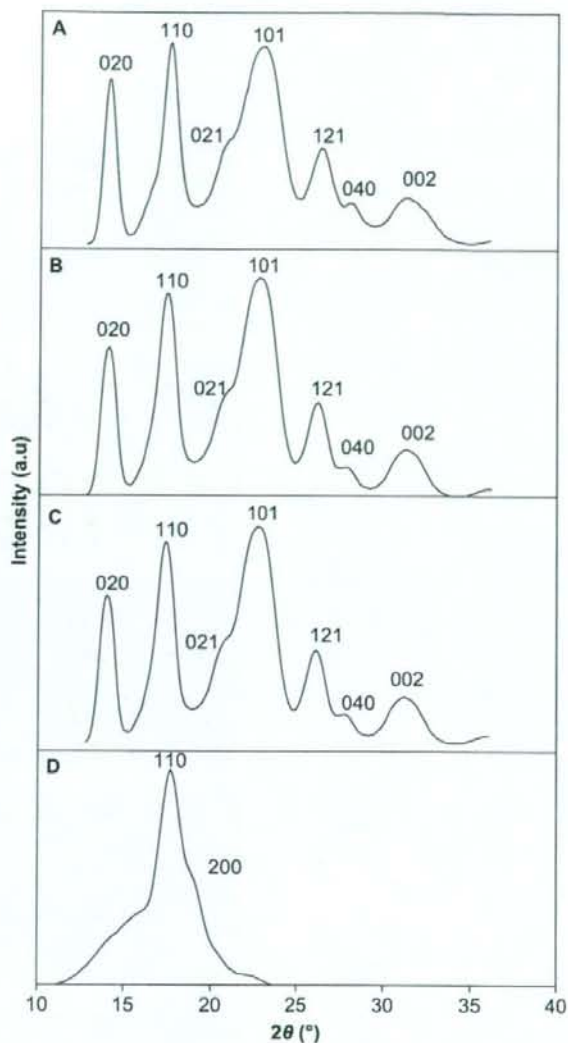


Fig. 5. Integrated 1D profiles from 2D WAXD patterns of as-spun: (A) P(3HB), (B) P(3HB-co-5 mol%-3HHx), (C) P(3HB-co-7 mol%-4HB) and (D) P(3HB-co-97 mol%-4HB).

PHA scaffolds. We described earlier that partial melting might occur during the sterilization of P(3HB-co-97mol%-4HB). Even if so, the crystallinity will surely recover after the sterilization. The crystallinities of the scaffolds after the *in vivo* and *in vitro* studies remained unchanged. But, it should be noted that the scaffolds for X-ray measurements are the retrieved or remained ones after *in vivo* and *in vitro* experiments. The crystallinity of the P(3HB-co-97mol%-4HB), which shows obvious bioabsorption or degradation in the macro-scale and SEM observations, also little changed even after implantation in rat. This means that the degradation of scaffolds progresses preferentially from the surface of the scaffolds or interface which contacts with the tissues of rat. It is deduced that some substance such as tissue enzymes facilitate the degradation [30].

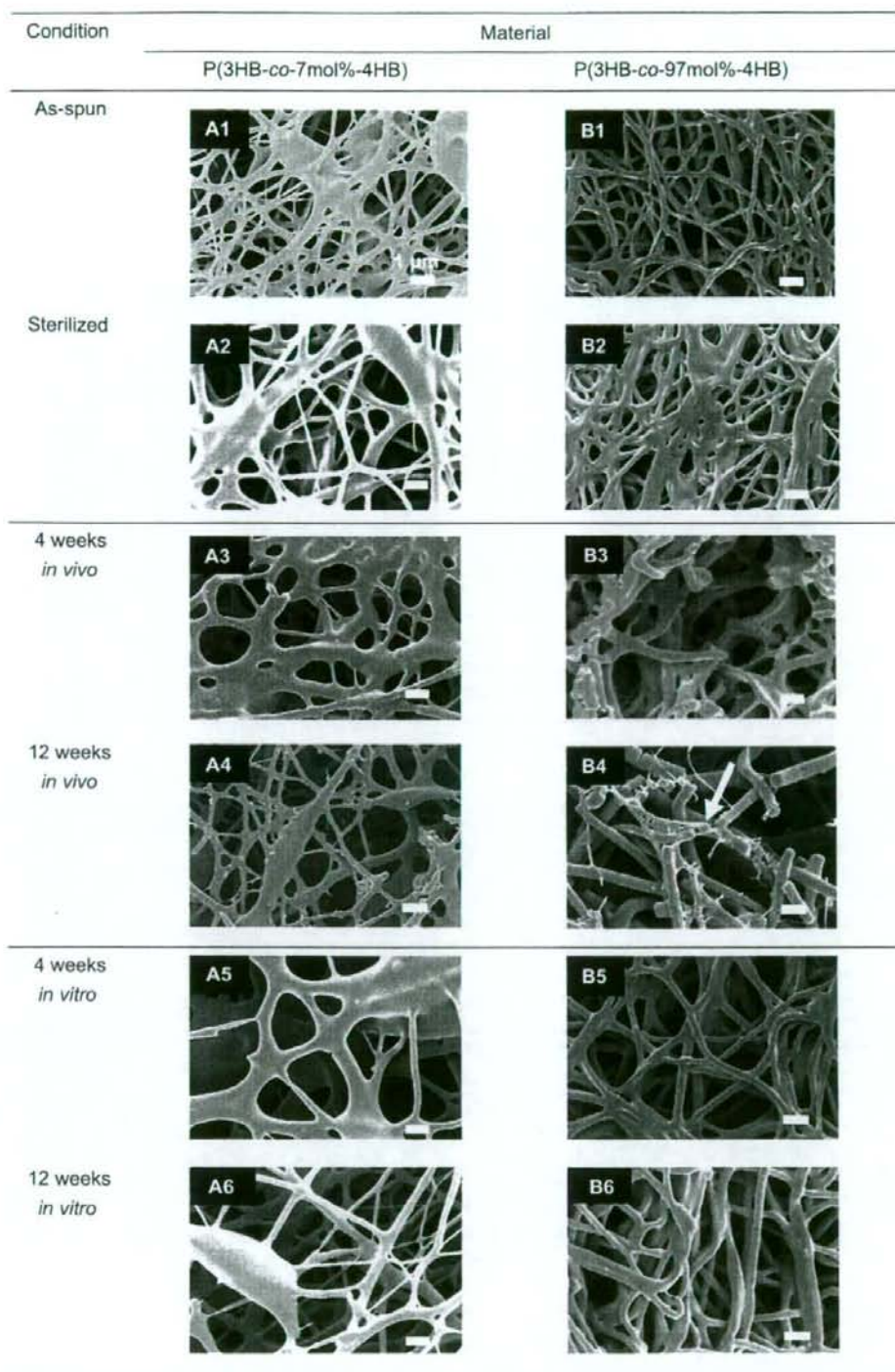


Fig. 4. SEM micrographs of the electrospun P(3HB-co-7 mol%-4HB) and P(3HB-co-97 mol%-4HB) in various conditions. The arrow shows the pores on the fiber.

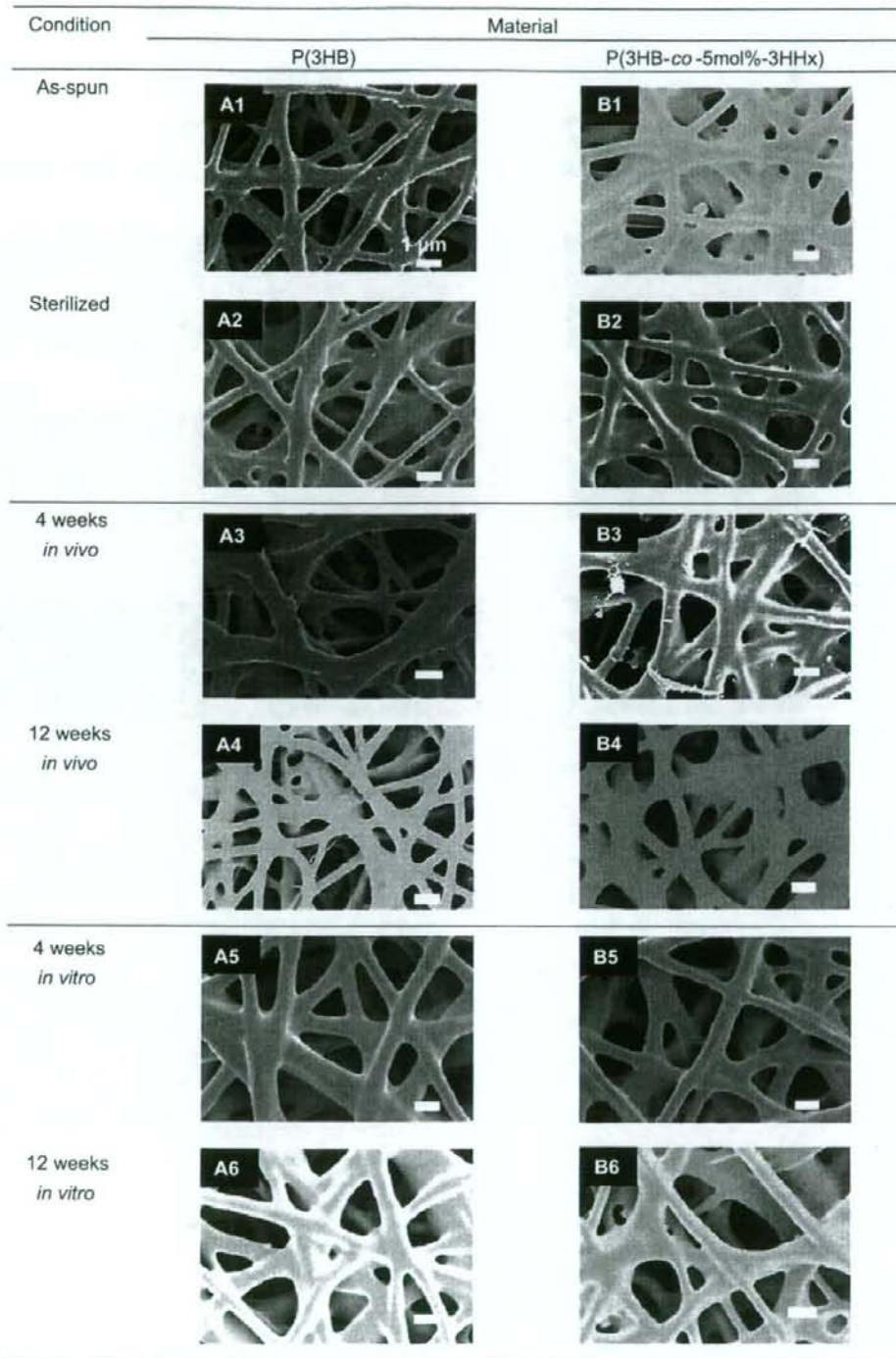


Fig. 3. SEM micrographs of the electrospun P(3HB) and P(3HB-co-5 mol%-3HHx) in various conditions.

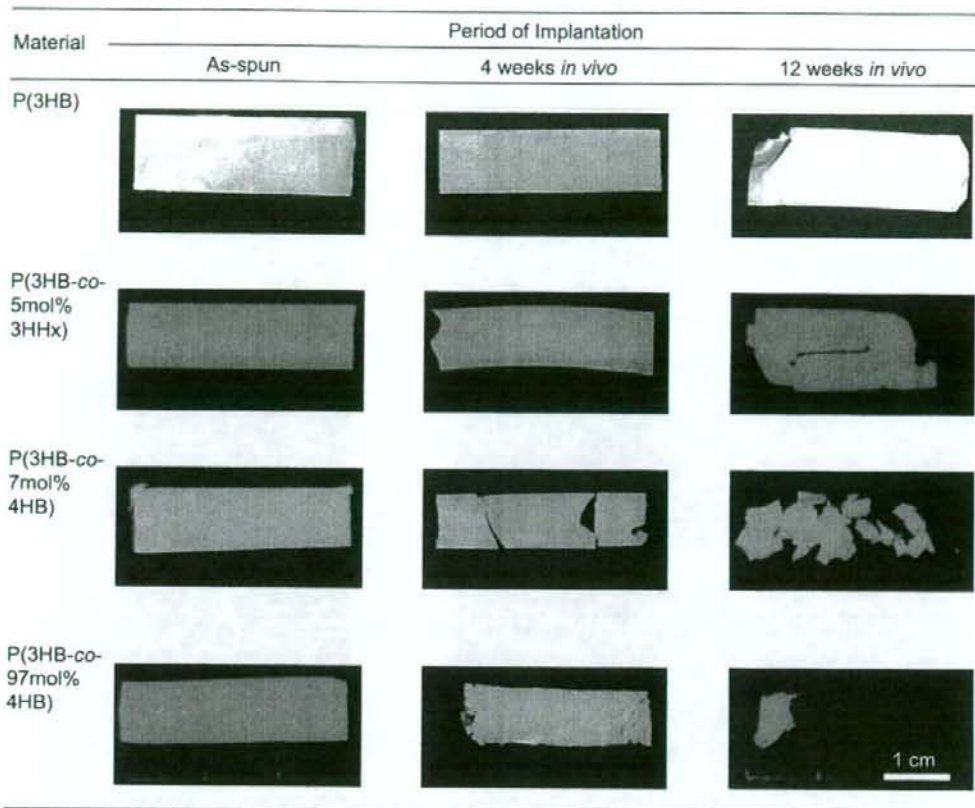


Fig. 2. Physical appearance of the electrospun PHA scaffolds before and after 4 and 12 weeks of subcutaneous implantation in rat.

(Fig. 4B2). The temperature (40 °C) of the EtO sterilization is close to the melting temperature of P(3HB-co-97mol%-4HB) ($T_m = 47$ °C), and thus led to the fusing of some fibers to each other.

3.1.1. *In vivo* study

In SEM, only the remaining scaffolds that could be retrieved from rat after the *in vivo* experiments were observed. Nevertheless, such observations provided the information on the morphological changes by implantation. For the P(3HB) and P(3HB-co-5mol%-3HHx) scaffolds, no remarkable change in their appearances by implantation during the period investigated here was observed (Fig. 3). This result is in good agreement with the macro-scale observation, as shown in Fig. 2. On the other hand, the fibers of the copolymers with 4HB unit were influenced by the implantation. At 4 weeks of implantation, the fibers of P(3HB-co-97mol%-4HB) showed fragmentation (Fig. 4B3). After 12 weeks, surface erosion became more evident as the density of the fibers decreased remarkably due to fragmentation of the fibers to shorter segments. The progression of bioabsorption was also evidenced by the formation of pores on the surface of these fibers as indicated by the arrow in Fig. 4B4. These evidences

indicate that the existence of 4HB monomer units enhances the degradability, or the bioabsorption of PHA.

After 4 and 12 weeks, the electrospun P(3HB-co-97mol%-4HB) fibers appeared to have swollen (Fig. 4B3 and B4). A slight decrease in the fiber density was also observed for the electrospun P(3HB-co-7mol%-4HB) (Fig. 4A4) at week 12. Previous studies have demonstrated that fibers of electrospun poly(D,L-lactic-co-glycolic acid), poly(D,L-lactic acid) and poly(butylene succinate) which were highly amorphous showed swelling after immersion in PBS [22–24]. Hence, it is possible that the swelling of the electrospun P(3HB-co-97mol%-4HB) fibers occurred due to the penetration of water into their amorphous regions.

3.1.2. *In vitro* study

SEM showed that there was no evidence of degradation on the surface of all the electrospun PHA scaffolds after 4 weeks of immersion in PBS (Figs. 3 and 4). All samples retrieved at 12 weeks also revealed that their structural integrities were maintained. This is because PHA hardly undergoes hydrolysis at pH value around neutrality. Similar to the observations for the *in vivo* study, the fibers of the electrospun P(3HB-co-97mol%-4HB) also demonstrated swelling.

2.4. Scanning electron microscopy

The PHA scaffolds were dehydrated in increasing concentrations of ethanol aqueous solutions containing 50%, 70%, 90%, 95%, 99.5% and 100%. They were then mounted on aluminum stumps and coated with gold in a sputtering device for 3 min at 5 mA. The scaffolds were observed with a scanning electron microscope (JSM-6330F, JEOL, Co. Ltd.) at an acceleration voltage of 5 kV and an emission current of 12 μ A.

2.5. Wide-angle X-ray diffraction (WAXD)

Two-dimensional (2D) WAXD patterns of the electrospun PHA scaffolds were acquired using an X-ray diffractometer (RINT UltraX 18, Rigaku Japan) equipped with an imaging plate (BAS-SR 127, Fuji Film Co., Japan). Ni-filtered Cu-K α radiation ($\lambda = 0.154$ nm) generated at 40 kV and 200 mA was collimated by a pinhole with a diameter of 1.0 mm. The distance from the scaffold to the imaging plate was 5 cm and the exposure time was 6 h. After converting the 2D images to 1D profile by circular-averaging, the crystallinity of the scaffolds was calculated from the ratio of the areas of crystalline reflections to the overall intensity in the range of $12^\circ \leq 2\theta \leq 35^\circ$ of the averaged 1D profile.

2.6. Tensile property

The non-sterilized scaffolds and the scaffolds before and after implantation were cut into 2 mm \times 5 mm strips for tensile test. Tensile test on the scaffolds was carried out on a tensile testing machine (SHIMADZU EZTest) at a cross-head speed of 1 mm/min under ambient conditions. The thickness of each scaffold was measured before testing. Tensile properties were calculated from the stress–strain curves as means of two measurements.

2.7. Gel permeation chromatography (GPC) analysis

The molecular weight of the scaffolds was measured with gel permeation chromatography at 40 $^\circ$ C, using a Shimadzu 10A GPC system equipped with a 10A refractive index detector and Shodex K-806M and K-802 columns. Chloroform was used as the eluant at a flow rate of 1.0 ml min $^{-1}$. The calibration curve was prepared by using polystyrene standards with narrow polydispersity.

2.8. In vitro degradation evaluation

Each of the 1 cm \times 3 cm sterilized scaffold was immersed in 10 mL phosphate buffered saline (PBS) at pH 7.4 in sterilized capped containers. These containers were then incubated at 37 $^\circ$ C without agitation. After 4 and 12 weeks, the scaffolds were recovered and characterized by SEM, tensile test, GPC and WAXD.

2.9. Histological observation

The surrounding tissues were excised together with the electrospun PHA scaffolds and fixed with 2.5% glutaraldehyde solution. A small piece of the tissue was then embedded in paraffin before subjecting it to microtome sectioning. Hematoxylin and eosin (HE) were used for staining the tissues. The tissue response to the scaffolds was evaluated from the coloration observed with a phase-contrast microscope.

3. Results and discussion

3.1. Morphological changes of electrospun PHA scaffolds

The retrieved electrospun PHA scaffolds showed various changes in appearances after subcutaneous implantation

(Fig. 2). After 4 weeks, both the electrospun P(3HB) and P(3HB-co-5mol%-3HHx) remained in their initial form. The electrospun P(3HB-co-7mol%-4HB) was fragmented into large pieces while the electrospun P(3HB-co-97mol%-4HB) shrunk and became thinner. Even after 12 weeks, the electrospun P(3HB) showed no morphological change. However, significant changes were observed for the other three electrospun PHA copolymers. The degree of degradation increased in the order of P(3HB-co-5mol%-3HHx), P(3HB-co-7mol%-4HB), and (3HB-co-97mol%-4HB). The electrospun P(3HB-co-5mol%-3HHx) displayed crevices on its surface while the electrospun P(3HB-co-7mol%-4HB) was degraded into small fragments. Only a small piece of the electrospun P(3HB-co-97mol%-4HB) scaffold was retrieved, indicating enhanced bioabsorption of this 4HB-rich copolymer.

To have a better understanding on the progress of biodegradation at a fiber scale, SEM was used to observe the morphological changes of the fibers of all the electrospun PHA scaffolds. SEM revealed that all the as-spun scaffolds consist of randomly oriented fibers (Figs. 3 and 4). Further morphological feature is that the fibers fuse together. Namely, the electrospun fibers organize into a three-dimensional network. This is probably because the solvent (HFIP) evaporation was incomplete when the fibers were deposited on the collection plate. The width of the fiber between the junctions was quite uniform. The width decreased in the order of P(3HB) = P(3HB-co-5mol%-3HHx) (520 nm) > P(3HB-co-97mol%-4HB) (220 nm) > P(3HB-co-7mol%-4HB) (190 nm). It has been reported that the width increases proportionally with the molecular weight of the polymer [18]. This is because higher degree of chain entanglement due to high molecular weight is assumed to make it harder for the electrostatic forces to pull, or extend individual chains [19]. Accordingly, the matrices of the electrospun P(3HB) and P(3HB-co-5mol%-3HHx) consisted of larger fibers compared to the electrospun P(3HB-co-97mol%-4HB) because of their high molecular weight (Table 1).

Interestingly, only the electrospun P(3HB-co-7mol%-4HB) formed fibers with irregular shapes with intermittent spindle-like beads on string (Fig. 4A). Possibly the formation of the beaded P(3HB-co-7mol%-4HB) fibers is the result of low net charge density. Previous studies have shown that higher net charge density favors the formation of bead-free fibers [20,21]. According to Ref. [20], the net charge density is inversely proportional to the mass of dry polymer (i.e. mass of scaffolds collected from electrospinning), if the other experimental conditions such as jet current, collecting time and polymer concentration are the same. The net charge density decreases in the order of P(3HB-co-5mol%-3HHx) (1058 C/l) > P(3HB) (1002 C/l) > P(3HB-co-97mol%-4HB) (778 C/l) > P(3HB-co-7mol%-4HB) (484 C/l). In this study, the P(3HB-co-7mol%-4HB) scaffold had the highest collected mass.

After sterilization, the morphologies observed for the electrospun P(3HB), P(3HB-co-5mol%-3HHx) and P(3HB-co-97mol%-4HB) remained unchanged (Figs. 3 and 4). The matrix of P(3HB-co-97mol%-4HB), however, became less porous

are well understood. For example, the monomeric component of P(3HB), (*R*)-3-hydroxybutanoic acid (3HB), is a ketone body present at concentrations of 3–10 mg per 100 mL blood in healthy adults [1,7]. The monomeric component of P(4HB), 4-hydroxybutanoic acid (4HB), can also be found widely distributed in the brain, kidney, heart, liver, lung and muscle of the mammalian body [8]. Furthermore, the hydroxyl acids released during PHA *in vivo* breakdown are found to be considerably less acidic and less inflammatory than many currently used synthetic absorbable polymers such as poly(lactic acid) (PLA) [9]. P(3HB) has, however, limited application due to its high brittleness, poor processability and slow degradation [10]. Therefore, researches in this field of interest had shown great progress over the past 20 years and it is now possible to design and synthesize various kinds of PHA (reviewed in Ref. [11]) to overcome the inferior properties of P(3HB).

New polymer processing approaches are in demand to create degradable porous scaffolds that can support the hierarchical structures of many tissues ranging between 0.1 and 1.0 mm [12]. Electrospinning has emerged as one of the methods offering simplicity and versatility in preparing such biomaterials [13]. Electrospun biomaterials facilitate better cell attachment and perfusion due to very high surface area-to-volume ratio and high porosity. Improved cellular response is also suggested because the morphology and architecture of electrospun structure are similar to those of some extracellular matrix (ECM) [14]. In addition, electrospinning may provide an alternative method to produce fibrous materials with improved mechanical properties compared to solid-walled equivalents [15].

Thus, in this study, we used electrospinning to develop scaffolds of fibrous PHA copolymers; P(3HB-*co*-5mol%-3HHx), P(3HB-*co*-7mol%-4HB) and P(3HB-*co*-97mol%-4HB) (Fig. 1), in aim of achieving enhanced biocompatibility, mechanical properties and bioabsorption. We also describe here the characterization and effects of sterilization on the physical properties of the electrospun PHA copolymers. Our study is the first to do a detailed comparison on the bioabsorption rate and the tissue response of electrospun PHA copolymers containing 3HB, 3HHx and 4HB monomers implanted subcutaneously in rat model.

2. Materials and methods

2.1. PHA synthesis

P(3HB-*co*-97mol%-4HB) was biosynthesized by *Delftia acidovorans* (formerly known as *Comamonas acidovorans*) using glucose and 1,4-butanediol as carbon sources, according to the method described in Ref. [16]. P(3HB-*co*-7mol%-4HB) was kindly provided by Dr. Toshihisa Tanaka of RIKEN Institute, Japan. The *in vitro* toxicity of P(3HB-*co*-4HB) derived from *D. acidovorans* has been determined recently [17]. P(3HB) and P(3HB-*co*-5mol%-3HHx) supplied by ICI Biopol and P&G, respectively, were purified before use. Briefly, the purification of the P(3HB) and P(3HB-*co*-5mol%-3HHx) was performed by dissolving the polymers in chloroform followed by precipitation in excess hexane and filtration.

2.2. Fabrication of fibrous implant and electrospinning

Each polymer solution of 1 wt% concentration was prepared by dissolving 100 mg of the polymer in 9900 mg of 1,1,1,3,3,3-hexafluoro-2-propanol

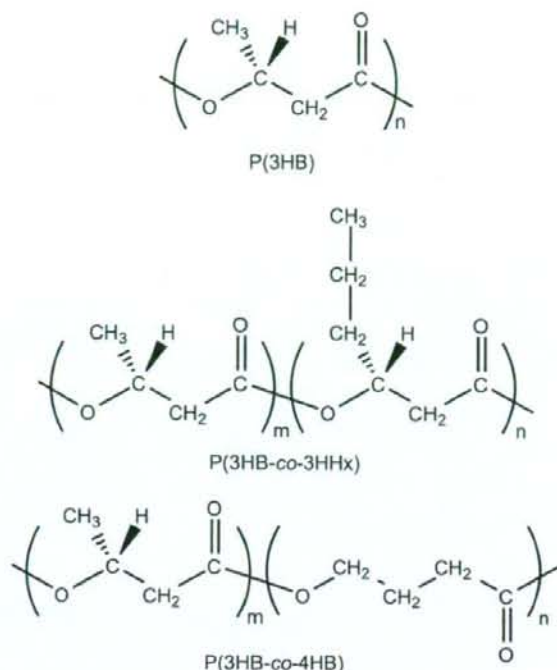


Fig. 1. Chemical structures of PHAs used in this study.

(HFIP). Electrospinning was performed using Esprayer ES-2000 (Fuence, Co. Ltd., Japan). The polymer solution was placed in a 1-mL glass syringe with an inner needle diameter of 0.5 mm. The syringe was set vertically at the support of the electrospinning device and the solution was extruded at a constant rate of 40 $\mu\text{l min}^{-1}$. A positive voltage of 15 kV was applied at a distance of 20 cm between the needle tip and the copper collecting plate. The jet current was in the range of 38.3–38.7 μA and the collecting time was approximately 8 h. For effective collection of the electrospun fibers, a square fiber-collecting area (3 cm \times 3 cm) was created on the copper plate by covering the rest of the plate with a non-conductive plastic. A sheet of aluminum foil was used to cover the collection area to ease the recovery of fibrous matrix from the copper plate. Each electrospun PHA scaffold was then cut into two different dimensions: 1 cm \times 1 cm and 1 cm \times 3 cm, respectively. All scaffolds were sterilized overnight with ethylene oxide (EtO) at 40 °C before implantation in rat.

2.3. Subcutaneous implantation in rat and retrieval

Four 12-week-old male Wistar rats were used for implanting the PHA scaffolds; two for scaffolds measuring 1 cm \times 1 cm while the remaining two for scaffolds measuring 1 cm \times 3 cm. The animals were cared for in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, approved by the Physiological Society of Japan. All experiment protocols had been reviewed and approved by the Animal Subjects Committee of the National Cardiovascular Center, Osaka, Japan. The rats were first injected with anesthetic before implanting the scaffolds. The 1 cm \times 1 cm scaffolds were implanted subcutaneously at one side of the backbone while the 1 cm \times 3 cm scaffolds were implanted subcutaneously at the backbone. The grouping of the rats was based on the duration of observation for 4 and 12 weeks. Upon explantation, the scaffolds measuring 1 cm \times 1 cm were stored in 2.5% glutaraldehyde solution until further analysis by SEM. The retrieved 1 cm \times 3 cm scaffolds were treated with 1.25 wt% trypsin to remove the surrounding tissues. They were then kept in tubes containing phosphate buffered saline (PBS) until further use.



Scaffolds from electrospun polyhydroxyalkanoate copolymers: Fabrication, characterization, bioabsorption and tissue response

Tang H. Ying^{a,b}, Daisuke Ishii^c, Atsushi Mahara^d, Sunao Murakami^d, Tetsuji Yamaoka^d, Kumar Sudesh^{a,b}, Razip Samian^b, Masahiro Fujita^{a,*}, Mizuo Maeda^a, Tadahisa Iwata^{c,**}

^a Bioengineering Laboratory, RIKEN Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan

^b School of Biological Science, Universiti Sains Malaysia, 11800 Penang, Malaysia

^c Department of Biomaterial Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

^d Department of Biomedical Engineering, Advanced Medical Engineering Center, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan

Received 10 September 2007; accepted 24 November 2007

Available online 21 December 2007

Abstract

Polyhydroxyalkanoate (PHA) copolymers of poly[(R)-3-hydroxybutyrate-co-5mol%-(R)-3-hydroxyhexanoate], poly[(R)-3-hydroxybutyrate-co-7mol%-4-hydroxybutyrate] and poly[(R)-3-hydroxybutyrate-co-97mol%-4-hydroxybutyrate] were electrospun to fabricate scaffolds with enhanced biocompatibility and bioabsorption. Subcutaneous implantation of the fibers in rats was performed to investigate their bioabsorption behavior and tissue response. The fibers before and after the *in vivo* experiments were characterized using gel permeation chromatography, scanning electron microscopy, X-ray diffraction and tensile test. Histological evaluation was also performed to determine the tissue response. The structures and properties of the electrospun PHA copolymers were compared with those of the electrospun poly[(R)-3-hydroxybutyrate]. The content and type of the second monomer and the diameter of fiber significantly influence the bioabsorption. The tissue response was found to improve with the high content of 4-hydroxybutyrate.

© 2007 Elsevier Ltd. All rights reserved.

Keywords: Electrospun fiber; Polyhydroxyalkanoate; Tissue response; Bioabsorption

1. Introduction

In response to the growing demand in the field of tissue engineering, the number of polyhydroxyalkanoates (PHAs) currently under evaluation as biomaterial has expanded to five, that is, poly[(R)-3-hydroxybutyrate] [P(3HB)], poly(4-hydroxybutyrate) [P(4HB)], poly[(R)-3-hydroxybutyrate-co-4-hydroxybutyrate] [P(3HB-co-4HB)], poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyvalerate] [P(3HB-co-3HV)] and poly[(R)-3-hydroxyoctanoate-co-(R)-3-hydroxyhexanoate]

[P(3HO-co-3HHx)] [1]. To date, PHA and its composites are thought to have good potentials as emerging materials for medical devices such as sutures, bone plates, surgical mesh and cardiovascular patches, just to name a few [2]. A recent major breakthrough for PHA as a new class of biomaterial is the clearance obtained from the Food and Drug Administration of the United States of America for the use of P(4HB)-derived Tephaflex[®] absorbable suture [3].

PHAs are a family of biopolyesters produced by numerous bacteria as intracellular carbon and energy compound under unfavorable growth conditions such as limitation of nitrogen, phosphorus, oxygen or magnesium in the presence of excess supply of carbon source [4–6]. PHAs are particularly attractive because they are bioabsorbable and biocompatible. The metabolism and excretion of some monomers incorporated into PHA

* Corresponding author. Tel.: +81 48 467 9312; fax: +81 48 462 4658.

** Corresponding author. Tel.: +81 3 5841 7888; fax: +81 3 5841 1304.

E-mail addresses: mfujita@riken.jp (M. Fujita), atiwata@mail.ecc.u-tokyo.ac.jp (T. Iwata).