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Basic Studies on Polymeric Materials to be Used for Hybrid-Type Artificial Organs

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Dedicated to

my parents

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Contents

Abbreviations

6F	Tetrafluoroethylene–hexafluoroethylene copolymer
BAEC	Bovine aorta endothelial cell
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
Coll	Type I collagen
DDS	Drug delivery system
DM	Diabetes mellitus
Eagle's MEM	Eagle's minimum essential medium
ECM	Extracellular matrix
ELISA	Enzyme–linked immunosorbent assay
FCS	Fatal calf serum
GA	Glutaraldehyde
HBSS	Hanks' balanced salt solution
HUVEC	Human umbilical cord vein endothelial cell
IgG	Immunoglobulin G
IL–1	Interleukin–1
MRPT	Mesh–reinforced PVA hydrogel tube
PAAm	Polyacrylamide
PBS	Phosphate buffered saline free of Ca^{2+} and Mg^{2+}
PE	Polyethylene
PET	Poly(ethylene terephthalate)
PGI_2	Prostaglandin I_2, Prostacyclin
PLLA	Poly(L–lactic acid)
PTFE	Polytetrafluoroethylene
PVA	Poly(vinyl alcohol)
S–D rat	Sprague–Dawley rat
TXA_2	Thromboxane A_2
VAECO	Vinyl alcohol–ethylene copolymer

General Introduction

If a patient has an irreversibly damaged internal organ, he or she might receive medical treatment for organ substitution. Today, organ transplantation is available in some areas of the world, but many problems are associated with the organ transplantation, including suppression of recipient's immunorejection, insufficient supply of donor organs,[1] and, especially in Japan, religious ill–feeling. The recipient should be treated with immunosuppressive agents and avoid infection throughout their life. Transplantation of animal organs, so called xenografts, is also attempted, but minimization of immunoreactions still remains unsolved. Therefore, many efforts have been directed to the development of artificial organs.

Figure I.1 represents artificial organs which are currently being investigated.[2] Almost all organs are targeted for artificial organ research, but only a few organs and tissues can at present be replaced by the artificial ones. These are dental implants, artificial joints, and vascular grafts of large size, but these still need improvement in efficiency and functionality. If the organ of target is simple in function, such as supporting body weight, or short in medical application, such as the cardiopulmonary bypass, artificial materials can be used for the replacement. However, it is very difficult to substitute metabolic internal organs such as the liver and pancreas with artificial materials, because these metabolic organs fulfil a lot of biofunctions to maintain the complicated body homeostasis. For example, the liver stores

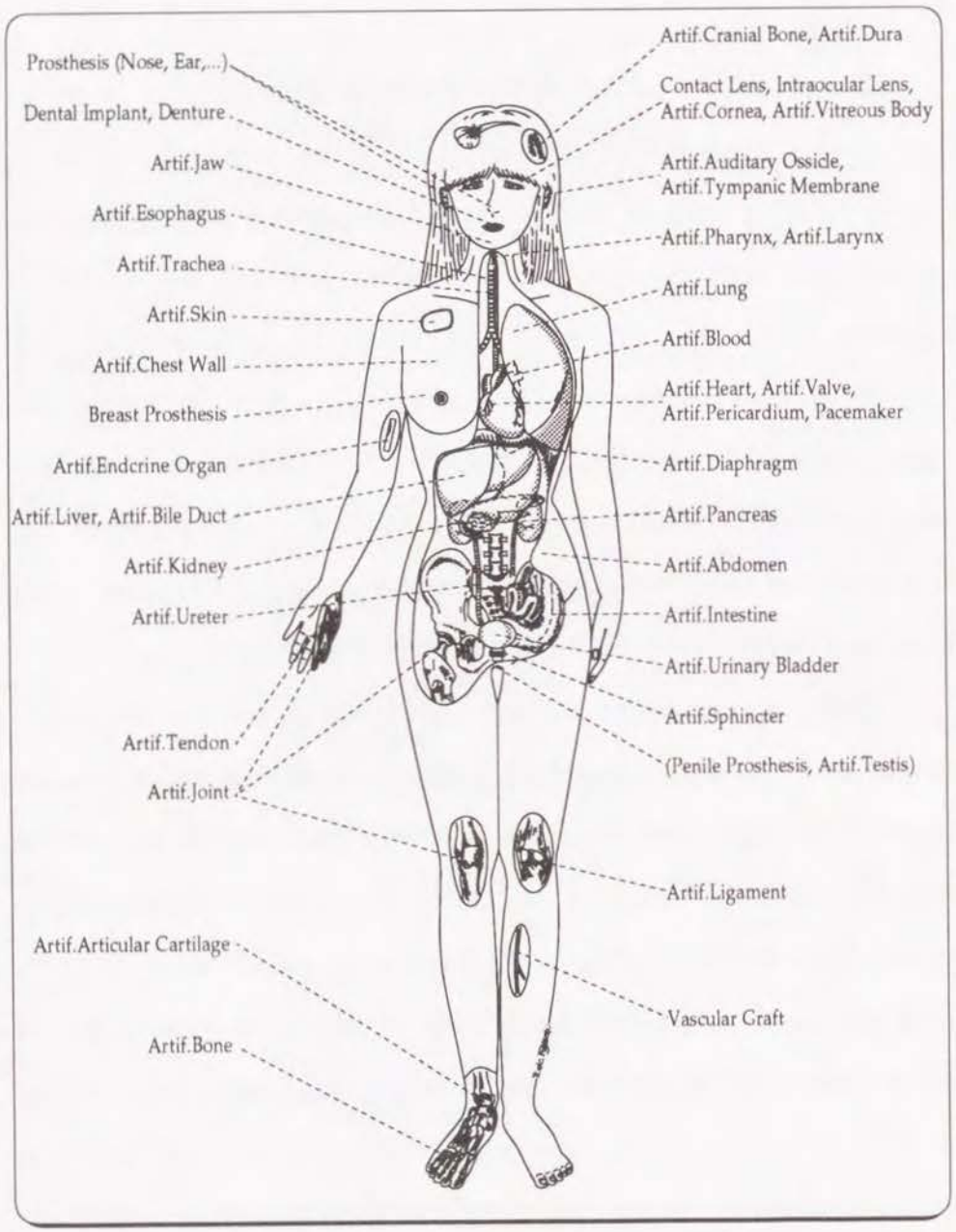


Figure I.1. Artificial tissues and organs.

glycogen, removes toxins, and synthesizes many lipids and proteins like blood coagulation factors.[3)]

Furthermore, it should be pointed out that even the currently used vascular graft is not completely artificial substitution, since its anticoagulation activity is obtained after conversion of the coagulated blood on the inner surface to the biological neointima which is the generated tissue of the patient. It is thought that the antithrombogenicity of natural blood vessels is maintained on the balance of prostacyclin (Prostaglandin I_2; PGI_2) and thromboxane A_2 (TXA_2) which are both secreted from the endothelial cells.[4)] Since our human body is controlled by highly balanced functions to keep homeostasis, it seems very difficult to rely on only artificial materials for organ substitution.

To solve this problem, the concept of hybrid-type artificial organs was proposed.[5-37)] The term 'hybrid' refers to the combination of artificial materials with biological components such as natural parenchymal cells and tissues which perform biofunctions and are protected from the host immune attack by the artificial substrate materials like membranes. This hybrid device may act as an intermediary between the totally artificial organ and the transplanted organ. Among the hybrid-type of artificial organs which are currently attracting much attention are vascular grafts of small diameters made from synthetic tubes and seeded with endothelial cells and skin substitutes made from collagen and a silicone film seeded with epidermal cells.[31)]

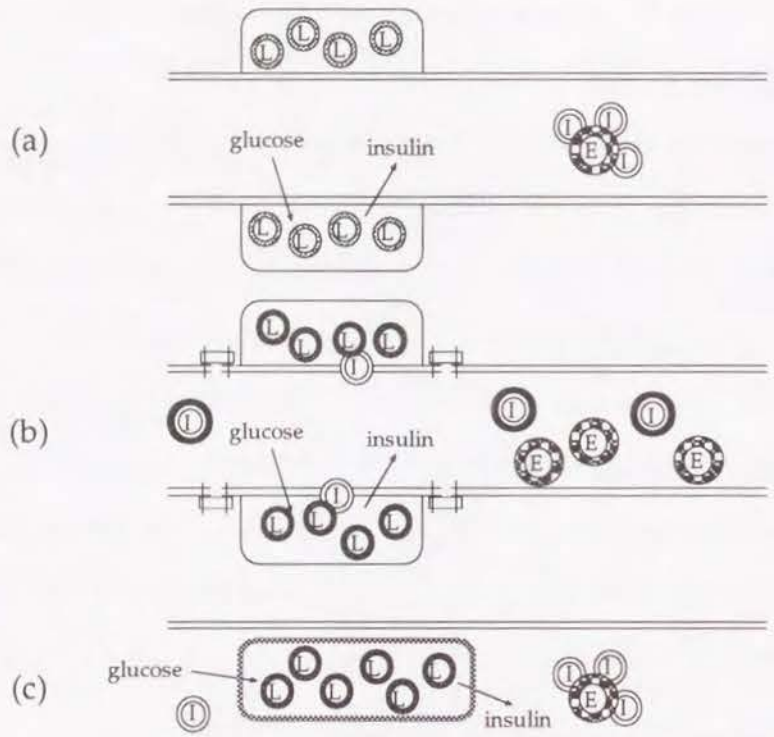
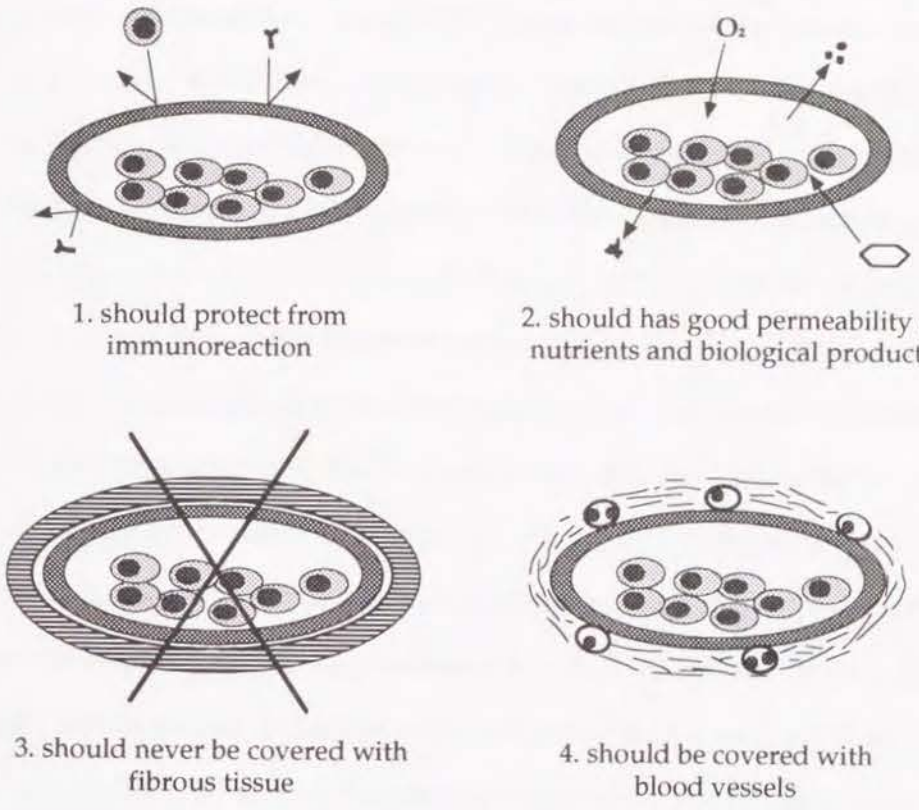


Figure I.2. Pancreas transplantation and hybrid-type artificial pancreas.
(a) Intact pancreas
Immunoagents (I) identify and attack the enemies (E).
(b) Transplantation
To protect transplanted islets (L), immunosuppressive agents are received, which diminish the resistance to the enemies.
(c) Artificial organ
To protect transplanted islets, polymeric membrane covers them, which does not influence the immunoagents.

However, such hybridization seems to be most effective when applied for metabolic organs such as the liver and pancreas. Figure I.2 shows the concept of a hybrid-type artificial pancreas.[38] To control the blood sugar concentration, β cells of Langerhans islet in the pancreas generally secret



1. should protect from immunoreaction

2. should has good permeability of nutrients and biological products

3. should never be covered with fibrous tissue

4. should be covered with blood vessels

Figure I.3. Membrane for hybrid-type artificial organs.

insulin in proportion to the glucose concentration. The patient of type I diabetes mellitus whose Langerhans islets are not effective enough for adjusting the blood sugar level, may be transplanted animal islets. However, these transplanted cells would be rejected by the recipient's immunoreactions, unless they are suppressed. Therefore, the recipient generally receives immunosuppressive agents, although these have many side-effects. A polymeric membrane encapsulating the islets might keep the

recipient under good healthy condition without immunosuppressive agents.

Figure I.3 illustrates how a polymeric membrane works for immunoisolation of the implanted cells. First, this membrane should protect the encapsulated cells from the host immune system, which consists of immunocells, such as lymphocyte and macrophage, and proteins such as immunoglobulin and complement. Second, it should allow rapid permeation of nutrients necessary for the encapsulated cells, such as glucose and oxygen, and also permeation of secreted substances from the cells, for example, insulin for the pancreas. Third, it should have such a surface that it prevents a thick covering of fibrous tissue and calcification from developing in order to maintain significant membrane permeation for a long period. Fourth, it should be covered with blood vessels to make a prompt response of the cells toward the stimuli coming from the blood possible.

On the other hand, the cells to be used for the hybrid-type artificial organs should be obtained on a large scale, grown to a high density *in vitro* culture, and kept functioning for a long time. For example, to fabricate a hybrid-type of artificial liver, at least 1.5×10^{10} cells with good cell functionality should be at hand since the weight of an adult's liver is about 1,500 g and the number of hepatocytes in the liver is 1.1×10^{8} cells/g.[39] As is well known, parenchymal cells are extremely difficult to culture *in vitro* for a long period without loosening their highly specified functions.

In this decade, hollow fibers with micropores or microspherical hydrogels have been used as the materials for immunoisolation of hybrid-

type artificial metabolic organs,[9-16,19,22,24] but it seems to take at least several years for their clinical trial because it is difficult to increase the membrane permeation and at the same time keep very excellent mechanical and interfacial properties of the membrane. This research field has been supported mainly by biologists and medical doctors because it requires cell cultures and animal experiments. However, the knowledge of polymer science must be input into the research of hybrid-type artificial organs, as the most important thing for the current hybrid-type artificial organs is the property of polymeric substrate.

Up to now, the biomaterials, used for medical applications, have mainly been estimated from the extent of cell adhesion and growth. It has been already reported that polymer surfaces with the water contact angle around 70° are most readily adsorbed with proteins and attached with cells so long as they have neither ionic groups nor biologically active moieties.[40] When parenchymal cells such as hepatocyte are used for hybrid-type artificial organs, the cells must attach on a substrate and exhibit their phenotype. In addition to cell number, the long-term maintenance of cell function should also be considered.

The primary purpose of the present work is to accumulate the fundamental information on the interaction of polymeric materials with cells and the protein permeation through the polymeric substrates that are required for the clinical application of hybrid-type artificial organs. This thesis consists of seven chapters which are summarized as follows:

Chapter 1 deals with the interaction between various parenchymal cells and polymeric substrates with different surface properties. Particular attention is paid to non-adherent surfaces, because the cells cultured on non-adherent substrates exhibit some interesting behavior. Cell morphology is observed with light microscopy. The cell functions to be estimated include glucose consumption and production of prostacyclin, thromboxane, collagen, and interleukin-1.

Chapter 2 deals with the behaviors of rabbit hepatocytes cultured on a number of polymeric substrates with different surface properties. Since hepatocytes must keep their functions on a polymeric substrate when used for hybrid-type artificial liver, cell morphology and functionality such as glucose consumption and albumin production is studied in addition to cell adhesion and morphology. Also, this chapter refers to the possible maintenance of the hepatocyte function for a long period.

Chapter 3 is concerned with the possibility of the scaffold for cell culture on a large scale and self-reconstruction of defective tissues such as the liver and cartilage which have the capacity to regenerate themselves. This chapter focuses on liver regeneration. After seeding hepatocytes on the scaffold of biodegradable polymer, the cell-polymer composites are

implanted in rabbits as the core for the reconstruction of the liver. The factors governing cell viability are attempted to be identified. Most of the results are histologically estimated.

Chapter 4 is focused on cartilage reconstruction using the cell-polymer composite. Chondrocytes-polymer scaffolds are implanted into nude mice who have immunodeficiency and new cartilage formation is observed. The assessment of the initial features of the cell's growth in the course of the generation is estimated.

Chapter 5 presents the results on poly(vinyl alcohol) (PVA) hydrogel membranes to be used for hybrid-type artificial organs. PVA is crosslinked with electron beam irradiation and glutaraldehyde (GA). The influence of crosslinking on the diffusive properties is estimated. Some general conclusions are drawn concerning the dependence of protein diffusion through hydrophilic matrix on the polymer network structure.

Chapter 6 is concerned with the protein permeation through PVA hydrogel membranes which microscopically contain the area available for the diffusion of solutes which is the open space in the macromolecular mesh. The diffusive properties of PVA hydrogel membranes to glucose, insulin, albumin, and immunoglobulin G are studied.

Chapter 7 deals with the application of PVA membranes for a hybrid-

type artificial pancreas. First, PVA hydrogel is implanted in a rat's peritoneal cavity and fibrous capsule formation is estimated. Second, it is determined by experimental transplantation of a hybrid-type artificial pancreas whether or not the device with pancreatic islets entrapped in the PVA membrane is effective.

It seems possible that the hybrid-type artificial organs will be available in the near future and help numerous patients suffering from heavy diseases of internal organs, provided there is further study in this specialized biomedical field.

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Chapter 1

Adhesion and Functions of Various Cells Cultured on Polymeric Substrates

INTRODUCTION

It is very important to clarify the interaction between cells and substrates for molecularly designing biomaterials, especially for hybrid-type artificial organs, in which parenchymal cells must attach onto a polymeric substrate and exhibit their phenotype and biofunction. In the conventional *in vitro* cell culture, polymeric substrates such as polystyrene are treated by plasma for cell adhesion. Generally, non-adherent substrates are thought to be unsuitable for cell culture, but effective for the biomaterials which come in contact with blood for very short times in order to prevent blood coagulation.[1-3] Recently, interactions between cells as well as between cell and extracellular matrix have actively been studied, and a lot of cell surface receptors such as integrin and cadherin families have been identified.[4-8] These recent investigations have revealed that such surface receptors influenced not only attachment of cells to the substrates, but also their biofunction and differentiation.[6,8]

There are several reports which indicate that cell attachment on

different polymer substrates becomes maximal when the water contact angle of substrate is around 70°.[9] The substrates which do not obey this rule are glass and those immobilized with proteins such as collagen, gelatin, and fibronection. In such a way, cell adhesion to substrates depends on their surface characteristics, but it is also likely that the substrate onto which the cells attach influences the cell function. This is supported by a finding that rat fibroblast produced larger amounts of collagen on non-adherent surfaces than on adherent ones.[10]

This work was undertaken in an attempt to study the behaviors and biofuctions of various parenchymal cells when cultured on different polymeric substrates. As a measure of cell functions, glucose consumption and production of prostacyclin, thromboxan, collagen, and interleukin-1 were determined.

EXPERIMENTAL

Substrate polymers

Cellulose, PVA, polyacrylamide (PAAm)-grafted polyethylene (PE), polytetrafluoroethylene (PTFE), and bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, MO, U.S.A.)-coated cell culture dish were used as non-adherent substrates, while poly(ethylene terephthalate) (PET), cell culture dish, glass, commercial sheet for cell culture (Wako Pure

Table 1.1. Contact angle of substrates used for cell culture.

Code	Substrate	Contact Angle (degree)
1	Cellulose	20
2	Glass [a)]	30
3	PVA	42
4	PAAm-grafted PE	43
5	Coll-coated cell culture dish [b)]	44
6	BSA-coated cell culture dish	48
7	Cell culture dish [a)]	54
8	VAECO	58
9	Commercial sheet for cell culture	67
10	PET	75
11	PE	95
12	Silicone	100
13	6F	105
14	PTFE	118

a) ionic surface
b) biologically active surface

Chemical Co. Ltd., Osaka, Japan), and type I collagen (coll; Nitta Gelatin Co. Ltd., Osaka, Japan)-coated cell culture dish were used as adherent substrates. PE, silicone (Dow Corning Corp., Midland, MI, U.S.A.), vinyl alcohol-ethylene copolymer (VAECO), and tetrafluoroethylene-hexafluoroethylene copolymer (6F) were selected as intermediate substrates (Table 1.1).

All of the film substrates except PAAm-grafted PE were commercially obtained. VAECO had a vinyl alcohol content of 33 mol%.

All of the films were purified by Soxhlet extraction with methanol for 24 hrs and dried under vacuum at room temperature. Grafting of PAAm onto PE film was carried out as follows.[11] Both sides of a polyethylene film were exposed to corona discharge at 15 kV for 1 min under dry air. The corona-treated film was immersed in 10 % aqueous solution of acrylamide in a glass test tube. After vigorous degassing, the test tube was sealed and kept at 50°C for 1 hr to allow graft polymerization of acrylamide to proceed. The ungrafted homopolymer was removed from the film by extraction with water at 60°C. The contact angle of films against water was measured by the sessile drop method at 25°C and 60 % relative humidity. Prior to the cell culture experiment, all of the films were punched off to disks of 1.5 cm diameter and sterilized with 70 % ethanol for 24 hrs, and then rinsed 3 times with phosphate buffered saline free of Ca^{2+} and Mg^{2+} (PBS; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan, pH=7.4) to completely remove the ethanol.

Cell isolation

The L cells were used as an established cell line, and the fibroblasts, endothelial cells, chondrocytes, and macrophages were used as primary cultured cells. Every cell was isolated each time at the primary cell culture experiment. All procedures were operated under a sterilized condition.

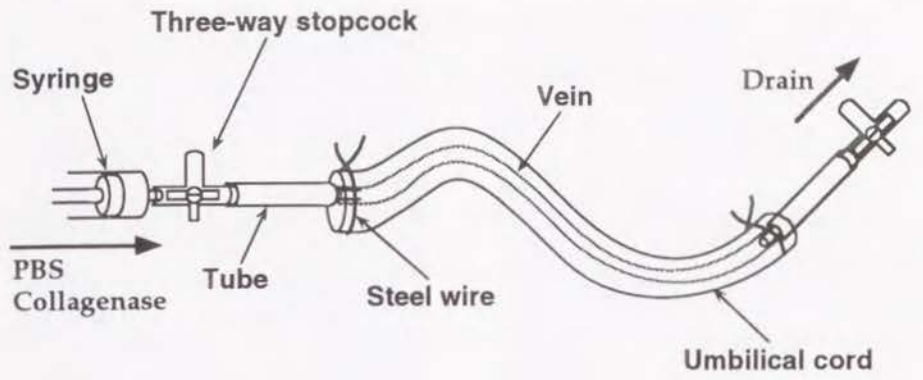


Figure 1.1. Method of isolation of human endothelial cells from umbilical cord vein.

Fibroblasts

Canine fibroblasts were isolated from the skin of mongrel dogs. A small skin area of 2×2 cm^2 was cut out under anesthetization and minced by surgical scissors, followed by immersion in the cell harvesting solution for 15 min (0.25 % trypsin, 0.02 % EDTA in PBS). The treated pieces of skin were put on a cell culture dish (Type 25020; Corning, NY, U.S.A.), incubated for about 3 weeks without floating in the medium, and then the migrated cells were collected by the cell harvesting solution.

Endothelial cells

Human endothelial cells (HUVECs) were isolated with the method of Jaffe *et al.*[12] from the human umbilical cord which was cut by about 20 cm length soon after birth. After removal of blood from the vein by washing with PBS, collagenase solution (0.1 % collagenase, 0.1 % $CaCl_2$, and 0.1 %

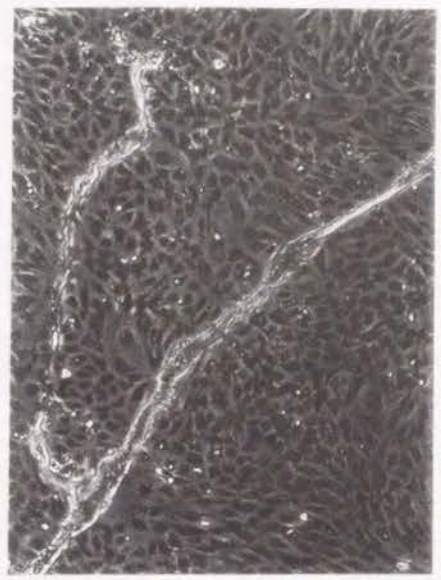
$MgCl_2$ in PBS) was inserted into the vein for 15 min as represented in Figure 1.1. Collagenase was kindly donated by Amano Pharmaceutical Company, Nagoya, Japan. Bovine aorta endothelial cells (BAECs) were isolated from the inner surface of the aorta which was immersed in the collagenase solution for 15 min. Both the isolated cells were washed by Eagle's minimum essential medium (Eagle's MEM; Nissui Pharmaceutical Co., Ltd.) and used for cell culture experiment.

Chondrocytes

Chondrocytes were isolated from rat costal cartilage.[13] Costae were removed from sacrificed rats and the cartilage was isolated. The isolated cartilage was minced by surgical scissors and immersed in 0.25 % trypsin–0.05 % collagenase solution for 1 hr. After washing 3 times with PBS, the treated pieces of cartilage were immersed in 0.02 % EDTA solution for 1 hr. After washing, they were put on a cell culture dish, incubated for about 3 weeks without floating in the medium, and then the migrated cells were collected by the cell harvesting solution.

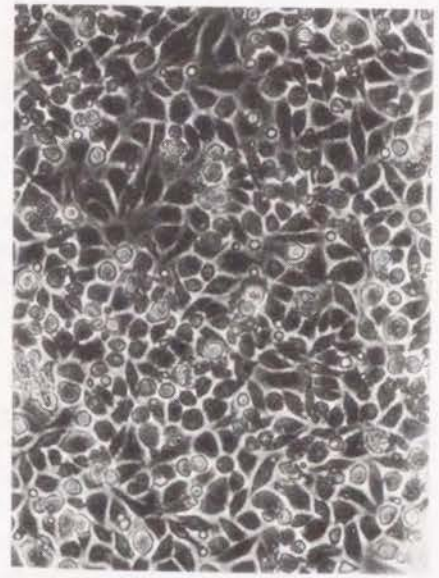
Macrophage

Macrophages were isolated with the method of Tabata and Ikada.[14] Four days prior to harvest, 5–7 week-old, male BALB/cCrSlc mice were intraperitoneally and aseptically injected with 2 ml of thioglycollate broth (Brewer's Medium; Difco Laboratories, Detroit, MI, U.S.A.). On the day of



HUVECs

Rat chondrocytes



Macrophages

L cells

Figure 1.2. Cells cultured on cell celture dish.

harvest, mice were exsanguinated by decapitation. The sacrificed mice were injected intraperitoneally with cold Hanks' balanced salt solution (HBSS; Nissui Pharmaceutical Co. Ltd.) and the peritoneal exudate cells were collected by syringe aspiration. After rinsing with HBSS, cells were seeded on a cell culture dish. After 2 hrs of incubation, the dish was washed with PBS and the adherent cells were collected with the cell harvesting solution and used for the cell culture experiment below.

Figure 1.2 shows the shape of cells used in this work adhered on cell culture dish.

Cell culture

Sterilized substrates were put on the 24 multi-well culture dish (Type 258201; Corning) and a sterilized thin platinum ring of 1.5 cm diameter was placed on each film to prevent floating in the medium. Type I collagen and BSA were coated on the 24 multi-well culture dish using the solutions. Every substrate was immersed in PBS to avoid drying before use.

The cells collected by the cell harvesting solution were seeded on each of the substrates at a density of 5.7×10^4 cells/cm^2. After predetermined periods of time, the cell appearance was photographed and the cell number was counted with a light microscope. Eagle's MEM with 10 % fatal calf serum (FCS) was used for the fibroblast and chondrocyte culture,[15] while Medium 199 with 10 % FCS, 10 U/ml heparin (Shimizu Pharmaceutical Co.

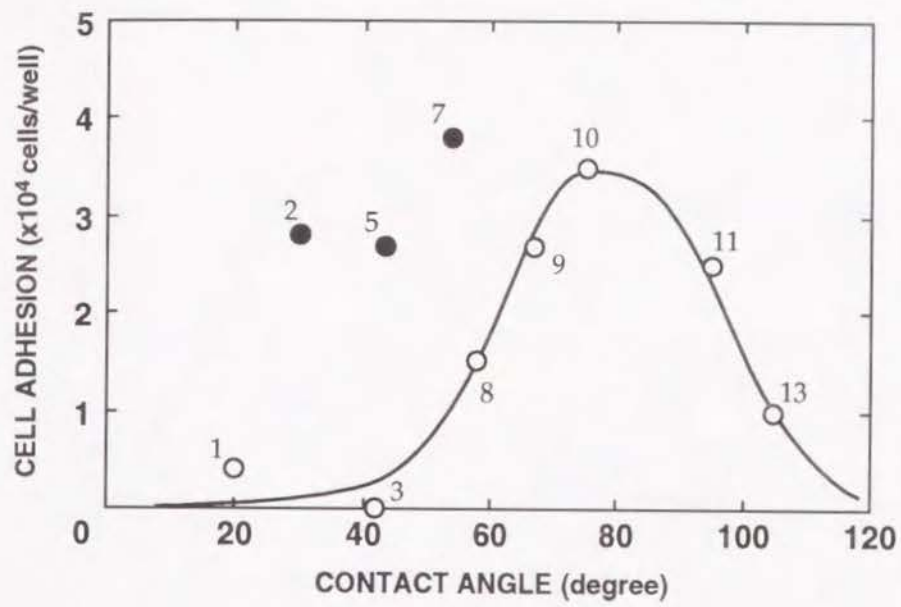


Figure 1.3. Effect of the water contact angle of film surfaces on endothelial cell adhesion. (See Table 1.1 for explanation of film material code.)

Ltd., Shimizu, Japan), and 20 μg/ml endothelial cell growth supplement (Collaborative Research Inc., Bedford, MA, U.S.A.) was used for the endothelial cell culture[16] and RPMI 1640 medium (Nissui Pharmaceutical Co. Ltd.) for the macrophage culture. Cultures were maintained in a 37 °C incubator equilibrated with 5 % CO_2 and 95 % air under a sterilized condition.

Measurement of cell activities

The number of cells adhering on the substrates was counted optically. The substrates were rinsed 3 times with PBS to remove all of the non-

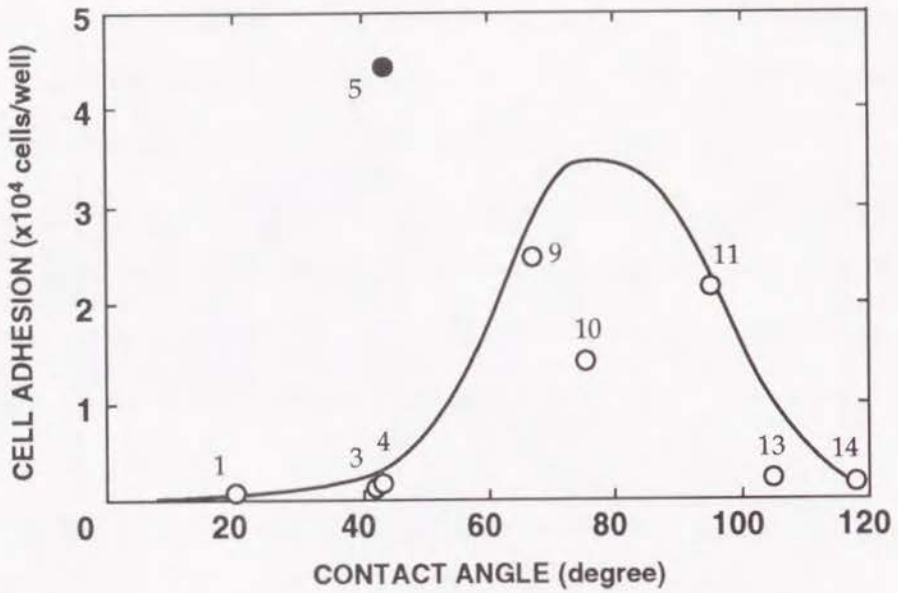


Figure 1.4. Effect of the water contact angle of film surfaces on chondrocyte adhesion. (See Table 1.1 for explanation of film material code.)

adherent cells, and then the cells were stained with Giemsa solution. Cell morphology was observed microscopically without rinsing the substrates.

Glucose consumption was estimated by determining the amount of glucose in the culture medium before and after the cell culture using the test kits for diagnosis provided by Wako Pure Chemical industries, Ltd. PGI_2 and TXA_2 were determined using radioimmunoassay kits (NEN Research, NY, U.S.A.). Type II collagen production was estimated using the enzyme-linked immunosorbent assay (ELISA).[17] Production of interleukin-1 (IL-1) by macrophages was studied with the method of Mizal *et al.*[18] using bioassay of mouse thymus cells.

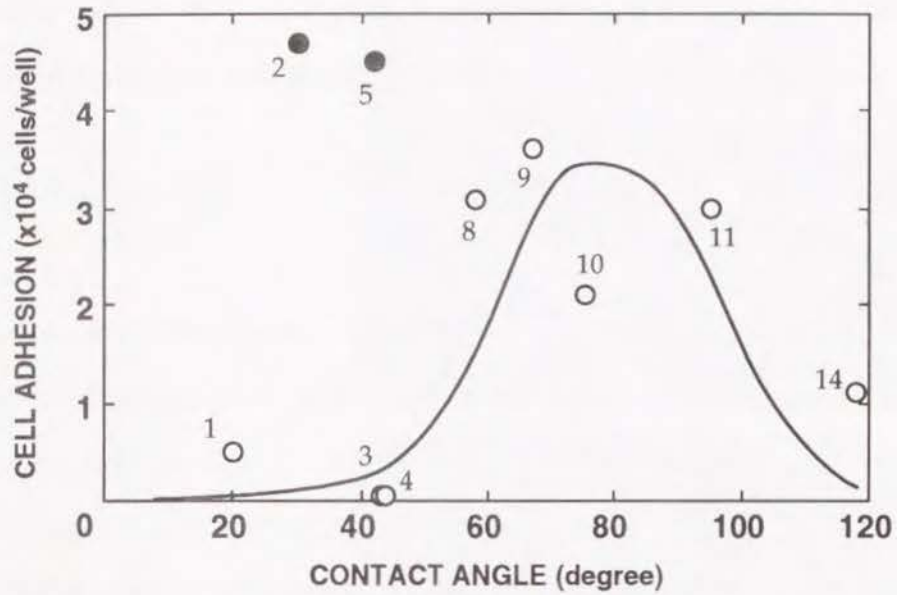


Figure 1.5. Effect of the water contact angle of film surfaces on macrophage adhesion. (See Table 1.1 for explanation of film material code.)

RESULTS

Cell adhesion

The dependences of the adhesion of endothelial cells, chondrocytes, and macrophages on the water contact angle of substrates are shown in Figures 1.3–1.5 The adhesion dependences on the contact angle are very similar to that found in a previous work.[9] L cells and HeLa S_3 cells did not adhere well, not only to highly hydrophilic surfaces such as PVA, but also to highly hydrophobic surfaces such as PTFE. On the other hand, significant

cell adhesion was noticed on the surfaces having the water contact angle around 70° such as PET and on the collagen coated surface, although this collagen surface had a low water contact angle.

Cell morphology

Figure 1.6 shows light microphotographs of the fibroblasts incubated on three different surfaces for 5 days. Apparently, cells aggregated each other on non-adherent surfaces, while they were present as a monolayer showing spreading cell shape on adherent surfaces. On the intermediate surfaces, cells exhibited the intermediate shape between the monolayered and the aggregated state.

Figure 1.7 shows the cell morphology of different kinds of cells on a non-adherent surface cultured for 5 days. The fibroblasts and chondrocytes had the three dimensional aggregated shape, but the endothelial cells and L cells did not reveal such three dimensional aggregation, but aggregated into a two dimensional layer.

The effect of the FCS addition to the cell culture medium is shown in Figure 1.8. Endothelial cells could attach on the BSA-coated cell culture dish, only when FCS was added. As FCS contains cell adhesion proteins such as fibronection and vitronection, these proteins might have adsorbed on the dish surface earlier than the cell attachment.[19]

PAAm-grafted PE

VAECO

Coll-coated cell culture dish

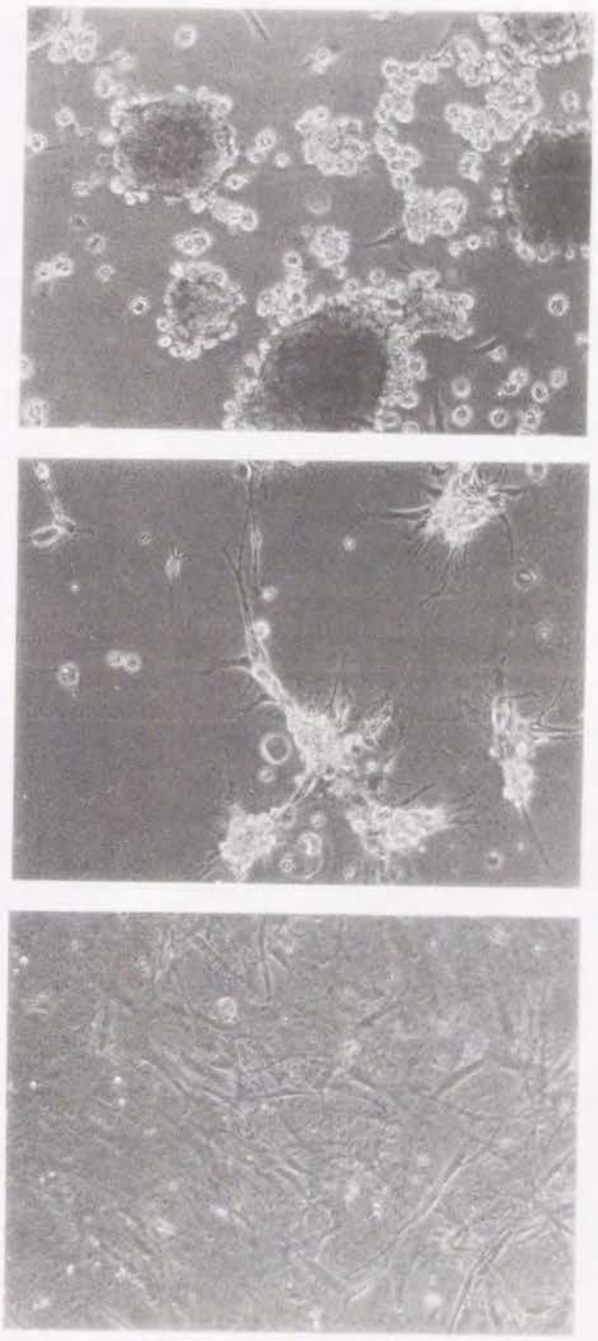
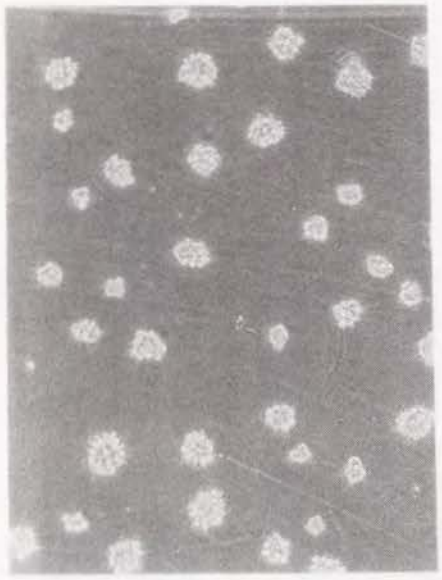


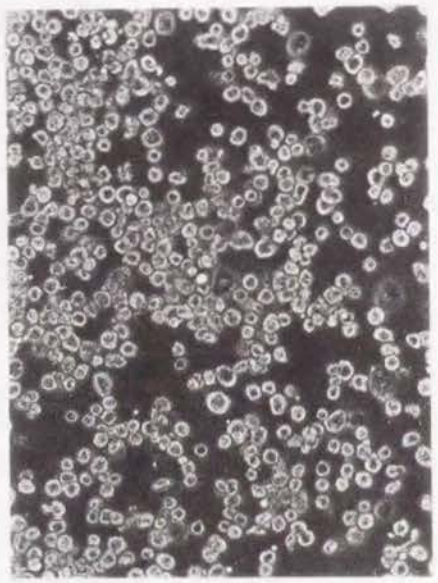
Figure 1.6. Canine fibroblasts cultured on three different films for 5 days.



HUVECs

Rat chondrocytes

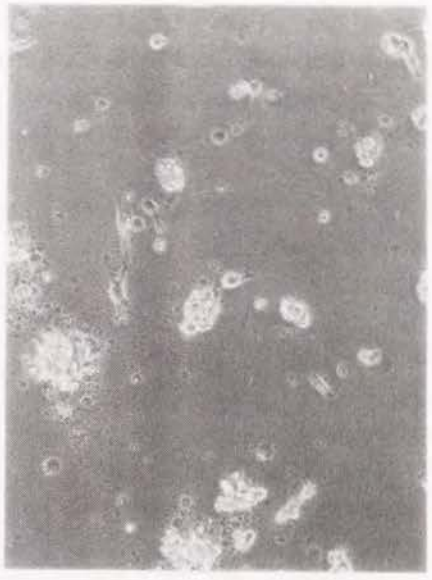
Macrophages



L cells

Figure 1.7. Shape of cells cultured on PVA for 5 days.

With FCS



Without FCS

Figure 1.8. BAECs cultured on BSA-coated cell culture dish.

Table 1.2. Glucose consumption of L cells and BAECs cultured on different substrates for 5 days.

Substrate	Glucose consumption (μg/well)		
	L cells		BAECs
	without FCS	with FCS	with FCS
PVA	158 ± 16	917 ± 55	54 ± 11
PAAm-grafted PE	162 ± 15	868 ± 43	99 ± 18
BSA-coated cell culture dish	384 ± 19	937 ± 47	212 ± 19
VAECO	256 ± 21	927 ± 51	84 ± 12
6F	379 ± 25	937 ± 53	128 ± 11
Coll-coated cell culture dish	216 ± 16	720 ± 44	153 ± 13

Table 1.3. Production of PGI_2 and TXA_2 from HUVECs cultured on various substrates in the presence of FCS for 24 hr.

Substrate	PGI_2 (ng/well)	TXA_2 (ng/well)
PVA	4.1	0.09
VAECO	4.7	0.25
6F	5.1	0.15
PET	4.8	0.16
Cell culture dish	3.2	0.19

Table 1.4. Production of type II collagen from rat chondrocytes cultured on various substrates for 3 days.

Substrate	Type II collagen (ng/cell)
PVA	0.48
PAAm-grafted PE	0.57
BSA-coated cell culture dish	0.03
6F	0.41
Cell culture dish	0.15
Coll-coated cell culture dish	0.03

Glucose consumption

Table 1.2 shows the result of glucose consumption by L cells and endothelial cells cultured for 5 days. It is clearly seen that cells consumed glucose even on the non-adherent substrate, indicating that cells could survive on this surface without attachment.

Table 1.5. Production of IL-1 in macrophages adhering on various substrates for 24 hr.

Substrate	^{3}H-thymidine incorporation (10^3 cpm/well)
PVA	850
PAAm-grafted PE	830
4F	1920
VAECO	1740
Cell culture dish	6030
Coll-coated cell culture dish	8090

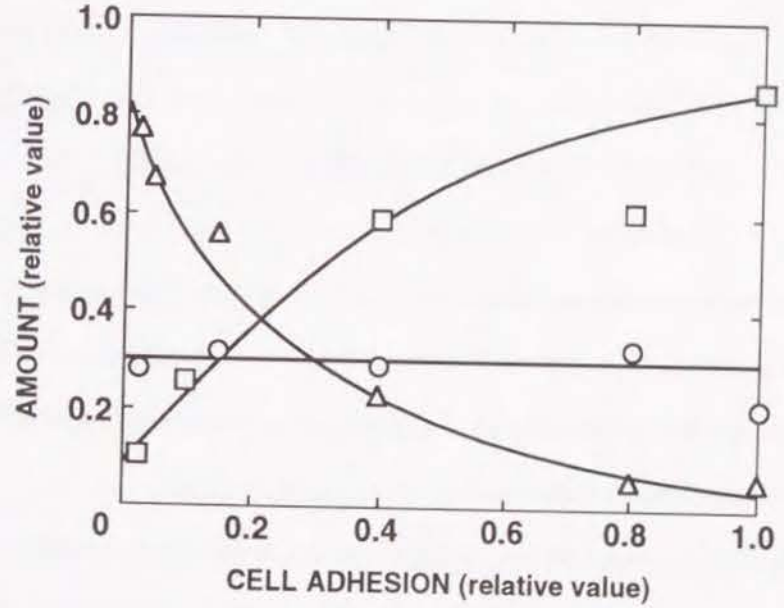


Figure 1.9. Production of (O) type II collagen from rat chondrocytes, (Δ) PGI_2 from HUVECs, and (□) IL-1 from mouse macrophages.

Cell functions

Production results of PGI_2 and TXA_2 from HUVECs are given in Table 1.3 when cultured on various substrates for 1 day. No clear production dependence on the substrates is seen for both PGI_2 and TXA_2. The amount of type II collagen produced by rat condrocytes cultured for 3 days is shown in Table 1.4. It is apparent that the chondrocytes on a less adherent surface produced a larger amount of collagen, whereas a better adherent surface produced less collagen. Table 1.5 shows the production of IL-1 in macrophages which adhered on various substrates for 1 day. It is clearly seen that the amount of IL-1 produced was proportional to the number of adherent macrophages.

DISCUSSION

A series of investigations performed by Ikada and his coworkers have already demonstrated that protein adsorption as well as cell attachment take place preferably on the polymer surfaces which have the water contact angle around 70°, unless they have ionic groups and biologically active moieties.[9] If the polymer substrates have ionic groups on their surfaces, cell adhesion will be greatly influenced, because most of the cell surfaces are negatively charged by sialic acid existing on the cell membrane. It is also likely that cells can attach directly to the substrate surface if it has biologically active

moieties such as collagen, fibronectin, and vitronectin, since the cell surface has a lot of cell adhesive receptors such as integrin and cadherin families.[4-8] Such preferable cell attachment on the surface with contact angle around 70° was observed also in this work with endothelial cells (Figure 1.3), chondrocytes (Figure 1.4), and with macrophages (Figure 1.5), which phagocytize water-soluble and water-dispersed substances which have invaded the body. It follows that this tendency may be observed for any kind of cell, as the surface property of cells does not differ much from cell to cell.

As demonstrated in Figures 1.5 and 1.6, the fibroblasts and chondrocytes underwent the three dimensional aggregation into spheroid on non-adherent substrates, whereas the L cells, endothelial cells, and macrophages did not reveal such spheroid formation, but simply two dimensional aggregation. This different dependence among cells may be explained in terms of the property inherent to the original organs from which the cells were obtained. The fibroblasts and chondrocytes exist in a three dimensional state in the skin and cartilage, respectively, while the endothelial cells are two-dimensionally lining the inner surface of the blood vessel and the macrophages work individually in the body without contacting with each other. Cells may aggregate on non-adherent substrates because they can attach to each other much easier than to the substrate which reject cell adhesion. If cells have the ability to form three-dimensional aggregates in tissues, they will form three-dimensional

spheroids also in cell culture.

The glucose consumption study denotes that cells can survive even on non-adherent surfaces at least in the initial stage of *in vitro* cell culture. The L cells and endothelial cells showed no clear dependence of glucose consumption on the contact angle of substrate.

It is well recognized that cells are influenced by the extracellular matrix (ECM).[20-22] In Figure 1.9, the dependence of the cell function on the surface property of the substrate is represented. The relative extent of cell adhesion onto the substrates was employed as a measure of the surface property. As can be seen, the cell functionality decreases with the increasing cell adhesion for the cells which formed spheroids on non-adherent substrates, such as chondrocyte and fibroblast. This trend probably has appeared because, in the state of high cell adhesion, the cells are existing as a monolayer on highly cell-adhesive substrates, leading to low intercellular communication, whereas they attach to each other to make spheroids on readily cell-adhesive surfaces, resulting in high intercellular communication. However, macrophage produced a lot of IL-1 on highly adhesive substrates. As IL-1 is an initial mediator of immune response, this interleukin may be produced upon macrophage stimulation which has occurred when the macrophage attached to the foreign materials. The endothelial cells exhibited no dependence of cell function to produce PGI_2 and TXA_2 on the surface property of substrates. It seems that PGI_2 and TXA_2 production from the cell does not depend on the intercellular communication.

As widely recognized, the cell function must be maintained to the same level as *in vivo* in order to develop hybrid-type artificial organs. The present study disclosed that the adherent surface is not always the best to keep a high level of cell function. It is very important for designing the surface properties of biomaterial suitable to the target tissue to fully understand for what purpose the biomaterial is to be used.

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Chapter 2

Behaviors of Rabbit Hepatocytes Cultured on Polymeric Substrates

INTRODUCTION

The liver is one of the most difficult organs to artificially substitute because it must carry out many functions in order to maintain complicated body homeostasis such as storage of glycogen, removal of toxins, and synthesis of many proteins including blood coagulation factors.[1] Currently, blood purification is available to improve the hepatitis and hepatic coma, but very good results have not yet been obtained because of its poor efficiency and functionality. Liver transplantation is also available for these diseases, but the recipient should receive immunosuppressive agents all through their life and avoid infection. Recently, much attention has been directed to hybrid-type artificial organs to replace irreversibly damaged metabolic organs such as the liver and pancreas.[2-20] An artificial liver with monolayered hepatocytes on collagen-coated glass plates was recently developed by Nakamura *et al.*[21] It was found that dogs lacking a liver could survive 3 days with such an artificial liver.

The general concept of hybrid-type artificial organs is to encapsulate

heterologous metabolic cells in a chamber constructed of a hydrogel membrane, in effort to protect them from immunological attacks of the recipient immune system.[22,23] However, many problems must be solved in the development of a clinically applicable hybrid-type artificial liver. One of them is to make possible the culture of hepatocytes on a large scale. It is well recognized that hepatocytes are extraordinarily difficult to culture *in vitro*, because they need many nutrients, growth factors, and oxygen. To develop hybrid-type artificial livers, we have to know the behavior and interaction between polymer substrates and hepatocytes. Koide and his coworkers[24] have reported that the primary culture of matured hepatocytes results in two distinct cell arrangements; a monolayer with a proliferative activity when cultured on a dish coated with collagen and a multicellular spheroid with poor proliferative activity when cultured on an extracellular matrix substrate.

Chapter 1 indicated that, for L cells, HeLa S_3 cells, endothelial cells, chondrocytes, and macrophages, the number of cells attaching on different substrates fitted on a single curve which exhibited a maximum at the water contact angle around 70° except for glass and substrates immobilized with biologically active proteins such as collagen, gelatin, and fibronection. These results suggest that cell adhesion to substrates depends on their surface characteristics, which, in turn, influence the cell function.

The purpose of this work is to study the behaviors of rabbit hepatocytes cultured on a number of polymeric substrates with different

surface properties. In addition to cell adhesion, cell morphology and functionality such as glucose consumption and albumin production will be studied.

EXPERIMENTAL

Polymeric substrates

As described in Chapter 1, eleven kinds of film were used after purification by Soxhlet extraction with methanol for 24 hr, followed by drying under vacuum at room temperature. All of them were commercially available. VAECO had a vinyl alcohol content of 33 mol%. Their water contact angle was measured by the sessile drop method for dried films at 25°C and 60 % relative humidity. For the cell culture on the film substrates, they were punched to disks of 1.5 cm diameter and sterilized by immersing them in 70 % ethanol for 24 hrs. To remove the ethanol completely, they were rinsed 3 times with PBS (pH=7.4).

Isolation of hepatocytes

Rabbit hepatocytes were isolated with a modified method of Seglen[25] developed for rat hepatocytes. Rabbits were carefully reared in the Research Center for Biomedical Engineering, Kyoto University according to the guidelines of Kyoto University for animal experiments. Collagenase was

kindly donated by Amano Pharmaceutical Company. A piece of rabbit liver was surgically isolated under anesthetization and perfused for 15 min with washing buffer solution (NaCl 8 g/l, KCl 0.4 g/l, $NaH_2PO_4 \cdot 2H_2O$ 0.078 g/l, $Na_2HPO_4 \cdot 12H_2O$ 0.151 g/l, Hepes 2.38 g/l, EGTA 0.19 g/l, $NaHCO_3$ 0.35 g/l, and glucose 0.9 g/l; pH=7.2). After complete removal of blood, the liver was perfused for 10 min with collagenase solution (collagenase 0.5 g/l, trypsin inhibitor 0.05 g/l, $CaCl_2$ 0.56 g/l, $MgCl_2 \cdot 6H_2O$ 0.1 g/l, and $MgSO_4 \cdot 7H_2O$ 0.1 g/l in washing buffer solution without EGTA; pH=7.5). After cutting the liver into pieces with surgical scissors in collagenase solution for 5 min, they were filtrated with a 140 mesh stainless filter. Following 5 times of washing with Eagle's MEM, the cells were seeded on a cell culture dish (Type 25020; Corning, NY, U.S.A.) coated with type I collagen. After 2 hrs of incubation, the dish was washed with PBS and the adhering cells remaining were collected with cell harvesting solution (0.25 % trypsin, 0.02 % EDTA in PBS) and then used for cell culture experiments. Primary cells were used for every experiment and all procedures were operated under a sterilized condition. Figure 2.1 shows the shape of hepatocytes used in this work.

Cell culture

Sterilized film substrates were placed in the 24 multi-well culture dish (Type 258201; Corning) and thin sterilized platinum rings of 1.5 cm diameter were put on the films to prevent their floating in the medium.

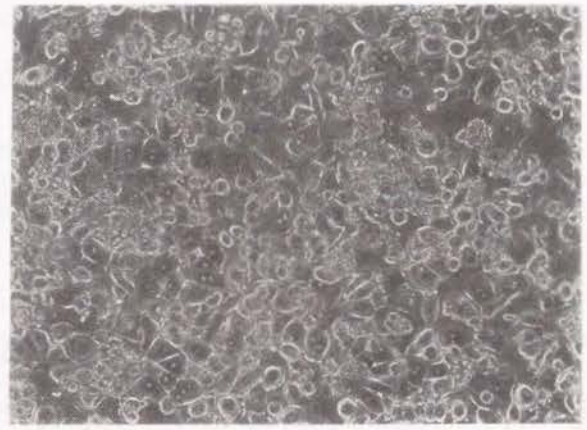

Figure 2.1. Hepatocytes cultured on cell culture dish.

Type I collagen and BSA were also coated on the 24 multi-well culture dish. All the substrates were immersed in PBS to avoid drying before use.

Cells collected by the cell harvesting solution were seeded on the substrates at a density of 5.7×10^4 cells/cm^2. After predetermined periods of time, the cell appearance was observed and the cell number was counted with light microscope. For the hepatocyte culture, William's E culture medium (Nissui Pharmaceutical Co. Ltd.) containing epidermal growth factor (10 μg/ml; Collaborative Research Inc., Bedford, MA, U.S.A.), insulin (10^{-8} M), dexamethasone (10^{-8} M), aprotinin (125 μg/ml), and 10 % FCS was used.[24] All of the cultures were maintained in a 37°C incubator equilibrated with 5 % CO_2 and 95 % air under a sterilized condition.

Measurement of cell activities

The number of cells adhering on the substrates was counted optically.

The substrates were rinsed 3 times with PBS to remove the non-adherent cells, and then the cells were stained with Giemsa solution. Cell morphology was observed microscopically without rinsing the substrates. Spheroid index, defined as the ratio of spheroid formation on the substrate, was estimated from the surface area of spheroid.

Glucose consumption was quantified from the amount of glucose in the culture medium before and after cell culture using a test kit of color reaction for diagnosis purchased from Wako Pure Chemical Industries, Ltd. Albumin production was measured from ELISA,[25] and LDH release was estimated from the amount of LDH in the culture medium using LDH monotest of Boehringer Manheim, Germany.

RESULTS

Cell adhesion

The dependence of the hepatocytes adhesion on the water contact angle of substrates is shown in Figure 2.2. Incubation was carried out for one day. The dependence is very similar to the previous result shown in Chapter 1 that cells did not adhere well not only to highly hydrophilic surfaces such as PVA and VAECO but also to highly hydrophobic surfaces such as PTFE, whereas significant cell adhesion was noticed on the surfaces with the water contact angle around 70° such as PET and on the collagen

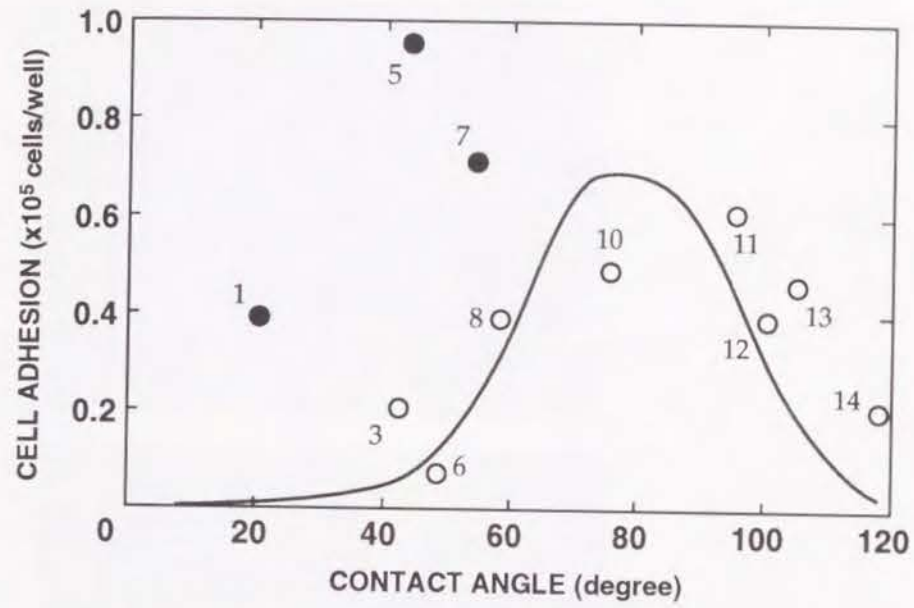

Figure 2.2. Effect of the water contact angle of film surfaces on hepatocyte adhesion. (See Table 1.1 for explanation of film material code.)

coated surface, although this surface had a low water contact angle.

Cell morphology

Figure 2.3 shows light microphotographs of the hepatocytes incubated on three different surfaces for 6 days. Apparently, cells form multicellular spheroids on the hydrated PVA surface, while they are present as a monolayer on the cell culture dish. Interestingly, hepatocytes exhibit the intermediate shape on the VAECO film. In other words, they are present both as spheroids and a monolayer on the VAECO film.

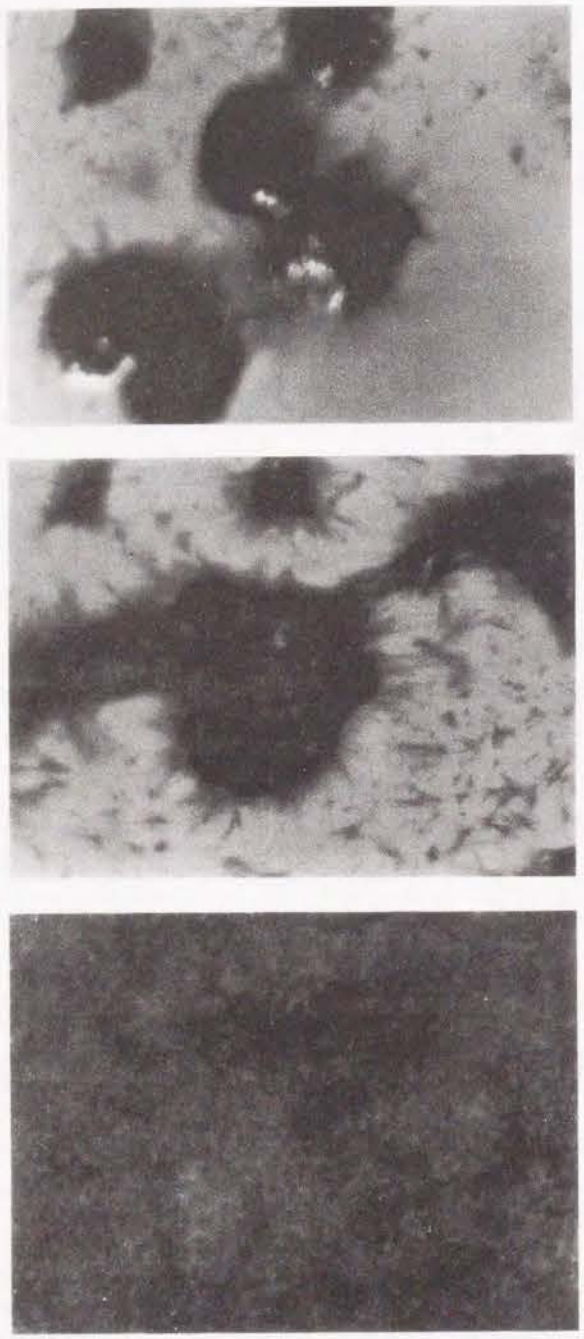

Figure 2.3. Rabbit hepatocytes cultured on three different films for 6 days.

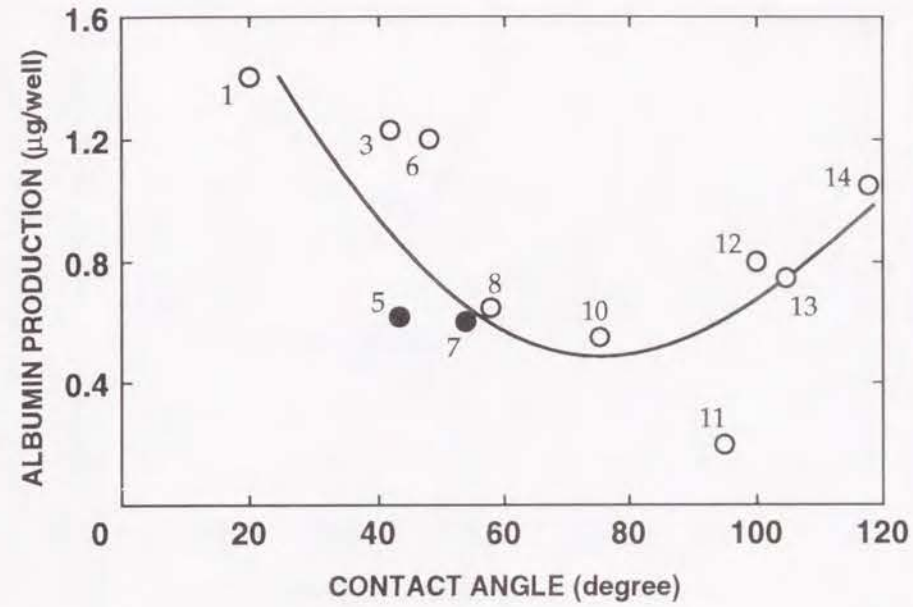

Figure 2.4. Production of albumin by rabbit hepatocytes cultured for 2-3 days on various substrates. (See Table 1.1 for explanation of film material code.)

Albumin production

The result of albumin production from the hepatocytes incubated on various substrates for 2–3 days is shown in Figure 2.4. It is seen that the albumin production depended on the contact angle of the substrate surfaces. Further, hepatocytes on less adherent surfaces produced larger amounts of albumin, while better adherent surfaces produced less amounts. Figure 2.5 shows the amount of albumin produced when incubation was carried out for 5–6 days. Apparently, the tendency observed for the above shorter culture became less significant.

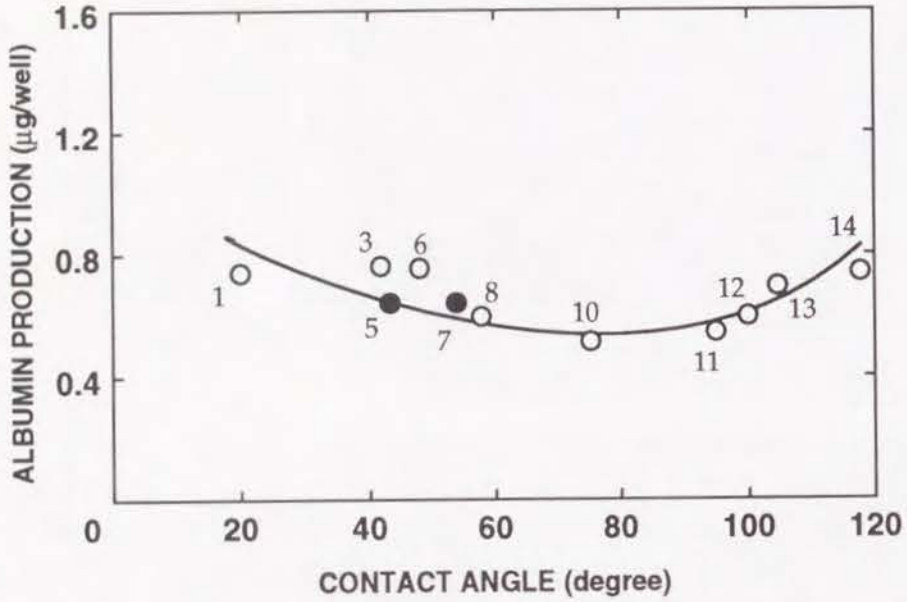

Figure 2.5. Production of albumin by rabbit hepatocytes cultured for 5-6 days on various substrates. (See Table 1.1 for explanation of film material code.)

Glucose consumption

A similar dependence on the contact angle of substrate was observed for glucose consumption of hepatocytes incubated for short periods of time. The result is given in Figure 2.6. For instance, hepatocytes on PET with a contact angle of 75°, belonging to well adherent surfaces, consumed two times less glucose than on PTFE with a contact angle of 118°, a poorly adherent substrate. However, this dependence of glucose consumption on the contact angle of substrate was reversed when cell culture was further continued. As is obvious from Figure 2.7, where the result of 5–6 day incubation is shown, hepatocytes incubated on PET consumed glucose twice

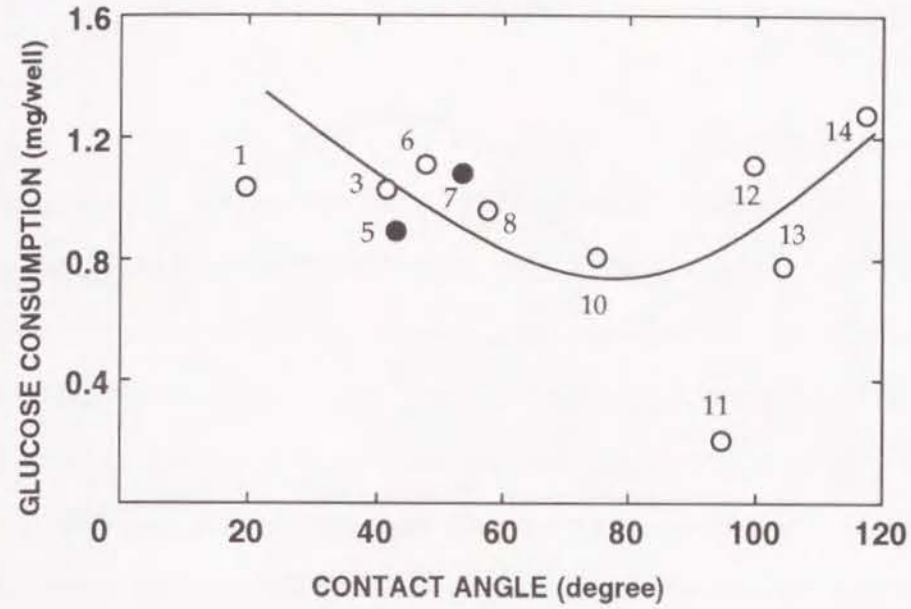

Figure 2.6. Glucose consumption by rabbit hepatocytes cultured for 2-3 days on various substrates. (See Table 1.1 for explanation of film material code.)

as much as those on PTFE.

LDH release

LDH release from the cells cultured for 5–6 days, is shown in Table 2.1. The LDH release is known as a measure of cellular necrosis. It is seen that less adherent surfaces exhibited higher LDH release.

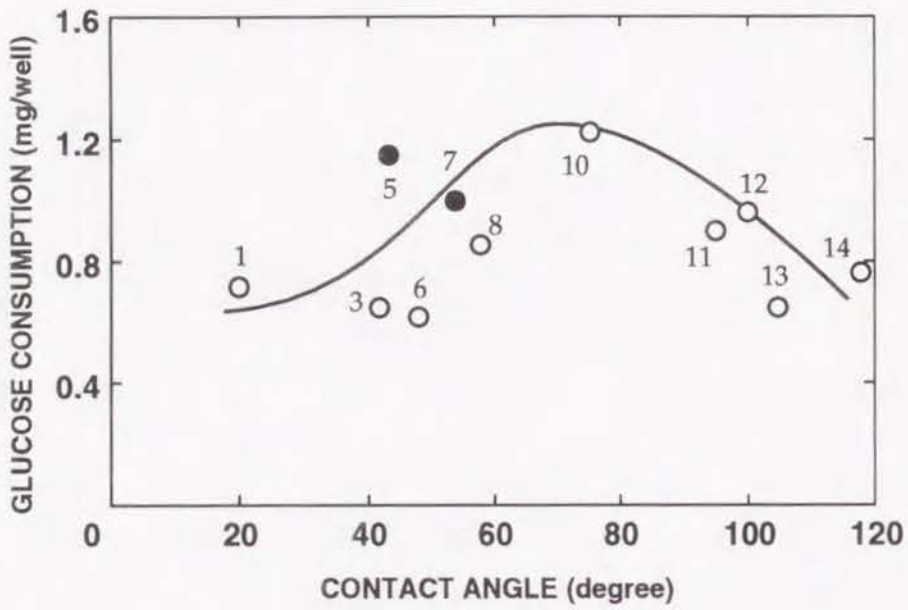

Figure 2.7. Glucose consumption by rabbit hepatocytes cultured for 5-6 days on various substrates. (See Table 1.1 for explanation of film material code.)

Table 2.1. Amount of LDH released from rabbit hepatocytes cultured on various substrates for 5-6 days.

Substrate	LDH (relative value)
PVA	1.86 ± 0.08
BSA-coated cell culture dish	1.43 ± 0.13
VAECO	1.80 ± 0.14
6F	1.88 ± 0.12
Coll-coated cell culture dish	1.00 ± 0.06

DISCUSSION

Tamada *et al.*[27] have already shown that the polymer surfaces preferable for both protein adsorption and cell attachment have a water contact angle around 70° as long as they have neither ionic groups nor biologically active moieties. This tendency was observed also in this work with hepatocytes (Figure 2.2). In general, cell surface is negatively charged because of sialic acid existing on the cell membrane. Therefore, if the substrate has ionic groups, especially cationic, cell adhesion should be enhanced. On the other hand, the cell surface also has many cell adhesion receptors such as integrin and cadherin families.[28-32] Through these surface receptors, cells can attach directly to the substrates having biologically active moieties such as collagen, fibronectin, and vitronectin. Furthermore, we should take into consideration that such receptors influence the cell function and differentiation.[30,32]

On the non-adherent substrates such as PVA, cells aggregate each other as demonstrated in Chapter 1. In the case of hepatocytes, when cultured on the extracellular matrix, the cells underwent multicellular spheroid and kept good cell function, as Koide and his coworkers[24] reported. In our work, a monolayer formation was discovered on adherent polymer substrates having the water contact angle around 70°, whereas clusters or multicellular spheroids were formed on non-adherent polymer surfaces which had very low or high contact angles against water. On the

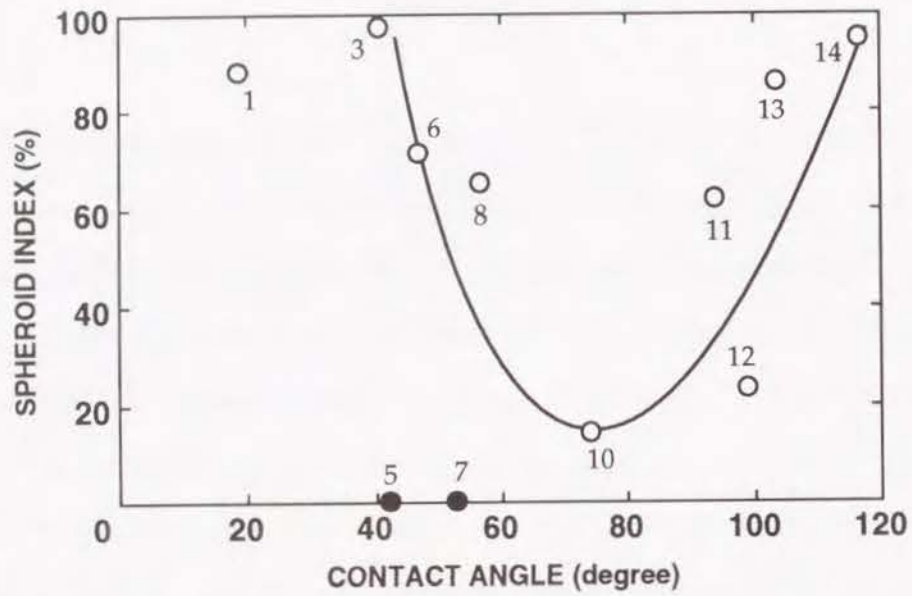

Figure 2.8. Surface area covered with rabbit hepatocytes cultured on various substrates. (See Table 1.1 for explanation of film material code.)

intermediate substrates, hepatocytes revealed both the characteristics. The typical substrate is VAECO to which hepatocytes could attach neither as a complete monolayer nor as complete spheroids (Figure 2.3). It is likely that the hepatocyte multicellular spheroid is formed as result of aggregation without adhering on the substrate. From the spheroid index plotted against the water contact angle in Figure 2.8, it is clear that the dependence of the spheroid index on the contact angle is opposite to that of cell adhesion.

Since cell function should be maintained almost at the same level as *in vivo* to develop hybrid-type artificial organs, we further investigated the hepatocyte functionality. The results given in Figure 2.4 clearly demonstrate

that the hepatocytes in multicellular spheroids have higher activity for albumin production than those at a monolayer state. However, after 5-6 day incubation, the albumin production was reduced (Figure 2.5). The results in Figures 2.6 and 2.7 and Table 2.1 suggest that necrosis increasingly occurred when culture was prolonged. Probably, insufficient supply of nutrients into the inside of the multicellular spheroids, caused by the large size of spheroid, resulted in cell necrosis.

Up to now, biomaterials have been estimated mainly on the basis of cell adhesion and growth. In the case of biomaterials to be used for hybrid-type artificial organs, parenchymal cells, such as hepatocytes, must attach on a substrate and exhibit their phenotype. Therefore, in engineering the biomaterials for this purpose, we should consider the maintenance of the cell function in addition to the cell number. Since the weight of an adult liver is about 1,500 g and the number of hepatocytes in the liver is 1.1×10^8 cells/g,[33] at least 1.5×10^{10} cells should be kept under good cell functionality in the device of the artificial liver for the clinical use. The present study suggests that hepatocytes might be used as a part of a hybrid-type artificial liver for short-term liver support. However, it does not seem realistic at present to use hepatocytes for long-term support because of the difficulty in keeping the hepatocyte functioning for a long period.

It may be concluded that spheroids are more readily formed on less adherent surfaces and produce more albumin with greater consumption of glucose at least in the initial stage of cell culture. However, increased

necrosis is observed when culture is prolonged, probably because the supply of nutrients into the inside of the multicellular spheroids becomes insufficient due to their large size.

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Chapter 3

Implantation of Hepatocyte-Seeded Biodegradable Polymers into Rabbit Peritoneal Cavity

INTRODUCTION

The aim of this chapter is to study biodegradable polymers as a scaffold for self-reconstruction of defective tissues which have the capacity to regenerate. They are, for instance, cartilages, blood vessels, bones, the peripheral nervous system, and the liver. The concept is to seed parenchymal cells of the target tissue on the polymer scaffold, followed by implantation of the cell-polymer composite as the core tissue for the organ reconstruction. There is another approach for this purpose, that is, the use of hybrid-type artificial organs, but they involve several problems such as poor viability and low capacity. In addition, as mentioned in Chapter 2, it is difficult to culture parenchymal cells, especially, hepatocytes on a scale large enough to maintain the biofuctionality for a long period. However, these problems may be solved if the tissue is self-reconstructable in the body on a scaffold seeded with the cells originated from the objective tissue.[1-4]

Poly(L-lactic acid) (PLLA), poly(glycolic acid), and their copolymers have already been used clinically as absorbable suturing materials for many

years, because they exhibit non-toxicity and minimal tissue reaction, and a wide range of physical properties and degradation rates can be varied by changing the molecular weight and the ratio of monomers in copolymer.[5,6] Lactic acid and glycolic acid, which are the biodegradation products, are metabolized to carbon dioxide and water in the body. They have also been utilized for other medical purposes such as bone plates, vascular grafts, wound covers, and carriers of the drug delivery system (DDS).[5-9]

In this study, the possibility of PLLA non-woven fabrics as the biodegradable scaffold for short-term implantation is explored. Rabbit liver is selected as the target organ, because it is easy to isolate the cells from the organ and to achieve autografting. The cell-scaffold composite is implanted into the rabbit and the factors governing cell viability are attempted to be identified.

EXPERIMENTAL

Polymer scaffold

PLLA non-woven fabrics with the fiber diameter of 5 μm were kindly supplied by Gunze Co., Ltd., Kyoto, Japan. Two sheets of fabrics were cut to have round shapes of 1.5 cm diameter, overlapping each other, and then sewn around by surgical suture. As a control, non-biodegradable PET non-woven fabrics (fiber diameter=1.5 μm; Nissho, Co., Ltd., Osaka,

Japan) were used. All the fabrics were sterilized by 70 % ethanol overnight, and then washed with PBS (pH=7.4).

Isolation of hepatocytes

Rabbit hepatocytes were isolated by the method described in Chapter 2.[10] A piece of rabbit liver was surgically excised under anesthetization without sacrificing the animal for later autograft implantation. After perfusing for 15 min with washing buffer solution, the liver was further perfused for 10 min with 0.5 % collagenase solution. The tissue was cut into pieces with surgical scissors in collagenase solution for 5 min and filtrated with a 140 mesh stainless filter. Following 5 times of washing with Eagle's MEM, the cells were seeded on a cell culture dish coated with type I collagen. After 2 hrs of incubation, the dish was washed with PBS and the adhering cells remaining were collected and then used for cell culture experiments. Primary cells were always used for every experiment and all procedures were operated under a sterilized condition.

Cell culture

For the *in vitro* study, the isolated hepatocytes were seeded on the PLLA fabrics in 24 multi-well cell culture dishes at a density of 5.7×10^4 cells/cm^2. After predetermined periods of time, the cell appearance was observed with a light microscope. Cells were counted by quantifying the activity of LDH in the cells after complete digestion using a test kit for

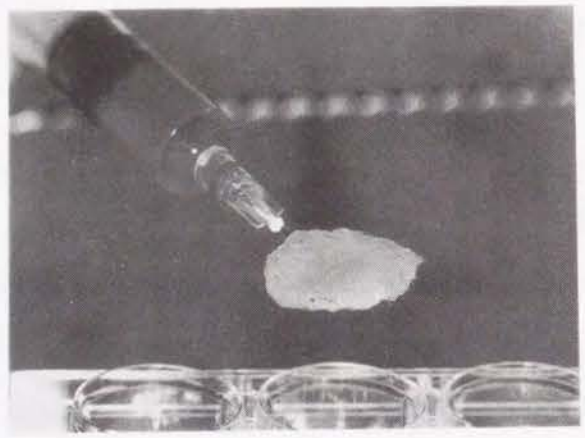


Figure 3.1. Injection of hepatocytes into PLLA non-woven fabrics.

clinical use (LDH monotest).[11] In addition, the concentration of serum albumin in the supernatant of culture dishes was measured using an ELISA technique.[12] Williams' E culture medium was used with FCS, insulin, and epidermal growth factor.[13]

Implantation

The cell suspension containing 1×10^6 cells was injected into non-woven fabrics with a needle (Figure 3.1). The hepatocyte-polymer composites were implanted into the rabbit peritoneal cavity as autograft, allograft, or allograft with a daily injection of an immunosuppressive agent (FK506; Fujisawa Pharmaceutical Company, Osaka, Japan[14]) (Figure 3.2). The composites were fixed to the peritoneal membrane by surgical suture. After predetermined periods of time, they were explanted and subjected to

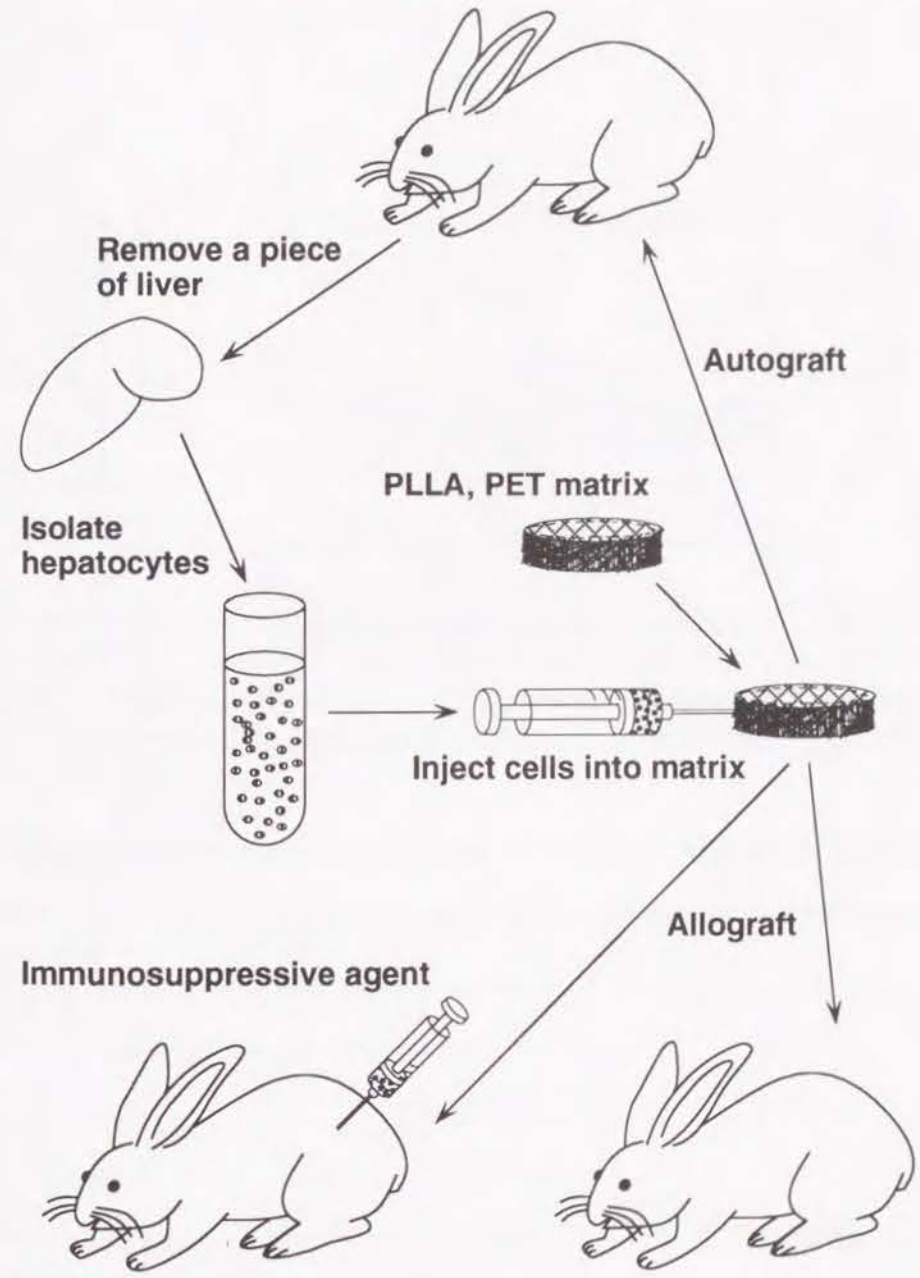


Figure 3.2. Implantation of rabbit hepatocyte-polymer composite.

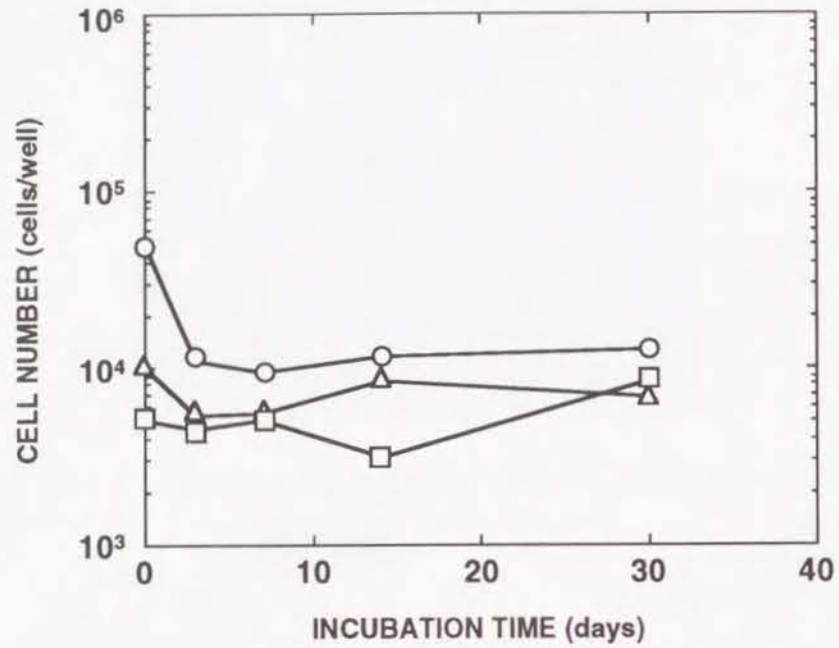


Figure 3.3. Growth curve of (O) $5x10^4$, (Δ) $1x10^4$, and (□) $5x10^3$ rabbit hepatocytes seeded into PLLA non-woven fabrics.

histological study. The minced liver tissues within PLLA scaffold were also implanted into the rabbit peritoneal cavity.

RESULTS

In vitro study

Figure 3.3 shows the *in vitro* growth of the hepatocytes seeded into the PLLA non-woven fabrics. It is clearly evident that the hepatocytes did not grow on the PLLA substrate. Albumin production from the hepatocytes

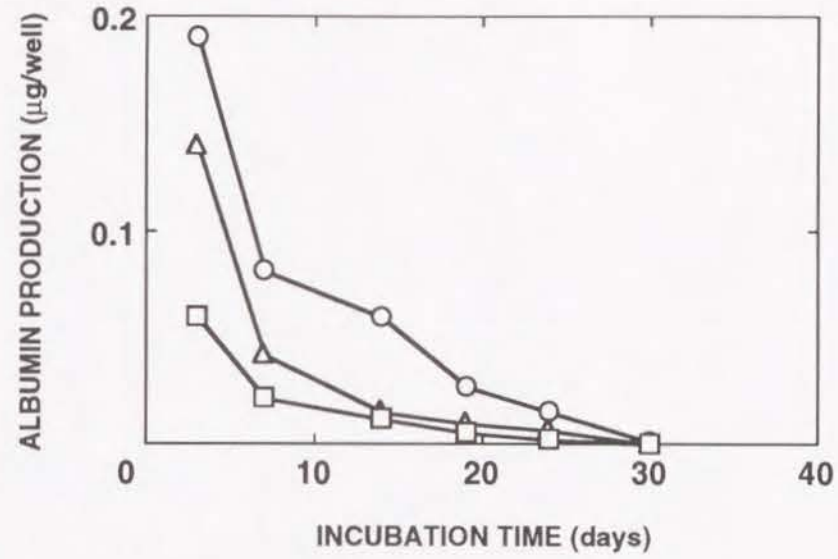


Figure 3.4. Time dependence of albumin production from (O) $1.3x10^5$, (Δ) $8.4x10^4$, and (□) $6.5x10^4$ rabbit hepatocytes in PLLA matrix.

incubated in PLLA fabrics for 1 month is shown in Figure 3.4. Obviously, albumin production decreased gradually as the incubation time became longer. This indicates that the hepatocytes in PLLA fabrics lost the function under this *in vitro* culture condition.

In vivo study

Minced liver tissue

Figure 3.5 shows the histological cross-section of minced liver tissues in PET non-woven fabrics implanted as an autograft. The liver tissues remained intact after 1 week of implantation, but many inflammatory cells

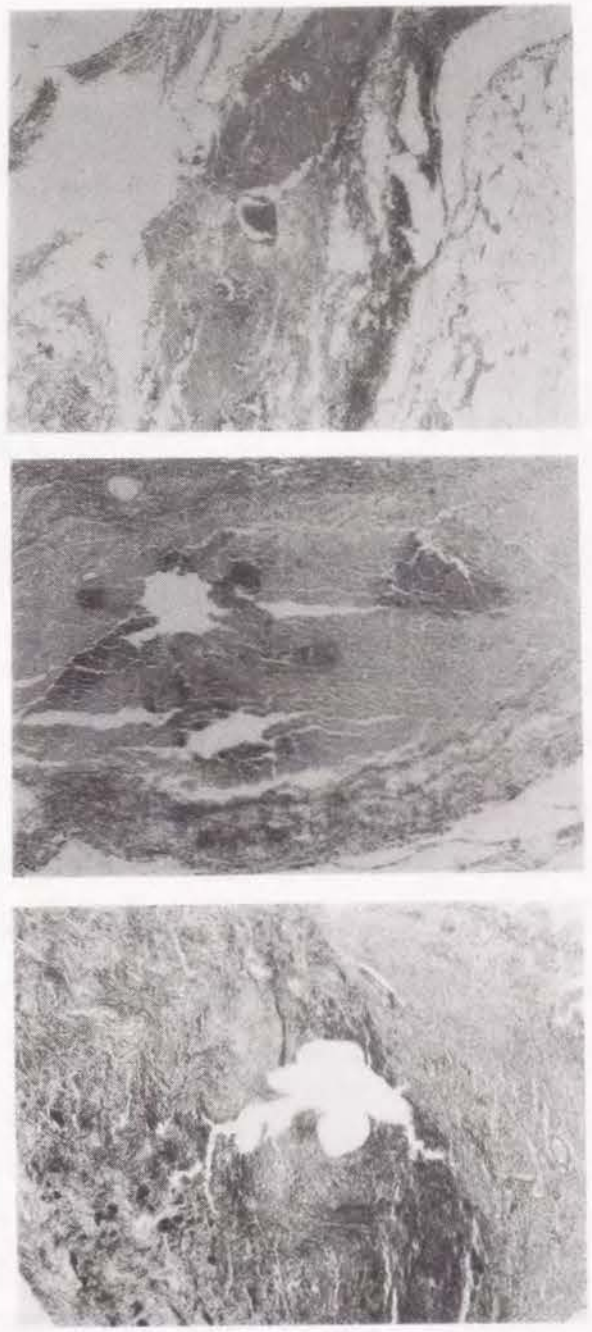


Figure 3.5. Cross-sections of minced liver tissues in PET non-woven fabrics implanted in rabbit peritoneal cavity.

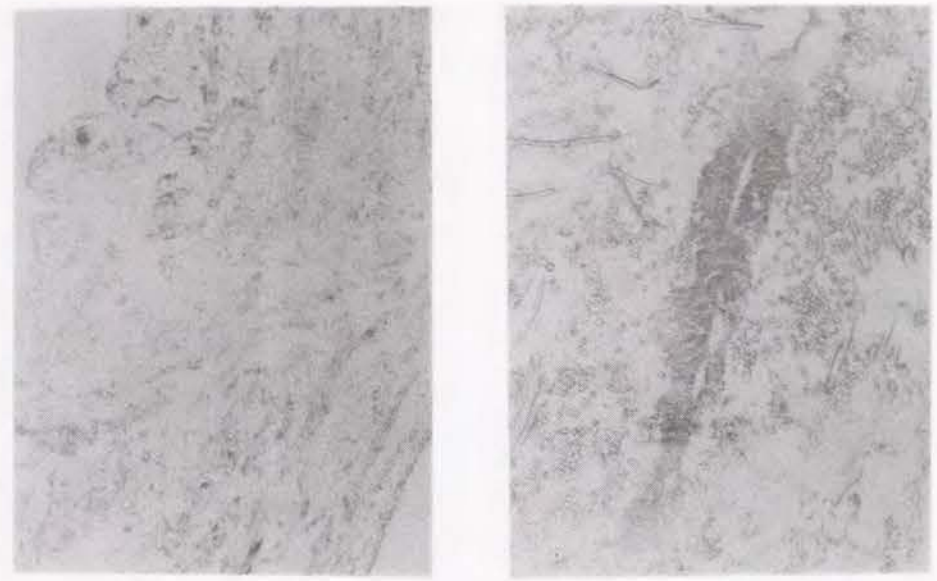
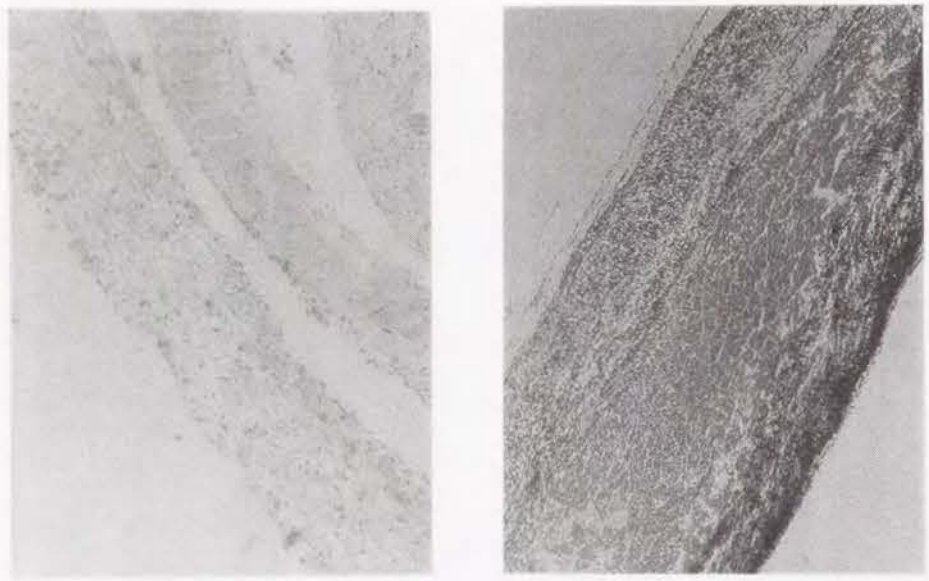


Figure 3.6. Cross-sections of PLLA non-woven fabrics 1 week after implantation.

such as macrophages and giant cells appeared around them after 2 weeks, and no liver tissues remained after 1 month. This suggests that the minced liver tissues could not survive likely because of the poor supply of nutrients and oxygen.

Autograft

Histologically stained cross-sections of hepatocyte-PLLA and PET composites autografted in the rabbit peritoneal cavity for 1 week are shown in Figures 3.6 and 3.7, respectively. Apparently, the cells remained in both types of the non-woven fabrics. This was more clearly seen for the PET



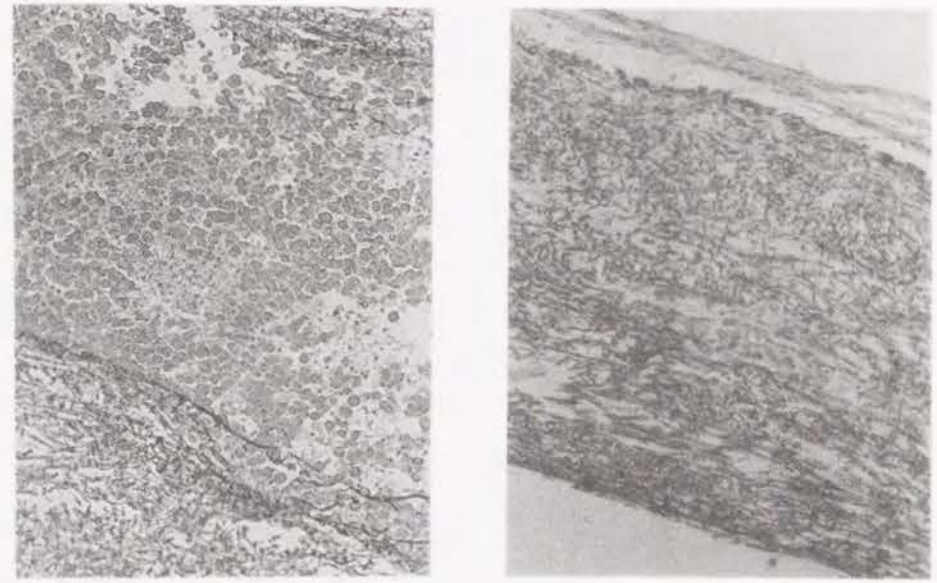
Non-seeded Hepatocyte-seeded

Figure 3.7. Cross-sections of PET non-woven fabrics 1 week after implantation.

fabrics. The difference between the PLLA and the PET matrix may be explained in terms of degradation of PLLA and the size and density of the fibers. Figure 3.8 shows the cross-section of hepatocytes in PET fabrics implanted for 2 and 4 weeks. As is seen, the inflammatory response was low even after 1 month of implantation.

Allograft

Figures 3.9 and 3.10 give the results of the histological study on allografting without any immunosuppressive agents. The hepatocyte-PLLA and PET composites were implanted in the rabbit peritoneal cavity as



2 weeks 1 month

Figure 3.8. Cross-sections of PET non-woven fabrics after autoglaft implantation.

allografts. Apparently, cells were attacked by the immune system and many small blood vessels were seen around the composites. After 1 month of implantation, almost all of the cells disappeared with a strong inflammatory reaction.

Allograft with immunosuppressive agent

Figure 3.11 shows the cross-section of a hepatocyte-PET composite matrix implanted as an allograft with an immunosuppressive agent for 1 month. It is seen that hepatocytes survived without immunorejection in spite of the allograft implantation, clearly indicating the effectiveness of the

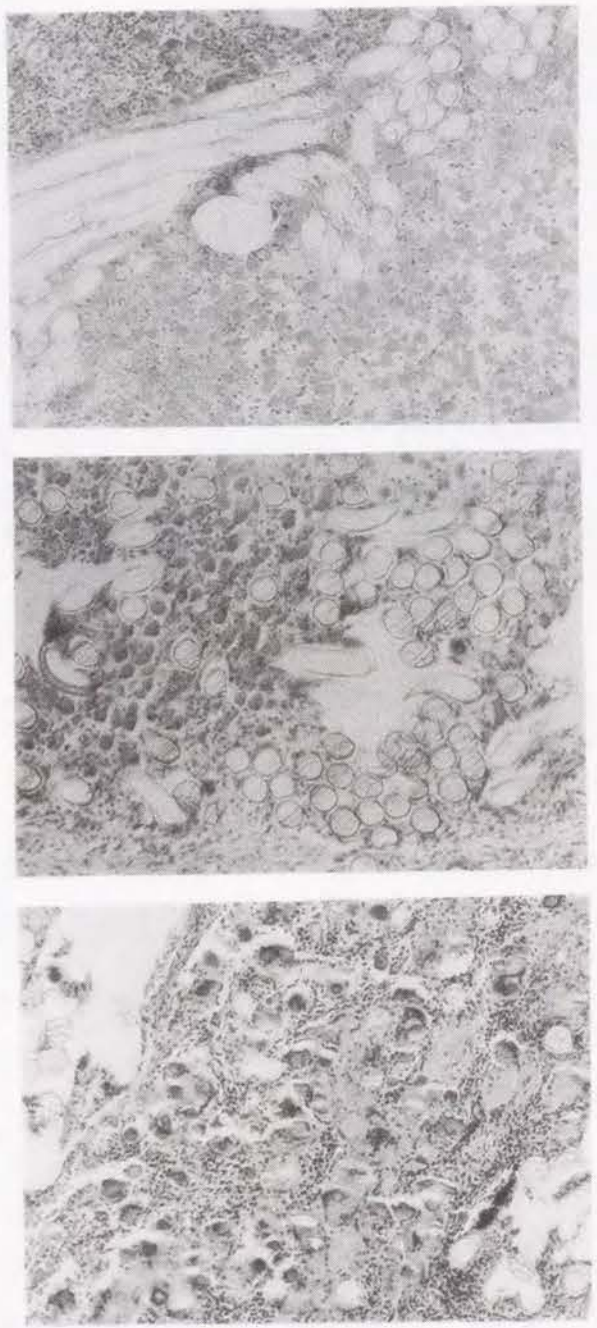


Figure 3.9. Cross-sections of hepatocyte-seeded PLLA non-woven fabrics after allograft implantation.

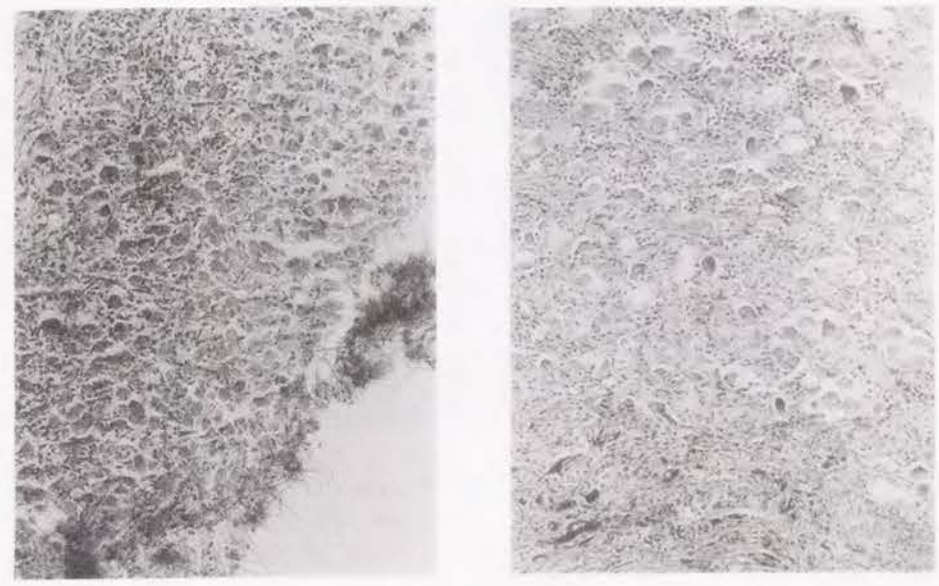


Figure 3.10. Cross-sections of PET non-woven fabrics after alloglaft implantation.

administered immunosuppressive agent.

DISCUSSION

There are many organs which have a regenerating capacity, such as the skin, cartilages, bones, the peripheral nervous system, and the liver. For example, the liver can regenerate within only a few weeks, even if 90 % of the organ has been removed. If any cell that is essential to replace the lost function of the organ is isolated from the patient and proliferated *in vitro*, the

Figure 3.11. Cross-section of hepatocyte-seeded PET non-woven fabrics 1 month after allograft implantation with immunosuppressive agent.

use of this cell would be very effective for the organ reconstruction.[1-4] Recently, skin reconstruction using collagen matrix and epidermal cells has been investigated.[15-17] However, the parenchymal cell is generally thought to be difficult to grow while keeping the biofunction under *in vitro* conditions because it needs lots of nutrients, growth factors, and oxygen. Hepatocyte injections into the portal vein and spleen without *in vitro* cultivation have been reported to support liver functions.

The present study was attempted to explore the possibility of the organ reconstruction by hepatocyte implantation using PLLA non-woven fabrics. As PLLA is biodegradable and relatively easy to control the degradation rate, this polymer has been widely used in the field of DDS. We embedded hepatocytes in the PLLA matrix which would be absorbed after cell proliferation in the body, if everything responded in the body as

expected.

As described in Chapter 2, hepatocyte attachment depends on the surface wettability of substrates so far as it has neither ionic groups nor biologically active moieties. A monolayer was formed on adherent polymer substrates, whereas clusters or multicellular spheroids were formed on non-adherent polymer surfaces. Many hepatocytes adhered *in vitro* on the PLLA surface with the intermediate cell shape, because the water contact angle of this polymer is about 55°, which indicates that the polymer surface is an intermediary between the non-adherent and the adherent one. However, the maintenance of cell function was very difficult on this substrate (Figure 3.4). This finding suggests that incubation of hepatocytes on a large scale while maintaining the biofunctionality *in vitro* is very difficult unless a special tissue culture medium or a stromal support layer is used.[18]

On the other hand, the present *in vivo* studies revealed that the composites of hepatocytes and biodegradable polymers show promise as the scaffold for liver reconstruction, if the hepatocytes are supplied with sufficient nutrients and oxygen. In the case of an allograft, an immunosuppressive agent was necessary for survival to avoid the immunorejection of the recipient. However, it should be pointed out that the present results were obtained only from short-term implantation and, in addition, that the cell function and cell growth were not yet evaluated *in vivo*. It seems likely that angiogenesis to the place where the composites are implanted is essential to maintain the seeded cells life and to allow rapid

tissue reconstruction. For this purpose, angiogenic factors such as the fibroblast growth factor (FGF) may be useful. A number of recent investigations on the relation between cell differentiation and the extracellular matrix (ECM) clearly demonstrate that FGF induces ECM digestion and parenchymal cell proliferation.[19] Further, it is highly possible that non-parenchymal cells may also play an important role in liver regeneration.[20,21] A pure population of parenchymal cells was used in this study, but cells other than the target cell may also be needed for complete tissue regeneration.

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Chapter 4

New Cartilage Formation *In Vivo* Using Chondrocytes Seeded on PLLA

INTRODUCTION

A potential need exists for the development of materials which help organ and tissue regeneration. In Chapter 3, it was revealed that biodegradable polymer was promising as the scaffold of hepatocytes for liver reconstruction. In that study, hepatocytes were seeded on PLLA fabrics and implanted in the rabbit peritoneal cavity. While hepatocytes remained alive after 1 month of implantation, appreciable proliferation was not observed, probably because the growth of hepatocytes is very difficult in environments different from the intact liver.

In this chapter, a chondrocyte–PLLA composite is employed in an attempt to regenerate cartilage. Chondrocytes are parenchymal cells like hepatocytes, but exhibit good proliferation and cartilage formation even *in vitro* if the culture condition is adequate.[1,2] Therefore, it may be easier to reconstruct cartilage tissue than liver. Cartilage replacements are required, especially in maxillofacial, orthopedic, and plastic surgery. Mostly, silicone prosthesis and autologous rib bones are used for this purpose, but some

problems are involved in the cartilage replacement, such as infection at the interface between the implanted material and the tissue, and lack of the donor site filled with fibrous cartilage tissue.[3] Therefore, a most ideal replacement would be to use the natural cartilage tissue,[4-7] especially the chondrocytes of the target region after isolation from the patient and proliferation *in vitro* culture.

This chapter studies the feasibility of rat chondrocytes grown on a synthetic polymer template to regenerate three dimensional neocartilage through assessment of the initial features of the cell growth in the course of the generation. As described in Chapter 3, an immunosuppressive agent was necessary in the case of allografts for survival to avoid the immunorejection of the recipient. In this chapter, nude mice are used to eliminate this problem. A nude mouse is a kind of mutant animal with immunodeficiency because it has no thymus and therefore no immunoreaction initiated by T cells. For comparison, rats are also used in this study.

EXPERIMENTAL

Polymeric scaffold

Similar to the study described in Chapter 3, PLLA non-woven fabrics were used as the polymer scaffold. They were cut to have round shapes of 1.5 cm diameter, overlapping each other, and then sewn around by surgical

suture. All of the fabrics were sterilized by immersing in 70 % ethanol overnight, and then washed with PBS. To promote blood vessel formation, the PLLA scaffold was coated with basic fibroblast growth factor (bFGF; Kaken Pharmaceutical Co. Ltd., Tokyo, Japan) by immersing in the bFGF solution.

Chondrocytes

Chondrocytes were isolated from rat costal cartilage with the method described in Chapter 1.[8] Costae were removed from sacrificed rats and the cartilage was isolated. The isolated cartilage was minced by surgical scissors and immersed in 0.25 % trypsin–0.05 % collagenase solution for 1 hr. After washing with PBS, the treated pieces of cartilage were immersed in 0.02 % EDTA solution for 1 hr. After washing, they were put on a cell culture dish, incubated for about 3 weeks without floating in the medium, and then the migrated cells were collected by the cell harvesting solution.

Cell culture

For the *in vitro* study, the isolated chondrocytes were seeded on various polymeric substrates in 24 multi-well cell culture dishes at a density of 5.7×10^4 cells/cm^2. The substrates used include PVA, coll-coated cell culture dish, commercial plastic sheet for cell culture, PE, PET, PTFE, and PLLA. After predetermined periods of time, the cells were counted by quantifying the activity of LDH in the cells after complete digestion using a

test kit for clinical use (LDH monotest).[9] In addition, the concentration of type II collagen in the supernatant of culture dishes was determined using an ELISA technique.[10] Eagle's MEM was used as culture medium with 10 % fatal calf serum.[2]

Implantation

Animals were carefully reared in the Research Center for Biomedical Engineering, Kyoto University according to the guidelines of Kyoto University for animal experiments. The chondrocyte suspension containing 1×10^6 cells was injected into the non-woven fabrics with a needle. The chondrocyte-polymer composite was implanted into the Wister male rat peritoneal cavity as an allograft or allograft with a daily injection of an immunosuppressive agent (FK506).[11] In the same way, chondrocyte-polymer constructs and controls without chondrocytes were surgically transplanted into the back of nude male mice subcutaneously, respectively. After predetermined periods of time, they were explanted and subjected to histological study to evaluate the cell adhesion, proliferation, and cartilage matrix secretion.

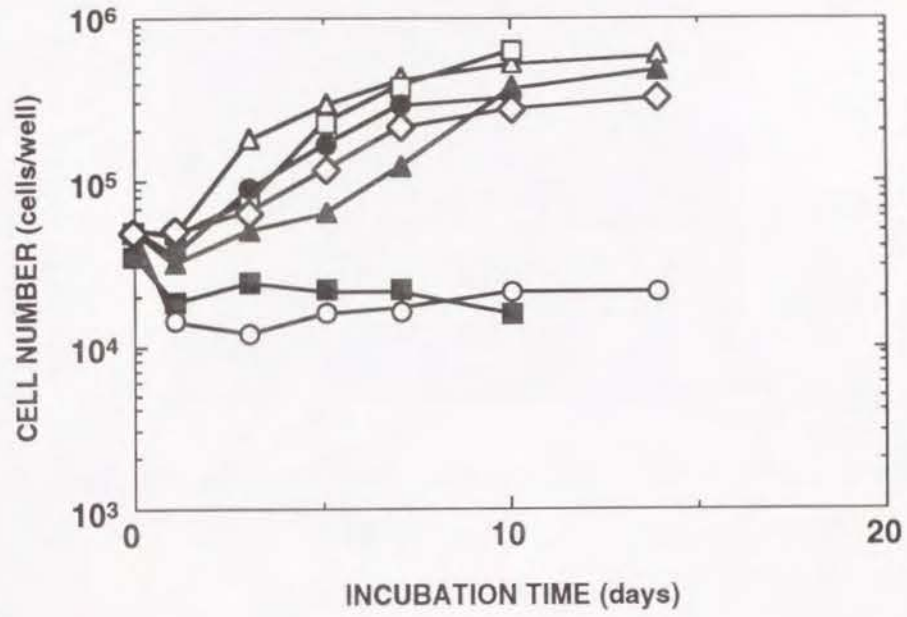


Figure 4.1. Growth curve of rat chondrocytes cultured on (○) PVA, (△) coll-coated culture dish, (□) commercial sheet for cell culture, (●) PE, (▲) PET, (■) 6F, and (◇) PLLA.

RESULTS

In vitro **study**

Figure 4.1 shows the growth curve of chondrocytes cultured on various polymeric substrates at the initial cell concentration of one tenth of the confluent. Apparently, chondrocytes proliferated on adherent substrates including PLLA, but not on non-adherent substrates such as PVA and PTFE. About 10 days after cultivation, they reached the confluent concentration in the culture dish.

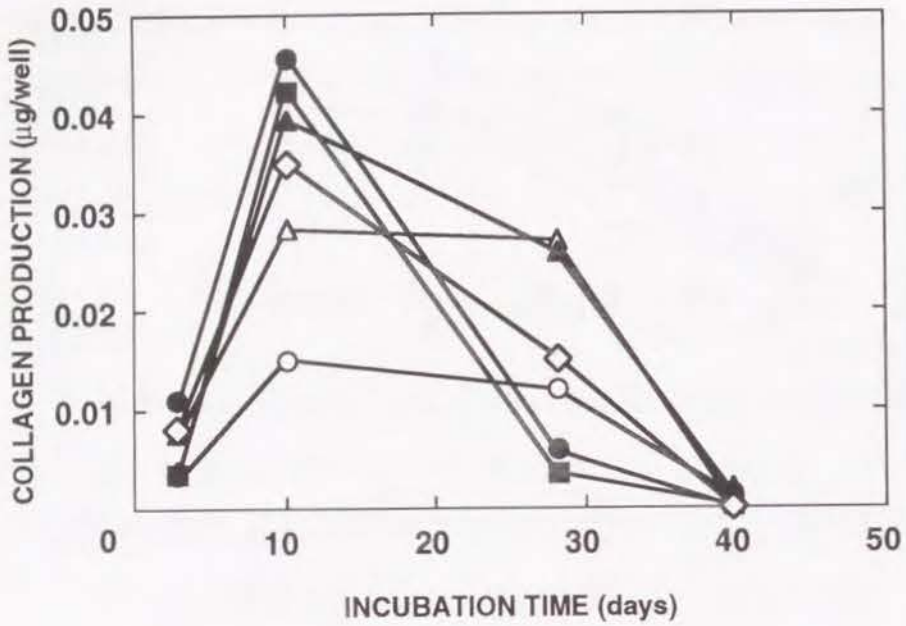


Figure 4.2. Time dependence of type II collagen production from rat chondrocytes cultured on (O) PVA, (Δ) coll-coated culture dish, (●) PE, (▲) PET, (■) 6F, and (◇) PLLA.

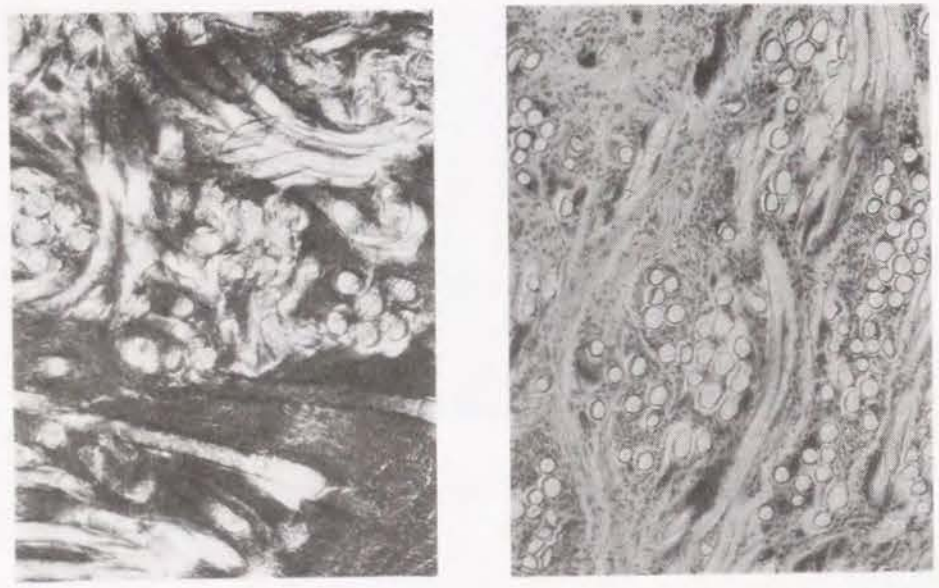
Figure 4.2 shows the time dependence of production of type II collagen, the most abundant protein in cartilage, from rat chondrocytes incubated on various substrates. The chondrocytes produced collagen on the largest amount 10 days after incubation on all the substrates, but after the 10th day the production diminished gradually. The time dependence of collagen production was not dependent directly on the cell adhesive property of the substrate surface.

The light and scanning electron microphotographs of cultured chondrocytes adhering to the PLLA scaffolds 2 hrs after seeding are given

Figure 4.3. Light microphotograph of the cultured chondrocytes attached to the PLLA scaffold.

Figure 4.4. Scanning electron microphotograph of the cultured chondrocytes attached to the PLLA scaffold.

in Figures 4.3 and 4.4, respectively. The attached cells appeared as round or flat shapes on the scaffold. It seems that the seeded chondrocytes adhere



Without immunosuppressive agent | With immunosuppressive agent

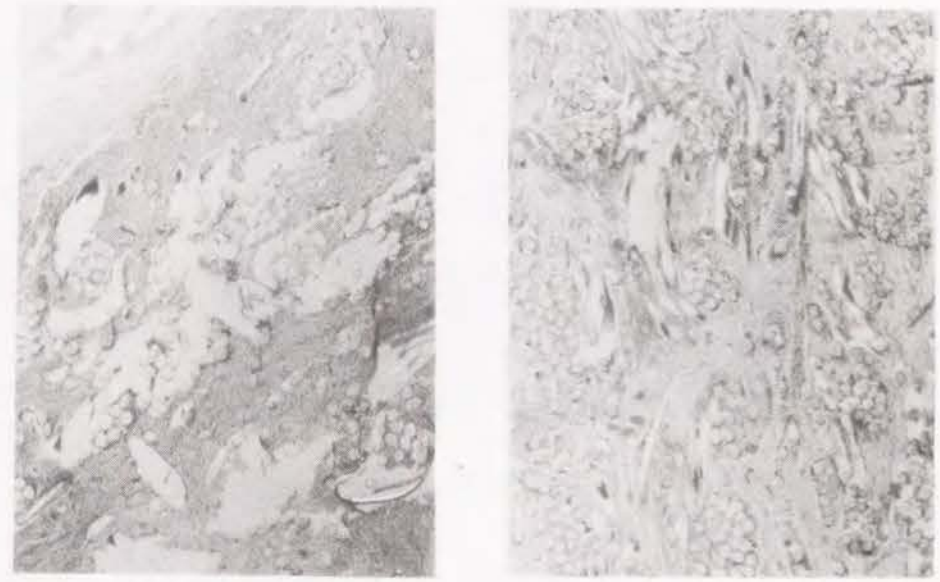
Figure 4.5. Cross-sections of chondrocyte-seeded PLLA non-woven fabrics 1 month after allograft implantation.

well onto the synthetic biodegradable scaffold.

***In vivo* study**

Implantation to rats

The result of the histological study on the allografting of chondrocyte–PLLA composite in the rat peritoneal cavity with and without the immunosuppressive agent after 1 month of implantation is given in Figure 4.5. Apparently, cells were attacked by the immune system if the immunosuppressive agent was absent, indicating no survival of chondrocytes. In contrast, chondrocytes could survive when the



Without bFGF | With bFGF

Figure 4.6. Cross-sections of chondrocyte-seeded PLLA non-woven fabrics 2 weeks after allograft implantation.

immunosuppressive agent was administered.

Figure 4.6 shows the cross–sections of a chondrocyte–PLLA composite matrix coated with bFGF after 2 weeks of implantation. It is clearly seen that the implanted matrix was surrounded by many blood vessels, if the fibroblast growth factor was present.

Implantation to nude mice

A nude mouse with a subcutaneous implant of chondrocytes seeded on the PLLA fabrics is shown in Figure 4.7. The implant could maintain the exact dimension of the original polymer scaffold. Histologically, the implant

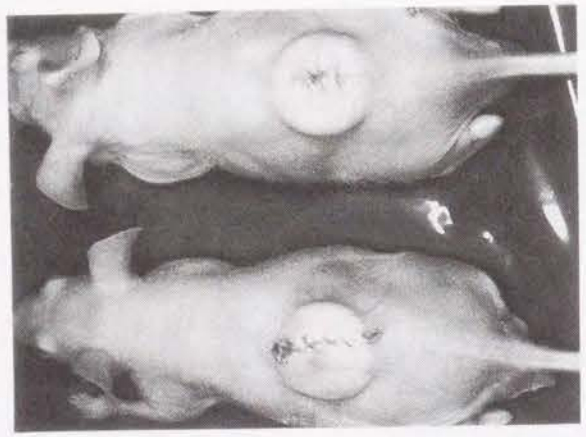


Figure 4.7. Nude mice with a subcutaneous implant of chondrocytes on a PLLA fabrics.

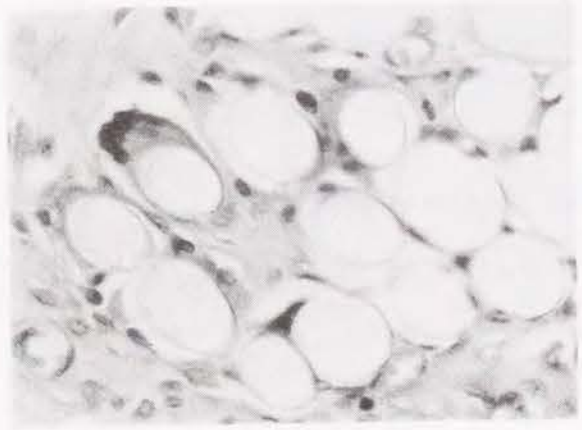


Figure 4.8. Explant histology of chondrocytes grown on PLLA for 3 weeks.

appeared infiltrated by host cells, such as erythrocytes in new blood vessels, lymphocytes, multinucleated giant cells, and fibroblasts. The result is given

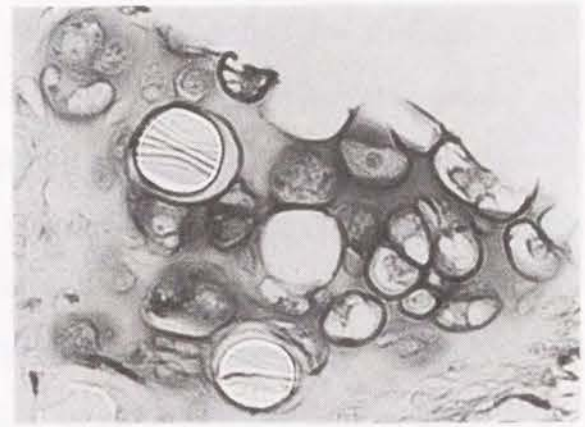


Figure 4.9. Chondrocyte-PLLA composite 2 weeks after implantation to nude mouse.

in Figure 4.8. A fibrous capsule with a thick 3–8 cell layer was observed surrounding all of them, indicating a mild inflammatory response of the host to the implant at the early implantation stage. Further, histological analysis of chondrocyte–polymer explants exhibited that the seeded cells spread dispersively throughout the scaffold. In particular, Figure 4.9 indicates that the specimen implanted for 3 weeks showed the formation of new matured cartilage. This is evidenced by the round chondrocytes in lacunae.

DISCUSSION

Very recently, the use of biodegradable polymers to regenerate metabolic organs such as the liver and pancreas and to reconstruct structural

tissues like cartilage and bone by cell transplantation has attracted attention.[5,12-14] To regenerate and maintain the organ function, individual cells are collected from the donor organ or tissue of the patient and the cells are attached to a polymer scaffold by a culture technique. The resulting cell–polymer composite is implanted at a site where the cells can grow and effectively express their function.

In Chapter 3, a hepatocyte–PLLA complex was studied to determine whether or not it was effective for liver regeneration. As demonstrated in that chapter, hepatocytes did not undergo any cell growth and gradually diminished their function of albumin production on the PLLA substrate *in vitro*. In this chapter, chondrocyte growth and function were estimated *in vivo*. It is possible that the factors necessary for the cell growth and function expression are provided automatically in the *in vivo* environment. As shown in Figures 4.1 and 4.2, rat chondrocyte showed good proliferation on adherent polymer substrates like PLLA and type II collagen production under the *in vitro* condition. The collagen production became maximal on the 10th day after cultivation. This is simply because the number of cells increased in this period to the highest level. It decreased gradually after the 10th day and the cells reached the confluent state. This indicates that chondrocytes produced less amounts of collagen under the confluent condition.

It is very difficult to transplant a cell–polymer complex as an autograft to a rat because the animal is too small for this experiment. In this

chapter, the complex was implanted as an allograft to another rat peritoneal cavity. The result was similar to that of hepatocyte–polymer composite described in Chapter 3, but an immunosuppressive agent was necessary for cell survival to avoid the immunorejection of the recipient. In the case of hepatocytes, a multicellular colony was seen in the scaffold, especially in PET non–woven fabrics, whereas seeded cells spread throughout the scaffold dispersively in the present study (Figures 4.5 and 4.8). Both hepatocytes and chondrocytes revealed multicellular three dimensional aggregation on non–adherent polymeric substrates as demonstrated in Chapter 1. This difference between hepatocytes and chondrocytes may denote that hepatocytes tend to form multicellular spheroids more than chondrocytes.

As mentioned in Chapter 3, quick formation of blood vessels around the transplanted tissue would be necessary to maintain the seeded cells life and to promote tissue reconstruction. Therefore, bFGF was used in this experiment as an angiogenic factor.[15] A lot of small blood vessels were observed around the matrix when bFGF was coated on the PLLA scaffold (Figure 4.6). This suggests that the use of angiogenic factor is very effective for blood vessel formation around the complex, but the influence of bFGF on the cell growth and function is not clear in this experiment. Many investigations recently reported that bFGF also induces parenchymal cell proliferation and differentiation.[16,17]

When the chondrocyte–PLLA composite was transplanted into nude mice, injection of an immunosuppressive agent was not necessary even in

the allograft implantation (Figure 4.8). A thin layer fibrous capsule was observed surrounding all of the implanted composites, and the inflammatory response of the host to the implants was mild, because the nude mouse does not have a thymus but is provided with the immunoprotein-mediated immunosystem. From the clear histological staining with thionin which can bind to negatively charged glycosaminoglycanes in cartilage,[18] it is obvious that the implanted cells exhibited their phenotype and formed new matured cartilage (Figure 4.9). On the contrary, implantation of polymer scaffolds without cultured chondrocytes did not result in formation of new cartilage.

These results suggest that the chondrocytes seeded onto the PLLA scaffold can proliferate, express their distinct phenotype, and mature gradually when they are implanted into the host for about 3 weeks. It may be concluded that the composites of chondrocytes and biodegradable polymer are very promising for cartilage reconstruction.

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Chapter 5

Preparation of PVA Hydrogel Membranes for Hybrid–Type Artificial Organs

INTRODUCTION

PVA hydrogels have been investigated for such medical applications as vascular grafts, artificial cartilages, and keratoprostheses,[1–5] because they are low in toxicity, protein adsorption, and cell adhesion in comparison with other conventional polymers.[5,6] Therefore, we selected PVA hydrogel as the immunoseparation membrane. To meet this requirement, crosslinks should be introduced on the chains of PVA as it is soluble in water unless there are microcrystalline regions. Several methods have been reported for preparation of the PVA hydrogel. They involve radiation, chemical, and physical methods.[7–9]

In this chapter, the PVA hydrogel to use for hybrid–type artificial organs is prepared by the chemical and radiation methods. As mentioned in the General Introduction, our hybrid–type artificial organs are fabricated by encapsulating heterologous metabolic cells in a chamber to protect them from immunological attacks by the recipient's immune system.[10,11] To ensure the proper functions of the cells, the membrane should be permeable to

substances which are essential for their viability. Simultaneously, the membrane should allow easy diffusion of some proteins secreted by the metabolic cells, such as insulin and albumin, but should be practically impermeable to the components of the recipient's immune system such as immunoglobulins, complements, and immune cells.

The purpose of this chapter is to prepare the PVA hydrogel membrane and study the influence of the parameters associated with the crosslinking density of the hydrogel network on the mesh size of PVA membranes. The crosslinking means employed here are electron beams and GA. Reinhart and Peppas[12] presented important evidence that the crosslinked structure of swollen PVA acts as a molecular screen for the diffusion of large solutes. They established that the mesh size of the crosslinked polymer network affects the rate of diffusion. In other words, with the decreasing mesh size the solute diffusion was slowed or even stopped, depending on the size of the solute.

EXPERIMENTAL

Preparation of membranes

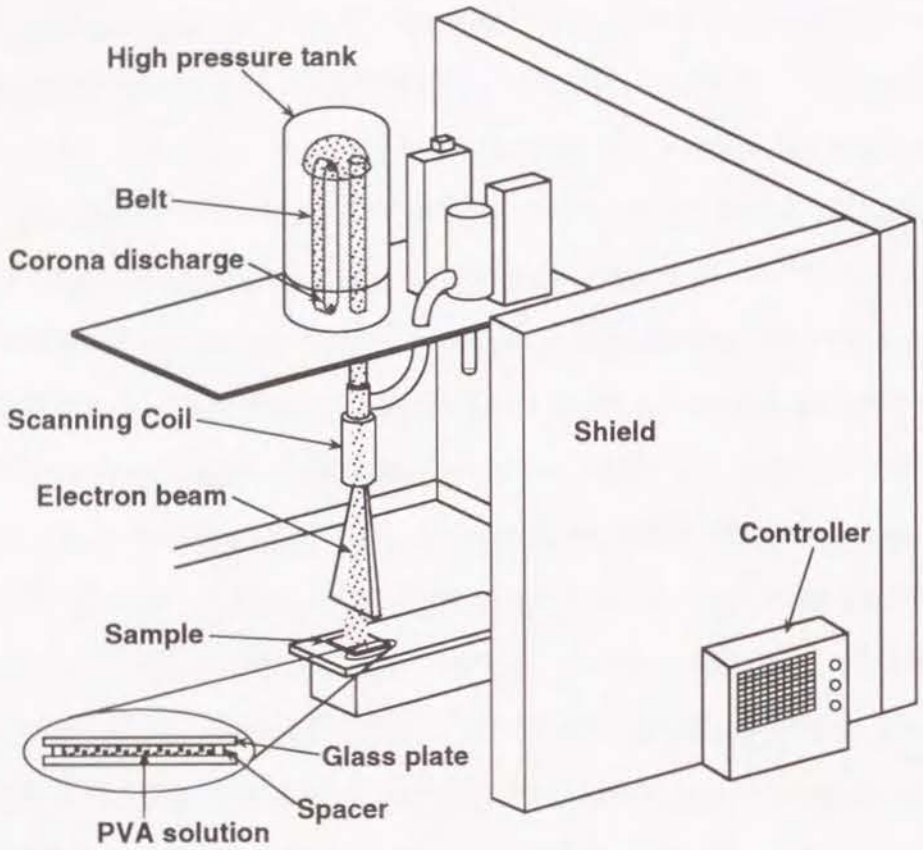
Radiation method

Atactic PVA supplied by Unitika Co., Ltd., Osaka, Japan and Kuraray Co., Ltd., Osaka, Japan had number-average molecular weights, $\overline{M}_{n(0)}$, of

0.48×10^5 and 2.47×10^5, and weight-average molecular weights, $\overline{M}_{w(0)}$, of 1.15×10^5 and 5.99×10^5, respectively. Both polymers had a polydispersity index of 2.4 and a degree of saponification of 99.5 mol%. 2-5 wt% aqueous solutions of PVA were prepared by mixing the PVA powders with double-distilled water and by heating the system in an autoclave at 120°C for 1 hr under a pressure of 1520 hPa. Prior to irradiation, the polymer solutions were carefully degassed to remove oxygen and thus avoid the degradation of PVA that might occur together with crosslinking.[8] The apparatus used for irradiation is illustrated in Figure 5.1. Flat molds consisting of two siliconized glass plates, located 1 mm apart, were filled up with the PVA solution by means of a syringe. A thin, coarse polyester mesh (150 mesh, 129 μm opening; Aicello Chemical Co. Ltd., Toyohashi, Aichi, Japan), subjected to corona discharge pretreatment of both sides (15 kV, 1 min, under dry air) was used to reinforce the hydrogel that formed upon irradiation of the polymer solution. Irradiation was carried out with electron beams from a 1.5 MeV Van de Graaff accelerator at a dose rate of 0.3 kGy/s. To avoid undesirable phenomena, such as formation of gas bubbles and cracking of hydrogels due to internal stress, the radiation dose was limited to about 30 kGy.

GA method

PVA membranes crosslinked with GA were prepared by impregnating the polyester mesh with the PVA solution containing GA (25



Fugure 5.1. Van de Graaff accelerator.

vol% solution, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and hydrochloric acid (0.5 M HCl), a catalyst, after carefully mixing and removing the air bubbles by 3–4 min centrifugation at 4°C and 3000 rpm. They were then placed in a container at room temperature and 100 % relative humidity and left for 24 hrs until the crosslinking reaction was completed. For each experiment, the GA concentration, expressed in mole of GA per mole of PVA repeating unit, varied from 0.007 to 0.037. In a

typical preparation of a hydrogel membrane at a GA concentration of 0.012, 10 μl of 25 vol% GA solution and 120 μl of 0.5 M HCl were added to 3 g of 3 wt% PVA solution successively while stirring.

Evaluation of the molecular weight between crosslinks

Hydrogel membranes were swollen in distilled water in a small beaker at 37°C until a thermodynamic equilibrium at a constant weight was attained, unless otherwise specified. The hydrogels were then dried at 80°C for 5 days to a constant weight, W_p. A crosslinked gel will swell up to an extent which is dependent on the number of effective chains per unit volume, V_e[13]:

$$V_e = -\frac{\frac{v}{V_1}[\ln(1-V_{2,s})+V_{2,s}+\mu V_{2,s}^2]}{V_{2,r}[(\frac{V_{2,s}}{V_{2,r}})^{\frac{1}{3}}-\frac{1}{2}(\frac{V_{2,s}}{V_{2,r}})]} \quad (1)$$

where v is the specific volume of bulk PVA in the amorphous state (=0.788 cm^3/g), V_1 is the molar volume of solvent (=18 cm^3/mol), $V_{2,r}$ and $V_{2,s}$ are the polymer volume fraction of the gel in the relaxed and the swollen state, respectively, and μ is the Flory–Huggins polymer–solvent interaction parameter for a corresponding value of $V_{2,s}$ at 37°C, calculated according to a literature.[14] The values of $V_{2,r}$ and $V_{2,s}$ can be calculated as:

where Q_p is the bulk density of polymer (=1.269 g/cm^3), V_p is the volume of

$$V_p = \frac{W_p}{Q_p} \; ; \quad V_{2,r} = \frac{V_p}{V_r} \; ; \quad V_{2,s} = \frac{V_p}{V_s} \tag{2}$$

the polymer, and V_r and V_s are the volume of the relaxed and the swollen gel, respectively. V_e is related to $\overline{M}_c$ (number-average molecular weight between crosslinks) and $\overline{M}_{n(0)}$ (number-average molecular weight of the starting polymer) through the following equation:

$$V_e = \frac{1}{\overline{M}_c} - \frac{2}{\overline{M}_{n(0)}} \tag{3}$$

Calculation of mesh size of network

Mesh size, ξ, was calculated on the basis of equation[15]:

$$\xi = V_{2,s}^{-\frac{1}{3}} (\overline{r_0^2})^{\frac{1}{2}} \tag{4}$$

where $(\overline{r_0^2})^{1/2}$ is the average end-to-end distance of the unperturbed (solvent-free) state:

$$(\overline{r_0^2})^{\frac{1}{2}} = l (2 \frac{\overline{M}_c}{M_r})^{\frac{1}{2}} C_n^{\frac{1}{2}} \tag{5}$$

where l is the C-C bond length (=1.54 Å), M_r is the molecular weight of the repeating unit of PVA (=44), and C_n is the characteristic ratio for PVA

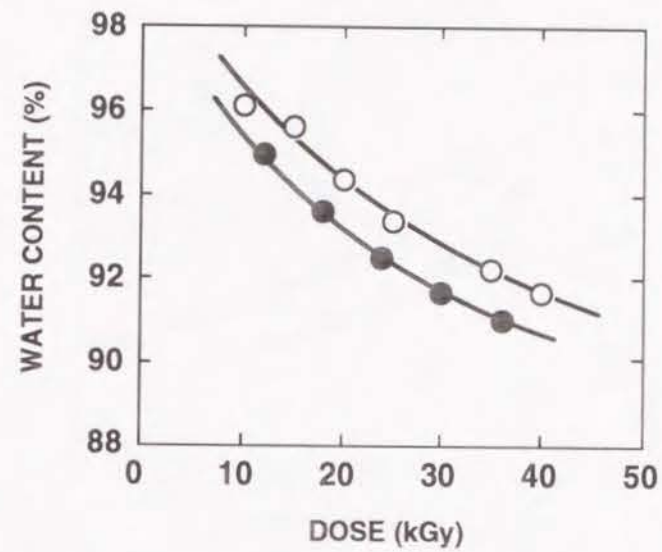


Figure 5.2. Dependence of the water content on the radiation dose for the radiation crosslinked hydrogels from 3 wt% PVA with a molecular weight of (O) 0.48×10^5 and (●) 2.47×10^5 (swollen at 37°C).

(=8.9).[15]

RESULTS

Radiation crosslinking

The dependence of the water content of the radiation crosslinked PVA hydrogel membranes, swollen at 37°C on the radiation dose is shown in Figure 5.2. The initial concentration of the PVA solution to be irradiated was 3 wt% and $\overline{M}_{n(0)}$ were 0.48×10^5 or 2.45×10^5. The higher the radiation

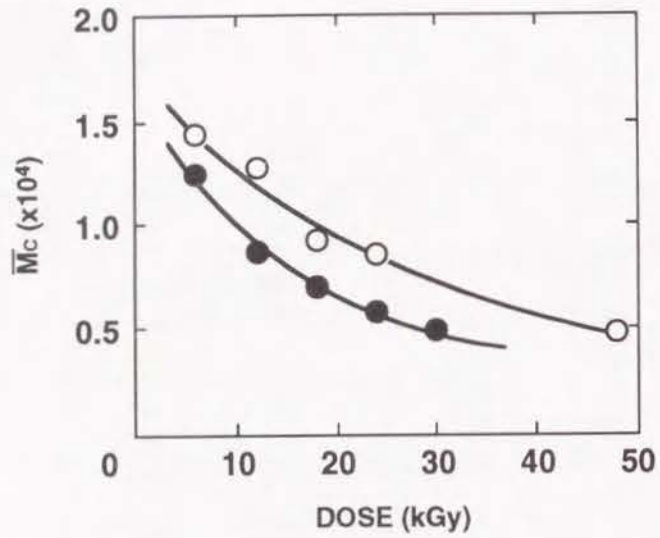


Figure 5.3. Dependence of number-average molecular weight between crosslinks, $\overline{M}c$, on the radiation dose for the radiation crosslinked hydrogels from 3 wt% PVA with a molecular weight of (O) 0.48x10^5 and (●) 2.47x10^5.

dose, the lower the water content. As can be seen, the water content depends not only on the radiation dose, but also on the initial molecular weight of PVA when compared at the same dose.

The effect of radiation dose on $\overline{M}_c$, calculated from the water content of PVA membranes, is shown in Figure 5.3. Apparently, the decrease in $\overline{M}_c$ with the dose was larger for the higher molecular weight PVA when compared at the same dose. The change in the molecular weight between crosslinks, caused by crosslinking, brought about the change in the size of macromolecular mesh which decreased with the increasing dose of ionizing radiation. The result is shown in Figure 5.4.

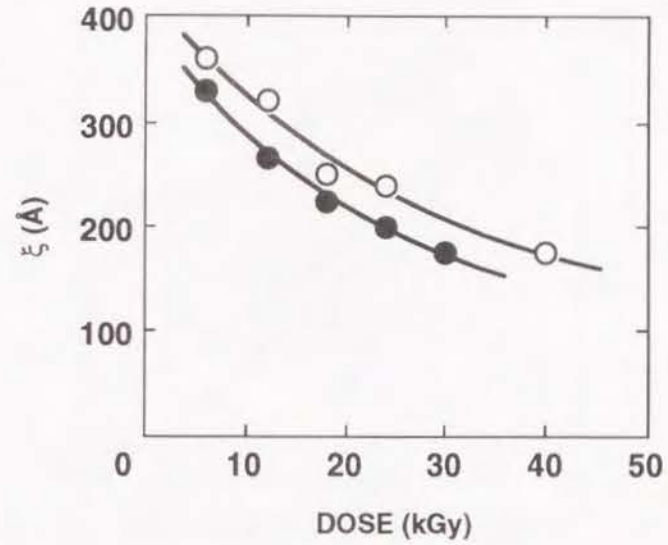


Figure 5.4. Mesh size, ζ, versus radiation dose for the radiation crosslinked hydrogels from 3 wt% PVA with a molecular weight of (O) 0.48x10^5 and (●) 2.47x10^5.

Chemical crosslinking

Figure 5.5 shows the dependence of the water content on the GA concentration for chemically crosslinked hydrogel membranes of PVA with $\overline{M}_{n(0)}$ of 2.47×10^5. The swelling was carried out at 25 and 37°C. Although the water content decreased with an increase in concentration of added GA, the PVA concentration before crosslinking had a more distinct effect on the water content of the resulting gels. The higher the initial concentration of PVA in the solution for crosslinking, the larger the decrease in the water content when compared to that of the same GA concentration. The lower the water content of gel found at a higher temperature might be ascribed to the well-known fact[16] that water becomes a poorer solvent for PVA as the

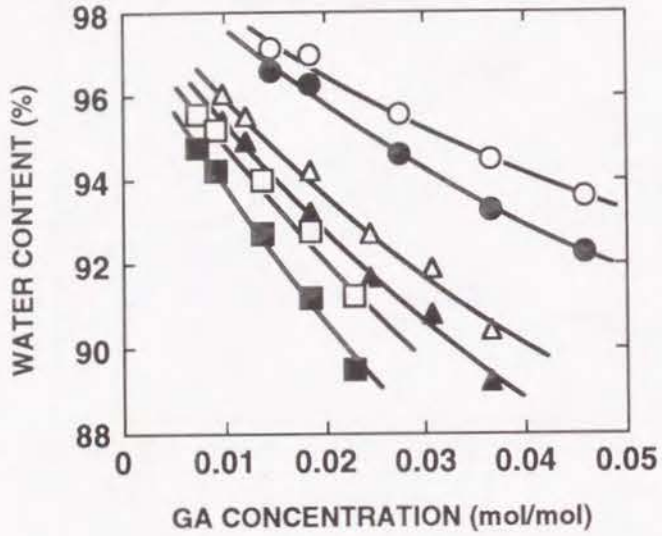


Figure 5.5. Dependence of the water content on the GA concentration for (O,●) 2 wt%, (Δ,▲) 3 wt%, and (□,■) 4 wt% PVA with a molecular weight of 2.47x10^5 (Open symbols show swelling at 25°C and closed symbols at 37°C).

temperature of the PVA–water system increases. Figures 5.6 and 5.7 show the effect of GA concentration on $\overline{M}_c$ and the mesh size of PVA hydrogels. The increase in the GA concentration led to the marked decrease both in $\overline{M}_c$ and mesh size. However, the change of these structural parameters was larger for the more concentrated PVA solution when compared to that of the same GA concentration.

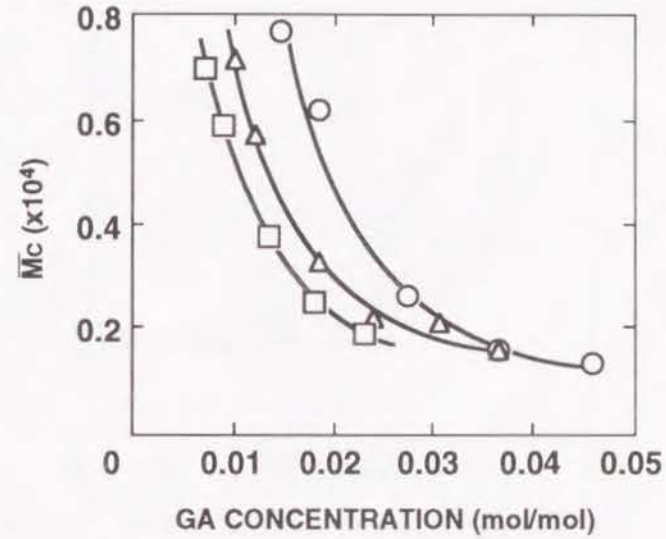


Figure 5.6. Effect of the GA concentration on the number-average molecular weight between crosslinks, $\overline{M}c$, for (O) 2 wt%, (Δ) 3 wt%, and (□) 4 wt% PVA with a molecular weight of 2.47x10^5 (swollen at 37°C).

DISCUSSION

The PVA hydrogel is macroscopically nonporous, but microscopically consists of networks of macromolecular chains (macromolecular mesh). The area available for the diffusion of solutes is the open space in the macromolecular mesh[17]. The crosslinking density affects not only the volume of the water phase through which solute diffusion occurs, but also the mesh size which controls the solute passing through the polymer network. Since the size difference among the penetrant molecules which are

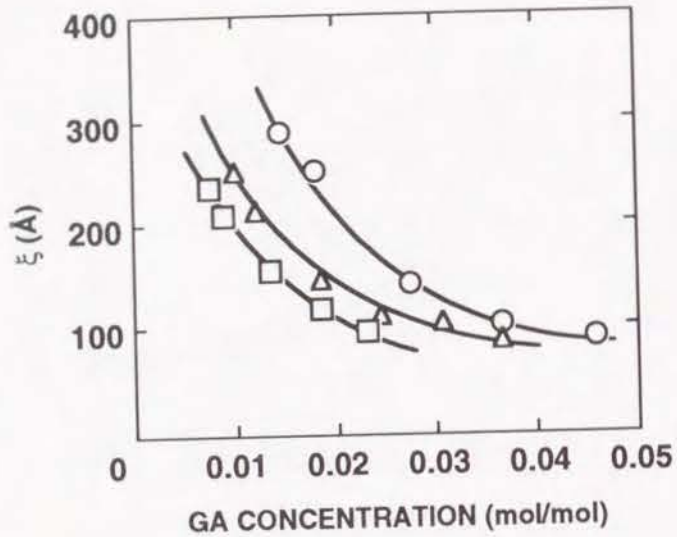


Figure 5.7. Mesh size, ζ, versus GA concentration for the chemically crosslinked hydrogel membranes from (O) 2 wt%, (Δ) 3 wt%, and (□) 4 wt% PVA with a molecular weight of 2.47×10^5 (swollen at 37°C).

very important for hybrid-type artificial organs is quite large, it seems possible that there is an optimal mesh size which can avoid permeation of large elements such as immunoglobulins and immune cells, yet still allow free transport of nutrients and low molecular weight proteins.

As demonstrated above, both the radiation and the chemical crosslinking of PVA in an aqueous solution resulted in formation of insoluble gel membranes. The possible reaction mechanisms of these crosslinkings are shown in Figure 5.8. The water content of gel decreased with the increase in the dose of radiation (Figure 5.2) and the concentration of GA (Figure 5.5). The water content of hydrogel must be related to the

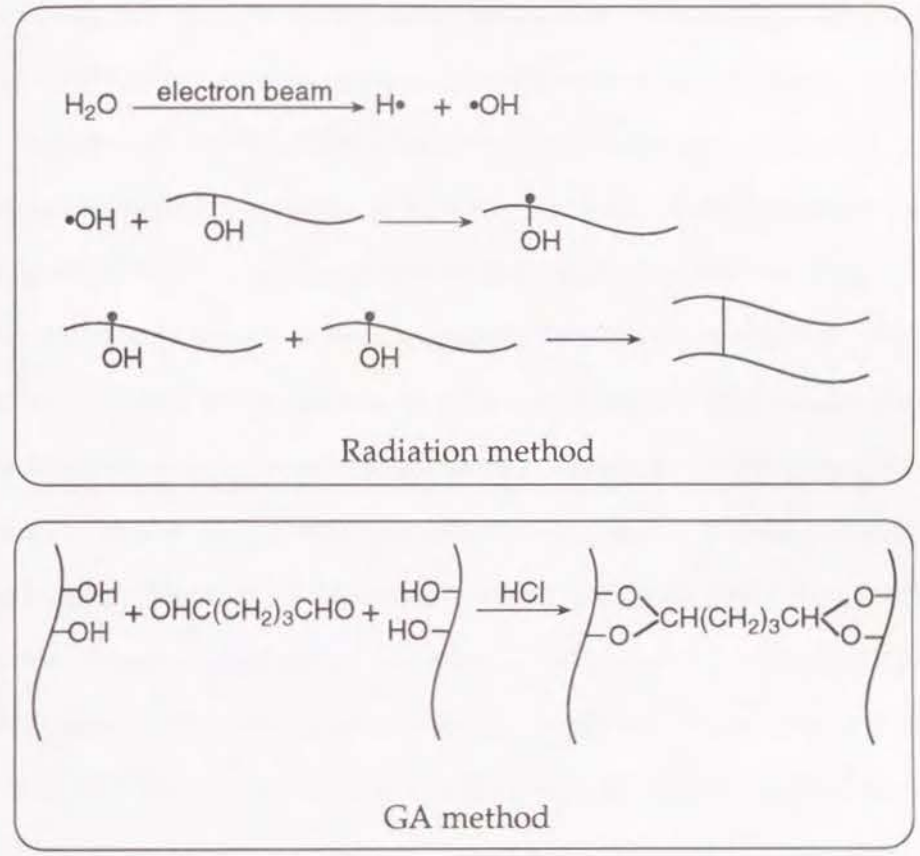


Figure 5.8. Reaction mechanisms of the crosslinking of PVA in aqueous solution.

morphological change in the polymeric network caused by the formation of crosslinks. The open spaces around the crosslinked macromolecular chains are filled with water. $\bar{M}_c$ and ξ are the parameters which describe the architecture of the hydrogel network. The increase in the density of crosslinks will thus lead to a decrease in $\bar{M}_c$ and ξ, thereby resulting in a decrease in the water content.

Figures 5.2 and 5.5 demonstrate that the increase in the dose of ionizing radiation or GA concentration forms more densely crosslinked PVA hydrogel networks with the lower amount of water in it. Furthermore, Figure 5.2 shows that the irradiation of PVA in aqueous solution leads to a more densely crosslinked hydrogel when PVA has a higher initial molecular weight. In this experiment the initial concentration of both the low and high molecular weight PVA in aqueous solution was the same. Although the same dose of the ionizing radiation should generate the same concentration of radical sites through the indirect effect, their number per macromolecular chain must be larger for higher molecular weight PVA. As a result, this should lead to the higher density of crosslinks within the hydrogel network and to the more significant change in the macromolecular architecture, as shown in Figures 5.3 and 5.4.

Figures 5.5 and 5.6 demonstrate how the GA concentration in the solution for crosslinking affects the water content, $\overline{M}_c$, and ξ of PVA hydrogel membranes. The finding that higher concentration of PVA led to a larger decrease in the water content, $\overline{M}_c$, and ξ at the same GA concentration suggests that the intermolecular crosslinking reaction between two different macromolecules took place more frequently and effectively in the more concentrated solution, where the distance between the polymer chains was shorter.

Although the macromolecular structure and thus the water content of PVA hydrogels can be influenced by many variables, such as polymer

concentration in solution, its molecular weight, the radiation dose, and the crosslinker concentration, the resulting PVA hydrogels always remained in a highly swollen state. Their water content did not decrease lower than 89 %, as determined on the basis of swelling at 37°C. It should be pointed out that the PVA hydrogel is a temperature-sensitive system, as demonstrated in Figure 5.5, where the water content determined at 25 and 37°C was plotted against the GA concentration. A lower water content was observed at 37°C than 25°C, since water became a poorer solvent for PVA when the temperature of the system increased, resulting in the shrinkage of the PVA networks. This fact should be taken into account when the PVA membrane is planned to be implanted into the living body as an encapsulating material. The decrease in the water content and hence the size of macromolecular mesh will change the diffusive characteristics of prepared membranes.

Generally, solute molecules are not able to diffuse through a gel membrane, unless the mesh size is larger than their own size. It has been demonstrated by Reinhart *et al.*[18] that the albumin diffusion coefficient greatly depends on $\overline{M}_c$ of PVA hydrogel membranes. They found that at the critical $\overline{M}_c$ of 3,750 (mesh size, ξ=38 Å) the diffusion of albumin was significantly prevented. The ξ values of our PVA hydrogel membranes, calculated on the basis of swelling experiments, never exceeded a value of 80 Å (Figures 5.4 and 5.7). Theoretically, such membranes should completely avoid the diffusion of IgG, as its hydraulic diameter is over 100 Å.[19]

The results of the swelling experiments done on the radiation and

chemically crosslinked PVA hydrogel membranes clearly show that any changes of the parameters having an influence on the crosslinking density of the polymer network generated hydrogels with different water contents and macromolecular mesh sizes. The parameters include radiation dose, polymer concentration, and polymer molecular weight, as well as concentration of the crosslinking agent in the chemical crosslinking process.

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Chapter 6

Protein Permeation through PVA Hydrogel Membranes

INTRODUCTION

Recently, several polymeric materials have been investigated as membranes of immunoisolation for hybrid-type artificial organs.[1–5] Sun and his coworkers reported that poly(alginic acid) combined with poly(L-lysine) is useful for a hybrid-type artificial liver, while Iwata *et al.* reported an agarose hydrogel membrane is applicable for an artificial pancreas.[4,5] On the other hand, Chapter 5 indicated that a hydrogel membrane could be prepared from PVA if crosslinked with a radiation or a chemical method.

Davis[6] studied the release of several proteins and protein hormones from implanted polyacrylamide and polyvinylpyrrolidone hydrogels and revealed a logarithmic dependence of the diffusion coefficients on the polymer concentration of implanted hydrogels and the solute molecular weight. Both the polymers showed almost a similar relationship between the solute diffusion coefficient and the solute molecular weight or the polymer concentration in the gel.

The purpose of this chapter is to study the diffusive properties of PVA hydrogel membranes. As mentioned in Chapter 5, the PVA hydrogel

is macroscopically nonporous but microscopically contains the area available for the diffusion of solutes which is the open space in the macromolecular mesh.[7] The crosslinking density affects not only the content of water accessible for the solute diffusion, but also the mesh size which controls the solutes to pass through the polymer network.

EXPERIMENTAL

Preparation of membranes

The PVA membranes were prepared by the method described in Chapter 5. Atactic PVA used had number-average molecular weights, $\overline{M}_{n(0)}$, of 0.48×10^5 and 2.47×10^5, and weight-average molecular weights, $\overline{M}_{w(0)}$, of 1.15×10^5 and 5.99×10^5, respectively. Both polymers had a polydispersity index of 2.4 and a degree of saponification of 99.5 mol%. Flat molds consisting of two glass plates were filled up with the PVA solutions and a polyester mesh was used to reinforce the hydrogels which formed upon irradiation of the polymer solutions. Irradiation was carried out with electron beams of a 1.5 MeV Van de Graaff accelerator at a dose rate of 0.3 kGy/s.

PVA membranes crosslinked by the chemical method were prepared by impregnating the polyester mesh with the PVA solution containing GA as a crosslinking agent and hydrochloric acid as a catalyst. They were then

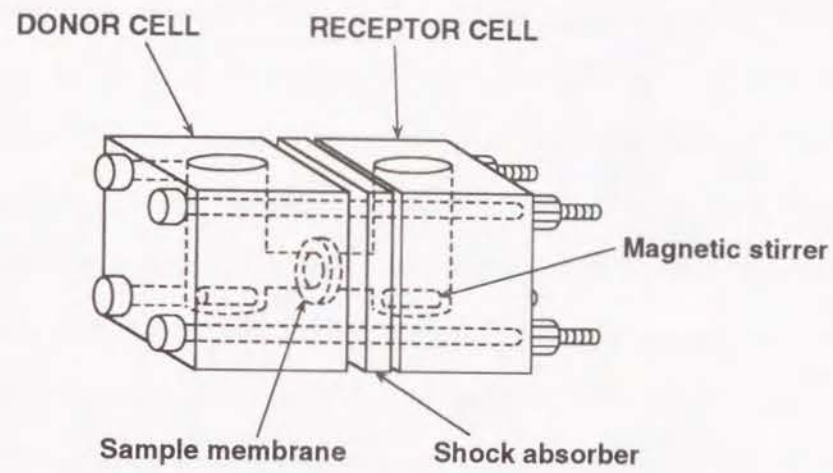

Figure 6.1. Scheme of the permeability device.

placed in a container at room temperature and 100 % relative humidity and left for 24 hr.

Prior to permeability measurements the PVA hydrogel membranes were subjected to extraction with distilled water at 50°C for 5 hrs and then swollen by water at 37°C.

Permeability measurements

The insulin used in the permeability measurements was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.; lot no. 79F-0092) with a nominal activity of 25.7 I.U./mg (bovine pancreas, I-5500) and used without further purification. Insulin used in the permeability experiments had a concentration almost similar to that *in situ*. Insulin is thought to

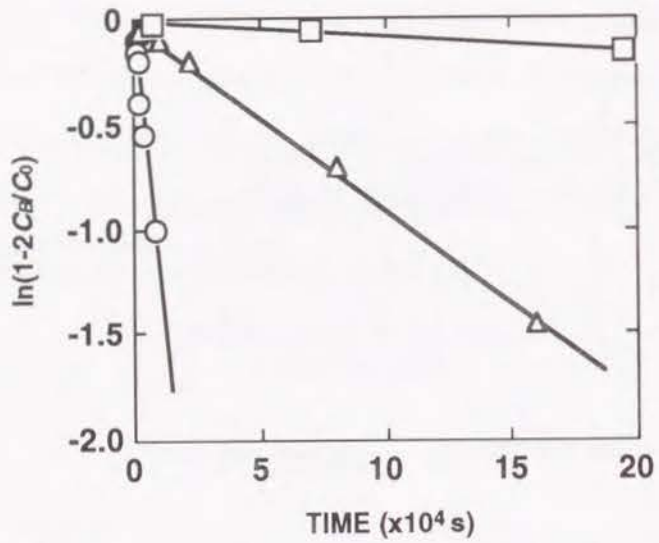

Figure 6.2. Plot of ln(1-2C_B/C_0) as a fuction of time for permeation of (O) glucose through PVA hydrogel membranes (initial concentration=2 wt%, radiation dose=15 kGy), (Δ) insulin (3 wt%, 24 kGy), and (□) IgG (4 wt%, 24 kGy).

circulate as a monomer at a concentration of 10^{-8} to 10^{-11} M in the blood.[8] Albumin permeability measurements were performed using BSA (fraction V, containing 96–99 % monomeric albumin; Sigma Chemical Company) having a molecular weight of 67,000. Immunoglobulin G (IgG, molecular weight=180,000) was also purchased from Sigma Chemical Company. Solutions of insulin, albumin, and IgG were prepared using phosphate buffer solution (0.15 M NaCl, 0.05 M phosphate, pH=7.4) to have concentrations of 500 μU/ml, 8 g/dl, and 1 g/dl, respectively. Glucose (molecular weight=180; Wako Pure Chemical Industries, Ltd.) was dissolved in distilled water to yield a solution with a concentration of 200 mg/dl. The solute

permeability was measured at 37°C using a two-compartment cell by placing the hydrogel membrane (9 mm in diameter) between the donor and the receptor compartment. The structure of the device used for the protein permeation is illustrated in Figure 6.1. To reduce the boundary layer effect and the aggregation of proteins, each of the compartments was stirred at a constant rate of 30 rpm with a magnetic chip (8 mm in diameter). At selected intervals of time, 20 or 100 μl was removed from both of the compartments of the permeability device to keep the fluid levels constant. The solutions from the receptor compartment were analyzed to determine the amount of the permeated solutes. Glucose, insulin, and albumin were quantified using the color reaction of test kits for diagnosis from Wako Pure Chemical Industries, Ltd., while IgG was determined using the ELISA method.[9]

Determination of diffusion coefficients

Diffusion of the solutes through the rubber-like polymer network of the PVA hydrogel membrane may follow the Fick's law,[10] which can be expressed by the following relation in the present cell system:

$$\ln(1-2\frac{C_B}{C_0})=2\frac{AD}{LV}\times t \qquad (1)$$

where C_B is the concentration of the solute in the receptor compartment, C_0 is the initial concentration of the solute in the donor compartment, t is the

diffusion time [s], A is the area of membrane (=0.64 cm^2), L is the thickness of membrane at the end of measurement [mm], V is the volume of compartment (=3 cm^3), and D is the diffusion coefficient of the solute [cm^2/s]. The thickness of the radiation crosslinked PVA hydrogel membranes was 0.15 to 0.5 mm, depending on the preparation conditions. The PVA hydrogel membranes obtained by the chemical crosslinking had a thickness of 0.07 to 0.1 mm. Typical examples of plots according to Equation (1) are given in Figure 6.2. It is seen that good linear dependence was obtained for three solutes passing across the hydrogel membranes prepared at different PVA concentrations and different radiation doses. Such a linear dependence with a high correlation coefficient was observed for all of the solutes and the membranes, regardless of the crosslinking method. The parameter s^2 given in Figure 6.2 is the correlation coefficients of the experimental points in relation to the linear dependence of $\ln(1-2C_B/C_0)$ against t. The diffusion coefficient of the solutes was calculated from the slopes of the straight lines.

Number of effective chains and mesh size of network

The number of the effective chains per unit volume, V_e, and mesh size, ξ, was calculated on the basis of the equation described in Chapter 5.

$$V_e = -\frac{\frac{\nu}{V_1}[\ln(1-V_{2,s})+V_{2,s}+\mu V_{2,s}^2]}{V_{2,r}[(\frac{V_{2,s}}{V_{2,r}})^{\frac{1}{3}}-\frac{1}{2}(\frac{V_{2,s}}{V_{2,r}})]} \quad (2)$$

$$\xi = V_{2,s}^{-\frac{1}{3}}(\overline{r_0^2})^{\frac{1}{2}} \quad (3)$$

RESULTS

Figure 6.3 shows the dependence of the diffusion coefficient of solute on the water content for the radiation crosslinked PVA hydrogel membranes. The decrease in water content in the hydrogels resulted in a reduction in the diffusion coefficient of all of the three solutes.

In Figures 6.4 and 6.5, the diffusion coefficients of insulin and IgG are plotted against the water content of the hydrogel membranes prepared by radiation crosslinking of PVA with two different molecular weights, respectively. As is clearly seen, the hydrogels prepared from PVA of the higher molecular weight gave the lower diffusion coefficient for IgG when compared to these of the same water content. However, no appreciable difference in diffusion coefficient was observed for insulin between the two

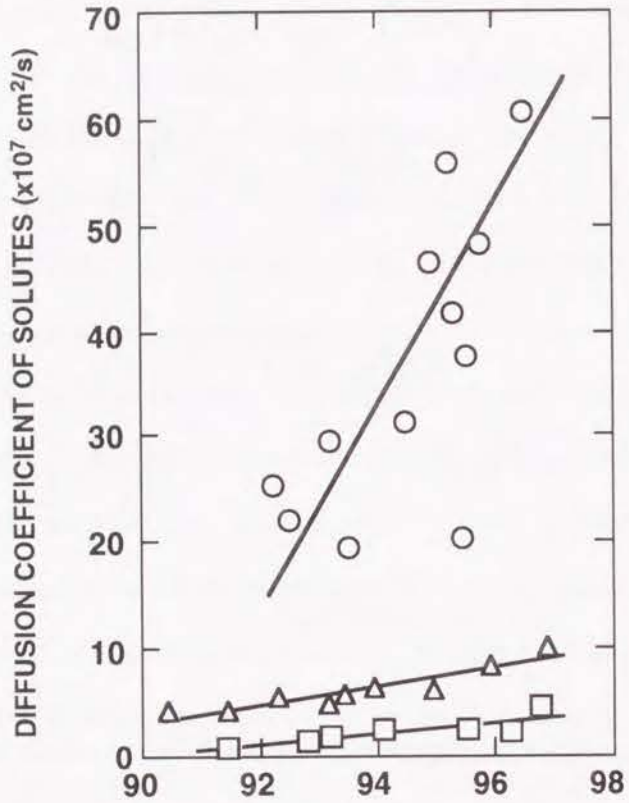

Figure 6.3. Dependence of the diffusion coefficient of (O) glucose, (Δ) insulin, and (□) IgG on the water content of PVA hydrogel membranes with a molecular weight of 0.48×10^5.

different PVA hydrogels. This must be due to the fact that insulin has a molecular weight 30 times lower than IgG, resulting in insignificant dependence of its diffusion on V_e of the hydrogel membrane, in marked contrast to IgG. The V_e value was dependent on the starting molecular weight of PVA, even though the PVA solutions of the same concentration were irradiated with the same dose. The result is shown in Figure 6.6.

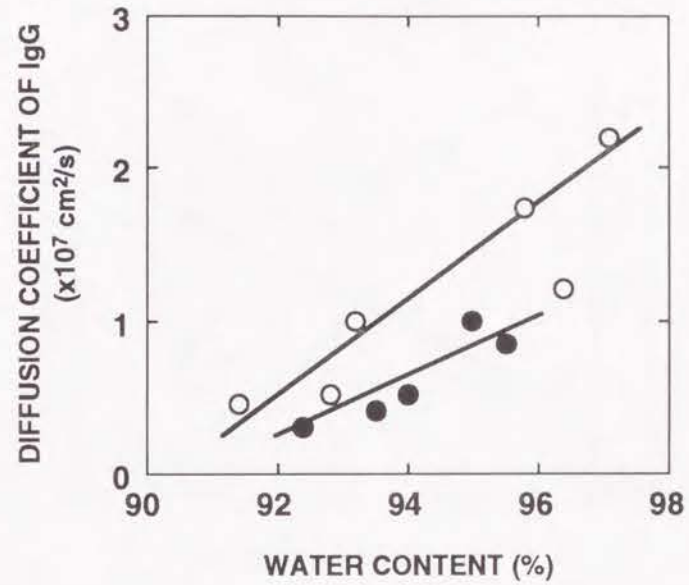

Figure 6.4. Dependence of the diffusion coefficient of IgG on the water content of PVA hydrogel membranes with a molecular weight of (O) 0.48×10^5 and (●) 2.47×10^5.

Clearly, the hydrogel network prepared from the higher molecular weight PVA had larger numbers of chains effective for network than those from the lower molecular weight PVA, when compared at the same radiation dose. The larger number of effective chains should lead to the smaller size of network meshes, thereby reducing the diffusion coefficient of the solute. As shown in Figure 6.7, the diffusion of IgG through the hydrogel membrane was greatly influenced by the size of macromolecular mesh. It should be pointed out that the diffusion coefficient becomes independent of the PVA molecular weight when plotted against the mesh size, as is evident in Figure 6.7. The diffusion coefficient of insulin hardly depended on the mesh size

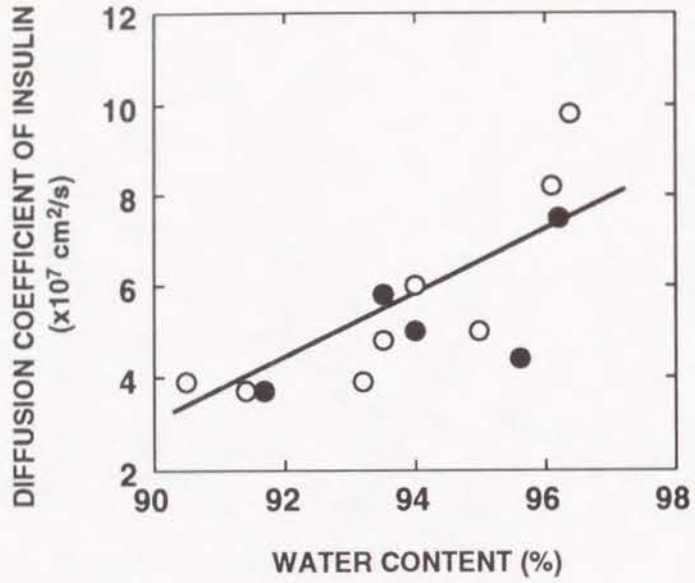

Figure 6.5. Dependence of the diffusion coefficient of insulin on the water content of PVA hydrogel membranes with a molecular weight of (O) 0.48x10^5 and (●) 2.47x10^5.

for all of the membranes studied here, probably because insulin molecules easily passed through the network as the mesh sizes were large enough for its permeation.

Figure 6.8 shows the dependence of the diffusion coefficient of three solutes on the gel water content for the chemically crosslinked PVA hydrogel membranes. Although the data scatter, almost a linear dependence of the diffusion coefficient was found for all of the solutes.

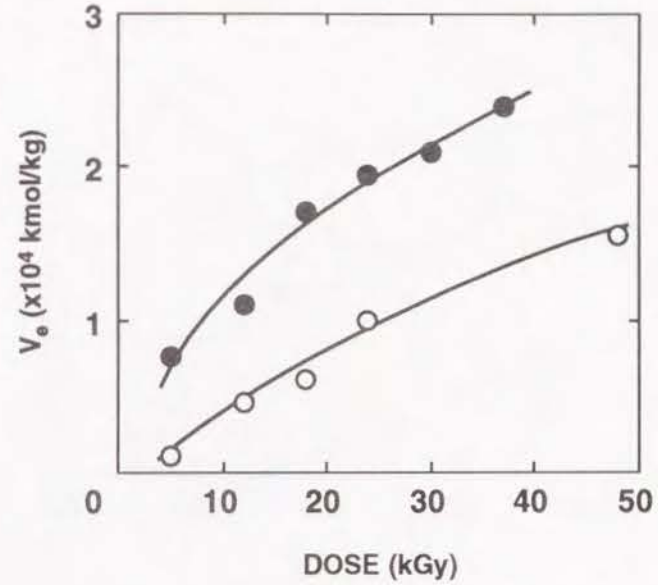

Figure 6.6. Dependence of the number of the effective chains, V_e, on the radiation dose of PVA hydrogel membranes with a molecular weight of (O) 0.48x10^5 and (●) 2.47x10^5.

DISCUSSION

PVA gel membranes with different water contents could be obtained by changing the dose of electron beams or the molar ratio of the chemical crosslinking agent (GA) to the repeating PVA units, as described in Chapter 5. The linear dependence of the diffusion coefficient on the water content of gel membranes found for all of the three solutes gives evidence that diffusion indeed occurred primarily through the water hydrating the polymer network (Figures 6.3,6.4,6.5, and 6.8). In addition, it was seen that

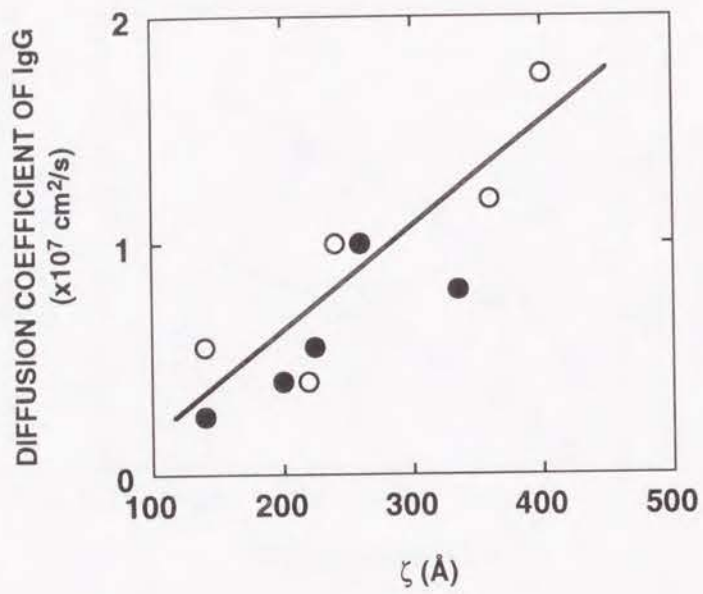

Figure 6.7. Dependence of the diffusion coefficient of IgG on the mesh size of PVA hydrogel membranes with a molecular weight of (O) 0.48x10^5 and (●) 2.47x10^5.

the diffusion coefficient of the solutes was practically the same for the radiation and the chemically crosslinked hydrogel membrane, when compared at the same mesh size (Figure 6.9). However, Figures 6.3 and 6.8 revealed that the dependence of the diffusion coefficient on the water content of the hydrogel membrane became less pronounced when the size of the solute molecule increased (note that the scale of the ordinate is logarithmic).

Generally, solute molecules may not be able to diffuse through the gel membrane, if the mesh size is smaller than that of their own. Thus, it is likely that, in addition to the water content of membrane, the mesh size of the hydrogel network will become a decisive factor in the case of the

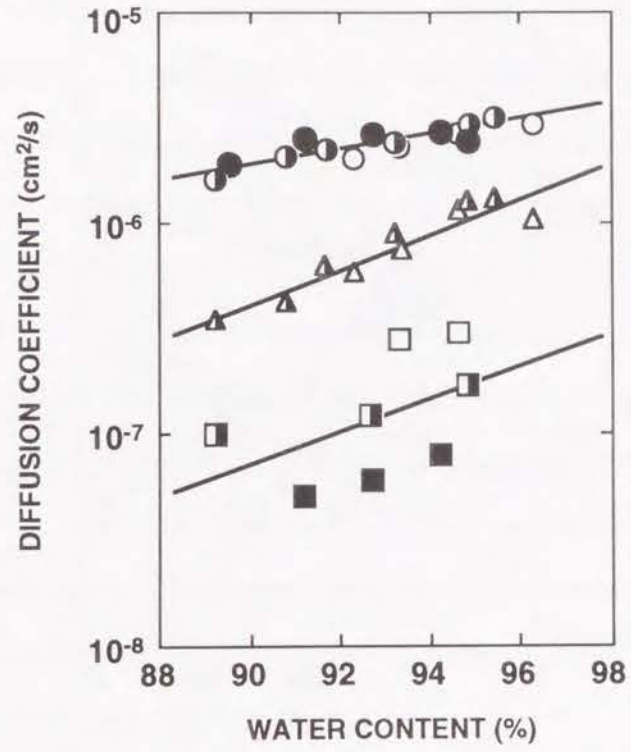

Figure 6.8. Dependence of the diffusion coefficient of (O,◑,●) glucose, (Δ,▲) insulin, and (□,◨,■) albumin on the water content in PVA hydrogels with a molecular weight of 2.47x10^5 crosslinked by GA. Open symbols show initial concentration of 2 wt%, half closed symbols of 3 wt%, and closed symbols of 4 wt%.

diffusion of high molecular weight solutes such as IgG. As described in Chapter 5, the ξ values calculated on the basis of swelling experiments never exceeded a value of 80 Å. Theoretically, such membranes should completely prevent the diffusion of IgG, which has the largest size among the proteins used in this permeability study. Its hydraulic diameter is over 100 Å (Table 6.1). However, as shown in Figure 6.9, IgG permeability through the PVA

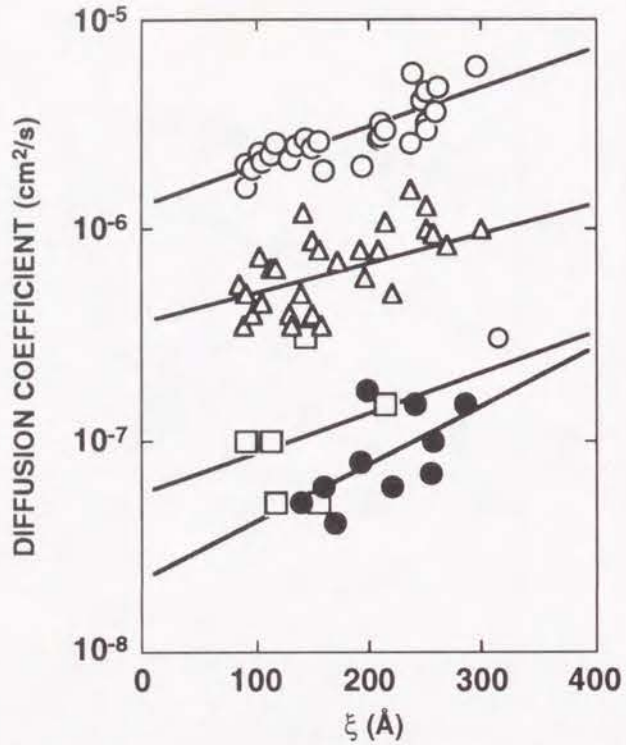

Figure 6.9. Dependence of the diffusion coefficient of (O) glucose, (Δ) insulin, (□) albumin, and (●) IgG on the mesh size of the PVA hydrogel network crosslinked by radiation and GA.

Table 6.1. Stockes radii or characteristic semiaxes and diffusion coefficients of insulin, albumin, and IgG in pure water at 20°C.

Solute	Stockes radius (Å)	Characteristic semiaxes (Å)	Diffusion coefficient (cm^2/s)
Insulin	11.7 - 12.8	-	-
BSA	-	25 x 75	$(5.9 - 6.0) \times 10^{-7}$ (9.09×10^{-7})*
IgG	51.9	-	4.0×10^{-7} (6.0×10^{-7})*

*Value extrapolated to 37°C using the well-known equation $D\eta/T$=constant

hydrogel membranes was not completely prevented. As the IgG molecule is not spherical,[11] it is likely that the membrane with a mesh size as low as 90 Å may not be as effective as a molecular sieve for this protein. Moreover, this mesh size is an average value and we have no information on the influence of the distribution of the mesh size because of the difficulty in measuring it. Although PVA hydrogel membranes employed here could not completely prevent the IgG permeation, they might be a good barrier for hybrid-type artificial organs because they greatly restrained IgG diffusion. This is evident from the comparison of the experimentally determined diffusion coefficient of IgG through the PVA membranes with that in pure water at 37°C (Table 6.1). The diffusion coefficient of IgG through even moderately crosslinked membranes amounts to about 1×10^{-7} cm^2/s, which is significantly lower than that in pure water at 37°C. It would likely be possible to obtain a more tightly crosslinked PVA hydrogel membrane that completely prevents IgG permeation, but the decrease in the mesh size must lead to a decrease in the water content. As mentioned earlier, to ensure the rapid response of encapsulated Langerhans islets, the encapsulating membrane should have high permeability of glucose. This may be achieved only when the water content of PVA membranes is very high, thus giving a rather high diffusion coefficient of glucose. A plot of the dependence of the glucose diffusion coefficient on the water content of radiation (Figure 6.3) and chemically (Figure 6.7) crosslinked PVA hydrogel membranes supports this suggestion. In this study, only the permeability of high water content

PVA was measured, but the permeability of low water content PVA may be nearly the same as that of Smith and Sefton,[14] who showed the diffusion coefficients for thrombin (molecular weight=37,000) and antithrombin III (molecular weight=65,000) through GA-crosslinked PVA gels with a water content of 75 % to be $6\pm4\times10^{-8}$ and $4\pm2\times10^{-8}$ cm^2/s, respectively. The more densely crosslinked PVA hydrogels, which would be able to refuse completely IgG diffusion, will also allow the diffusion of glucose significantly. It has been found that glucose permeability through hydrogel-type intraocular lenses decreased drastically when the water content decreased from 80 % ($D_{glucose}$= 1.28×10^{-6} cm^2/s) to 38 % ($D_{glucose}$= 1.21×10^{-8} cm^2/s).[15]

All of the above results led to a conclusion that parameters having an influence on the crosslinking density of the polymer network such as radiation dose, concentration of the crosslinking agent, polymer concentration, and polymer molecular weight, also effect the protein permeation. These may be helpful to obtain hydrogel membranes with fairly accurately identified diffusive properties.

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Chapter 7

Application of PVA Hydrogel Membranes for Hybrid–Type Artificial Pancreas

INTRODUCTION

Although there has been increasing evidence that pancreatic islet transplantation may offer an ideal endocrine replacement therapy for patients with type I (insulin–dependent) diabetes mellitus (DM), this islet transplantation has a major problem, that is, recurrence of the original disease in the unprotected islet cells.[1] Immunosuppressive therapy is at present indispensable when islet transplantation is clinically applied in spite of its toxicity. A possible solution to these problems is encapsulation of donor islets in a biocompatible polymer capsule membrane that constitutes a physical barrier to the host's immune system. The need for any kind of immunosuppression might be eliminated even in the future clinical application of islet transplantation if a unique immunoisolation system is developed for islets.

In previous chapters of this thesis, PVA hydrogel was found to reveal minimal cell adhesion and good solute permeation, which are very beneficial to hybrid–type artificial organs. This chapter is conducted to examine

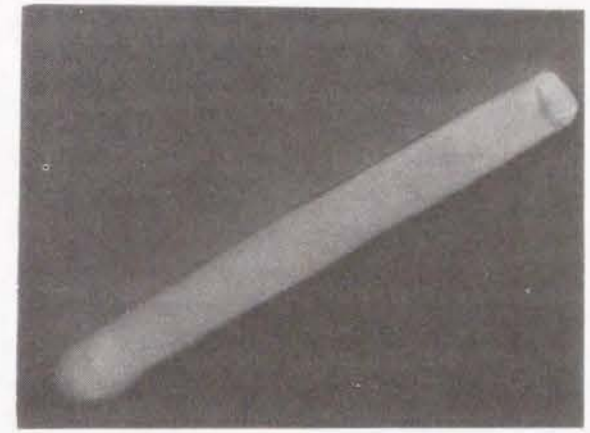
whether the PVA hydrogel membrane can prevent immunorejection of islet cells and allows free passage of insulin, glucose, and nutrients in experimental transplantation of the PVA membrane entrapping pancreatic islets.

EXPERIMENTAL

Preparation of a mesh-reinforced PVA hydrogel membrane tube

The PVA hydrogel membrane was prepared by crosslinking of PVA in aqueous hydrochloric acid solution containing GA, as described in Chapter 5. The water content of these membranes was varied from 75 to 97 % by changing the molecular wight and the concentration of PVA and GA.

The procedure for making the mesh-reinforced PVA hydrogel membrane tube (MRPT) is as follows: A polyester mesh tube was immersed in PVA solution (number-average molecular weight=2.47×10^5; concentration=3 %) to which 0.083 % GA in 0.1 N HCl was added and stored at 100 % relative humidity for 24 hrs. After complete gelation, the tube was washed in boiling water for 30 min. The water content of the resulting product, MRPT, was 97 %, the surface area was 2.5 cm^2, the width was 2 mm, the length was 4 cm, and the volume was 0.125 cm^3. Figure 7.1 shows the diagram and the photograph of the MRPT.



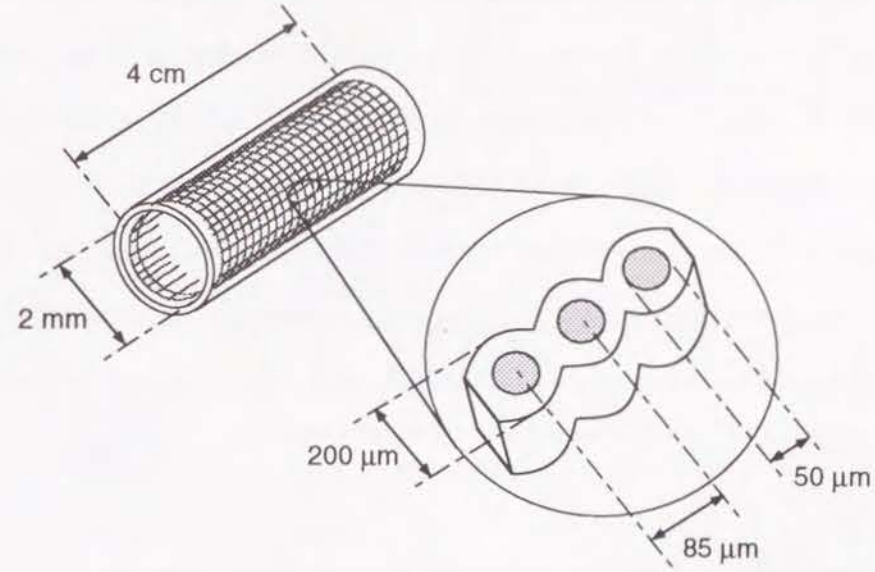


Figure 7.1. Mesh-reinforced PVA hydrogel membrane tube (MRPT).

Implantation of polymer rods

A PVA hydrogel rod was implanted in the peritoneal cavity of Wister male rats. Rods of polyethylene, silicone, and PTFE were also implanted in a similar way for comparison. Eighteen weeks after implantation, encapsulation with fibrous tissues around each rod was compared histologically.

Isolation of rat Langerhans islets

Rats were reared in the Animal Laboratory Center of Kyoto University. Pancreatic islets were isolated from donor Sprague–Dawley (S–D) male rats weighing 220–260 g by a modified method of Sutherland's group.[2] Briefly, ductal collagenase (1,000 U/dl, 6 ml, Type XI; Sigma Chemical Company, St. Louis, MO, U.S.A.) distension, static incubation for 20 min at 37°C, filtration through wire mesh (850 μm), and separation in a dextran discontinuous density gradient were performed as represented in Figure 7.2. The recovery rate was 85 to 95 % after purification of pancreatic islets.

Experimental transplantation of hybrid-type artificial pancreas

Diabetes was induced in Wister rats (200–250 g) by intravenous injection of streptozotocin (60 mg/kg) into the tail vein, and these rats were used as the transplant recipients. Only such rats that showed high persisting nonfasting serum glucose levels (>400 mg/dl) for at least 7 days after

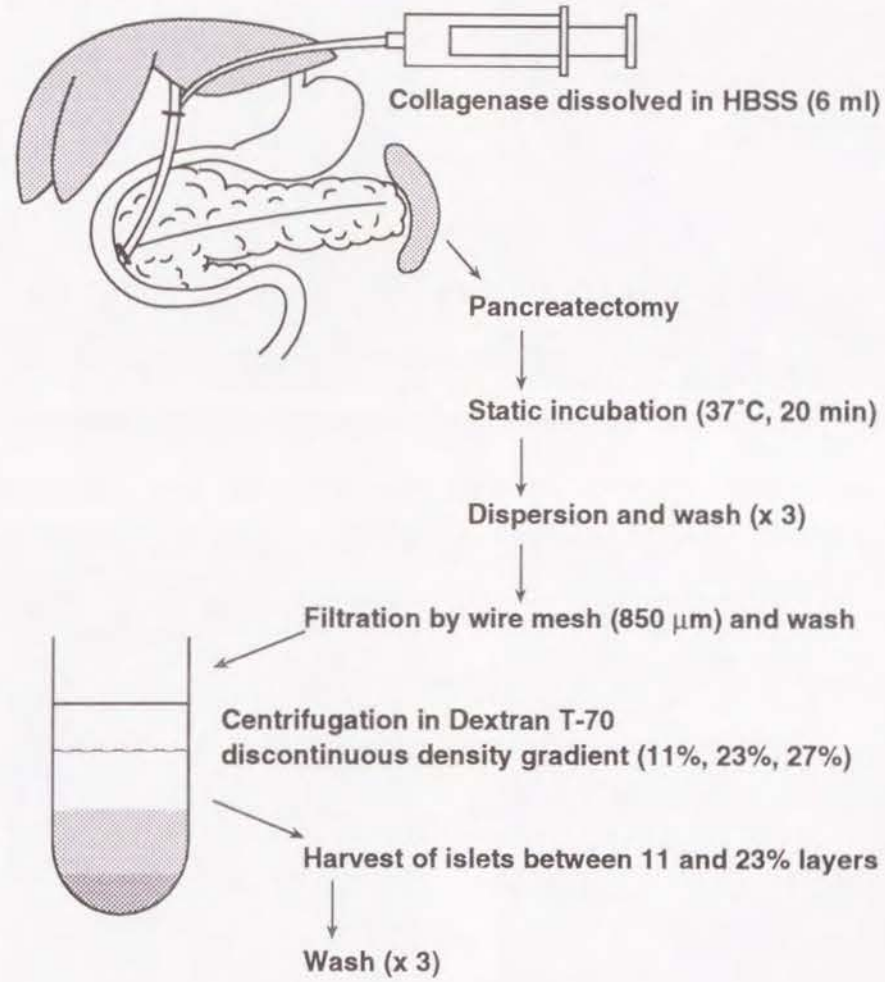


Figure 7.2. Isolation of rat pancreatic islets.

injection of streptozotocin were used. Approximately 2,000 isolated islets were obtained from three S-D rats and enclosed in the MRPT. This hybrid-type artificial pancreas with entrapped pancreatic islets was transplanted into the peritoneal cavity of six Wister rats with diabetes mellitus under ether anesthesia.

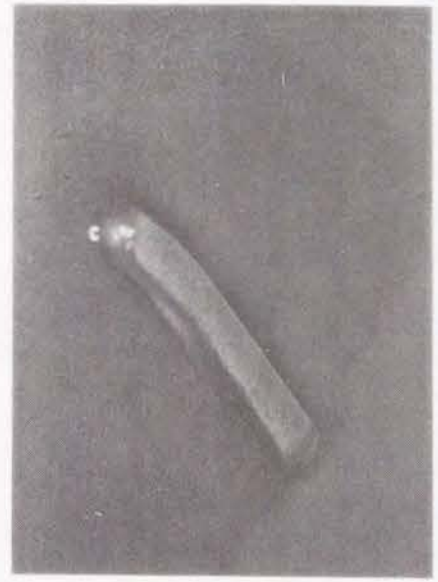
In six control diabetic Wister rats, the same number of islets (approximately 2,000) from three S-D rats, but not entrapped in MRPT (free islets), were transplanted directly into the peritoneal cavity. The nonfasting serum glucose levels of the transplant recipients (diabetic Wister rats) were monitored nearly every day following the intraperitoneal transplantation of either hybrid-type artificial pancreas with MRPT or free islets. For comparison, the nonfasting serum glucose levels in diabetic Wister rats without transplantation were also measured (n=10).

Serum glucose levels were expressed as mean±SEM. Student's *t* test or analysis of variance was used for statistical analysis. Differences with a *p* value less than 0.05 were considered as significant.

RESULTS

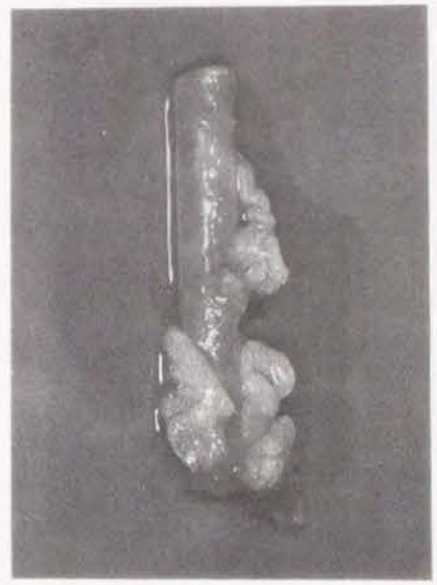
Assessment of PVA hydrogel as membrane material for hybrid-type artificial pancreas

Figure 7.3 shows the light microphotographs of a PVA hydrogel rod



PVA hydrogel

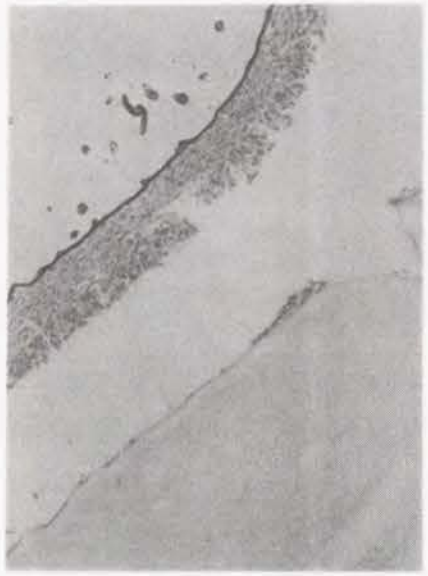
PTFE



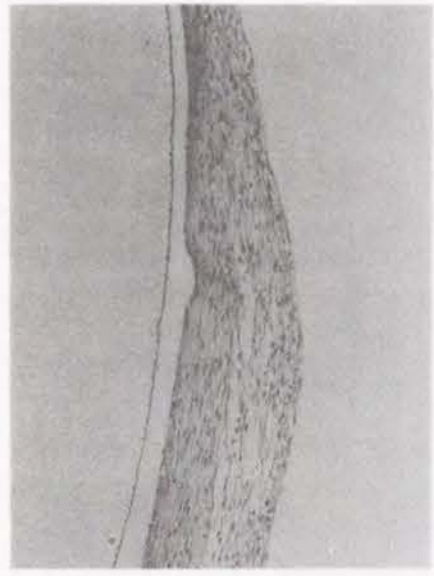
PE

Silicone

Figure 7.3. Light micrographs of polymer rods intraperitoneally implanted in rat for 18 weeks.



PVA hydrogel



PTFE

PE

Silicone

Figure 7.4. Photomicrographs of polymer rods intraperitoneally implanted in rat for 1 week.

made of 97 % water content intraperitoneally implanted for 18 weeks, together with those of other synthetic polymers. Clearly, the PVA hydrogel was almost free of adhered tissues in contrast to silicone and polyethylene rods, which were covered with fibrous tissues. PTFE also exhibited no detectable tissue covering. Encapsulation around the PVA and PTFE rods was insignificant in comparison with the other materials.

Figure 7.4 shows the histologically stained cross-section of implanted polymer rods. Apparently, the PVA hydrogel was surrounded by only a few cell layers.

Experimental transplantation of hybrid pancreatic islets

Nonfasting serum glucose levels in the recipient diabetic Wister rats significantly decreased for at least 12 days after the intraperitoneal transplantation of the hybrid-type artificial pancreas with entrapment of islets in MRPT (Figure 7.5). Since any animal that achieves a blood glucose level less than 200 mg/dl after implantation qualifies as a technical success, four of six rats were technically successful. The lowest values obtained were 97 (14th day), 184 (12th day), 131 (10th day), and 153 mg/dl (10th day). The nadir of the glucose values in two rats that failed to achieve this level of glycemic control was 250 (9th day) and 260 mg/dl (10th day). On the contrary, diabetic Wister rats receiving intraperitoneal transplantation of free islets without entrapment in MRPT (IPT rats) showed slight but significant decreases in nonfasting serum glucose levels for only 3 days. In the diabetic

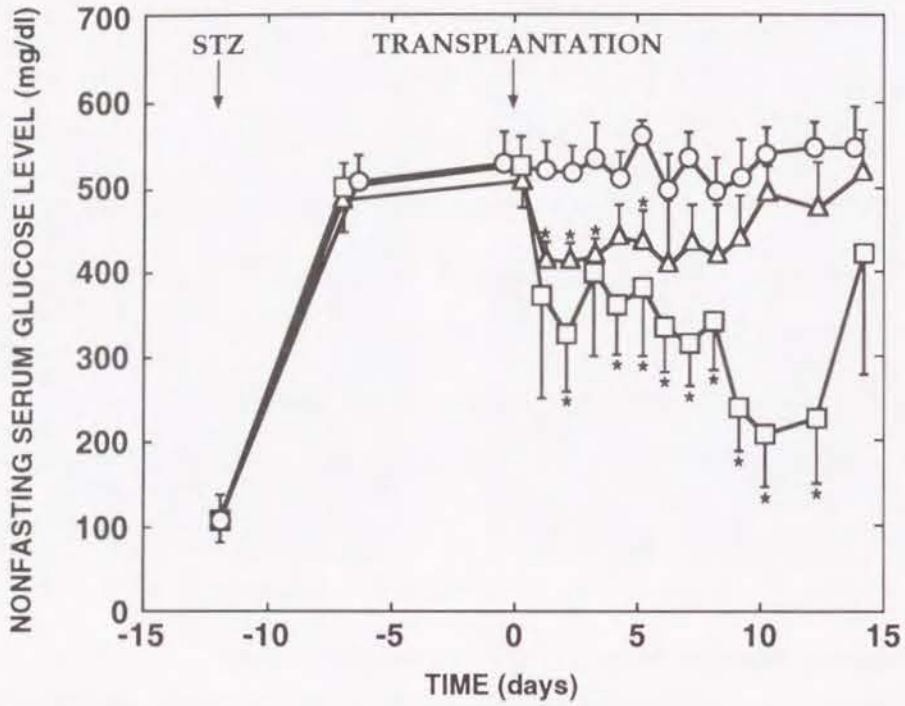


Figure 7.5. Comparative study on changes in nonfasting blood glucose levels among (O) DM rats (n=10), (Δ) IPT rats (n=6), and (□) MRPT rats (n=6). $*p < 0.05$; significant difference in glucose levels from those in DM rats. Each value represents M ± SEM.

Wister rats without any peritoneal transplantation (DM rats), nonfasting serum glucose levels showed no significant changes (Figure 7.5). Figure 7.6 shows a nonfasting Wister rat after intraperitoneal transplantation of the hybrid-type artificial pancreas made from MRPT. Nonfasting serum glucose levels in this diabetic recipient rat decreased from pretransplant levels of 440-500 to 100-200 mg/dl and this decrease to a mean value of 162±13 mg/dl was maintained for over 3 months following intraperitoneal

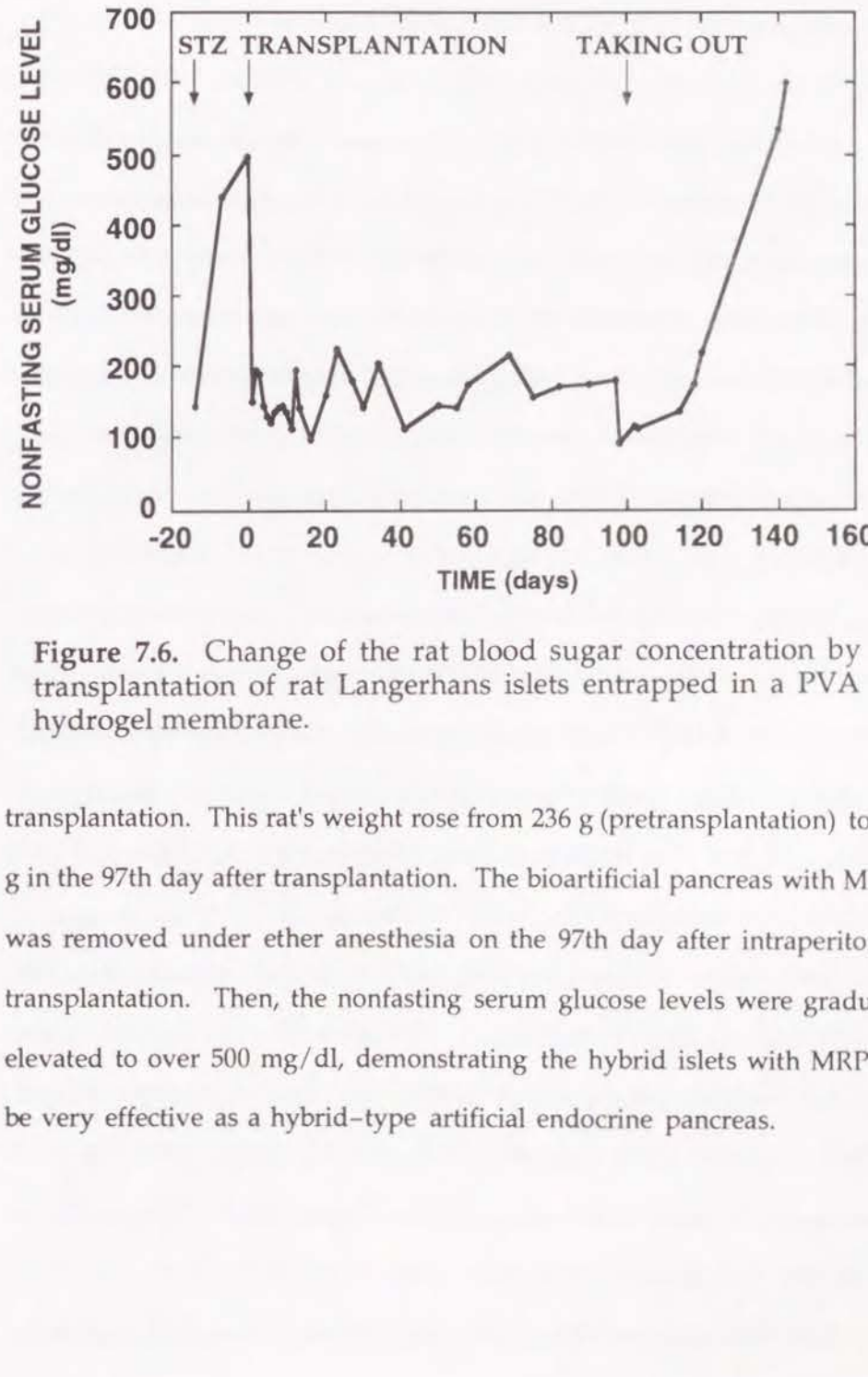


Figure 7.6. Change of the rat blood sugar concentration by transplantation of rat Langerhans islets entrapped in a PVA hydrogel membrane.

transplantation. This rat's weight rose from 236 g (pretransplantation) to 374 g in the 97th day after transplantation. The bioartificial pancreas with MRPT was removed under ether anesthesia on the 97th day after intraperitoneal transplantation. Then, the nonfasting serum glucose levels were gradually elevated to over 500 mg/dl, demonstrating the hybrid islets with MRPT to be very effective as a hybrid-type artificial endocrine pancreas.

DISCUSSION

A hybrid-type artificial endocrine pancreas should retain biological functions of the donor islet cells, be bioinert, and avoid both encapsulation by connective tissue and rejection by recipient immunological cells. To this end, both necessary nutrients, such as glucose, and substances secreted by the cells, such as insulin and albumin, need to pass freely through the biomaterial membrane. On the contrary, immunological macromolecules such as immunoglobulins and immune cells must not pass through the membrane.

The *in vitro* and *in vivo* studies carried out in this and previous chapters have demonstrated that PVA hydrogel provides a promising membrane that satisfies these requirements for the hybrid-type artificial endocrine pancreas. These are good permeability for glucose, insulin, and albumin but not for immunoglobulin G (Chapter 6), minimal IL-1 production by macrophages adhering to this membrane (Chapter 1), and insignificant encapsulation around the hydrogel membrane after intraperitoneal implantation (Figures 7.3 and 7.4). An in vitro static incubation study on insulin release revealed that the hybrid-type artificial pancreas with entrapped islets in MRPT showed a significantly greater insulin response to incubation with higher concentrations (16.7 mM) of glucose than was observed during incubation.[3)]

Data obtained from an in vivo experimental transplantation study

supported the aforementioned assessment and clarified that entrapment of pancreatic islets in MRPT was much more effective than the transplantation of free islets in inducing a sustained decrease in the nonfasting blood glucose level in recipient diabetic rats without the use of immunosuppressive therapy. This suggests that immunoisolation of grafted cells by entrapment in the PVA membrane could protect islet cells from both graft rejection and autoimmune destruction while eliminating the need for immunosuppression.

A sustained decrease in the nonfasting blood glucose level was observed in one recipient diabetic rat following intraperitoneal transplantation of the hybrid-type artificial pancreas with entrapped islets in the PVA membrane in the absence of immunosuppression until removal of the bioartificial pancreas on the 97th day.

Clinical trials of islet transplantation were shown to be unsuccessful in the registry report of 1985.[4)] However, clinical application of islet transplantation has very recently been increasing with a few successful cases.[5,6)] Even if islet transplantation becomes more clinically applicable, difficulty will still remain in meeting the requirements of islet tissue on the basis of human organ donation as well as the problem of immune rejection.

In contrast, hybrid-type artificial pancreases are extremely attractive and could be the ideal approach to overcome this problem. Among immunoisolation techniques, there are hollow fiber devices to function as mechanical barriers between islets and the host's immune cells and molecules.[9-10)] Promising hybrid-type artificial pancreases with hollow fiber

have recently been reported.[11,12] Microencapsulated islets have also been well known as an immunoisolation technique.[13-16] Although the hybrid-type artificial pancreas with hollow fiber devices has demonstrated an excellent result in experimental implantation studies,[11,12] one of the problems associated with hollow fiber units includes the small surface area of devices, which necessitates the use of large units that would be rather bulky for clinical application. The hollow fiber device consists of a chamber connecting to standard vascular grafts. For implantation of the device, invasive surgical procedure must be performed because of the anastomosis of the arterial limb of the device to the common iliac artery and anastomosis of the venous limb to the common iliac vein.[11,12] Great care should be taken to maintain long-term patency of the vascular shunt and hence good biocompatibility with respect to thrombosis. In contrast, our tube type of hybrid-type artificial pancreas with PVA membrane provides a simple device with relatively small size (Figure 7.1), and can be easily constructed and implanted into the peritoneal cavity of the recipient without an invasive procedure. One of the merits of the membrane used in the present study is that PVA hydrogel membranes with fairly accurately identified diffusive properties can be obtained by changing the parameters having an influence on the crosslinking density of the polymer network (Chapter 5). To our knowledge, the PVA hydrogel is the first biomaterial membrane to demonstrate good permeability to glucose, insulin, and albumin with no permeation to immunoglobulin G in vitro (Chapter 6).

Excellent results observed for experimental transplantations of microencapsulated islets might be due to the smaller diffusion lag of the microencapsulated islets, since each capsule contains one or two islets with a small diameter ranging from only 0.5 to 0.7 mm.[13-16] However, this microencapsulation technique has problems because the preparation of perfect capsules is far from simple and such capsules tend to collapse a few days after transplantation unless the capsules are correctly prepared.[15] For clinical islet transplantation, roughly 200,000-500,000 isolated islets must be prepared.[5,6] It might not be practical to prepare such a huge number of perfect microencapsulated islets for clinical treatment of type I diabetes. Our tube type of hybrid-type artificial pancreas can entrap at least 2,000 islets. The volume of the MRPT is approximately 0.125 cm^3 (Figure 7.1). It has to be determined in a further study how many islets should be entrapped in the tube. Nonfasting serum glucose levels significantly decreased for at least 12 days after implantation of our hybrid-type artificial pancreas in the peritoneal cavity. Five rats, however, became hyperglycemic thereafter (Figure 7.5). The occurrence of this hyperglycemic state might be partly due to hypoxia and not due to rejection, since one diabetic rat with the transplanted hybrid-type artificial pancreas had a significant and sustained nonfasting serum glucose level of 162±13 mg/dl for over 3 months without any immunosuppression (Figure 7.6). It is of importance to improve the relatively ischemic environment in the tube to ensure long survival of the enclosed islets. This situation may be improved, for instance, by choosing

a suitable implantation site with sufficiently high blood circulation and by preventing the kinking of the tube in the peritoneal cavity.

In conclusion, this study suggests that our hybrid-type artificial pancreas with entrapped islets in MRPT is a promising therapeutic approach to diabetes mellitus.

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Summary

Chapter 1

Adhesion and functions of various cells cultured on polymeric substrates were studied. Fibroblasts and chondrocytes underwent three dimensional aggregation into spheroid on non–adherent substrates, whereas L cells, endothelial cells, and macrophages did not reveal such spheroid formation, but simply two dimensional aggregation. The glucose consumption study denoted that cells could survive even on non–adherent surfaces at least in the initial stage of *in vitro* cell culture. The cell functionality decreased with the enhanced cell adhesion for the cells which formed spheroids on non–adherent substrates, such as chondrocytes and fibroblasts. However, macrophages produced a lot of IL–1 on highly adhesive substrates. The endothelial cells exhibited no dependence of cell function to produce PGI_2 and TXA_2 on the surface property of substrates.

Chapter 2

The behaviors of rabbit hepatocytes cultured on a number of polymeric substrates with different surface properties were estimated. A monolayer was found to form on adherent polymer substrates having the water contact angle around 70°, whereas clusters or multicellular spheroids were formed on non–adherent polymer surfaces which had very low or high contact angles against water. On the intermediate substrates, hepatocytes

revealed both of the characteristics. The dependence of spheroid index on the contact angle was opposite to that of cell adhesion. The hepatocytes in multicellular spheroid had higher activity for albumin production than those at a monolayer state. However, after 5–6 days of incubation, the albumin production was reduced. Probably, insufficient supply of nutrients into the inside of the multicellular spheroids, caused by the large size of spheroid, resulted in cell necrosis. Hepatocytes might be used as a part of hybrid-type artificial liver for short-term liver support. However, it seems unrealistic at present to use hepatocytes for long-term support because of the difficulty in keeping the hepatocyte functioning for a long period.

Chapter 3

The possibility of PLLA non-woven fabrics as the biodegradable scaffold for short-term implantation was explored. The cell-scaffold composite was implanted into rabbit and the factors governing the cell viability were attempted to be identified. Many hepatocytes adhered *in vitro* on the PLLA surface with the intermediate cell shape. However, the maintenance of cell function was very difficult on this substrate. On the other hand, *in vivo* studies revealed that the composites of hepatocytes and biodegradable polymers were promising as the scaffold for liver reconstruction, if hepatocytes were supplied with sufficient nutrients and oxygen. In the case of an allograft, an immunosuppressive agent was necessary for survival to avoid the immunorejection of recipient.

Chapter 4

A chondrocyte-PLLA composite was employed in an attempt to regenerate cartilage. Rat chondrocyte showed good proliferation on adherent polymer substrates like PLLA and type II collagen production under the *in vitro* condition. The collagen production became maximal on the 10th day after cultivation. From the *in vivo* study, seeded chondrocytes spread throughout the scaffold dispersively and a lot of small blood vessels were observed around the matrix when bFGF was coated on the PLLA scaffold. When the chondrocyte-PLLA composite was transplanted into nude mice, injection of an immunosuppressive agent was not necessary even in an allograft implantation. A thin layer fibrous capsule was observed surrounding all of the implanted composites, and the inflammatory response of the host to the implants was mild. From the clear histological staining with thionin, it was obvious that the implanted cells exhibited their phenotype and formed new matured cartilage.

Chapter 5

PVA hydrogel membranes were prepared by irradiation with electron beams and GA. The influence of the parameters associated with the crosslinking density of the hydrogel network on the mesh size of PVA membranes was studied. The water content of gels decreased with the increase in the dose of radiation or the concentration of GA. It was demonstrated that the increase in the dose of ionizing radiation or GA

concentration formed a more densely crosslinked PVA hydrogel network with the lower amount of water in it. The GA concentration in the solution for crosslinking affected the water content, $\overline{M}_c$, and the mesh size, ξ, of PVA hydrogel membranes. The intermolecular crosslinking reaction between two different macromolecules took place more frequently and effectively in the more concentrated solution. The ξ values of PVA hydrogel membranes never exceeded a value of 80 Å. Any changes of the parameters having an influence on the crosslinking density of polymer network generated hydrogels with different water contents and macromolecular mesh sizes.

Chapter 6

Effects of PVA concentration, PVA molecular weight, and radiation dose as well as concentration of GA in the case of chemical crosslinking procedure, on the permeation of insulin, albumin, and immunoglobulin through the membranes were investigated. Glucose permeation was also studied. The diffusion coefficient linearly increased for all of the solutes with the increasing water content in the PVA hydrogels, indicating that diffusion occurred primarily through the water hydrating the polymer network. The permeability study showed that the water content as well as the mesh size had an influence on the diffusion of low molecular weight glucose and insulin. Although the diffusion of higher molecular weight solutes, such as albumin and IgG, was not so much affected by the mesh size of elaborated PVA hydrogel membranes, the diffusion of these proteins was

very low.

Chapter 7

PVA hydrogel was implanted into rat peritoneal cavity. Insignificant encapsulation was found around the hydrogel membrane after intraperitoneal implantation. Approximately 2,000 islets collected from three Sprague-Dawley rats were enclosed in MRPT and transplanted into the peritoneal cavity of six Wister rats with streptozotocin-induced diabetes. Their nonfasting serum glucose levels significantly decreased for at least 12 days. Six diabetic rats receiving intraperitoneal transplantation of free islets without the tube showed a slight but significant decrease in nonfasting serum glucose levels for only 3 days. One diabetic rat with transplantation of the bioartificial pancreas had significant and sustained nonfasting glucose levels from pretransplanted levels of 440-500 mg/dl to a mean value of 162±13 mg/dl for over 3 months without immunosuppression. The results suggested that a hybrid-type artificial pancreas with entrapment of islets in a PVA membrane could be a promising therapeutic approach to diabetes mellitus.

List of Publications

Chapter 1. In preparation

Chapter 2. In preparation

Chapter 3. Mat. Res. Soc. Symp. Proc., **252**, 359–365 (1992).

Chapter 4. In preparation

Chapter 5. Proc. Jpn Acad., **67**(B), 83–88 (1991).

Chapter 6. Biomaterials, in press.

Chapter 7. Pancreas, **7** (5), 562–568 (1992).

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