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H. 知的財産権の出願・登録状況

特になし。

Ⅲ. 研究成果の刊行物

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Suppression of the inflammatory response from adherent cells on phospholipid polymers

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Abstract: The expression of interleukin-1β (IL-1β) messenger RNA (mRNA) in macrophage-like cells cultured on phospholipid polymers was evaluated to determine the extent of the inflammatory response. As phospholipid polymers, poly(2-methacryloyloxyethyl phosphorylcholine(MPC)-*co*-*n*-butyl methacrylate(BMA)s (PMBs) were synthesized. Poly(ethylene terephthalate) (PET), poly(2-hydroxyethyl methacrylate) (PHEMA), and segmented poly(ether urethane) (Tecoflex® 60) were used as reference biomedical polymers. The protein adsorption onto the polymer surfaces from a cell culture medium was determined. The amount of the total protein adsorbed onto the PMBs was lower than that adsorbed onto the reference polymers, and the amount of adsorbed protein decreased with an increase in the MPC units in the PMBs. Human premyelocytic leukemia cell line (HL-60) was used, and the expression of IL-1β mRNA was investigated with the reverse transcription polymerase chain reaction (RT-PCR) method. When HL-60 cells were cultured on PMBs, the expression of IL-1β mRNA in the cells was much less than that on the reference polymers. In particular, the expression of IL-1β mRNA in HL-60 cells cultured on the PMBs containing more than 10 mol % MPC units was not detected. This corresponded to the reduced amount of adsorbed proteins on the PMB surfaces. These results suggest that the PMBs effectively suppressed the activation and inflammatory response of adherent macrophagelike cells. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 64A: 411–416, 2003

Key words: messenger RNA (mRNA) expression; reverse transcription polymerase chain reaction (RT-PCR); interleukin-1β (IL-1β); phospholipid polymer; biocompatibility

INTRODUCTION

An understanding of the interactions between cells and biomaterials is indispensable for developing implantable biomedical devices. The response of cells in contact with biomaterials is composed of various biological processes.[1] Cell behavior has been actively researched with different kinds of cells, materials, and techniques, such as the counting of adherent cells, morphological observations, and the evaluation of bioactive substances secreted from adherent cells.[2–4]

The macrophage is considered to be an important cell in the initial nonspecific host response against implantable biomedical devices. Macrophages mediate inflammation by the secretion of inflammatory mediators, such as coagulation factors, lysosomal enzymes, cytokines, and growth factors. The proinflammatory cytokines, interleukin-1β (IL-1β), interleukin-6, and tumor necrosis factor-α are multifunctional, soluble mediators that affect various types of cells. IL-1β mediates the activation and proliferation of lymphocytes, fibroblasts, and endothelial cells.[5] Recently, researchers have carried out numerous studies of the interactions between macrophages and materials to gain insight into material biocompatibility.[6–12] In these reports, cytokine production from macrophages was evaluated with enzyme-linked immunosorbent assay.[7–9]

We synthesized MPC polymers as novel biomedical materials.[13] Platelet adhesion and activation were suppressed on the surface of the MPC polymers.[14,15] Fibroblast adhesion was also prevented on the MPC polymer surfaces.[16] These results correlate with a reduction in the amount of protein adsorbed onto the MPC polymer surfaces.

We have hypothesized that MPC polymers reduce not only cell adhesion but also the inflammatory re-

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sponse of adherent inflammatory cells. The expression of IL-1β mRNA in cells cultured on MPC polymers was evaluated with RT-PCR analysis.[17,18] HL-60 cells were selected because they can differentiate into macrophage-like cells. In addition, we assessed cell adhesion and protein adsorption so that we could consider their relationship to the expression of IL-1β mRNA.

MATERIALS AND METHODS

Materials

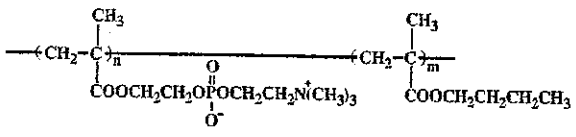
MPC was synthesized by a previously reported method.[13] Butyl methacrylate was purchased from Nacalai Tesque, Inc. (Tokyo, Japan) and purified by distillation under reduced pressure in an argon atmosphere. The chemical structure of the PMBs is shown in Figure 1. PHEMA was synthesized by the radical polymerization of 2-hydroxyethyl methacrylate (HEMA; Nacalai Tesque; bp = 62°C at 3.5 mmHg) with azobisisobutyronitrile (AIBN) as the initiator in 2-propanol. Tecoflex® 60 was obtained from Thermedics, Inc. (Woburn, MA) and used after purification by reprecipitation. A PET plate 185 μm thick was obtained from Toray Industries, Inc. (Tokyo, Japan).

Preparation of the polymer plates

The PET plates (14 or 34 mm in diameter) were cleaned by sonication in acetone and hexane. Each PET plate was dipped in an EtOH solution containing 0.5 wt % PMB; the solvent was slowly evaporated under an EtOH vapor atmosphere at room temperature for 30 min. The plate was soaked in a polymer solution again, stored under an EtOH vapor atmosphere at room temperature for over 1 day, and dried *in vacuo*. For the coating of PHEMA and Tecoflex® 60 on the PET plates, methanol and tetrahydrofuran were used as solvents, respectively.

Surface analysis of the polymer plates

The surface analysis of the PET plates coated with various polymers was performed with X-ray photoelectron spectroscopy (XPS; ESCA-200, Scienta, Uppsala, Sweden). The photoelectron takeoff angle was 90°.



Poly(MPC-co-BMA) (PMB)

Figure 1. Chemical structure of PMB.

Measurement of the total amount of adsorbed plasma protein

The PET plates (34 mm in diameter) were immersed in a phosphate-buffered solution (PBS; pH 7.4) overnight at room temperature for equilibration of their surfaces. They were immersed in a cell culture medium (RPMI1640, Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco BRL) and an antibiotic/antimycotic mixture (Gibco BRL) for 180 min at 37°C. They were removed from the cell culture medium and rinsed with PBS. They were then immersed in a 1 wt % aqueous solution of sodium dodecyl sulfate (SDS) and sonicated for 5 min. The concentration of proteins in the SDS solution was determined by calibration with bovine serum albumin (BSA) standard solutions with a clinical analysis protein assay kit (MicroBCA protein assay reagent kit, Pierce, Rockford, IL).

Detection of a specific protein adsorbed onto the surface

The bovine serum fibronectin and BSA adsorption onto the polymer surface from the cell culture medium was detected by a method based on the antigen–antibody reaction with enzyme-labeled immunoglobulin.[19] The PET plates (14 mm in diameter) were placed in a 24-well tissue culture plate and secured with a silicone rubber ring. They were in contact with PBS overnight at room temperature for equilibration of the surface. The cell culture medium was added to each well after the removal of PBS and kept there for 180 min at 37°C; then, the PET plates were rinsed with PBS. For the determination of fibronectin adsorption, the PET plates were soaked in a BSA (Sigma Chemical, St. Louis, MO) solution (1 wt % BSA in PBS) to inhibit any undesirable reactions and nonspecific adsorption with the following antibody. An ovalbumin (Sigma Chemical) solution (1 wt % ovalbumin in PBS) was used as a blocking reagent for the examination of the adsorbed BSA from the cell culture medium. The PET plates were kept at 4°C overnight and then rinsed with PBS. They were incubated with one of the primary antibodies (antibovine fibronectin rabbit polyclonal antiserum or antibovine albumin rabbit polyclonal antiserum; Yagai Co., Ltd., Yamagata, Japan) for specific proteins for 3 h at 25°C. The primary antibody was reacted with the adsorbed proteins and then rinsed with PBS. The secondary antibody was applied to the PET plates. Horseradish peroxidase (HRP)-conjugated immunoglobulin (antirabbit immunoglobulin G peroxidase conjugate, Sigma Chemical) was used as the secondary antibody. After sufficient rinsing with PBS for the enzyme-linked immunoassay, a solution containing the substrate for HRP, 3,3',5,5'-tetramethylbenzidine (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), was added, and the absorbance of the solution at 450 nm after 15 min was recorded. Measurements were carried

out with triplicate samples for each polymer. A comparative analysis was done with an analysis of variance and a Student *t* test.

Culture of the cells on the polymer surfaces

The HL-60 cells (JCRB0085; Health Science Research Resources Bank) were differentiated to macrophage-like cells by 50 n*M* phorbol 12-myristate 13-acetate. The PET plates were set in a 6-well tissue culture plate. The HL-60 cells were added to each well at a concentration of 3.0×10^5 cells per well in the cell culture medium with 10% FBS. The cells were incubated for given time at 37°C, 5% CO_2, and 95% humidity. The morphology of the adherent cells was observed with a phase-contrast microscope (IMT-2, Olympus Optical Co., Ltd., Tokyo, Japan). The number of adhered cells was determined by lactate dehydrogenase (LDH) assay. After incubation, the PET plates were rinsed with PBS and then placed in 0.5% Triton X-100 for lysing of the adhered cells. The LDH activity in the lysed cell suspensions was measured with an LDH-cytotoxic test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The measurements were carried out with triplicate samples for each polymer.

mRNA isolation and RT-PCR

The procedure was based on that reported by Kishida and coworkers.[17,18] HL-60 cells were cultured on polymer plates for 24 h. The total RNA was isolated from the cells by the acid guanidine method with ISOGEN (Nippon Gene, Tokyo, Japan) and was dissolved in diethylpyrocarbonate-treated water. The RNA concentration was spectrophotometrically measured at 260 nm. The RNA purity was determined from the ratio of the absorbance at 260 nm to that at 280 nm. First-strand complementary DNA (cDNA) synthesis was carried out with 1 μg of total RNA. The RNA samples were reversibly transcribed to cDNA with a Ready-To-Go T-primed first-strand kit (Pharmacia Biotech, Uppsala, Sweden). The PCR reaction was carried out in a volume of 25 μL at final concentrations of 10 m*M* Tris–HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.2 m*M* of each dNTP, and 0.5 μ*M* of each primer and contained 1.5 units of Taq DNA polymerase (Pharmacia Biotech). The primer pair sequences used were obtained from published sequences and were purchased from Takara Biochemicals (Kyoto, Japan). The PCR procedure consisted of 94°C for 30 s, 54°C for 1 min, and 72°C for 45 s with the oligonucleotide primer sets shown in Table I. The products of the PCR reaction were fractionated by electrophoresis on an agarose gel in a Tris–acetate–ethyl enediaminetetraacetic acid buffer at 100 V and were visualized with ethidium bromide staining. The amplified products were confirmed to be those of the gene transcripts by the detection of an 821-bp band (β-actin mRNA) and a 566-bp band (IL-1β mRNA). The values of each band were measured by NIH image program (NIH, Bethesda, MD),[20] and the expression of the IL-1β mRNA was determined as the relative value to that obtained for β-actin. In general, amplified β-actin mRNA was used as an internal standard for the semiquantification.[21,22]

RESULTS

Characterization of the polymers coated on the PET plates

Table II shows the synthetic results for the MPC copolymers. The MPC unit composition in the polymer was the same as that in the feed. The XPS analysis indicated that the surface composition of the MPC unit increased with an increase in the composition of the MPC unit in the PMB.

Protein adsorption on the polymer surfaces

Figure 2 shows the total amount of protein adsorbed onto the various polymer surfaces after contact with the cell culture medium for 180 min. The protein that adsorbed onto PMB was effectively reduced in comparison with that adsorbed onto PET, Tecoflex® 60, and PHEMA. On PMB30, the lowest amount of adsorbed protein was detected. Interestingly, the amount of protein adsorbed onto tissue culture polystyrene (TCPS) was small and the same as that adsorbed onto PMB5 and PMB10.

Figure 3(a,b) shows the results of the enzyme-linked immunoassay for fibronectin and BSA adsorbed onto the polymer surfaces. The vertical axis is the product absorbance in the reaction solution that is proportional to the amount of fibronectin. The amount of fibronectin adsorbed onto PMB5 was the same as that adsorbed onto PET, Tecoflex® 60, and TCPS; however, it decreased with an increase in the MPC unit composition in the PMBs.

The amount of BSA adsorbed onto TCPS was lower than that adsorbed onto PET. The amount of BSA adsorbed onto the PMBs decreased with an increase in the MPC unit composition.

TABLE I
Oligonucleotides of 5′ and 3′ Primers for Targets

Transcript	5′ Primer	3′ Primer	Size of Product (bp)
β-Actin	ACTACCTCATGAAGATCCTC	CTAGAAGCATTTGCGGTGGACGATGG	566
IL-1β	AAGCAGCCATGGCAGAAGTACC	TCTTTAGGAAGACACAAATTGC	821

TABLE II
Synthetic Results of the MPC Polymers

Abbreviation	MPC Molar Fraction			M_w (10^5)[d]	M_w/M_n[d]	Yield (%)[a]
	In Feed	In Copolymer[b]	At Surface[c]			
PMB5	0.05	0.05	0.05	1.0	1.7	59
PMB10	0.10	0.10	0.10	1.6	1.8	57
PMB30	0.30	0.24	0.27	2.4	2.1	80

[a]The polymerization was carried out at 60°C for 15 h in ethanol. [Monomer] = 1.0 mol/L; [AIBN] = 5 mmol/L.
[b]Determined by phosphorus analysis.
[c]Determined by XPS.
[d]Determined by gel permeation chromatography with poly(methyl methacrylate) standard. M_n = number-average molecular weight; M_w = weight-average molecular weights.

HL-60 cell adhesion on the polymer surfaces

The density of HL-60 cells cultured on the various polymers for 24 h is shown in Figure 4. On PET, PHEMA, and TCPS, the number of adherent cells was clearly greater than that on the PMBs. The number of cells on Tecoflex® 60 was similar to that on PET. On the PMBs, the number of adherent cells decreased with an increase in the MPC unit composition. PMB30 dramatically suppressed the cell adhesion.

Phase-contrast microscopy images were recorded after a 24-h culture. The cells that adhered to the PMBs were round in shape, but those that adhered to other polymers and TCPS had a spreading morphology.

Expression of IL-1β mRNA

The expression of IL-1β mRNA was determined by RT-PCR analysis. Figure 5 shows the relative expression of IL-1β mRNA in HL-60 cells cultured on various polymers for 24 h. Depending on the polymers, a significant difference in the expression of IL-1β mRNA from the HL-60 adherent cells was observed. On PET, Tecoflex® 60, PHEMA, and TCPS, a high expression of IL-1β mRNA was detected. However, the expression of mRNA in PMB5 was significantly lower than that in the reference polymers. Moreover, the expression of IL-1β mRNA could not be detected PMB10 or PMB30.

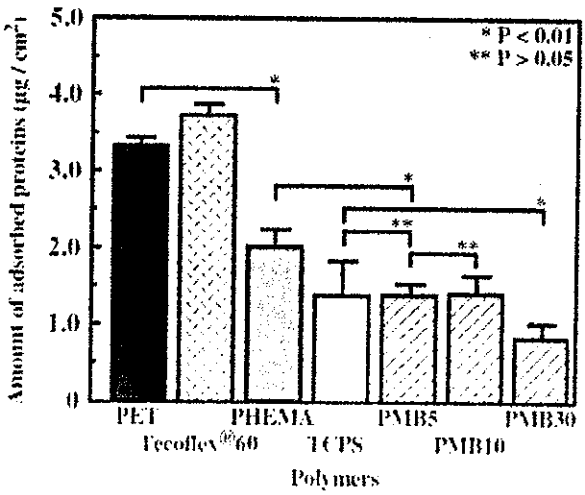


Figure 2. Amount of total protein adsorbed onto the polymer surfaces from the cell culture medium. The mean values of six measurements and standard deviations are indicated.

DISCUSSION

In our previous articles, protein adsorption and blood cell adhesion on MPC polymer surfaces were examined and reported.[13–15] The MPC polymers inhibited platelet adhesion and aggregation. In studies with fibroblasts, the number of adherent fibroblast cells on the MPC polymers decreased with an increase in the MPC unit composition.[16] Protein adsorption is an important factor for determining biocompatibility and thrombogenicity on the materials.[23] Plasma proteins were hardly adsorbed onto the MPC polymer.[24] In particular, the adsorption of cell-adhesive proteins such as fibronectin and von Willebrand factor were suppressed on the MPC polymer in comparison with conventional polymers.[16,23]

The behavior of monocytes with MPC polymers has been studied,[25] and substrates coated with MPC polymers definitely reduce monocyte adhesion. Tegoulia and Cooper[26] examined neutrophil adhesion on phosphorylcholine (PC)-modified polyurethanes and self-assembled monolayers of a PC derivative under flow conditions. Only a slight neutrophil adhesion was observed at all flow conditions for the PC surfaces, but other surfaces showed a large number of adherent neutrophils. That is, the introduction of a PC group contributed to suppressing cell nonspecific adhesion on the surface.

As shown in Figure 2, the amount of total protein adsorbed onto the PMBs was lower than that adsorbed onto PET and Tecoflex® 60. However, there was no sig-

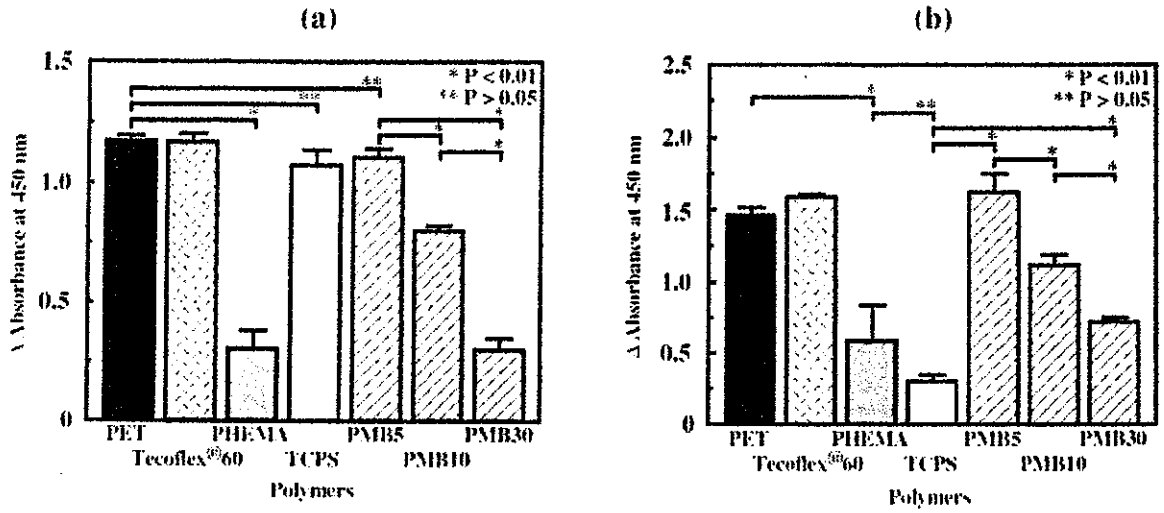


Figure 3. Amount of protein adsorbed onto the polymer surfaces from the cell culture medium (as determined by enzyme-linked immunoassay): (a) fibronectin and (b) BSA. The mean values of three measurements and standard deviations are indicated.

nificant difference between the amount of protein adsorbed onto TCPS and that adsorbed onto PMB5 and PMB10. According to the increment in the MPC unit molar fraction to 0.30 (PMB30), the amount of adsorbed protein was effectively reduced. This result suggests that the MPC units in PMB play an important role in reducing protein adsorption.

PMB effectively reduced HL-60 cell adhesion, as shown in Figure 4. On PMB, the adherent cells were round in shape and not spread. The cell-adhesive proteins adsorbed onto polymer surfaces such as fibronectin can support cell adhesion and spreading, and fibronectin receptors are present on the HL-60 cells.[27,28] On PMB10 and PMB30, the fibronectin adsorption was reduced, and the amount decreased with an increase in the MPC unit composition. BSA adsorption onto the PMB was similar to that of fibronectin. We have already reported that the interactions between the surface of PMB30 and proteins are weak, and proteins can be reversibly adsorbed.[29]

RT-PCR analysis is a highly sensitive method that can detect various mRNAs from a small number of cells in contact with polymeric materials. As shown in Figure 5, adherent HL-60 cells on the PMBs showed a lower expression of IL-1β mRNA than those on other reference polymers. Kishida et al.[17] reported the ex-

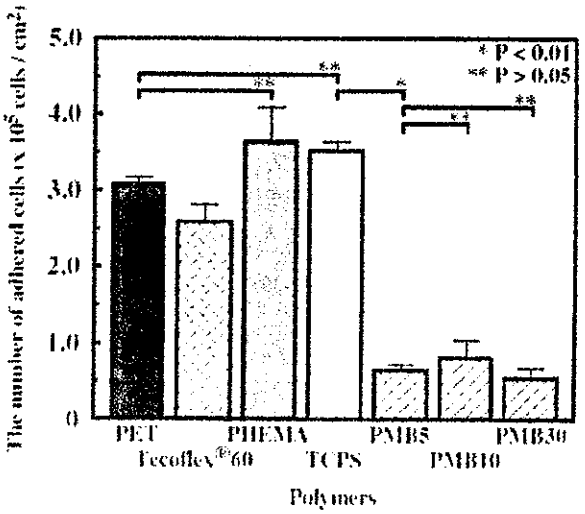


Figure 4. Number of HL-60 cells adhering to the polymer surfaces after a 24-h incubation. The mean values of three measurements and standard deviations are indicated.

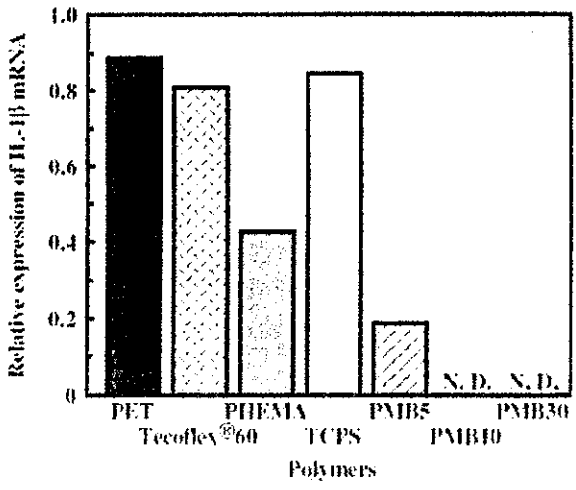


Figure 5. Expression of IL-1β mRNA transcripts in HL-60 cells on the polymer surfaces as a standard of β-actin by RT-PCR after a 24-h incubation. *N.D.* indicates that the expression was not detected.

pression of IL-1β mRNA from HL-60 cells adhered to various polymer surfaces. They described how the expression of IL-1β mRNA was affected by surface wettability and protein adsorption. In this study, the amount of protein on TCPS and PHEMA was the same as that on PMB5 and PMB10. However, the expression of IL-1β mRNA on TCPS and PHEMA was high in comparison with that on PMB.

On the basis of this study, MPC polymers suppress the inflammatory reaction of adherent HL-60 cells. Therefore, MPC polymers are useful as coating materials for implantable biomedical devices.

The authors express their appreciation to Dr. Yoichi Shindo (Toho University) and Dr. Mitsuru Akashi (Kagoshima University) for their helpful discussions. The authors also thank Mr. Akihiko Watanabe (Tokyo Medical and Dental University) for help with the cell culture and Dr. Shinya Kato (Kagoshima University) for help with the RT-PCR procedure.

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Change in cell adhesion property on cytocompatible interface using phospholipid polymer grafted with poly(D,L-lactic acid) segment for tissue engineering

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Abstract

Tissue engineering is a multi-disciplinary science that utilizes basic principles from materials engineering and molecular biology to reconstruct tissues from polymer matrices and cellular components. Artificial skins were well known as one of the concrete examples. Technological innovation of the tissue engineering must be contributed to improve quality of life. From the viewpoint, design of cytocompatible materials for tissue engineering would be the most important candidate to reconstruct tissue. 2-Methacryloyloxyethyl phosphorylcholine (MPC), *n*-butyl methacrylate, and polylactic acid (PLA) macromonomer were polymerized for the preparation of cytocompatible interface. The polymer may involve following novel properties: (i) cytocompatibility by phospholipid groups, and (ii) enhancement of cell adhesion by PLA segment. The results of X-ray photoelectron spectroscopy showed the MPC unit and PLA segment on the membrane, which was prepared by dip coating. The surface mobility by contacting water was estimated with static contact angle measurement. The contact angle by water decreased after contact with water due to the chain rearrangement of hydrophilic MPC unit. Fibroblast cells adhesion and protein adsorption on the membranes were studied. The number of cell adhesion and cell proliferation on the membrane was well correlated with each other. Furthermore, the number of cell adhesion was proportional to the PLA macromonomer (MaPLA) composition. The adherent cell morphology showed round shape, because of the existence of MPC unit. However, the cell morphology would be spread after the cell proliferation. These findings suggest that the change in the polymer composition by combination of MPC and MaPLA could regulate the number of cell adhesion and the morphology.

Keywords: Phospholipid polymer; Polylactic acid; Tissue engineering; Cytocompatibility; Cell adhesion; Bio-interface

1. Introduction

Tissue engineering consists of a multi-disciplinary science, including fundamental principles from materials engineering and molecular biology. Langer et al. reported a leading work on tissue engineering; cells, bioactive molecules, and polymer matrices are required to regenerate tissue [1]. Recent biological achievements regarding cell culture using bioactive molecules are promising techniques to regenerate tissue. Therefore, materials for tissue engineering play a great important role towards the development of biomaterials. As for the biomaterials on tissue engineering, biodegradable polymers have been used, for example, synthetic polymers such as poly(α-hydroxy acid) and also natural polymers such as hyaluronan and collagen. The synthetic and natural polymers were molding for the membrane, plate, sponge-form, and hydrogel. The conventionally using polymers involve excellent molding process, however, no guarantee regarding the cytocompatibility exists on the polymers because the conventional polymers are not properly designed for tissue engineering. From this, preparation of cytocompatible polymer material is quite important on further development for tissue engineering.

Cytocompatibility on the biomaterials was generally evaluated using lactate dehydrogenase (LDH) assay [2] and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [3]. The LDH assay provides the information regarding cellular membrane damage by the release of the cytosolic enzyme, reflecting short incubation time with biomaterials. On the other hand, MTT assay shows detrimental intracellular effects on mitochondria and

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metabolic activity. The obtained information was limited after suffering damage from the biomaterials. Anderson et al. systematically evaluated the cytokine production from macrophages using cytotoxic activity and enzyme-linked immunosorbent assay (ELISA) [4]. Though the ELISA technique was detectable, the information on contacting cell on the biomaterials, however, sensitivity was major and dominant problems due to the detection of quite low amount of secreted proteins. Kishida et al. proposed a novel methodology for the cytocompatibility, the evaluation of one of the well-known cytokines, interleukin-1β (IL-1β) mRNA expression from the cells contacting with the biomaterials [5]. The expression of IL-1β indicated inflammatory response in the cells.

Among the numerous kinds of polymer materials, a series of phospholipid polymer, poly(2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-*n*-butyl methacrylate (BMA)), would be good candidates for the cytocompatibility. Originally, the MPC was strongly inspired by cellular membrane structure, which was assembled phospholipid molecules [6], and then synthetic route was developed in high purity [7]. The most advantageous characteristics of the phospholipid polymer showed excellent blood and biocompatibility in contact with biological components in vivo and in vitro [8,9]. Furthermore, the phospholipid polymer prevented the cells on the biomaterials from inducing the inflammatory response, estimation by IL-1β mRNA expression [10,11]. Taking these characteristics into account, the phospholipid polymer are promising cytocompatible materials for tissue engineering. However, the number of cell adhesion via adsorbed protein layer was quite low, because the phospholipid polymer surface suppressed protein adsorption. Then, the following cell adhesion would not happen. Therefore, cytocompatible interface with cell adhesion property was designed; a novel macromonomer with poly(D,L-lactic acid) (PLA) short segment was used for the cytocompatible interface [12]. In this paper, the cytocompatible polymers were systematically prepared by changing monomer composition in feed, and the obtained polymers were coated on the cell culture substrates. The number of cell adhesion was discussed in relation to the surface properties.

2. Experimental

2.1. Materials

D,L-lactide was kindly supplied by Musashino Chemical Laboratory (Tokyo, Japan) and was recrystallized from ethyl acetate. *n*-Butyl methacrylate (BMA, Wako Pure Chemical Co., Ltd, Osaka, Japan) and 2-isocyanate ethyl methacrylate (IEMA, Showa Denko Co., Tokyo, Japan) were distilled at reduced pressure, and the following fractions were used (BMA: 50 °C/20 mm Hg, IEMA: 60 °C/2.5 mm Hg). *n*-Dodecanol, stannous octoate ($Sn(oct)_2$), and dibutyltin dilaurate (DBTL) were purchased from Wako Pure Chemical Co., Ltd and used without further purification. MPC was synthesized and purified by a method previously reported [7]. Other reagents were commercially available and used without further purification.

2.2. Preparation of phospholipid polymer membranes

The preparation of phospholipid polymer was already reported in our previous paper [12], the synthetic outline was briefly described. PLA macromonomer (MaPLA, Fig. 1(a)) was synthesized, following two steps:

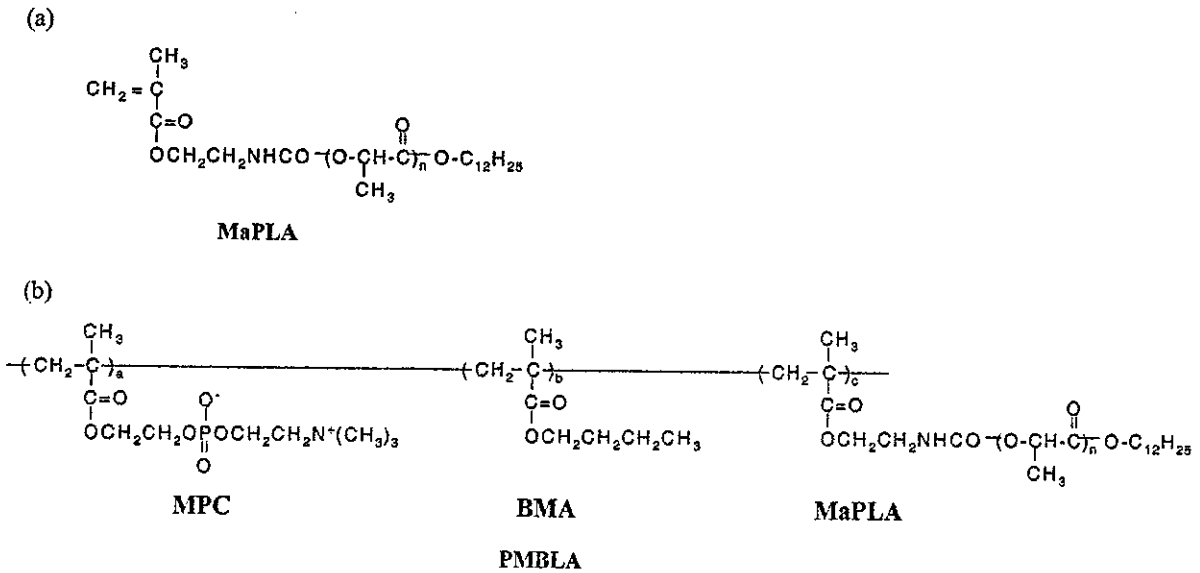

Fig. 1. Chemical structure of (a) poly(D,L-lactic acid) macromonomer (MaPLA) and (b) phospholipid polymer PMBLA.

(i) ring-opening polymerization of D,L-lactide using *n*-dodecanol as an initiator, and (ii) methacrylation of the terminal hydroxyl group in PLA with IEMA. The MaPLA was copolymerized with MPC and BMA to prepare the phospholipid polymers (Fig. 1(b)).

The phospholipid polymer was dissolved in chloroform (1 w/v%), and was coated on poly(ethylene terephthalate) (PET) membrane (diameter: 14 mm, Wako Pure Chemical Co., Ltd) as a substrate. The membrane prepared by dip coating was dried in vacuo (before contacting water). For the surface equilibration, the membranes were immersed in distilled water for overnight (after contacting water). Poly(D,L-lactic acid-*co*-glycolic acid) (PLGA) was used as a conventional polyester, purchased from Aldrich Chem. Co., WI, USA.

2.3. Characterization of surface properties

The surface properties were characterized in terms of elemental analysis and molecular mobility. X-ray photoelectron spectroscopy (XPS, AXIS-HSi, Shimadzu/KRATOS, Kyoto, Japan) with MgKα was carried out. The analyzer was placed perpendicular to the surface of the membrane. Static contact angle by water was measured using an automatic contact angle meter apparatus (CA-W, Kyowa Interface Science Co., Ltd, Saitama, Japan) at 25 °C. The drop of pure water was introduced on the membrane using a micro syringe.

2.4. Protein adsorption and cell culture on the membranes

Protein adsorption was evaluated using bovine serum albumin (BSA, A-8022, SIGMA, MO, USA), bovine gamma globulin (BγG, G-5009, SIGMA), and bovine plasma fibrinogen (BPF, F-8630, SIGMA). The concentrations of the proteins were adjusted to 4.5, 1, and 0.3 mg/ml, respectively. The membranes were firstly equilibrated by phosphate buffer saline (PBS) for overnight, and were incubated in each protein solution (pH 7.4) for 3 h. After rinsing with PBS, the adsorbed proteins were removed by 1 wt% of *n*-sodium dodecyl sulfate (SDS). The recovered proteins from the membranes were evaluated using Micro BCA kit (Pierce, No. 23235, IL, USA).

Mouse fibroblast (L-929) cells were purchased from RIKEN cell bank (Saitama, Japan) and were routinely cultured in Eagle's Minimum Essential Medium (E-MEM, Nissui, Tokyo, Japan), supplemented with 5% calf serum (CS, Gibco, NY, USA) at 37 °C in a 5% CO_2 atmosphere. After treatment with 0.25% trypsin, the cell density was adjusted to 6×10^3 cells/ml and the cells were then seeded on the membranes. After 6 h, 1 day, 2 and 5 days, the number of adherent cells were treated with 0.1 wt% SDS solution, and then evaluated by LDH assay kit (Wako Pure Chemical Co., Ltd). The cell morphology was observed by phase contrast microscope (IX71, OLYMPUS, Tokyo, Japan).

3. Results and discussion

3.1. Surface properties on membranes

Repeating number of lactic acid unit in the PLA segment was determined ca. 40 using ^{1}H NMR. The polymer composition is summarized in Table 1. The PLA unit was quantitatively incorporated into each phospholipid polymer. The compositions of the MaPLA unit in the polymers were 5, 10, 29, and 53 mol%. Each polymer was abbreviated as PMBLA5, PMBLA10, PMBLA30, and PMBLA50, respectively. On the other hand, MPC unit was incorporated in the range of 10–20 mol%. All of the polymers were dissolved in non-polar solvent such as chloroform.

The polymer membranes were prepared by dip coating onto the PET substrates. Interference fringe was observed on the substrates; therefore, nano-scale polymer coating layer (below 100 nm) was then prepared. The PMBLA involved hydrophilic monomer unit (MPC) and hydrophobic graft segment (PLA). Generally, block- and graft-type copolymer would spontaneously form domain structure. Thus, the possibility of the domain formation would be undeniable. On the XPS measurement, the releasing angle of the photoelectron for each atom was fixed 90°. The detecting depth was about 10 nm, which is thinner than the thickness of the polymer membrane by dip coating. Therefore, obtained results indicated surface properties regarding the coating polymer. Fig. 2 shows XPS results of the membranes after contact with water. In the case of N_{1s} core level spectra of PMBLA5, nitrogen peaks of both urethane bonds and trimethyl ammonium groups were observed at 399 and 402 eV, respectively. Phosphorus peak corresponding to the phosphate ester in PMBLA5 was also found at 133 eV. However, the differences on peak intensity attributed to the MaPLA and MPC units were not clearly observed. In the case of PET substrate, no nitrogen and phosphorus peaks were found as expected. These results indicate that the phospholipid moiety and PLA segment were located onto the surface after contacting water.

In order to estimate the mobility of the polymer segment on the membrane surface, static contact angle by water was

Table 1
Synthetic results of phospholipid polymers

Abbreviation	Monomer unit composition[a] (mol%)						Yield (%)
	In feed			In polymer			
	MPC	BMA	MaPLA	MPC	BMA	MaPLA	
PMBLA5	5	90	5	10	85	5	40
PMBLA10	5	85	10	13	77	10	46
PMBLA30	5	75	20	20	51	29	63
PMBLA50	5	65	30	16	31	53	53

Preparative condition: [Monomer] = 0.5 mol/l, [AIBN] = 2.5 mmol/l, 60 °C, 24 h.

[a] Determined by ^{1}H NMR.

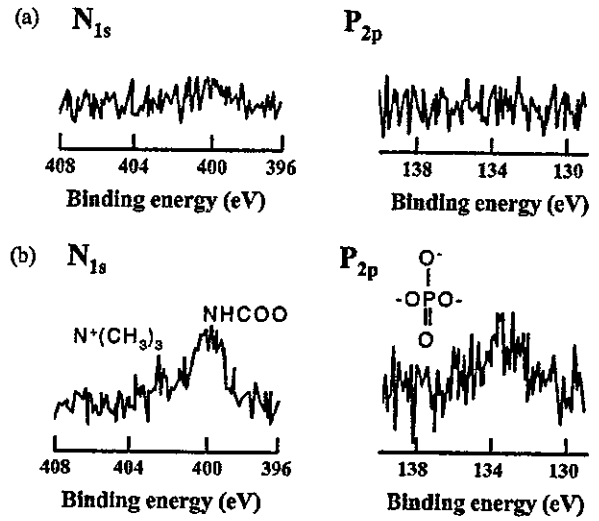

Fig. 2. N_{1s} and P_{2p} core level spectra with XPS; (a) PET and (b) PMBLA5.

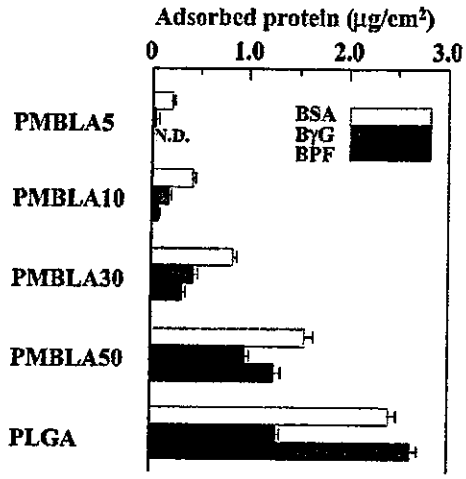

Fig. 3. Results of protein adsorption on the PMBLA and PLGA membranes. Mean values of three measurements and standard deviation are indicated. ND means not detected.

measured as shown in Table 2. For example, 88.9° was found on the PMBLA10 before contacting water. After contacting water, the contact angle decreased, and the hysterysis was then ca. 17°. The other PMBLAs were also observed of the hysterysis, 7–10°. This result indicated that spontaneous rearrangement of the hydrophilic–hydrophobic segment on the membrane occurred to reduce surface free energy. It was suggested that MPC unit was located onto the membrane after contacting water. In the case of PLGA coating surface, the contact angle did not change, indicating relatively higher hydrophobic surface.

3.2. Protein adsorption on membranes

Protein adsorption was evaluated using BSA, BγG, and BPF. These proteins are very popular for evaluation of biocompatibility on the surface. The amount of protein adsorption is summarized in Fig. 3. In the case of PMBLA5, all kinds of protein adsorption were below 0.2 μg/cm^2, in particular, BPF adsorption was not detected (roughly zero). The amount of adsorbed proteins increased with composition of PLA unit in the polymer, indicating enhancement of affinity between proteins and polymer membranes. In PMBLA50 surface, the amount of adsorbed BSA, BγG, and BPF increased to be ca. 1.6, 1.0, and 1.3 μg/cm^2, respectively. Generally, the amount of maximum protein adsorption was calculated as mono-layered adsorption on the surface, and obtained as follows: BSA: 0.9 μg/cm^2, BγG: 1.8 μg/cm^2, and BPF: 1.7 μg/cm^2. From these calculated values, it is suggested that multi-layer BSA adsorption was located on the PMBLA50 and the other proteins (BγG and BPF) were adsorbed within mono-layer on the surface. On the other hand, 1–2.5 μg/cm^2 of protein adsorption was observed on PLGA membrane, indicating multi-layer protein adsorption. From this, it was considered that amount of protein adsorption on the PMBLA was relatively lower than that of PLGA surface, even if higher MaPLA content (PMBLA50), reflecting protein adsorption resistant MPC unit. The major factor on protein adsorption was to be PLA unit, which functional unit effectively increased protein adsorption. Cell adhesion would be favorable with increasing PLA composition in the polymer.

Table 2
Static contact angle by water

Abbreviation	Contact angle (°)[a]	
	Before contacting water	After contacting water
PMBLA5	85.4 ± 1.2	75.8 ± 2.3
PMBLA10	88.9 ± 1.1	71.8 ± 1.1
PMBLA30	82.5 ± 1.1	74.8 ± 1.8
PMBLA50	81.3 ± 1.6	74.0 ± 0.9
PLGA	97.8 ± 2.0	101.4 ± 2.2

[a] Mean values of the 10 measurements and standard deviations are indicated.

3.3. Cell adhesion on membranes

L-929 cell adhesion was evaluated as shown in Fig. 4. Primary seeding density was 6×10^3 cells/cm^2 in each membrane. In the case of PMBLA5 membrane, the number of cell adhesion was determined to be below 500 cells/cm^2, then the data was shown using asterisk (*) in Fig. 4. PMBLA10 also showed low cell adhesion at 6 h. The PMBLA5 surface showed very low affinity to the cells. From the protein adsorption data, the PMBLA5 surface would be very inert to the BPF adsorption. The dominant factor regarding the cell adhesion was discussed as follows. Generally, cell adhesion on the membrane was induced via protein adsorption. The protein adsorption, particularly, fibrinogen and fibronectin such as cell adhesive proteins, were quite important, because receptors on the cell

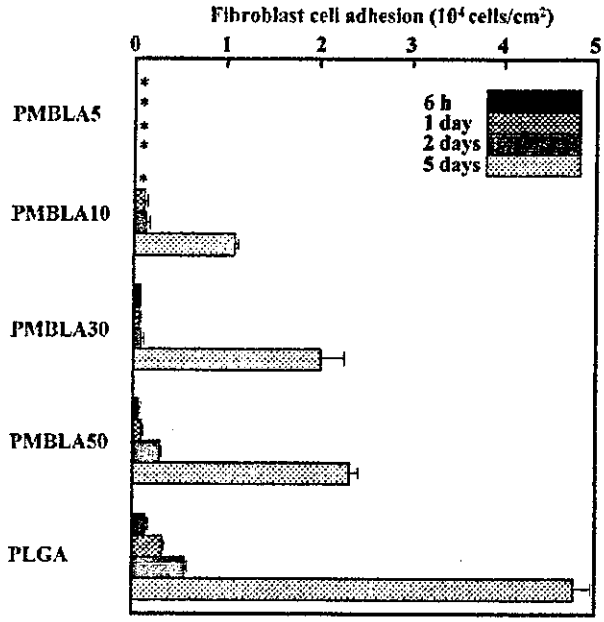

Fig. 4. Fibroblast cells adhesion on the PMBLA and PLGA membranes after 6 h, 1, 2, and 5 days. Mean values of three measurements and standard deviation are indicated. (*) Below 500 cells/cm^2.

membrane recognized the adhesive proteins via RGDS (arginine–glycine–aspartic acid–serine) moiety, and the cell attached on the surface via protein adsorption layer. Therefore, the relationship between amount of protein adsorption and number of cell adhesion was observed with similar tendency. Taking these results into account, the number of cell adhesion was dependent on the amount of protein adsorption. It is considered that over 0.5 μg/cm^2 of protein adsorption was onset for cell adhesion at primary stage.

On the other hand, large number of primary cell adhesion was observed after 6 h; 1000 cells/cm^2 of the fibroblast adhered on the PMBLA30 and PMBLA50, and PLGA showed good adherent property (3600 cells/cm^2). Among them, the difference on the primary adhesion was considered effect of phospholipid unit. However, cell proliferation after 5 days was observed except for PMBLA5. This result indicated that the phospholipid polymer surface with PLA segment showed biologically inert on primary stage. The adherent cells on the inert surface (PMBLA) would show nice cell proliferation as same as on PLGA. It is suggested that the protein adsorption from the serum in the medium, produced proteins from the adherent cells were very important for the cell growth. Furthermore, the cell proliferation was proportional to the PLA content in the polymer. The cell proliferation was also dependent on the amount of protein adsorption. These results suggest that the biologically inert surface on the phospholipid polymer is changeable by incorporation of MaPLA.

The cell morphology was observed with phase contrast microscopy as shown in Figs. 5 and 6. The cell morphology was clearly shown as a round shape on MPC incorporated membrane after 6 h. On the other hand, spread morphology has already observed on PLGA membrane after 6 h. The difference on the cell morphology was based on the difference of cell–material interaction via protein adsorption layer. It is considered that the adhesive properties between the membrane surface and the cells would be weaker due to the small amount of protein adsorption. After 5 days, spread morphology was observed on the membrane except for PMBLA5. Cell proliferation was also observed on the PMBLA10, PMBLA30, and PMBLA50, which surfaces showed the small amount of cell adhesion at 6 h. From these results, the phospholipid polymer with PLA

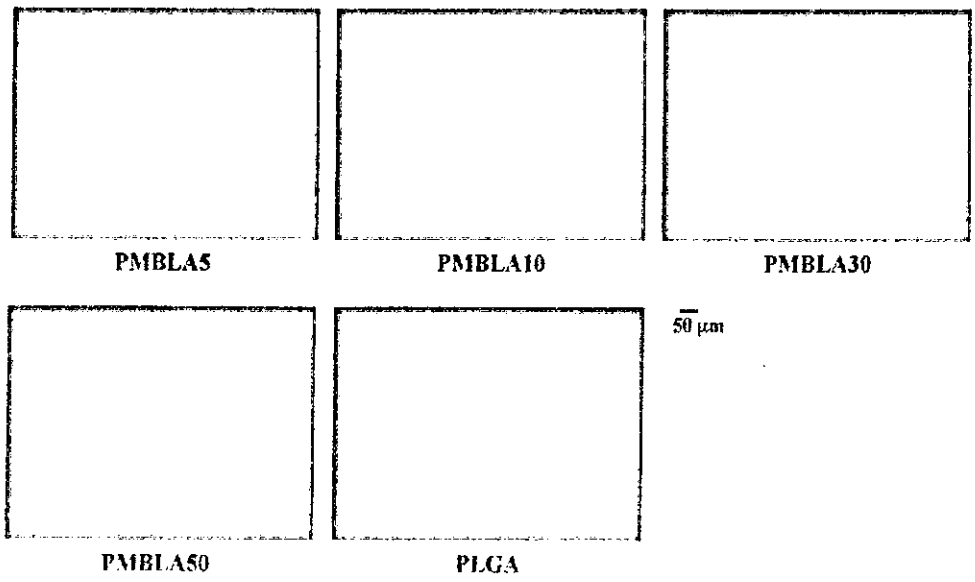

Fig. 5. Cell morphologies of fibroblast on the PMBLA and PLGA membranes after 6 h.

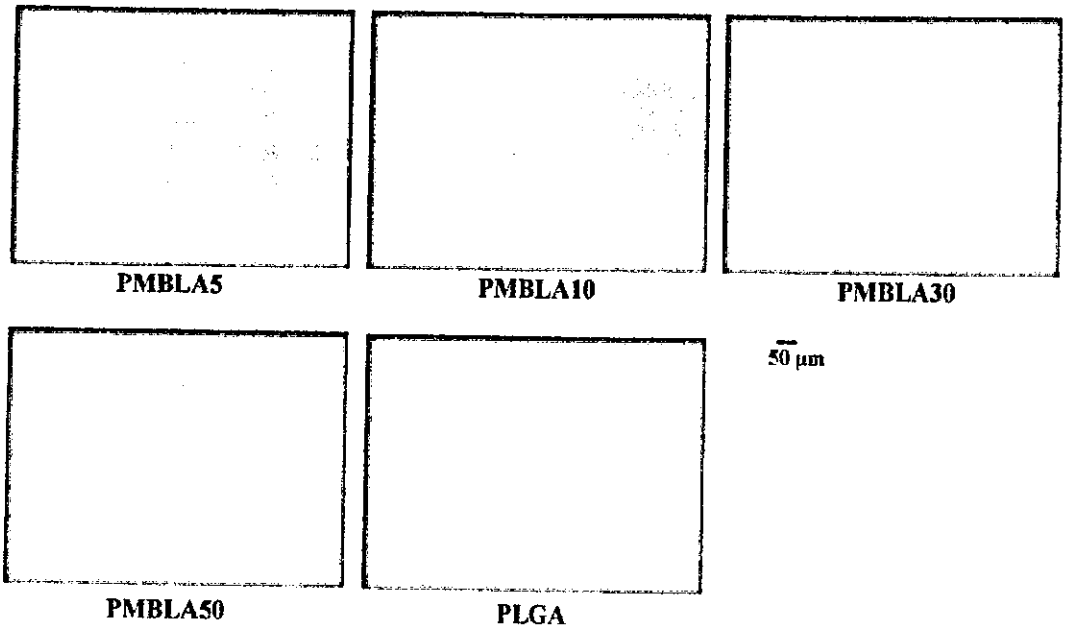

Fig. 6. Cell morphologies of fibroblast on the PMBLA and PLGA membranes after 5 days.

segment showed changeable cell adhesion property by PLA composition, and the surface property on phospholipid polymer (PMBLA) would reach PLGA surface as well-known PLA polymer.

4. Conclusions

The novel phospholipid polymers with PLA segment were synthesized for the preparation of cytocompatible interface. The polymer membrane surfaces involved the MPC unit and PLA segment, indicating the higher hysterysis on contact angle measurement and XPS analysis. The surface properties were spontaneously changed by chain rearrangement of MPC unit after contacting water. The number of cell adhesion increased with incorporation of PLA composition. And the membrane with higher PLA composition showed good cell proliferation, even when the small number of cell adhesion at primary stage. The phospholipid polymer membrane surface would be active with increasing MaPLA content for cell adhesion. These results are of great important for the design of cytocompatible interface.

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Phosphorylcholine and Poly(D,L-lactic acid) Containing Copolymers as Substrates for Cell Adhesion

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Abstract: Fibroblast cell culture was performed to evaluate cell adhesion and cell morphology on novel hydrolyzable copolymers composed of poly(D,L-lactic acid) (PDLA) macromonomer, 2-methacryloyloxyethyl phosphorylcholine (MPC), and *n*-butyl methacrylate. The copolymers were used as cell culture materials for regulating the interaction between the cells and the polymer surface. The results of X-ray photoelectron spectroscopy (XPS) confirmed that the PDLA chains and MPC units were present in the copolymer coating on PET films. Cell adhesion and morphology of adherent cells on coatings of the copolymers were studied. The number of cells on the surface increased with the PDLA content of the copolymer. As for the cell morphology, a round shape was observed on copolymers containing MPC units. These findings suggest that the cells recognize the PDLA and MPC units on the surface via changes in protein adsorption and/or conformation, and that the numbers of adhering cells and the cell morphology can be regulated by the composition of the copolymer. **Key Words:** Poly(D,L-lactic acid)—2-Methacryloyloxyethyl phosphorylcholine—Fibroblast—Cell adhesion—Cell culture.

Cell adhesion on polymer surfaces is facilitated by the adsorption of adhesive proteins such as fibronectin and vitronectin. The morphology of adhering cells can be classified into round, spread, and intermediate stages (1,2). It is considered that the morphology of cells is regulated by the following two dominant factors: cell-protein interaction and protein-polymer surface interaction. Generally, cells are cultured on surface-treated poly(styrene) dishes such as tissue culture poly(styrene) (TCPS). Excellent cell growth is observed on TCPS. However, a serious problem was recently reported (3). An inflammatory reaction of the cells on conventional polymer-coated surfaces was observed by evaluating the messenger RNA (mRNA) expression of inflammatory cytokines. Control of the interaction between cells and polymer surfaces is necessary for developing novel biomaterials that do not induce inflammatory reactions (4).

2-Methacryloyloxyethyl phosphorylcholine (MPC), which has been synthesized because of its resemblance to the chemical structure of phospholipids in biomembranes, was copolymerized with *n*-butyl methacrylate (BMA) (5,6). The MPC copolymers were widely investigated and used as an excellent biomaterial suppressing protein adsorption (7–9) and cell adhesion (10,11). In order to prepare novel cell culture materials, a copolymer consisting of MPC, BMA, and poly(D,L-lactic acid) (PDLA) macromonomers were designed. With this copolymer it should be possible to regulate the cell-material interaction by changing the copolymer composition. Other advantages of these copolymers for culturing cells may be suppression of inflammatory reactions by the MPC unit, adequate cell adhesion via the PDLA chains, and tissue recovery from copolymer-coated surfaces after hydrolysis of the PDLA side-chains. The objective of this article is to study the interaction of cells with copolymer-coated surfaces. In the synthesis of the copolymers, the characterization of the surfaces and the adhesion and

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