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Cytocompatible Graft Polymer Materials Composed of Phospholipid Polymer and Poly(lactic acid)

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Cell culture graft-polymer materials were designed and synthesized by using 2-methacryloyloxyethyl phosphorylcholine (MPC), *n*-butyl methacrylate, and poly(lactic acid) (PLA) macromonomers. The polymers may involve following novel properties; (i) cell compatibility with phospholipid groups, and (ii) adequate cell adhesion by poly(lactic acid) segment. The results of X-ray photoelectron spectroscope showed the MPC unit and poly(lactic acid) segment on the coating membrane. 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 MPC unit. Fibroblast cells adhesion and the morphology on the membranes were studied. The number of cell adhesion and cell growth on the membrane including poly(D-lactic acid) and poly(L-lactic acid) segment were larger than that of poly(D,L-lactic acid) segment. The cell morphology was observed after seeding 48 h. The adherent cell morphology showed round shape by the existence of MPC unit. The morphology would be spread with increasing the macromonomer content in the polymer. These findings suggest that the change in the polymer composition by combination of MPC and PLA macromonomer could regulate the number of cell adhesion and the morphology.

Key words: Phospholipid polymer, poly(lactic acid), tissue engineering, cytocompatibility, cell adhesion.

1. INTRODUCTION

Tissue engineering is a multidisciplinary 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. Langer *et al.* reported a pioneer work; three-dimensional tissue reconstruction by using porous polymer matrix [1]. Requirements of the polymer materials may involve: (i) cytocompatibility, (ii) biodegradable properties, and (iii) mechanical properties. Poly(lactic acid) (PLA) have been widely discussed as tissue engineering material due to the excellent mechanical property and biodegradability [2]. However, inflammatory response was observed from the cultured cells on the conventional polymers such as PLA. Iwasaki *et al.* reported the improvement of the inflammatory response [3]. A series of phospholipid polymers, which is composed of 2-methacryloyloxyethyl phosphorylcholine (MPC), show excellent blood- and bio-compatibility *in vivo* and *in vitro* [4-5]. And also, MPC polymer prevents from inducing the inflammatory response by gene expression [3].

In this paper, cytocompatible graft polymer materials were designed by using the MPC. A novel macromonomer which has PLA short segment (D,L-form, D-form, and L-form) in side chain was polymerized with the MPC. The polymer was utilized as coating regent to prepare cytocompatible membranes. Cell adhesion, morphology, and protein adsorption on the membrane surface were discussed.

2. MATERIALS AND METHOD

2.1 Materials

D,L-Lactide was kindly supplied by Musashino Chemical Laboratory (Tokyo, Japan) and was recrystalized from ethyl acetate. L-Lactide and D-lactide were kindly supplied by Dainippon Ink and Chemicals, Inc. (Tokyo, Japan) and were used as received. *n*-Butyl methacrylate (BMA), *n*-Dodecyl alcohol, stannous octoate (Sn-(oct)$_2$), and dibutyltin dilaurate (DBTL) were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan), and BMA was distilled at 50 °C (20 mmHg). 2-Isocyanate ethyl methacrylate (IEMA, Showa Denko Co., Tokyo, Japan) were distilled at reduced pressure (60 °C/ 2.5 mmHg).

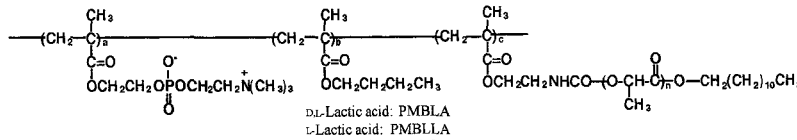

Figure 1. Chemical structure of phospholipid polymers.

2-Methacryloyloxyethyl phosphorylcholine (MPC) was synthesized and purified by a method from a previous report [6]. The other reagents were commercially available and used without further purification.

2.2 Synthesis of phospholipid polymers

The macromonomers were synthesized following two steps: (i) polymerization of D,L- or L-(D-)lactide by *n*-dodecyl alcohol as an initial point (prepolymer), and (ii) methacrylation of the prepolymer by IEMA. The phospholipid polymers were synthesized by copolymerization of MPC, BMA, and corresponding macromonomer. Preparative condition in detail was reported previous paper [7-8].

2.3 Preparation of phospholipid polymer membranes

The phospholipid polymer was dissolved by using chloroform, and adjusted the concentration to 1 wt%. To prepare the thin polymer membrane, poly(ethylene terephthalate) (PET, diameter=14 mm) was used as base materials. The membrane prepared by dip coating was dried *in vacuo* (before contacting water), and immersed in distilled water or phosphate buffered saline (PBS) for surface equilibration (after contacting water). Poly(D,L-lactic acid-co-glycolic acid) (PLGA) was purchased from Aldrich Chem. Co., WI, USA, and was coated on the PET films using same procedure.

2.4 Characterization of the membrane 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 by using an automatic contact angle meter apparatus (CA-W, Kyowa Interface Science Co., Ltd., Saitama, Japan) at 25°C. The drop (10 μL) of pure water was introduced on the membrane by using a micro syringe.

2.5 Protein adsorption and cell culture on the membranes

Protein adsorption was evaluated by 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 membranes were firstly equilibrated by immersion in PBS for over night, and were incubated in each protein solution (pH 7.4) for 3 hours. The adsorbed proteins were evaluated by using Micro BCA kit (Pierce, #23235, IL, USA). Mouse fibroblast (L-929) cells were used and were routinely cultured in Eagle's Minimum Essential Medium (E-MEM, Nissui, Tokyo Japan), supplemented with 10% fetal bovine serum (FBS, Gibco, NY, USA) at 37°C in a 5% CO_2 atmosphere. After treatment with 0.25% trypsin, the cell density was adjusted to $3x10^4$ cells/mL and the cells were then seeded on the membrane. After 24 h and 48 h, the numbers of adhering cells and the cell morphology were evaluated.

3. RESULTS AND DISCUSSION

3.1 Synthesis of macromonomer and phospholipid polymers

Molecular weight of the macromonomers was estimated by gel permeation chromatography (poly styrene standard), and was to be *ca.* 7900 (D,L-form), 3970 (L-form), and 3680 (D-form). As an alternative method, ^{1}H-NMR measurement of the macromonomers was carried out, and the degree of polymerization in poly(lactic acid) segment was 40 (D,L-form), 23 (L-form), and 27 (D-form). Concentration of the terminal methacryloyl group would relatively decrease in comparison with co-monomers. Based on this consideration, reactivity of the macromonomer would be lower than that of co-monomers. From the viewpoint of the polymer sequence, BMA was used as a role of spacer, because there are bulky side chains in the MPC and PLA macromonomer. Taking both the lower reactivity and the bulky side chain of the macromonomer into account, it is suggested that the sequence of the MPC, BMA, and the macromonomer in the polymer would be random-type polymer.

The phospholipid polymers, PMBLA, PMBLLA, and PMBDLA were obtained. Here, M, B, and LA (LLA and DLA) refer to the MPC unit, BMA unit, and D,L- (L-form and D-form) lactic acid unit, respectively. Synthetic results regarding monomer content are shown in Table I. The polymerization was carried out for 24 hours in methylene chloride/ethanol=1/1 (by volume). MPC unit and macromonomer content in the polymer were 13-16 mol% and 10-16 mol%, respectively. This result indicated that the polymerization was quantitatively proceeding as feeding monomer content.

Table I. Synthetic results of phospholipid polymers.

Code	In polymer (mol%)			DP*	Yield (%)
	MPC	BMA	Macromonomer		
PMBLA	13	77	10	40	46
PMBLLA	16	72	12	23	69
PMBDLA	16	68	16	27	55

*DP: Degree of polymerization in poly(lactic acid) segment determined by ^{1}H-NMR.

3.2 Surface properties of the membranes

On the XPS measurement, the releasing angle of the photoelectron for each atom was fixed 90 degree. The detecting depth was within 10 nm which is well correlate with the thickness of the polymer membrane by dip coating. This result indicated that XPS measurement would reflect the membrane by the polymer. Figure 2 shows XPS results of the membrane after contact with water. In the case of N1s core level spectra of PMBLA, nitrogen peaks of both urethane bonds and choline methyl groups were observed at 399 and 402 eV, respectively. Phosphorus peak corresponding to the phosphate ester in PMBLA and was found at 133 eV. In PMBLLA, the spectra attributed to each functional group were obtained at 400 eV (N1s), 402 eV (N1s), and 134 eV (P2p). Similar spectra were observed on PMBDLA membrane (data not shown). In the case of PET as a base material, no nitrogen and phosphorus peaks were found as expected. These results indicate that the chemical composition on the membrane surfaces corresponds to the polymer composition.

In order to estimate the mobility of the segment in the membrane surface, static contact angle by water was measured as shown in Table II. The contact angle of PMBLA was found to be 88.9° before contacting water.

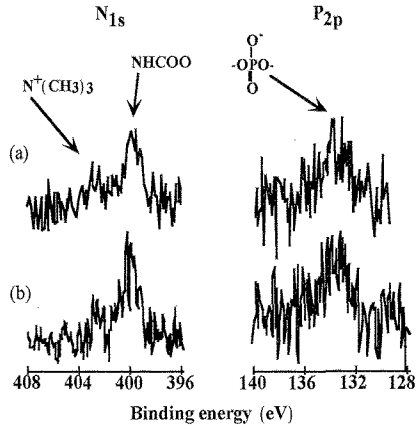

Figure 2. X-ray photoelectron spectroscopy of (a) PMBLA and (b) PMBLLA membranes after contact with water.

Table II. Results of static contact angle on the membranes.

Code	Before contact with water	After contact with water
PMBLA	88.9 ± 1.1	71.8 ± 1.1
PMBLLA	77.6 ± 0.8	70.0 ± 2.3
PMBDLA	78.4 ± 1.4	68.1 ± 1.3
PLGA	97.8 ± 2.0	101.4 ± 2.2
PET	82.4 ± 1.8	76.4 ± 1.4

n=10, Mean ± SD.

In the case of PMBLLA and PMBDLA, the contact angle showed around 78.0°. The difference among them would be based on chemical composition; especially degree of polymerization in the PLA segment. After contacting water, the contact angle uniformly decreased around 70°. In this case, significant difference among the membranes (PMBLA, PMBLLA, and PMBDLA) was not observed. This result indicated that spontaneous rearrangement of the hydrophilic-hydrophobic component on the membrane surface was occurred to reduce surface free energy. Ishihara *et al.* reported that the contact angle decreased with increasing the MPC unit in the copolymer [9]. It is considered that the spontaneous rearrangement was dominated by the strong hydrophilicity of the phosphorylcholine group. The time to reach complete rearrangement was quite short period within a few minutes. The interface on the membrane would show good cell attachment. Ikada *et al.* systematically examined regarding to the relationship between cell adhesion and surface wettability [10]. In the report, cell adhesion appears to be maximized on the surface with adequate wettability, which is shown around 70° as contact angle by water. Taking these reports into account, the phospholipid polymer membranes with poly(lactic acid) segment would show good cell adhesion.

3.3 Protein adsorption behavior on the membranes

Protein adsorption was evaluated by using BSA, BγG, and BPF. These proteins are very popular for characterization of property on biomaterials surface; especially biocompatibility. The amount of protein adsorption is shown in Figure 3. In the case of PMBLA, protein adsorption was below 0.5 μg/cm^2. On the other hand, 1-2 μg/cm^2 of BγG and BPF adsorption and over 4 μg/cm^2 of BSA adsorption were observed on the PMBLLA and PMBDLA. PLGA and PET also show similar protein adsorption (over 4 μg/cm^2). Generally, it is reported that 0.9-1.8 μg/cm^2

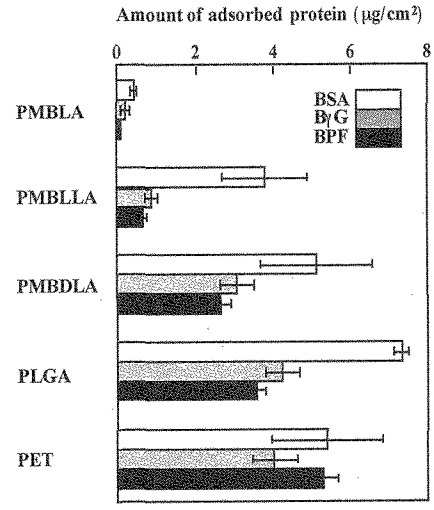

Figure 3. Protein adsorption on the membranes at 37°C. Mean values of three measurements and standard deviation are indicated.

of protein adsorption was formed on the membrane surface as mono-layer [11]. From this report, it was considered that adsorbed proteins on the membranes were multi-layer except for PMBLA. In the viewpoint of monomer composition, MPC unit content was higher than the macromonomer content in PMBLA and PMBLLA. On the other hand, MPC and the macromonomer were equally incorporated in PMBDLA (MPC=macromonomer). This monomer content would regulate the protein adsorption on the phospholipid polymers with PLA segment. Furthermore, it is considered that stereoregularity was also important factor regarding the protein adsorption. In the case of PMBLA and PMBLLA, protein adsorption on PMBLLA was higher than that of PMBLA. It is considered that the PMBLLA surface was fully covered with each protein in spite of similar monomer content (MPC>macromonomer). The difference on protein adsorption between PMBLA and PMBLLA was based on stereoregularity in PLA segment. PMBLLA with poly(L-lactic acid) (PLLA) segment shows crystalline structure, on the other hand PMBLA with poly(D,L-lactic acid) shows amorphous. This surface morphology would regulate the amount of protein adsorption.

3.4 Cell adhesion on the membranes

L-929 cells adhesion were evaluated as shown in Figure 4. The number of cell adhesion on each membrane was 3000-5000 cells/cm^2 after 24 hours. Even the PET membrane for cell culture, 3000 cells/cm^2 of cells was adhered. Therefore, it is considered that no significant difference was observed. The adhered cells were doubled after 48 hours except for PMBLA membrane. In the case of poly(styrene) dish, the time to reach double cell number is also 24 h. From this, it is considered that cell growth on the membrane was equal in comparison with poly(styrene) dish. The cell number on the PMBLA was not changed from 24 hours to 48 hours. The dominant factor regarding the cell growth was discussed as follows. Generally, cell adhesion on the membrane was induced by protein adsorption. The protein adsorption; especially

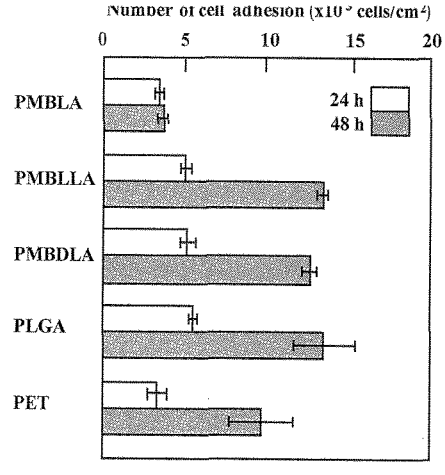

Figure 4. Fibroblast cell adhesion on the membrane after 24 and 48 hours. Mean values of three measurements and standard deviation are indicated.

fibrinogen and fibronectin such as cell adhesive proteins, was quite important. Because receptors on the cell membrane recognized the adhesive proteins with RGDS (arginine-glycine-aspartic acid-serine) moiety in the protein, and the cell attached on the surface via protein adsorption layer. From the viewpoint of the amount of protein adsorption, few protein adsorptions were observed on the PMBLA membrane. The amount of fibrinogen adhesion on the PMBLA was closely background level. Taking these results into account, the number of cell adhesion was dependent on the amount of protein adsorption. It is considered that over 1 $\mu g/cm^2$ of protein adsorption was onset for cell growth.

The cell morphology was observed with phase contrast microscopy as shown in Figure 5. The cells showed round shape on the membranes with MPC unit. Especially, all of the attached cells were rounded. In the case of PLGA and PET, all of the adhered cells showed spread. The cell morphology is good correlated with cell adhesive properties via protein adsorption. 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. In the case of PMBLLA and PMBDLA, some of the cells show spread. Though the MPC unit in the PMBLLA and PMBDLA is larger than that of PMBLA, the macromonomer content is also larger (PMBLLA=12 mol%, PMBDLA=16 mol%). From this, the cell morphology, the protein adsorption, and the cell adhesion were dependent on the macromonomer content in the polymer. Based on these results it is considered that the cell morphology could be regulated by the phospholipid polymer composition.

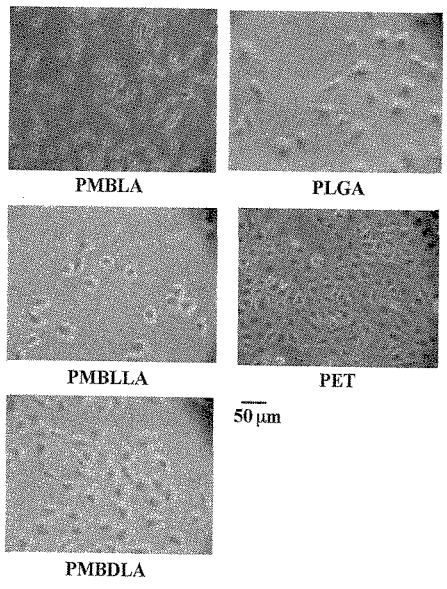

Figure 5. Morphology of the fibroblast cell adhesion on the membrane after 48 hours.

4. CONCLUSIONS

The novel phospholipid polymers composed of MPC, BMA, and PLA macromonomers were synthesized as cell compatible polymer materials for tissue engineering. The degree of polymerization of the lactic acid in the macromonomers was tailored. The MPC unit and the macromonomer content in the polymers were determined by ^{1}H-NMR and to be the range of 13-16 and 10-16, respectively. The mobility of the coating membranes was confirmed by static contact angle measurement. Spontaneous rearrangement of the polymer chain on the membrane surface was observed on the phospholipid polymer, and the surface would be adequate surface for cell adhesion. L-929 fibroblast cells were used for the evaluation of cell adhesion. The number of cell adhesion and the cell morphology were dependent on the protein adsorption, which is regulated by polymer composition.

5. ACKNOWLEDGEMENT

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