Biocompatibility of Poly(MPC-co-EHMA)/Poly(l-lactide-co-glycolide) Blends

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Abstract: Poly(l-lactide-co-glycolide) (PLGA) was blended with poly[ω-(methacryloyloxyethyl phosphorylcholine-co-ethylhexylmethacrylate] (PLGA/PMEH) to endow with new functionality i.e., to improve the cell-, tissue- and blood-compatibility. The characteristics of surface properties were investigated by measurement of contact angle goniometer, Fourier-transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) and electron spectroscopy for chemical analysis (ESCA). NIH/3T3 fibroblast and bovine aortic endothelial cell were cultured on control and PLGA/PMEH surfaces for the evaluation of cell attachment and proliferation in terms of surface functionality such as the concentration of phosphorylcholine. Also, the behavior of platelet adhesion on PLGA/PMEH was observed in terms of the surface functionality. The contact angles on control and PLGA/PMEH surfaces decreased with increasing PMEH content from 75° to about 43°. It was observed from the FTIR-ATR spectra that phosphorylcholine groups are gradually increased with increasing blended amount of MPC. The experimental P percent values from ESCA analysis were more 3.28 7.4 times than that of the theoretical P percent for each blend films. These results clearly indicated that the MPC units were concentrated on the surface of PLGA/PMEH blend. The control and PLGA/PMEH films with 0.5 to 10.0 wt% concentration of PMEH were used to evaluate cell adhesion and growth in terms of phosphorylcholine functionality and wettability. Cell adhesion and growth on PLGA/PMEH surfaces were less active than those of control and both cell number decreased with increasing PMEH contents without the effect of surface wettability. It can be explained that the fibronectin adsorption decreased with an increase in the surface density of phosphorylcholine functional group. One can conclude the amount of the protein adsorption and the adhesion number of cells can be controlled and nonspecifically reduced by the introduction with phosphorylcholine group. Morphology of the adhered platelets on the PLGA/PMEH surface showed lower activating than control and the number of adhered platelets on the PLGA/PMEH sample decreased with increasing the phosphorylcholine contents. The amount of fibrinogen adsorbed on the PLGA/PMEH surface demonstrated that the phospholipid polar group played an important role in reducing protein adsorption on the surface. In conclusion, this surface modification technique might be effectively used PLGA film and scaffolds for controlling the adhesion and growth of cell and tissue, furthermore, blood compatibility of the PLGA was improved by blending of the MPC polymer for the application of tissue engineering fields.

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Introduction

Recently, poly(lactide-co-glycolide)s (PLGA) are extensively used or tested for a wide range of medical applications as a bioerodible material due to good biocompatibility, controllable biodegradability, and relatively good processability. PLGA is biodegradable polyesters belonging to the group of poly (α-hydroxy acids). This polymer and its homopolymers (poly(lactide (PLA) and polyglycolide (PGA)) degrade by nonspecific hydrolytic scission of their ester bonds. The hydrolysis of PLA yields lactic acid which is a normal byproduct of anaerobic metabolism in human body and is incorporated in the tricarboxylic acid (TCA) cycle to be finally excreted by the body as carbon dioxide and water. PGA biodegrades by a combination of hydrolytic scission and enzymatic (esterase) action producing glycolic acid which can either enter the TCA cycle or be excreted in urine and be eliminated as carbon dioxide and water. The degradation time of PLGA can be controlled from weeks to over a year by varying the ratio of monomers and the processing conditions. It might be a suitable biomaterial for use in tissue engineered repair systems in which cells are implanted within PLGA films or scaffolds and in drug delivery systems in which drugs are loaded within PLGA microspheres.

However, it is more desirable to endow with new functionality for the medical applications such as cell and tissue engineering and drug delivery systems. For example, hydrophobic surfaces of PLGA possess high interfacial free energy in aqueous solutions, which tend to unfavorably influence their cell-, tissue- and blood-compatibility in initial stage of contact.

We synthesized 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers with excellent biocompatibility, especially blood-compatibility, and applied them to biomedical fields. The MPC polymer was designed taking into account the surface structure of the biomembrane. Recently, the phospholipid-accumulated surfaces have been prepared by various methods, and it has been reported that the phosphorylcholine group plays an important role showing excellent blood compatibility. Particularly, the MPC not only has a phosphorylcholine group but also a methacryloyl group. The MPC units can be then introduced to conventional polymers by the various modification methods. For example, the cellulose and polysulfone hemodialysis membrane surface modified with poly(MPC). The poly(MPC) was grafted onto the cellulose membrane using Ce4+ as an initiator. Also, the solution blend polysulfone with poly(MPC) was carried out. The cellulose and polysulfone membrane modified with poly(MPC) effectively reduced protein adsorption, platelet adhesion, and complement activation. The segmented polyurethane/poly(MPC) blends were prepared and tested the blood compatibility for the application of artificial vascular grafts. The polyethylene (PE) sheet grafted with the poly(MPC) was gradually prepared following corona discharge treatment, and plasma protein adsorption and fibroblast cell adhesion evaluated taking into account the chemical structure, surface density, mobility, and orientation of the grafted poly(MPC).

In this study, PLGA was blended with poly[(ω-methacryloyloxyethyl phosphorylcholine-co-ethylhexylmethacrylate (PMEH)] (PLGA/PMEH) to endow with new functionality i.e., to improve the cell-, tissue- and blood-compatibility. The surface properties were investigated by measurement of water contact angle, Fourier-transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) and electron spectroscopy for chemical analysis (ESCA), NIH/3T3 fibroblast and bovine aortic endothelial cell were cultured on control and PLGA/PMEH surfaces for the evaluation of cell attachment and proliferation in terms of surface functionality such as the concentration of phosphorylcholine. Also, the behavior of platelet adhesion on PLGA/PMEH was observed in terms of the surface functionality.

Experimental

Preparation of PLGA and PMEH. PLGA (75:25) was synthesized by direct condensation from 75 mole percent of l-lactide (Boehringer Ingelheim, Ingelheim, Germany) and 25 mole percent of glycolide (Boehringer Ingelheim, Ingelheim, Germany) under low pressure nitrogen
atmosphere with 150 ppm of catalyst as stannous octoate (Wako Chem. Co., Osaka, Japan) in toluene.\textsuperscript{15, 16, 20} This reaction was carried out at 165°C and for 4 hrs. The weight-average molecular weight ($M_w$) was 90,000 g/mole by the measurement of gel permeation chromatography (GPC) using standard polystyrenes.

MPC was synthesized with a previously described method.\textsuperscript{35, 36} PMEH polymer with 30 mole percent of MPC and 70 mole percent of 2-ethylhexylmethacrylate (EHMA) were prepared by a conventional radical polymerization technique and purified twice by reprecipitation from acetonitrile. The chemical structures of PMEH and PLGA are shown in Figure 1. PMEH is water-insoluble but can be dissolved in ethanol (EtOH). The $M_w$ was 20,000 g/mole by the measurement of GPC using polystyrenes standard sample.

**Preparation of PLGA Film Blended with the PMEH Polymer.** 10 w/v percent solutions of both PLGA in dichloromethane (MC) and PMEH polymer in EtOH were prepared separately. The solutions were mixed in a given composition as 0, 0.2, 0.5, 1.0, 5.0 and 10.0 wt% of PMEH. Two grams of PLGA/PMEH solution was cast onto PE sheet onto custom-made kit on a horizontal level in order to get 400-600 μm thickness of PLGA/PMEH films. After evaporation of the MC and EtOH at room temperature, samples were cut 7×7 cm square. The PLGA/PMEH films were dried in vacuo overnight to remove residual solvents and were ultrasonically washed in water. The PLGA/PMEH films were kept in a vacuum until use.

![Figure 1: Chemical structure of PLGA and PMEH.](image)

**Surface Characterizations.** The surfaces with control and PLGA/PMEH were characterized by the water contact angle, FTIR-ATR, and ESCA.

The water contact angle, a parameter of hydrophilicity of the surfaces, was measured at 25°C using a contact angle goniometer (model 100-0, Rame-Hart, Inc., U. S. A.) for both side such as air and PE. Contact angles were measured at least five different parts for each specimen. For each part, 3 mL of deionized water drop was deposited onto the surface. The degree of reproducibility for the different specimen was within ± 4.0°.

The FTIR-ATR spectra were obtained using a Magna-IR Spectrophotometer 550 (Nicolet, U. S. A.) equipped with a KRS-5 internal reflection element. The control and blended samples were cut 1.0×2.0 cm size and the characteristics absorption bands as N-H peak observed at 3409.4 cm\(^{-1}\) were compared for the each films.

To determine the introduction of the functional groups on the PLGA/PMEH films, the surfaces of the control and blended samples were also analyzed by ESCA (ESCALAB MK II, V. G. Scientific Co., U. K.) with AlKα at 1487 eV and 300 W power anode (incidence angle 30°). Survey scan spectra of N1s and P2p were taken at 403.2 and 134.1 eV, respectively.

**Fibroblast and Endothelial Cell Culture on the PLGA/PMEH Surfaces.** NIH/3T3 fibroblast cells (KCLB 21658, Seoul, Korea) and CPAE bovine aortic endothelial cells (KCLB 10209, Seoul, Korea) were obtained from Korean Cell Line Bank and were used to study the effects of surface wettablility and functionality of PLGA/PMEH on the behavior of cultured cells. The fibroblast and endothelial cells\textsuperscript{41, 42} routinely cultured in tissue cultured polystyrene flasks (Corning, U. S. A.) at 37°C under 5% CO\(_2\) atmosphere were harvested after the treatment with 0.25% trypsin (GIBCO Laboratories, U. S. A.). The control and PLGA/PMEH surfaces (size: 1.5×1.5 cm) were placed in 12 well polystyrene culture plate (Corning, U. S. A.). The PLGA/PMEH film surfaces placed in the culture plate were equilibrated with Dulbecco's phosphate buffered saline (PBS, pH 7.3-7.4; Sigma, U. S. A.) free of Ca\(^{2+}\) and Mg\(^{2+}\) for 30 min. After removing the PBS solution from the chambers by pipetting, the cells
(4×10⁶/cm²) were seeded to the surfaces. The culture medium used was RPMI 1640 nutrient mixture (GIBCO Laboratories) containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL gentamycin sulfate.

The cell culture on the PLGA/PMEH surfaces was carried out for up to 4 days. The culture medium was changed once after 1 day. After incubation at 37°C under 5% CO₂ atmosphere, the surfaces were washed with PBS. The cells attached on the surface were fixed with 2.5% glutaraldehyde (GIBCO Laboratories) in PBS for 24 hrs at room temperature. After thorough washing with PBS, the cells on the surface were dehydrated in EtOH graded series (50, 60, 70, 80, 90, and 100%) for 10 min each and allowed to dry in a clean bench at room temperature. The cell-attached surfaces were gold deposited in vacuum (Emscope, Model SC 500A, U. K.) and examined by SEM with a tilt angle of 45 degree. The cell density on the surfaces was estimated by counting the number of attached cells with different wettability. Different fields for each section were randomly counted and the results were expressed in terms of the number of cells attached per cm².

**Platelet Adhesion on the PLGA/PMEH Surfaces.** In order to evaluate blood compatibility of control and PLGA/PMEH films, platelet adhesion experiments were carried out with platelet rich plasma (PRP). PRP was separated from citrated canine blood which consists of mixture of fresh canine blood and 3.8% sodium citrate aqueous solution with the volume ratio of 9:1 by centrifugation at 1,100 rpm (100 g) for 20 min at 4°C.¹⁷⁻¹⁹

For the evaluation of platelet adhesion on the PLGA/PMEH films, the parallel flow chamber was used as used previous study.¹⁷, ⁴³⁻⁴⁵ It consists of three parts: (i) a series of control, PLGA/PMEH films (each sample size is 0.5 mm × 4 mm × 2.54 cm) on a 0.2 cm × 2.54 cm × 7.62 cm size of glass slide, (ii) a 1 mm thick spacer with silicon rubber gasket (Dow Corning, Midland, MI, U. S. A) which has a 1 cm × 7 cm cut out space (ca., 0.7 mL volume) as blood chamber, and (iii) polymeric cover plate which had inlet and outlet silicon tubes (3 mm diameter each) placed 6 cm apart. Silicon grease was pasted both side of silicon spacer and clips were used to prevent leaking of PRP during experiment. Parallel flow chamber was assembled just before experiment.

PBS was used to fill the parallel flow chamber first to avoid air-blood contact. Then, 3 mL of PRP was carefully and slowly replaced by inserting end of the pipette tip into hole. After incubation for 30 and 120 min at 37°C, the parallel flow chamber was carefully and slowly rinsed by PBS three times to remove unadhered platelet. Adhered platelets on the PLGA/PMEH samples were fixed by using 2.5% glutaraldehyde for 4 hrs, followed by a dehydration procedure using a series of EtOH-water mixtures (50, 60, 70, 80, 90, and 100 vol%) of ethanol for 20 min each. Finally, samples were dried under N₂ atmosphere. Four SEM photographs magnified 1,000 times were taken from each sample surface randomly after gold sputtering. Also, 4,000 magnification of SEM photographs with 45° tilted were retaken from the same region to observe more closely the morphology of adhered platelets. Platelets in each photograph whose actual area was 8,541 µm² were counted and normalized (#/mm²).

**Protein Adsorption of the PLGA/PMEH Blend Films.** PLGA/PMEH blend films with 15 mm diameter size was immersed in 0.03 g/dL concentration of human plasma fibrinogen solution for 60 min. The films were gently taken off and rinsed 5 times with PBS, and then placed in a 1 wt% aqueous solution of sodium dodecyl sulfate (SDS) and kept for 60 min at room temperature to remove the fibrinogen adsorbed on the surfaces. A protein analysis kit (MicroBCA protein assay reagent kit, #23235, Pierce, Rockford, IL, U. S. A.) based on the bicinchoninic acid (BCA) method was used to determine the concentration of the proteins in the SDS solution. The amount of the proteins adsorbed on the polymer surfaces was calculated from the concentration in the SDS solution.¹⁷, ³⁸

**Results and Discussion**

**Surface Characterization of PLGA/PMEH Films.** In order to evaluate the biocompatibility of the PLGA films as well as study cell and platelet adhesion onto PLGA in terms of functionality of phosphorylcholine and wettability, PMEH poly-
mers was blended by means of solution method. This method is a simple and convenient tool to physically mix functional groups onto polymeric surfaces. The amount of phosphorylcholine and wettability can be controlled by initial loading of PMEH polymer.

Water contact angles of control and PLGA/PMEH films with 0.5 to 10 wt% to PLGA were measured and plotted in Figure 2. The contact angles on control and PLGA/PMEH surfaces decreased with increasing PMEH content from 75° to about 43°. It can be observed that contact angle of air side was lower than that of PE side, i.e., the air side of PLGA/PMEH surface was more wettable than that of PE side. It can be explained by the hydrophobicity of the EHMA unit. This EHMA unit was more miscible to PLGA hydrophobic chain than hydrophilic phosphorylcholine unit. So, it might be suggested that the MPC units in the PLGA/PMEH blends were located at the near-surface region.\(^{26}\) Also, Ishihara explained that the mobility of the polymer chains and/or the polar groups increased because the mobility factor increased with an increase in the MPC polymer composition in the polysulfone/poly(MPC) blend system.\(^{35,36}\)

Figure 3 shows FTIR-ATR spectra of the surface of PLGA/PMEH blends. The absorption band at \(\sim3409.4\,\text{cm}^{-1}\) corresponds to a N-H band derived from phosphorylcholine moiety in the PMEH polymers. It was observed from the spectra that phosphorylcholine groups are gradually increased with increased blended amount of MPC.

In order to study more detailed changes in the composition of MPC on the PLGA/PMEH surfaces, analysis of ESCA was performed. The N1s and P2p core level spectra of the PLGA/PMEH blend surfaces with different concentration of PMEH are shown in Figure 4. In Table I, the percent of the ESCA signal intensities of phosphorous was calculate to estimated the surface composition of the PLGA/PMEH blends. The theoretical value of the P was also calculated based on the blending composition of the PLGA and the MPC polymer. The experimental P values were more 3.28 ~ 7.4 times than that of the theoretical values.

![Figure 2](image1.png)

**Figure 2.** Changes in the water contact angle of PLGA/PMEH blend surfaces with different concentration of PMEH.

![Figure 3](image2.png)

**Figure 3.** FTIR-ATR spectra of control and PLGA/PMEH blend surfaces with different concentration of PMEH.

![Figure 4](image3.png)

**Figure 4.** ESCA survey scan spectra of control and PLGA/PMEH blend surfaces with different concentration of PMEH.
for each blend films same as we have already observed on the PSi/poly(MPC-co-2-methacyrloyloxyethyl butylurethane) [poly(MPC-co-MEBU)] blends system. These results clearly indicated that the MPC units were concentrated on the surface of PLGA/PMEH blend. The compatibility of the hydrophobic EHMA part of MPC polymer and PLGA was maybe good, whereas the hydrophilic MPC moiety was separated for the matrix polymer, PLGA, as predicted the water contact angle experiment. It might be suggested that the MPC moieties are concentrated at the surface of the PLGA/PMEH films as shown in Figure 5.

**Fibroblast and Endothelial Cell Adhesion and Growth on the PLGA/PMEH Films.** The control and PLGA/PMEH films with 0.5 to 10.0 wt% concentration of PMEH were used to evaluate cell adhesion and growth in terms of phosphorylcholine functionality and wettability. SEM microphotographs of cultured fibroblast and endothelial cells on the control and PLGA/PMEH films for 1 day are shown in Figure 6. Cell adhesion and growth on PLGA/PMEH surfaces were less active than those of control, that is to say, the morphology of cells were more round. It can not be seen pseudopods and lamellipodium from the cells with increasing PMEH concentration. In case of control sample, cells were adhered onto the PLGA surface while cells were rarely adhered and spread onto PLGA/PMEH surfaces. The results of number of fibroblast and endothelial cells adhesion and growth for 1, 2 and 4 days are shown in Figure 7 and 8, respectively. The adhered cell number of fibroblast and endothelial cell increased with culture time, however, the both cell number decreased with increasing PMEH contents without the effect of surface wettability. Iwasaki explained that the fibronectin adsorption decreased with an increase in the surface density of phosphorylcholine functional group in the system of poly(MPC) grafted onto PE surfaces. One can conclude the amount of the protein adsorption and

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Figure 5. Possible schematic diagram of PLGA/PMEH blends.

Figure 6. SEM microphotographs of fibroblast and endothelial cell attached on PLGA/PMEH blends surfaces with different concentration of PMEH after 1 day culture (original magnification, x 400).
the adhesion number of cells can be controlled and nonspecifically reduced by the introduction with phosphorylcholine group. This surface modification technique can be used PLGA film and scaffolds for controlling the adhesion and growth of cell and tissue.

**Platelet Adhesion on the PLGA/PMEH Surfaces.** Canine platelet adhesion tests on the control and PLGA/PMEH surfaces were carried out for 30 and 120 min and analyzed by SEM to compare number of adhered platelets and their morphological changes. SEM micrographs of the platelet adhesion on the control and PLGA/PMEH surfaces with different composition of phosphorylcholine functionality are shown in Figure 9.

The five stages of shape change for activated platelets on the polymeric surfaces can be classified. An inactivated platelet has a round shape. A few pseudopods are extruded (dendritic shape) and reacted from a platelet depending on whether the platelet is activated. An activated platelet spreads in an irreversible process, first becoming flatter, losing its rigidity, and then acquiring a spread-dendritic shape. The contents of the cell then move outward between the pseudopods, eventually reaching a fully spread shape. The shape of platelets adhered onto surface can be used to evaluate the relative degree of platelet.
activation on the modified surface.

Morphology of the adhered platelets on the PLGA/PMEH surface showed lower activation than control. Many activated pseudopods were connected to neighboring platelets on the control PLGA surface whereas inactivated round shape platelets were observed on the PLGA/PMEH surface. The number of adhered platelets with varying composition of the PMEH polymer are plotted in Figure 10. The number of adhered platelets on the PLGA/PMEH sample decreased with increasing the phosphoryl contents. It is believed that platelet adhesion is preceded by protein adsorption such as fibrinogen. Protein adsorption is a thermodynamically spontaneous process by lowering free energy of the system and the important interaction and force in protein adsorptions is hydrophobic dehydration interaction. The amount of fibrinogen adsorbed on the PLGA/PMEH surface with 0.2 and 0.5 wt% composition of PMEH are listed in Table II. This result demonstrated that the phospholipid polar group played an important role in reducing protein adsorption on the surface. The amount of fibrinogen adsorbed on the PLGA/PMEH surface was decreased with an increase in the PMEH content of the blends. In our previous works, the blending of the MPC polymer with segmented polyurethane and PSf, and the blood compatibility of the blended membrane were studied. The MPC polymer functioned very well as a blood compatible additive, that is, protein adsorption and platelet adhesion were effectively reduced even when the composition of the MPC polymer was only 1–10 wt% relative to control. In conclusion, blood compatibility of the PLGA was improved by blending of the MPC polymer.

Acknowledgments. This work was supported by grants from KOSEF 996-0800-002-2 for International Research Program and KMOST 98-N-02-05-A-02.

Table II. Amount of BPF Adsorbed on the Control and PLGA/PMEH Blends

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<th>Content of PMEH</th>
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References


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