Bone Cell Viability on Collagen Immobilized Poly(3-hydroxybutrate-co-3-hydroxyvalerate) Membrane: Effect of Surface Chemistry

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ABSTRACT: The effect of surface chemistry on proliferation and morphology of bone cells cultured on surface modified poly(3-hydroxybutrate-*co*-3-hydroxyvalerate) (PHBV) and untreated PHBV was evaluated. The surface of cast PHBV film was physically and chemically immobilized with collagen. For preparing chemically immobilized collagen surface, PHBV film was ozone treated followed by grafting of PMAA chains and the immobilization of collagen. The surface roughness and hydrophilicity of PHBV film were determined by atomic force microscopy (AFM) and contact angle measurements, respectively. It was found that the duration of ozone exposure and monomer concentration used for grafting PMMA chains influenced the amount of collagen immobilized. The cell proliferation on PHBV surfaces with chemically and physically immobilized collagen was compared with untreated PHBV using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The bone cell activity on chemically and physically immobilized collagen PHBV films was found to be 246 and 107% for UMR-106 and 68 and 9% for MC3T3 cell lines, respectively. Although the results are very preliminary, the chemically grafted collagen on PHBV surface provided a favorable matrix for cell proliferation. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 93: 2445–2453, 2004

Key words: biodegradable; functionalization of PHBV; atomic force microscopy (AFM); biocompatibility

INTRODUCTION

During the last two decades, a wide variety of biodegradable materials have been studied as biomaterials in the field of tissue engineering. The biodegradable materials include synthetic polymers such as polyglycolic acid (PGA), polylactic acid (PLA), poly(lactic-*co*-glycolic acid) (PLGA), and poly(caprolactone), and natural polymers such as poly(3-hydroxybutrate-*co*-3-hydroxyvalerate) (PHBV).[1,2] Among these materials, PHBV has received enormous attention lately due to its possible application in drug delivery system, and as substrate for cellular support.[3–6]

Polyhydroxybutyrate (PHB) is a polyester that is made by several microorganisms when provided with a nitrogen-deficient feed.[7] One of the attractive aspects of microbial synthesis of PHB is the ability to obtain high yield of the final product. Among the host of PHB copolymers, it is the copolymer of hydroxybutyrate and hydroxyvalerate (PHBV) that is widely used in biomaterials and packaging application. By varying the hydroxyvalerate content of PHBV, the flexibility, impact strength, processability, and the degree of degradation of the final copolymer can be altered.[8,9] A number of investigators have studied the degradability of PHB and PHBV in several environments including bacteria, enzyme, acid, and buffer medium.[10,11] The less crystalline component (hydroxyvalerate) in the copolymer system was found to exhibit higher degradation rate than the highly crystalline component (hydroxybutyrate). By adjusting the percent composition of the hydroxyvalerate content in the copolymer, the degradation of PHBV can be tuned to achieve desired rates. Furthermore, *in vivo* degradation of PHBV yields a low toxic product, D-3-hydroxybutyrate, which is a normal constituent of blood.[12] It is the combination of useful properties of PHBV such as natural origin, biodegradability, biocompatibility, stereospecificity, thermoplasticity, and the ability to tune the characteristics of PHBV to achieve desired mechanical properties and rate of degradation that makes the copolymer extremely attractive biomaterial for use in tissue engineering applications.

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The use of biodegradable polymer in the field of tissue engineering and drug delivery has led to some specific requirements on the use of biomaterial. Biomaterial should favor cell adhesion, promote cell growth, provide directionality to cell growth, promote in growth of viable cells by allowing nutrients and medium to percolate, and should degrade without releasing any toxic byproducts. These requirements are closely related to the environment in which the biomaterial is implanted. For a given environment, the cellular level interaction (cell behavior, cell growth, cell migration, and cell differentiation) with polymer is strongly dependent on the surface characteristics (chemistry and topography) of biodegradable polymer.

An approach commonly used to promote cell adhesion on a foreign polymer surface is to provide a surface structure close to that of the natural cell environment. To mimic the natural cell environment on polymer surface, extra-cellular matrix proteins such as collagen, laminin, and fibronectin are immobilized on polymer surface.[14–18] Collagen is one of the most abundant structural proteins found in animal connective tissues and is important protein for anchoring cells such as fibroblasts or epithelium.[19] Depending on the final application (cell carrier/scaffold), the natural protein is either grafted (chemically immobilized) or dip coated (physically immobilized) on the polymer surface.[20,21] Although a number of studies have been conducted to investigate the cytocompatability of grafted and dip coated natural proteins on polymeric surface, a comparative cytocompatability study of these surfaces is not well known. The comparative data can be valuable since dip coated proteins (e.g., collagen) on polymeric surface are not covalently immobilized and may not be available to promote cell adhesion on the surface.

The objective of this study is to compare the bone cell viability on grafted (chemically immobilized) and dip coated (physically immobilized) collagen on treated and untreated PHBV surface. Because PHBV lacks functional groups to covalently anchor extracellular matrix protein, several approaches have been tried to perform functionalization of polymer surface. These approaches include plasma or gamma radiation grafting of acrylic acid or the oxidation of chemically grafted chains and the ozone induced grafting of chains to PHBV film.[22–24] Some of these approaches allow endowing solid polymer surfaces with a rich chemical functionality while keeping the polymer surface flat on a micrometer scale.

This article reports the graft polymerization of hydrophilic poly methyl methacrylic acid (PMAA) onto ozone activated PHBV surface, and the further immobilization of collagen using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC) as the condensing agent to further the reaction between the carboxyl groups of the grafted PMAA and amino groups of collagen. In this article, AFM results are used along with results from contact angle measurements and chemical assay to assist in interpreting the changes in PHBV membrane due to modification. The culture of mouse osteoblastic cell line MC3T3-E1 subclone 14 and rat osteosarcoma cell line UMR-106 grown *in vitro* on physically or chemically immobilized collagen PHBV surface was compared to untreated PHBV film. MC3T3-E1 subclone 14 and UMR-106 cells were utilized in this study because both cell lines have been studied extensively and exhibit expression products characteristic of differentiated osteoblasts.[25,26] MTT assay combined with optical microscopic measurements provides information about the cells' viability on PHBV modified surfaces.

MATERIALS AND METHODS

Materials

Poly(3-hydroxybutrate-*co*-3-hydroxyvalerate), containing 8 wt % hydroxyvalerate (PHBV), type I collagen (calf skin), methacrylic acid (MAA), 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and Bradford reagent, was purchased from Sigma Aldrich (St. Louis, MO). Prior to use, MAA was purified by distillation under reduced pressure. Toluidine BlueO (TBO) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC) were supplied by Fisher Scientific. The mouse osteoblastic cell line MC3T3-E1 subclone 14 (ATCC CRL-2594) and the rat osteosarcoma cell line UMR-106 (ATCC CRL-1661) were purchased from the American Type Culture Collection (Manassas, VA).

Preparation of PHBV membrane

The PHBV powder was dissolved at 60°C in chloroform to obtain a concentration of 50mg/mL. Prior to the application of the solution; the glass petri dish was cleaned first with acetone, then with methanol, and dried with nitrogen. Free standing PHBV films were made from solution in chloroform by flooding the glass dish with a known volume of polymer solution. The solvent was allowed to gradually evaporate for 12 h leaving behind the film. The resulting film was conditioned for 1 day at 24 ± 2°C under vacuum before further use. The resulting membrane was washed with methanol and then dried under vacuum at 24 ± 2°C for 12 h to yield membrane with thickness of 0.34 ± 0.01 mm.

Ozone treatment of PHBV membrane for peroxide formation and determination of surface peroxide density

Ozone was generated at room temperature by passing oxygen at a rate of 2.2 g/h through an ozone generator

(Ozonology, Model #L-25, Evanston, IL). Strips of PHBV membrane of dimension (1×4 cm^2) were placed in Pyrex fritted glass chamber. The ozone along with oxygen was allowed to purge the system for a predetermined time interval (e.g., 1 to 3 h). After discontinuing the ozonolysis experiment, fresh oxygen was allowed to purge for a brief duration (10 min) so that the system is free of unreacted ozone. The peroxide formed on the ozone treated membrane was determined by the iodide method spectrophotometrically. The ozone treated films were immersed in 7 mL of benzene : isopropanol mixture (1 : 6 vol %) containing 60 mg of sodium iodide and 1 ppm ferric chloride for 10 min at 60°C ± 2°C. Immediately after the reaction, the oxidized iodine was measured as triiodide anion by recording the absorbance of the solution at 360 nm using a HP-8453A diode array spectrophotometer.[27]

Graft polymerization of MAA on PHBV membrane

It was ensured that there was no considerable lag time between ozonolysis and the graft polymerization of PHBV membrane. Strips of ozone treated membrane of dimension (1×4 cm^2) were placed in a Pyrex glass tube that contained aqueous 5 to 20 wt % MAA, 0.2*M* H_2SO_4, and 1mM $FeSO_4$. The solution was degassed by purging with a stream of nitrogen gas followed by performing grafting at 65°C ± 2°C in a constant temperature water bath for 1 h. Then the membrane was retrieved and repeatedly rinsed with distilled water to remove the remnants of the unreacted MAA and the adsorbed homopolymer. Soxhlet extraction was performed on the grafted membranes at 75°C for 24 h using water as the solvent.[28,29]

Determination of surface grafting density of carboxyl groups on PHBV membrane

The surface density of the carboxyl group grafted on the PHBV (PMMA-g-PHBV) membrane was determined by reacting with 0.5 mM TBO at pH 10 and 30°C for 5 h. The membrane was then rinsed with distilled water and immersed in a solution that contained an excess amount of 0.1mM NaOH to remove the noncomplexed dye. The complexed TBO on PHBV membrane was desorbed in 50% (v/v) acetic acid. The dye absorbance was recorded at 633 nm using a spectrophotometer, and the surface density of carboxyl groups on PHBV membrane was determined from the calibration curve.[30,31]

Protein immobilization on PMMA grafted PHBV membrane

PMAA grafted PHBV membranes were cut into circular pieces of 10mm diameter and were placed in a solution (containing 10mg/mL of EDAC in phosphate buffer solution (PBS) maintained at pH 4.5) for 24 h at 2–4°C. The EDAC activated membrane was then retrieved and washed repeatedly with PBS solution and dried under vacuum. EDAC treated membrane was then placed in a solution of type I collagen (4mg/mL in 0.3% acetic acid) for 24 h at 2–4°C. The collagen-immobilized membrane was sonicated with deionized water for 3 min to remove the physically immobilized collagen. Since one of the intents of the study was to compare chemically immobilized collagen PHBV surface with physically immobilized collagen, collagen was dip coated on untreated PHBV film at room temperature in type I collagen (4mg/mL in 0.3% acetic acid) for 24 h. The surface density of the collagen physically (Coll/PHBV) and chemically immobilized (Coll-g-PMMA-g-PHBV) on PHBV films was determined as described by Shu et al.[27] and Bradford.[32]

Contact angle measurements

To estimate the change in the polarity of PHBV membrane due to PMMA grafting and collagen immobilization, contact angles of cast PHBV film exposed to different conditions were measured using a video contact angle system (model VCA-2500, Advanced Surface Technology Inc., Billerica, MA). 1μL of water droplet was placed on the surface and the water droplet image was captured, from which the contact angle was estimated. A minimum of six readings was recorded for each sample.

Atomic force microscopy (AFM)

Tapping mode AFM was performed with a Dimension 3100 (Digital Instruments, Santa Barbara, CA) scanning probe microscope. Commercial silicon nitride cantilever probes, each with a nominal tip radius of 5 nm to 10 nm and spring constant in the range of 20*N*/m to 40*N*/m (values provided by manufacturer), were oscillated at their fundamental resonance frequencies, which ranged between 250 kHz and 350 kHz. Topographic (height) and phase images of the casted and treated membrane were recorded simultaneously at ambient conditions. Roughness analysis was determined using the DI software for image analysis.

Preparation of cells for cell viability and proliferation studies

UMR-106 and MC3T3-E1 cells were grown in 0.22 μm filtered Dulbecco's modified essential medium (DMEM) supplemented with 10% (w/v) heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL (w/v) streptomycin (Quality Biological, Inc.) and 50 μg/mL L-ascorbic

acid (Fisher Scientific) at 37°C in 5% CO_2. Confluent cell cultures were washed with Hank's balanced salt solution (Sigma Aldrich) and trypsinized with Trypsin-EDTA (0.05% trypsin, 0.1% EDTA) (Quality Biological, Inc.) for 15 min to obtain a cell suspension. Trypsin activity was inhibited upon the addition of FBS at a final concentration of 10%. Next, cells were centrifuged and washed with 0.45 μm filtered sterilized serum-free DMEM three times to remove residual FBS. The concentration of the resulting cell suspension was determined with the use of a hemocytometer. Care was taken during the culture preparation and viability studies to use sterile techniques.

Proliferation assay

Washed cells were seeded at a density of 1.4×10^4 cells/cm^2 (UMR-106) and 1.2×10^3 cells/cm^2 (MC3T3-E1) into a 24 well tissue culture plate (Costar) that contained untreated or modified PHBV membranes in 1 mL serum-free DMEM. The cells were incubated overnight at 37°C in 5% CO_2. Next, all wells were washed with serum free DMEM and the nonadherent cells were aspirated. The remaining adherent cells were cultured in 10% FBS supplemented DMEM for five days at 37°C in 5% CO_2 to facilitate the proliferation of cells. At day six, cells were washed with serum free DMEM; some samples were saved to be viewed by optical microscope at 100× magnification while MTT assay was performed on the remaining samples. Briefly, 100μL of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each culture well containing 1 mL serum free DMEM and incubated for 6 h. The cultured media was aspirated and the membranes were washed with phosphate buffered saline PBS (Quality Biological, Inc). Next, the membranes were placed into eppendorf tubes containing 0.1*N* HCl in isopropyl alcohol to extract the formazan dye from the living cells and the absorbance was then measured by Titertek Multiskan 310C plate reader using a 595 nm filter.[33,34] A minimum of three samples was used for individual membrane type to account for variation in data for a sample type.

RESULTS AND DISCUSSION

Surface modification

The oxidation of PHBV membrane in ozone environment is expected to generate peroxide and other functional groups on the surface of PHBV membrane. The peroxide groups have the ability to initiate the grafting of MAA on the surface of PHBV, leading to homopolymer of PMMA (poly methyl methacrylic acid) graft brushes on PHBV surface. Then the PMMA-g-PHBV film was used to covalently immobilize type I collagen.

TABLE I
Water Contact Angle (deg.) and Mean Roughness (nm) of PHBV and Modified PHBV Surfaces

Membrane type	Contact angle (deg.)	Mean roughness (nm)
PHBV	59.0 ± 0.59	16.0 ± 0.14
PHBV (1 hr O_3 exposed)	52.0 ± 0.74	106.0 ± 1.73
PHBV (2 hr O_3 exposed)	46.0 ± 0.25	121.0 ± 2.85
PHBV (3 hr O_3 exposed)	—	144.0 ± 3.12
PMMA-g-PHBV (5%)	41.0 ± 0.26	102 ± 1.53
Coll-g-PMMA-g-PHBV	33.0 ± 0.71	62.0 ± 0.87
Coll/PHBV	36.0 ± 1.3	65.0 ± 1.2

Table I summarizes the static water contact angle measured at room temperature for untreated and modified PHBV films. The activated PHBV film showed a lower water contact angle than the cast PHBV film. For example, cast PHBV film had a water contact angle of 59.0 ± 0.59° while 2 h ozone treated film had a water contact angle of 46.0 ± 0.25°. The decrease in the water contact angle of PHBV film with ozone exposure indicates that ozone activation has introduced a number of polar functional groups such as peroxides, hydroxyl, and carbonyl groups, etc. on the surface of the PHBV films.[35,36] In particular, we are interested in determining the peroxide build up on PHBV membrane using iodide method spectrophotometrically. It must be mentioned that the peroxide measured by iodide method has contribution of peroxide from the bulk and surface of the film.

Figure 1 shows the dependence of the surface density of peroxide as a function of exposure time. The standard deviation for the individual data points is shown as error bar. The surface density of peroxide on the PHBV membrane reached a maximum at 2 h and then decreased with further ozone exposure. Except for minor variations, the trend closely mirrors the observation reported in the literature for peroxide build up on PHBV surface when air containing 10.2 g/m^3 ozone was used to activate the PHBV film; the peroxide concentration produced on the PHBV membrane after 20 min reached a maximum value of 7.2 ± 0.54 nmol/cm^2.[15] In this study, it was noticed that when oxygen was fed at a rate of 2.2 g/h through an ozone generator, the peroxide concentration produced on the 10 mm diameter PHBV membrane after 2 h reached a maximum value of 11.0 ± 0.61 nmol/cm^2. The results indicate that the concentration of peroxide build up on the PHBV membrane is not only dependent on the exposure time; it is also dependent on the overall concentration of ozone fed to activate the surface. Because the dosage of ozone present in our system is lower than that used by Hu et al.,[15] the PHBV surface had to be exposed for longer duration so as to

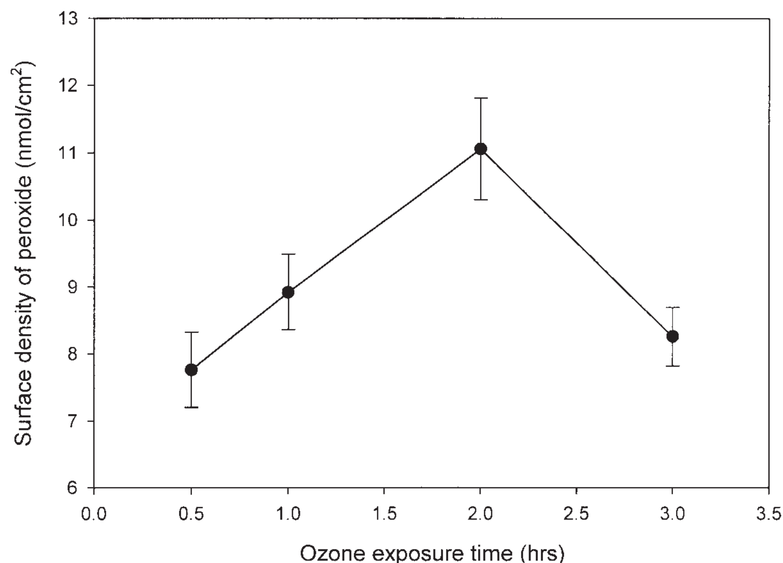

Figure 1 The formation of peroxide on the PHBV surface by ozone oxidation at different oxidation times (iodide method).

build up considerable peroxide density. Although the hydrophilicity of PHBV membrane as measured by water contact angle and the peroxide surface density as measured by iodide method are only qualitatively comparable, all information is consistent with chemical changes of the PHBV film upon exposure to ozone.

To determine morphological changes to the PHBV film due to ozone treatment, AFM was used to characterize the topography of the PHBV film before and after ozone exposure. Mean square roughness of the film was determined from the topographic (height) image data. During ozone treatment, there is grafting of peroxide groups to the surface as well as chain scission of the polymer backbone. Roughness was used as an indicator to measure changes (chemical and physical) on the surfaces of the PHBV film.[15,21] The root mean square roughness value (R_q) is defined as the standard deviation of height value about an average height. For example, the untreated PHBV membrane was relatively smooth and the root-mean-square (RMS) roughness was 16.0 ± 0.14 nm, after 1 h ozone exposure it increased to 106.0 ± 1.73 nm while after 2 h ozone exposure it increased to 121.0 ± 2.85 nm and upon an additional hour ozone exposure the roughness increased to 144.0 ± 3.12 nm. The results indicate that the duration of exposure of PHBV to

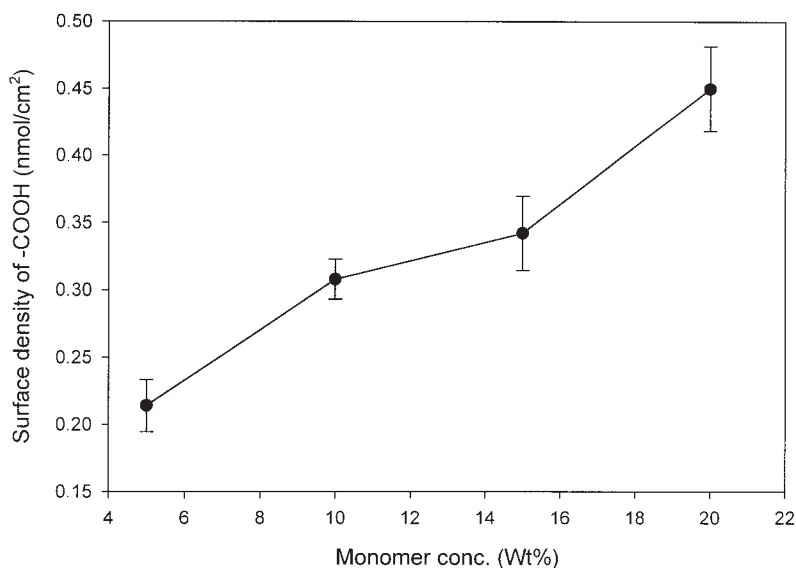

Figure 2 Effect of the monomer concentration on the ozone-induced graft polymerization of MAA onto PHBV membrane (60°C for 1 h).

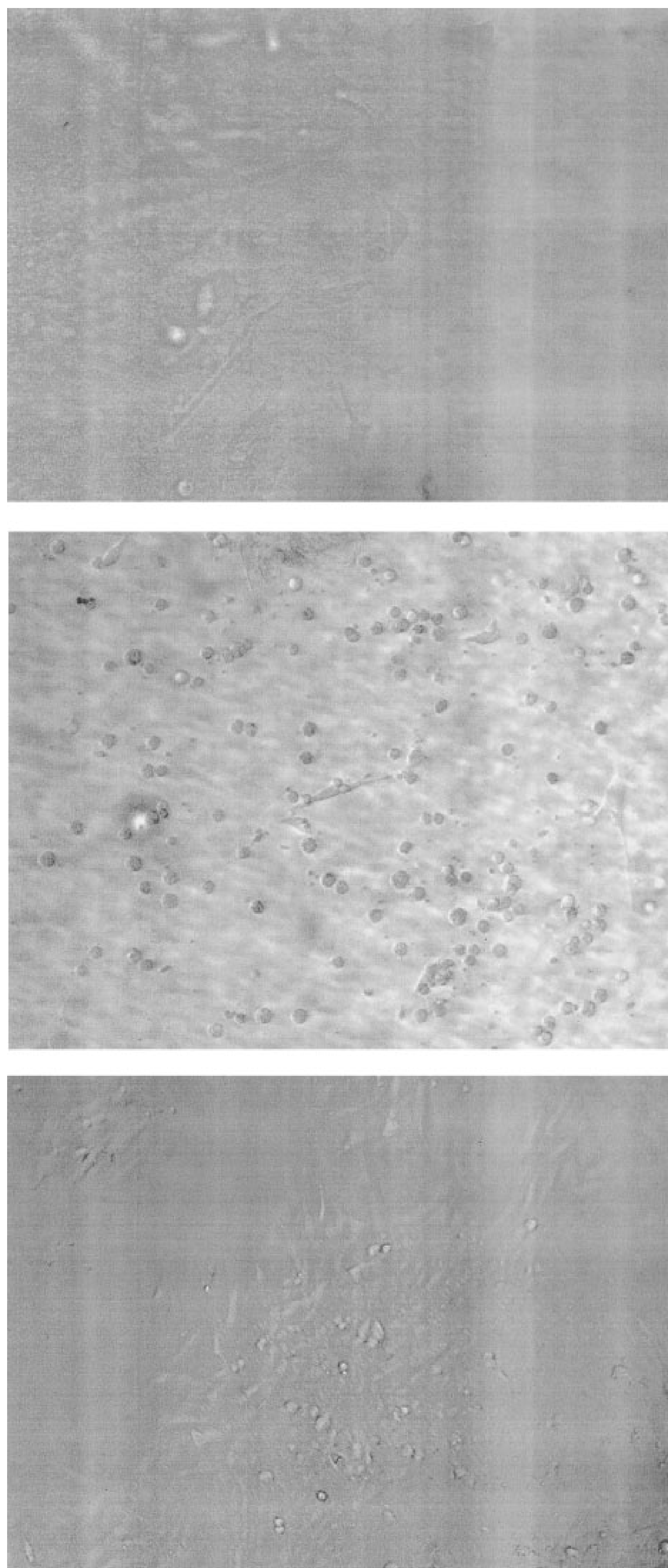

Figure 3

ozone will dictate the degradation of PHBV and buildup of peroxide.

Grafting copolymerization of MAA and influence of monomer concentration

In the second step, the grafting of MAA on PHBV membrane was performed immediately after the activation of PHBV film so as to minimize surface contamination. Activated PHBV film was allowed to react with monomer of varying concentration (5–20 wt % MAA in water) for a reaction time of 1 h at 65°C. The grafting of MAA to PHBV film is expected to change the chemical characteristics of the film. To establish that the characteristics of PHBV film have indeed changed due to grafting of MAA chains, the water contact angle of the film before and after grafting was measured by contact angle goniometer. The 1 h ozone activated film had a water contact angle of 52.0 $\pm$ 0.74°, which decreased to 41.0 $\pm$ 0.26° when the film was grafted with 5 wt % MAA. These results suggest that the surface of PHBV film has been rendered hydrophilic due to grafting of MAA brushes on the film.

The quantitative density of -COOH groups grafted on PHBV membrane was determined by the reaction with TBO, generating the absorbance at 633 nm. Results on the dependence of the surface density of –COOH groups on monomer concentration are presented in Figure 2. For the individual monomer concentration, the standard deviation is shown as error bar. These observations were found to be consistent with the general trend reported in the literature for the plasma-induced grafting of 2-hydroxyethylmethacrylate onto silicone rubber and acrylic acid grafting on PHBV membrane.[37]

As expected, the surface becomes acidic with an increase in the density of PMMA brushes grafted on PHBV film. Especially when PHBV film was grafted with 10 wt % or higher monomer concentration, the surface became acidic. We noticed that the surface was highly acidic as experienced by the pH change of the cell culture media. Similarly, Gupta et al.[38] and Gao et al.[39] reported that at higher grafting density of acrylic acid and poly(methacrylic acid) to polymeric surface, the surface became acidic and less conducive for cell adhesion. Therefore, the cell activity study was limited to membranes grafted with 5 wt % or lower MAA concentration.

Immobilization of collagen on PMMA-g-PHBV membrane

The PMMA-g-PHBV membrane was employed to covalently immobilize collagen by the formation of amide bonds by reaction of the carboxyl groups from PMAA with the amino groups from collagen in the presence of a condensing agent. Immobilization of collagen onto the PMAA-g-PHBV film was performed at 2–4°C for 24 h with the carbodimide as the condensing agent.

It was noticed that Coll-g-PMMA-g-PHBV surface showed relatively lower water contact angle than PMMA-g-PHBV film. The decrease in water contact angle of collagen-immobilized surface is in general agreement with similar trend observed for type I atelocollagen grafted onto ozone treated polyurethane and poly L-lactic acid membranes.[21,27] The decrease in water contact angle of type I atelocollagen grafted PU membrane has been attributed to the presence of a thick hydrated layer at the interface of grafted polymer brushes and bulk water. Similar explanation can be extended to the collagen-immobilized surface because collagen has the ability to adsorb water and behave as a hydrophilic membrane.

To establish that the collagen was indeed chemically immobilized, the surface density of collagen chemically immobilized on PHBV film was determined as described by Shu et al.[27] and Bradford method.[30] A collagen density of $0.25 \mu g/cm^2$ (7.29×10^{-13} mol/cm^2) was found for Coll-g-PMMA-g-PHBV (5wt % MAA) while only $0.17 \mu g/cm^2$ (4.96×10^{-13} mol/cm^2) of collagen was found on Coll/PHBV film. The evidence for anchoring of collagen to carboxyl groups on PHBV film as noted by chemical assay and contact angle measurement data were further supported with mean square roughness AFM measurement data. The collagen immobilized PHBV film was found to be relatively smooth compared to ozone treated film and PMMA grafted PHBV film. It is believed that the pore and the rough terrain that were created during ozone oxidation and thermal grafting of PMMA chains are more readily accessible for collagen to occupy and create a relatively uniform film.

Cell activity

In this study, mouse MC3T3-E1 and rat osteosarcoma cell lines were seeded on Coll-g-PMAA-g-PHBV, Coll/PHBV, and PHBV membranes. Figures 3a–c show phase contrast micrographs of rat osteosarcoma cells cultured on untreated and collagen immobilized PHBV membranes, after incubation in the culture medium for six days. Some areas on the surface of PHBV membrane modified by chemical immobilization of collagen had several spherical cells in a group, while other areas of the membrane had few isolated cells. It must be mentioned that we performed the cell adhe-

Figure 3 Phase contrast micrographs of UMR-106 cells on (a) PHBV membrane, (b) Coll-g-PMMA-g-PHBV, and (c) Coll/PHBV after 6 days of incubation. Magnification 100×.

TABLE II
Cell Activities Measured on Modified and Unmodified PHBV Films

Membrane type	Cell type	MTT assay after 6 days mean OD_{595}	% cell activity relative to PHBV
PHBV	UMR-106	0.071 (0.012)[a]	-
Coll-g-PMMA-g- PHBV	UMR-106	0.246 (0.046)	246[b]
Coll/PHBV	UMR-106	0.147 (0.018)	107
PHBV	MC3T3	0.069 (0.020)	-
Coll-g-PMMA-g-PHBV	MC3T3	0.116 (0.016)	68
Coll/PHBV	MC3T3	0.075 (0.019)	9

[a] Standard deviation is shown in bracket.
[b] Coll-g-PMMA-g-PHBV = % cell activity relative to PHBV.

sion studies in serum free media so as to eliminate the contribution of other proteins in the cell culture media to the number of cells adhered to the surface of the film. After six days of incubation, some of the cells with extended filopodia, that is, typical polygonal morphology, a characteristic of osteoblastic cells, were observed on Coll-g-PMMA-g-PHBV surface. However, for the same duration, the osteoblast cells on untreated PHBV as well as on Coll/PHBV surface retained their spherical morphology. These results suggest that collagen chemically immobilized on PHBV membrane could provide more conducive environment for cell growth.

Table II summarizes the cell activities as measured by MTT assay at 595nm (OD_{595}) on various membranes after six-day incubation. The improvement in the cell viability of the PHBV membrane due to collagen immobilization was obtained by determining the % increase in cell activity compared to that of untreated PHBV. Coll-g-PMAA-g-PHBV showed 246% UMR-106 cell activity (relative to PHBV) while Coll/PHBV showed 107% UMR-106 cell activity (relative to PHBV). The lower cell activity of MC3T3-E1 on Coll-g-PMAA-g-PHBV and Coll/PHBV (relative to PHBV) of 68 and 9%, respectively, can be attributed to the initial low seeding density of MC3T3-E1 cells and the lower cell attachment to the surfaces. The adhesion and growth of cells on a surface is strongly influenced by the surface chemistry, microstructure, and roughness. Meredith et al.[40] has shown that UMR-106 cells showed enhanced alkaline phosphatase expression over a wider range of micro structural sizes than the MC3Tc-E1 cells.

To determine whether there were statistically significant differences in the cell activity due to the physical or chemical immobilization of collagen on PHBV film, pooled standard deviation was used. Any confidence level lower than 95% was equivalent to no statistically significant difference.[41] Generally, it was found that the cell activity on Coll-g-PMAA-g-PHBV surface was statistically greater than that on Coll/PHBV surface, which in turn is greater than untreated PHBV membrane. The differences in the surface chemistries of these membranes are believed to influence the cell activities. Previously, small difference in the surface structure of polymer surface has been attributed to differences in cell proliferation.[42] Studies are underway to investigate the effect of concentration, distribution, and conformation of type I collagen molecules anchored to PHBV surface on cell activity.

CONCLUSION

In this study, the grafting of hydrophilic PMAA onto ozone activated PHBV membrane surface and the chemical immobilization of collagen was reported. Water contact angle measurement of PHBV films demonstrated that the ozone activated, grafted, and collagen immobilized PHBV membranes are relatively hydrophilic. The cellular activity (as measured by MTT assay) was used to compare the viability of bone cells on collagen chemically immobilized PHBV, collagen physically immobilized PHBV, and untreated PHBV film. Our very preliminary results suggest that in a favorable biological environment, the collagen chemically immobilized on PHBV is a better material for supporting bone cell growth.

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