# Bone Cell Viability on Methacrylic Acid Grafted and Collagen Immobilized Porous Poly(3-hydroxybutrate-*co*-3-hydroxyvalerate)

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**ABSTRACT:** Porous poly(3-hydroxybutrate-*co*-3-hydroxyvalerate) (PHBV) film was prepared by solute leaching of salt/PHBV cast film. The surface chemistry of the PHBV membrane was modified by performing graft polymerization of methacrylic acid (MAA) on ozone treated porous PHBV film, followed by immobilization of type I collagen. The surface characteristics of the modified and nonmodified porous films were measured by water contact angle. The rat osteosarcoma cell line UMR-106 osteoblast like cells were used as model cells to evaluate the cell viability on surfaces. The initial cell attachment, growth pattern, and proliferation as measured by MTT assay were used to evaluate the bone cell viability on the modified and nonmodified films. Among the PHBV films studied, the nonmodified porous PHBV and the porous PHBV film type I collagen dip coated showed no significant difference in cell attachment and proliferation, while the porous PHBV membrane that was collagen immobilized after MAA grafting showed considerable activity of osteoblast like cells. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 98: 1916–1921, 2005

**Key words:** biocompatibility; chemical grafting; osteoblast cell activity; porous polymeric membrane

## INTRODUCTION

Biodegradable polymers have gained considerable importance in the biomedical industry as scaffold and in the development of biomaterials for therapeutic applications. In particular, poly(3-hydroxybutrate-*co*-3-hydroxyvalerate) (PHBV) has received enormous attention for biomedical application in part due to the ability to tune the characteristics of PHBV to achieve desired mechanical properties and rate of degradation.[1] Since PHBV is relatively hydrophobic, the low surface energy of the PHBV affects cell attachment and growth.[2–4]

For a given environment, the cellular interaction with a polymer is strongly dependent on the surface characteristics (i.e., topography and chemistry) of the polymer.[5–8] Polymer surface chemistry, especially the wettability of the surface, has been shown to influence the initial cell attachment through the adsorption of proteins derived from serum used in the culture medium. In the absence of natural recognition sites of cells on the surface of PHBV, commonly surface treatment techniques are used to functionalize the polymer surface so as to promote favorable cellular and physiological response. The cell attachment is believed to involve the adhesion receptors on the cell surface and extracellular matrix (ECM) proteins adsorbed to the polymer surface. To mimic the natural environment on polymeric surfaces, the treated polymeric surfaces have been modified by coating or grafting extracellular matrix (ECM) proteins (fibronectin, vitronectin, collagen) that have a cell-binding domain containing the RGD sequence.[9–14]

The anchoring of extracellular matrix protein to the polymer surface and its influence on osteoblastic cell attachment and growth was a part of our recent investigation. A collagen coated surface can perform significantly different from the collagen anchored surface because collagen can be removed from the dip coated surface when exposed to culture medium with time. Our preliminary results suggest that in a favorable biological environment, collagen chemically immobilized on PHBV is a better material for supporting bone cell growth than collagen dip coated PHBV and untreated PHBV.[15]

In addition to our interest in understanding the role of the immobilization of proteins in supporting osteoblastic cell proliferation on a polymer surface, we consider it pertinent to examine the combined effect of porosity and immobilization of proteins in cell attachment and growth for the development of tissue engineered medical products. High porosity and high interconnectivity of pores are required to minimize the

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amount of polymer usage, to increase the amount of surface area available for cell anchoring, and to assist in the transport of nutrient and cellular byproducts across the porous membrane.[16]

Several methods have been developed to prepare highly porous scaffolds. They include phase inversion techniques such as liquid-liquid and liquid-solid phase separation,[17] leaching of the soluble phase from the multiphase polymer blend, or leaching of soluble particulates from particulate composite.[18,19] For achieving complete removal of the particulate, the particulate fraction should exceed the percolation threshold (about 40 wt %) so that a number of significant pathways for the diffusion of the leaching solvent are generated. In recent studies, we showed that when particle concentration in the composite exceeds the percolation threshold, with the exception of very few particles in the bulk of the film, the particle leaching in the composite is very high.[20,21] Depending on the particle cluster size and the film thickness, one can obtain localized percolation pathways, which is an interesting thin-film effect of porous material.

To further enhance the pore structure and pore interconnectivity of the porous biomaterial, several studies have tried to combine particulate leaching with freeze-drying, gas foaming, and solvent casting.[22–24] Among these various methods, solvent casting in combination with particle leaching was chosen to fabricate three-dimensional porous scaffold.[25] The objective of the study was to address whether porous film with collagen chemically immobilized or physically immobilized impact osteoblastic cell attachment and growth. In this study, confocal microscopy was used to map the cell infiltration after 5 days on modified and unmodified porous PHBV film.

## METHODS

### Materials

Poly(3-hydroxybutrate-*co*-3-hydroxyvalerate) containng 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 were purchased from Sigma Aldrich (St. Louis, MO). MAA was purified by distillation under reduced pressure prior to use for the grafting experiment. 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) were obtained from Fisher Scientific. Rat osteosarcoma cell line UMR-106 (ATCC CRL-1661) was purchased from the America Type Culture Collection.

### Membrane preparation

Porous PHBV membranes were prepared by using a combination of solvent casting followed by solute leaching techniques. Sieved sodium chloride particles of size 75–150 $\mu$m (1.5 g) were hand mixed with 1.0 g of PHBV powder and dissolved in 10 mL of $CHCl_3$ at 60°C. The resulting dispersion was cast in a 9 cm petri dish, and the solvent was allowed to gradually evaporate over 24 h by covering the petri dish partially with a lid. The resulting PHBV/NaCl composite membrane was conditioned for 1 day at 24 $\pm$ 2°C under vacuum before further use. The membrane was immersed in 100 mL of distilled water in a shaker at 25°C for 2–5 days (the water was changed every 2 h for the first 12 h, then 2–3 times a day) to leach out the salt. The salt-free PHBV membrane was air and vacuum dried for 24 h and stored in a dessicator until further use. The drying process was continued until constant weight of the film could be obtained.

### Ozone treatment and PMAA grafting of porous PHBV film

Details about ozone activation and grafting of MAA of porous PHBV film can be found elsewhere.[15] Briefly, a rectangular piece of membrane 2 $\times$ 4 $cm^2$ of thickness 1.2 mm was flushed with air containing 2.2 g/h ozone generated using an ozone generator (Ozonology, Model # L-25, Evanston, IL) for a predetermined time interval. The chamber was then purged with oxygen for 10 min to remove unreacted ozone. Contact angle measurements of activated and untreated porous PHBV film were collected in triplicate.

The activated membrane was retrieved and placed in a Pyrex glass tube that contained 5 wt % methacrylic acid (MAA), 0.2*M* $H_2SO_4$, and 1mM $FeSO_4$. The grafting experiment was performed at 65°C for 1 h in a nitrogen environment, and the grafted membrane was retrieved and rinsed with double-distilled water to free it of residual MAA. The amount of carboxyl groups grafted onto porous PHBV membranes was determined by simple acid base titration using a literature procedure.[26]

### Collagen immobilization on PMAA grafted PHBV membranes

MAA grafted porous PHBV membranes were cut into discs of 10 mm diameter and placed in a solution (containing 10 mg/mL of EDAC in a phosphate buffer solution (PBS) maintained at pH 4.5) for 24 h at 2–4°C. The EDAC treated membrane was then placed in a solution of type I collagen (4 mg/mL in 0.3% acetic acid) for 24 h at 2–4°C. The collagen-grafted membrane was retrieved and sonicated with double-distilled water to free the membrane of physisorbed collagen. In addition to preparing collagen grafted PHBV membrane, collagen physisorbed PHBV film was prepared by dip coating untreated porous PHBV film into a 4 mg/mL collagen solution (prepared in 0.3% acetic

acid) for 24 h. The collagen density on the individual PHBV membranes was determined as described by Shu et al.[27] and the Bradford method.[28]

### Culture and preparation of cells for cell attachment and proliferation studies

UMR-106 cells were cultured in 0.22 $\mu$m 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 $\mu$g/mL (w/v) streptomycin (Quality Biological, Inc.), and 50 $\mu$g/mL L-ascorbic acid (Fisher Scientific) at 37°C in 5% $CO_2$. At termination, cells were harvested and 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 $\mu$M 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.

To asses cellular attachment and proliferation on the PHBV membranes, cells were seeded at $3 \times 10^4$ cells/cm$^2$ and $2 \times 10^4$ cells/cm$^2$, respectively, in 96 well tissue culture plates in 200 $\mu$L serum-free DMEM. Cells were also seeded on tissue culture polystyrene (TCPS) at identical conditions, a positive control for the study. The cells were incubated for 1, 3, and 5 h at 37°C in 5% $CO_2$. For the cell attachment study, the film was retrieved from the well after 1, 3, and 5 h incubation and washed with serum free DMEM and the number of adhered cells to the film was determined by MTT assay.[29] For the cell proliferation study, the film was retrieved from the well after 24 h incubation, washed with serum free DMEM, and resuspended in 10% serum containing DMEM media. After 5 days, the films were collected and washed with serum free DMEM and MTT assay was performed.

Briefly, 100$\mu$L of MTT (5 mg/mL) was added to each culture well containing 200 $\mu$L serum free DMEM, and the membrane was allowed to incubate for 3 h at 37°C with 5% $CO_2$. During incubation, the viable cells are involved in the mitochondrial reduction of MTT to a dark blue formazan product. Following incubation, the MTT solution was removed and the membranes were rinsed twice in PBS. The water insoluble formazan product was dissolved by adding 200 $\mu$L of dimethyl sulfoxide (DMSO) and transferred to a 96 well plate. The absorbance was measured at 595 nm using a Titertek Multiskan 310 C plate reader.

**Figure 1** Optical micrograph of porous PHBV film after salt leaching by deionized water over 5 days. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

### Confocal microscopy characterization

Confocal microscopy and 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) were used to visualize viable adherent cells within the porous membrane. Cellular esterases in viable cells convert the non fluorescent 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) into the fluorescent 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein BCECF compound.[30–32] Briefly, viable cells were stained by incubating the membranes containing cells with 5 mg/mL BCECF-AM in serum-free media for 1 h followed by rinsing in PBS. The membranes were examined using an upright laser confocal microscope (Olympus). The key feature of confocal microscopy is that only light from a narrow objective focal plane is detected. Intracellular BCECF was excited at 488 nm using an Ar+ laser light, and light emitted (505–550 nm) from the film was detected with a photomultiplier tube. The 10× objective was used for the study. Images were acquired by focusing the laser beam at 300 $\mu$m beneath the surface of the film.

## RESULTS AND DISCUSSION

Porous PHBV film was prepared by solvent casting of a salt : PHBV solution followed by solute leaching over a duration of 2 to 5 days. Figure 1 shows images of PHBV film that were prepared with pores having a characteristic dimension in excess of 75 $\mu$m with a maximum of 150 $\mu$m (based on optical micrograph data). The micropores formed were cuboids and were dispersed throughout the film. The size and shape of the pores are in general agreement with the salt particle dimension used for the study. Although we restricted the porous membrane preparation to a single

**TABLE I**
**Amount of Carboxyl Group Introduced After Grafting, Amounts of Collagen Immobilized, and Water Contact Angles on Porous PHBV Surfaces**

| | -COOH introduced (nmol/cm$^2$) | Collagen density ($\mu$g/cm$^2$) | Water contact angle (°) |
|---|---|---|---|
| PHBV | — | — | 65 ± 1.3 |
| PHBV-g-PMAA | 0.08 ± 0.06 | — | 52 ± 2.1 |
| PHBV-g-PMAA-COLL | — | 3.4 ± 0.91 | 54 ± 0.9 |
| PHBV-COLL | — | 1.1 ± 0.87 | 55 ± 1.4 |

salt composition, in a related study we have shown that interconnectivity of the pores is strongly related to the particulate composition and particle size range. For example, when the leachable component exceeds 40 wt % of the overall composition of the composite, 95 wt % of leachable component in the particulate composite is leached, leaving behind pores in the composite, because of the well established interconnecting percolation pathways for solvents to access the leachable component.[20,21] For this study, we limited our work to porous membranes prepared from 60 wt % salt : 40 wt % PHBV film.

In our earlier publication, we described the procedure for grafting collagen on PHBV film.[15] Similarly, microporous PHBV membranes were ozone treated and MAA grafted followed by collagen chemical immobilization. The water contact angle data of the individual membranes is shown in Table I. Water contact angle values of the membranes were measured by the sessile drop method. As expected, the contact angle of MAA grafted PHBV membranes was significantly lower (more hydrophilic) than the unmodified PHBV film. A word of caution is in order that measuring the contact angle of porous material is not an accurate reflection of the hydrophilicity of the membrane because of the irregularity of the film. However, these results follow the trend noticed in PHBV film, where the contact angle of PHBV film was shown to decrease with exposure of PHBV film to ozone, grafting of MAA, and collagen immobilization. The exposure of the porous PHBV membrane to ozone gives rise to the formation of a variety of functional groups, including peroxides and hydroperoxides.

To establish that indeed polar functional groups (as evidenced by water contact angle data) are formed during ozone activation, the amount of collagen present on dip coated and chemically immobilized membranes were compared. As mentioned in Table I, the collagen density on collagen immobilized membrane was found to be higher than that of collagen dip coated membrane. The PMAA grafted membrane provided anchoring sites for collagen immobilization, because of the strong interaction of negative charge of the carboxylate group of MAA grafted chains with the cationic groups of the collagen molecule.[33,34]

Washing the membranes resulted in some loss of the collagen molecules from the grafted porous PHBV membrane (about 15%), and significant loss (about 50%) of the collagen molecules from the dip coated porous PHBV membrane. PMMA grafting of the PHBV membrane surface was beneficial in strongly binding collagen molecules to the film surface.

The membranes that were seeded with cells were used in the measurement of cell attachment, cell proliferation, and infiltration of cells into the porous film network structure. To assess cell viability on the porous membranes, cell density was assayed by the MTT optical density assay, and the cell morphology on the PHBV membrane was mapped using a vital fluorescence microscopy technique. Figure 2 shows UMR-106 osteoblastic cell attachment data on various membranes relative to incubation time. Cell attachment is expressed as a ratio of cells attached on a specific membrane relative to tissue culture polystyrene (TCPS) by performing cellular measurements under identical conditions. The results are an average of multiple measurements for all three sample types. It must be mentioned that the cell adhesion studies were performed in serum free media to eliminate the contribution of other proteins in the cell culture media to the number of cells adhered to the porous film. The rate of cell attachment on each of the three membranes was very similar. There is a gradual increase in the number of cells attached to the dip coated PHBV-COLL porous membrane or the chemically modified PHBV-g-PMMA-COLL porous membrane. However, the greatest number of attached cells was observed on the collagen immobilized PHBV porous membrane relative to the collagen dip coated PHBV or the nonmodified PHBV porous membrane.

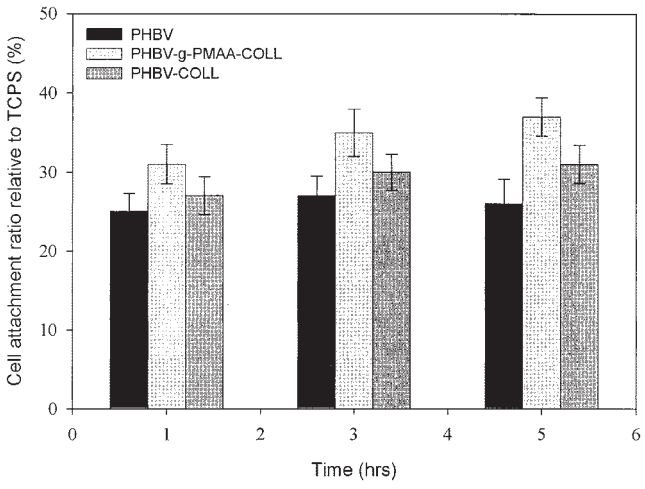


**Figure 2** Cell attachment ratio of porous PHBV, collagen dip coated PHBV, and COLL-PMMA-g-PHBV membrane relative to TCPS.

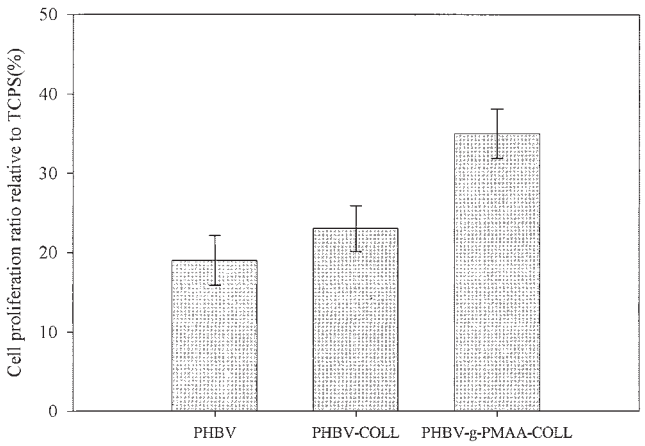


**Figure 3** Cell proliferation ratio of porous PHBV, collagen dip coated PHBV, and COLL-PMMA-g-PHBV membrane relative to TCPS.

Figure 3 provides the cell proliferation data on the individual membrane types. The number of cells attached after 24 h effectively formed the nucleation point for further cell proliferation study, since any nonadherent cells in the attachment assay were removed via washing prior to the transfer of the membrane to a serum containing a culture medium that facilitates growth. Chemically grafted COLL-PMMA-g-PHBV porous membranes exhibited the highest cell proliferation potential when compared to the collagen dip coated PHBV or the nonmodified PHBV porous membrane films. Cells on untreated porous PHBV membrane exhibited ~ 50% of the cell proliferation observed on the collagen immobilized porous membrane.

The fluorescence images in Figure 4 show UMR-106 cells on porous PHBV film at 300 $\mu$m beneath the surface. Osteoblast cells seem to have proliferated the various membranes during incubation. Abundant viable cells (as observed by a high percentage of the cells exhibiting fluorescence intensity) were observed for the collagen immobilized membrane when compared to either the collagen dip coated or the unmodified porous PHBV membranes. From the confocal microscopy characterization results and MTT assay, it seems that the porous PHBV membrane after PMAA grafting and collagen immobilization provides a favorable surface for UMR-106 cell attachment and subsequent proliferation.

## CONCLUSIONS

The following conclusions can be drawn from the current work:

1. Porous PHBV membranes were prepared by the conventional salt leaching technique. The

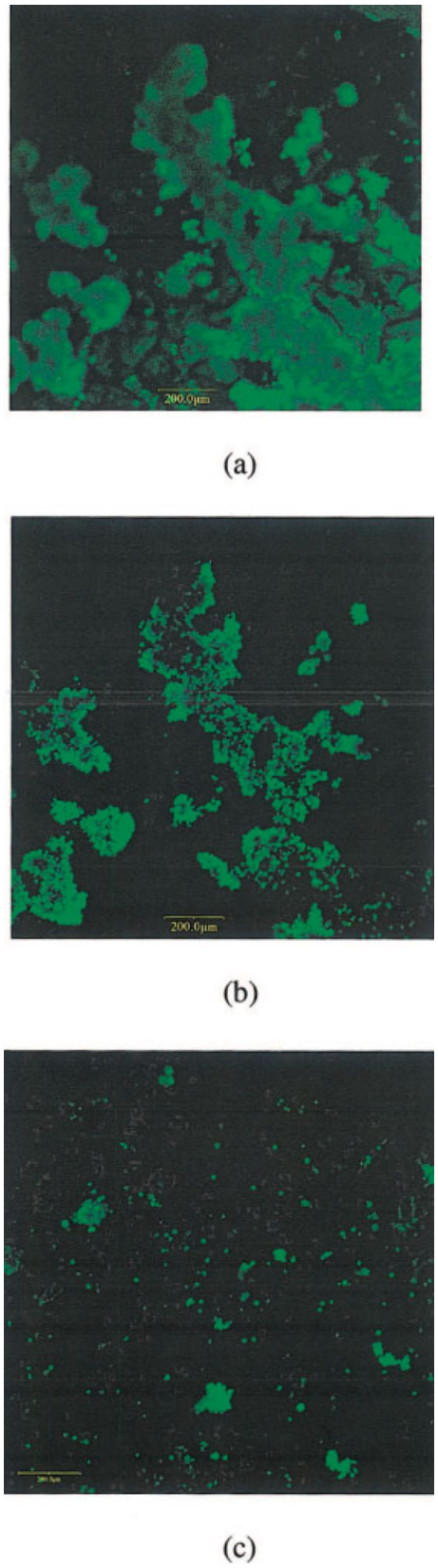


**Figure 4** Confocal micrograph of 3 day incubated: (a) COLL-PMMA-g-PHBV, (b) collagen dip coated PHBV, and (c) porous PHBV membrane. Micrographs taken at 300$\mu$m beneath the surface. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

membranes were dip coated or chemically grafted with collagen. The chemically grafted PHBV membrane had a higher density of collagen than the dip coated PHBV membrane.
2. Collagen immobilized PHBV membrane provided a more favorable matrix for cell proliferation than either dip coated collagen PHBV porous membranes or unmodified PHBV porous membranes.
3. Confocal microscopy imaging is a valuable tool to map viable cells buried in the porous PHBV matrix.

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