Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

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To cite this Article Wang, Ling-Yun , Wang, Ying-Jun and Cao, De-Rong(2009) 'Surface Modification of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) Membrane by Combining Surface Aminolysis Treatment with Collagen Immobilization', Journal of Macromolecular Science, Part A, 46: 8, 765 — 773
To link to this Article: DOI: 10.1080/10601320903004517
URL: http://dx.doi.org/10.1080/10601320903004517

Journal of Macromolecular Science®, Part A: Pure and Applied Chemistry (2009) **46**, 765–773

ISSN: 1060-1325 print / 1520-5738 online
DOI: 10.1080/10601320903004517

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Surface Modification of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) Membrane by Combining Surface Aminolysis Treatment with Collagen Immobilization

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Received January 2009, Accepted February 2009

Amino groups were introduced onto a poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) surface by applying 1,6-hexanediamine treatment. The effects of aminolysis time and 1,6-hexanediamine concentration on hydrophilicity of the treated PHBV were investigated using contact angle measurement. The occurrence of the aminolysis and the introduction of NH_2 groups were verified by X-ray photoelectron spectroscopy (XPS) and ninhydrin method. By use of the NH_2 groups as active sites, collagen was further immobilized on the aminolyzed PHBV (NH_2-PHBV) membrane via a cross-linking agent, glutaraldehyde. The increase of nitrogen content and further decrease of water contact angle after immobilization of collagen suggested that the surfaces became more hydrophilic. Mouse bone marrow stromal cells (BMSc) cultured on untreated PHBV and treated PHBV films were evaluated by cell attachment, cell proliferation, and morphological observation under scanning electron microscope (SEM). The order of cytocompatibility is Coll-PHBV > NH_2-PHBV> PHBV, indicating coll-PHBV was a promising material in future tissue-engineering application.

Keywords: PHBV, collagen, immobilization, hydrophilicity, aminolysis

1 Introduction

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), commonly referred to PHBV, has received enormous attention lately due to its possible application in the drug delivery system, and as substrate for cellular support (1–3). 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 (4). It is the combination of useful properties of PHBV such as natural origin, biodegradability, biocompatibility, thermoplasticity, and desired mechanical properties that makes PHBV an extremely attractive biomaterial for use in tissue engineering applications. However, the surface properties of PHBV are not ideal for cell growth. PHBV is relatively hydrophobic compared to the natural extra cellular matrix (ECM), is unable to interact specifically with cells, and does not possess any functional groups for the attachment of biologically active molecules.

It is well known that 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, etc) of biodegradable polymer. Therefore, surface modification gives useful surface characteristics to the polymer, without changing the properties of the bulk. Many approaches have been taken to modify the surface of PHBV to date. For example, surface hydrophobicity and surface chemistry were altered using oxygen plasma treatment (4–7), perfluorohexane plasma (8), allylamine plasma (9), and ammonia plasma (10). In addition, studies investigated gamma irradiation induced graft polymerization of methyl methacrylate and 2-hydroxyethyl methacrylate (11) or acrylic acid (AAc) (12) onto PHBV. As described by Grøndahl and coworkers (12), the advantages of using gamma irradiation induced grafting rather than plasma treatment is the ability to modify the internal surface of a three-demensional scaffold and to maintain surface functionality as the polymer

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surface erodes. However, since PHBV is known to degrade when exposed to gamma irradiation, only low doses were used (13).

In studies by Hu et al. (14, 15), carboxylic acid groups were introduced onto PHBV by ozone treatment followed by AAc grafting. Subsequently, chitosan was attached to the modified material to improve antibacterial activity. Recently, we used plasma and photografting polymerization to treat PHBV film to get functionalized surface, which promoted cells growth and proliferation (16, 17).

On the other hand, study on aminolysis using ethylenediamine (ED) in basic aqueous solution for the introduction of amino groups on PHBV has been investigated (18). However, it was found that ED treated PHBV led to degradation of the polymer during aminolysis and became brittle due to an increase of crystallinity after three weeks of storage. So, the use of controllable surface aminolysis to activate the surface of PHBV film with amino groups is necessary.

A desirable property of any surface modification technique is that it is inherently limited to the very surface of the substrate. In this study, we report on the use of 1,6-hexanediamine aminolysis in 2-propanol to produce amino groups on the surface of PHBV in controlled manner, with minimal erosion. In addition, as an important protein for direct tuning of biophysical parameters (19), collagen is chemically immobilized on the newly formed aminolyzed PHBV (NH_2-PHBV) film via glutaraldehyde (GA). To the best our knowledge, collagen immobilization has so far not been applied to PHBV using aminolysis. The hydrophilicity and surface chemical states are characterized by water contact angle measurement and X-ray photoelectron spectroscopy (XPS), respectively. The existence of amino groups is verified quantitatively by ninhydrin analysis method. The surface morphologies are characterized by scanning electron microscope (SEM). The culture of mouse bone marrow stromal cells (BMSc) *in vitro* shows that the cytocompatibility of collagen-immobilized PHBV (coll-PHBV) membranes is obviously improved.

2 Experimental

2.1 Materials

1,6-hexanediamine was purchased from Guangzhou Chem. Co. (China, Guangzhou) and purified by vacuum sublimation. Other reagents were obtained from Tianjin Chemical Reagent No. 1 Plant (China, Tianjin) without further purification. Deionized water (MilliQ) was used throughout.

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from Gibco (USA). 3-(4,5-Dimethyl-2-thiazoly)-2,5-dipheny-2H- tetrazalium bromide (MTT) was obtained from Amresco (USA) and dimethyl sulfoxide (DMSO) was received from Sigma (USA).

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) with 8% 3-hydroxyvalerate content (M_n = 185000, PDI = 2.2) was purchased from Aldrich Chemical Co., Inc. (USA). The PHBV was dissolved at 60°C in chloroform to obtain a concentration of 60 mg/mL in a round-bottom flask fitted with a stirrer and reflux condenser. Prior to the application of the solution; the glass Petri dish was cleaned first with acetone, then with methanol, and dried with nitrogen. The resulting solution was immediately poured into a glass Petri dish and covered; ensuring a minimum amount of condensate formed. 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 PHBV film with average thickness of 0.15 mm was removed from the glass dish with a plier and cut into 20×20 mm^2 pieces. The resulting film was further dried under vacuum for another 24 h at 30°C before further use.

2.2 Preparation of Acid-Soluble Bovine Tendon Collagen Type

Bovine tendons were purchased from a local market in Guangzhou, China. After the residual meat and tissue were removed manually, tendon were descaled and washed with distilled water. The cleaned tendons were then minced into small pieces (less than 4 mm) by a cutting mill. Then the tendon pieces were frozen at –20°C until use. 10 g of tendon were precisely weighed. The following experiments were done in a room at 4°C. To remove non-collagenous proteins, the tendon was mixed with 0.1 M NaOH at a sample/alkaline solution ratio of 1:10 (w/v). The mixture was stirred for 6 h. The alkaline solution was changed every 2 h. Then, the alkali-treated tendons were washed with cold water until neutral wash water was obtained.

Pretreated tendons were soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) for 24 h, followed limited hydrolysis with pepsin at 50 Unit/mg of tendon for 48 h in 0.5 M acetic acid. The pepsin amount was calculated on the basis of its activity. Sodium acetate–acetic acid buffer was used to regulate the pH of the bulk solution. The extract was filtered with Whatman No. 1 paper (Whatman, Maidstone, UK). Then collagen solutions were precipitated by adding NaCl to a final concentration of 2.6 M in the presence of 0.05 M tris(hydroxymethyl)aminomethane (pH 7.0). The resulting sediment was collected by centrifugation at 10,000 g for 50 min 100 mL of 0.5 M acetic acid were used to dissolve the sediment, then the solution was dialysed against 0.1 M acetic acid and then distilled water, three times, respectively, and freeze-dried. The dried collagen was dissolved in 0.5 M acetic acid to give 3 mg/mL collagen solution.

2.3 Preparation of NH_2-PHBV Films

The PHBV film was washed with a large amount of deionized water, then was immersed in 1,6-hexanediamine/

2-propanol solution for the given time at 37°C, rinsed with deionized water for 24 h at room temperature to remove free 1,6-hexanediamine, and finally dried in a vacuum at 30°C for 24 h to constant weight.

2.4 Preparation of coll-PHBV Films

The NH_2-PHBV membrane was immersed in 1 wt% GA solution for 3 h at room temperature, followed by rinsing with a large amount of deionized water for another 24 h to remove free GA. The membrane was then incubated in 3 mg/mL collagen solution (pH 3.4) for 24 h at 2–4°C. The coll-PHBV membranes were rinsed with 1.0% acetic acid solution for 24 h to remove free collagen, followed by three washes with deionized water. The collagen density on the coll-PHBV membranes was determined as described by Shu et al. (20).

2.5 Determination of the Amino Groups

The ninhydrin analysis method was employed to quantitatively detect the amount of NH_2 groups on the NH_2-PHBV film, as previously described (21). The membrane was immersed in 1.0 mol/L ninhydrin/ethanol solution for 1 min and then was placed into a glass tube, following with heating at 80°C for 15 min to accelerate the reaction between ninhydrin and amino groups on PHBV membrane. After the adsorbed ethanol had evaporated, 5 mL of 1,4-dioxane was added into the tube to dissolve the membrane when the membrane surface displayed blue. Another 5 mL of 2-propanol was added to stabilize the blue compound. The absorbance at 450–650 nm of this mixture was measured on a UV-Vis spectrophotometer. A calibration curve was obtained with 1,6-hexanediamine in 1,4-dioxane/isopropane (1:1, v:v) solution.

2.6 Characterization

The wettability of untreated and treated PHBV films was determined by measuring the contact angle of distilled water at room temperature, using a video contact angle system (model VCA-2500, Advanced Surface Technology Inc., Billerica, MA). 1 μL 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.

X-ray photoelectron spectroscopy (XPS) was used to evaluate the surface elemental composition and atomic concentration of untreated and treated samples, and was performed on a VG Scientific ESCALAB MkII electron spectrometer employing MgKα radiation. The charging shift was referred to the C1s line emitted from the saturated hydrocarbon. Analysis of the spectra was done by peak fitting using the data processing unit of the instrument.

Surface morphologies were observed with a Philips (Holland) XL 30 scanning electron microscope (SEM) with an accelerating voltage of 10 kV. The films were then mounted on metal stubs and coated with gold through sputter (HITACHI E-1010). An Olympus IX70 inverted microscope equipped with a SPOT Diagnostic Instruments camera was used to visualize samples.

2.7 *In vitro* Cytocompatibility

The seeding cell suspension was prepared from mouse bone marrow stromal cells (BMSc). The control or modified PHBV films were placed in 24-well plates and 1×10^5 cells/well were seeded onto the films. Then the cells were cultured in an incubator (Thermo Forma, USA) with 5% CO_2 at 37°C and prepared for testing.

2.7.1. *SEM analysis*

When the cells were cultured for 7 days, the cells on the films were fixed with 2.5% glutaraldehyde and dehydrated by sequential dipping in increasing concentrations of alcohol, then dried at critical temperature for SEM observation to visualize the manner of cell attachment and spread.

2.7.2. *Cell attachment*

Study of cell attachment was on two groups of four 3-cm polystyrene Petri dishes without biomaterial deposit: untreated PHBV, and modified membranes. Each specimen was thoroughly washed with PBS. BMSc were seeded on top of each film, and cultured at 37°C for 6 h. unattached cells were removed by washing with PBS, and the number of attached cells was determined by MTT test.

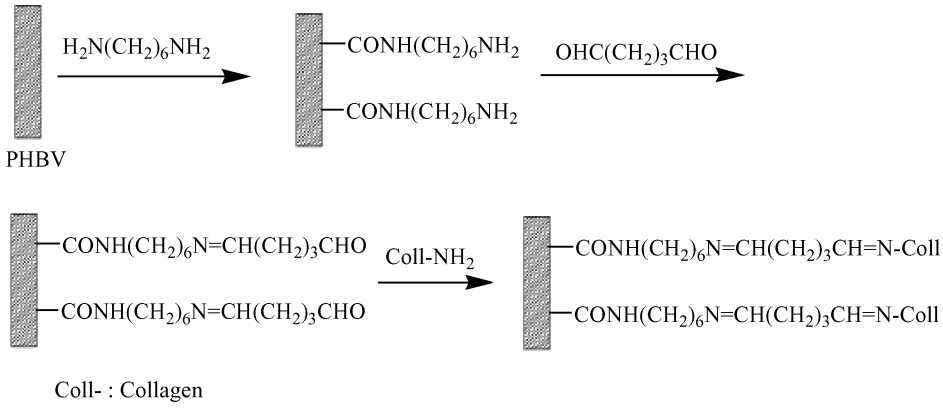
2.7.3. *Cell proliferation*

BMSc were plated on the specimens and the medium was changed every 24 h during incubation in a CO_2 incubator. Cell proliferation on each specimen was determined after 7 days. To remove unattached cells, specimens were gently washed with PBS. The attached cells were separated from the substrate by incubation in 50 μl of 0.25% w/v trypsin solution for 10 min at 37°C, and 100 μl of media was added. After centrifugation, cells were placed in the fresh medium. An aliquot of the resulting cell suspension was stained with trypan blue and counted by using a Neubauer hemacytometer on an inverted light microscope.

3 Results and Discussion

3.1 Aminolysis and Collagen Immobilization

Aminolysis is a technique which has been used in the textile industry for many years to improve the dyeability, wettability characteristics. However, its applicability to surface modification of biodegradable polyesters for tissue engineering has only recently been noticed (18, 21–23). A large



Sch. 1. The schematic representation of aminolysis and further immobilization of collagen on PHBV films.

amount of work was carried out in the past to elucidate the mechanism of aminolysis of model esters (24–27), which indicated the reaction proceeded via nucleophilic attack on the carbonyl carbon to form a positively charged tetrahedral intermediate. Under basic conditions, the tetrahedral intermediate is deprotonated, leading to the extremely unfavorable R-NH^- leaving group (pKa > 30), and hence, the reaction proceeds to the formation of an amide and an alcohol. Therefore, aminolysis is generally carried out either in basic aqueous solution (pH > pKa of the amine) (18, 24, 26) or in an aprotic, polar solvent with a high degree of π basicity, such as an alcohol (27).

In this study, aminolysis of PHBV was carried out by use of 1,6-hexanediamine in 2-propanol, which was different from that performed in basic aqueous solution with ED (18). The overall route taken to functionalize PHBV via aminolysis is shown schematically in Scheme 1. The aminolysis process may be that one amino group of 1,6-hexanediamine reacts with the -COO- group of PHBV to form a covalent amide bond, while the other amino group is unreacted and free.

The introduction of NH_2 groups onto PHBV film can not only modify the poor hydrophilicity but also provide the necessary active sites through which other biocompatible components can be further immobilized. To achieve the covalent coupling of collagen, the NH_2-PHBV film is treated with a large amount of glutaraldehyde (GA) first. The reaction between NH_2 groups and GA yields a bonding via –N=CH-$(CH_2)_3$CHO, and one free aldehyde group can react with NH_2 groups existing in collagen. Samples are rinsed thoroughly with 1.0% acetic acid solution after treatment to ensure that only strongly bound collagen remains attached. Therefore, collagen is covalently immobilized to surface of PHBV.

3.2 Water Contact Angle

Figure 1 summarizes water contact angle of treated PHBV films on various 1,6-hexanediamine concentration. It can be seen that aminolysis method induces drastic changes of the polymer surface. The hydrophobic nature of the untreated PHBV surface shows relatively high contact angle of 76.4 ± 1.6°, which compares favorably previous studies (16, 17). For the NH_2-PHBV, the data of contact angle decreases rapidly with the increase of 1,6-hexanediamine concentration up to 10 wt%, reaching the value of 45.3 ± 1.7°. The possible reason may be that the aminolysis becomes faster and more hydrophilic groups are introduced at higher 1,6-hexanediamine concentration. When the 1,6-hexanediamine concentration is 12 wt%, NH_2-PHBV films reaches equilibrium contact angle value (43.8 ± 1.5°). It must be indicated that the concentration of 1,6-hexanediamine should be preferably lower than 14 wt% because of its poor solubility in 2-propanol (23). Therefore, the optimal concentration of 1,6-hexanediamine was chosen as 10 wt%. For coll-PHBV, contact angle further decreases with the increase of 1,6-hexanediamine concentration, indicating more hydrophilicity surface is formed

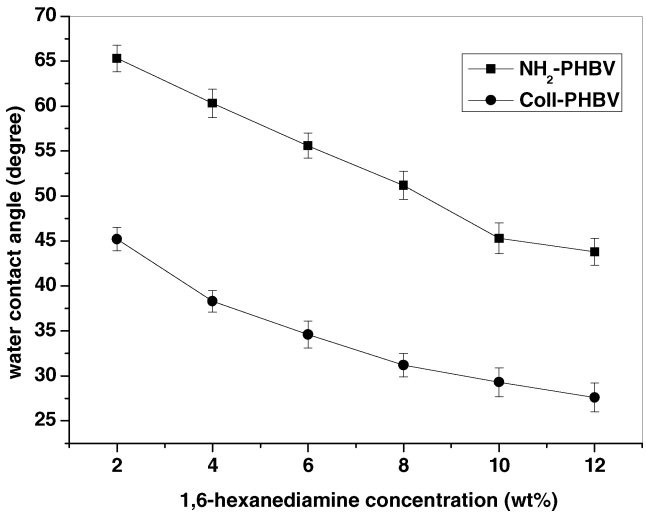


Fig. 1. The influence of the 1,6-hexanediamine concentration on the water contact angle of treated PHBV films (Treatment time: 60 min).

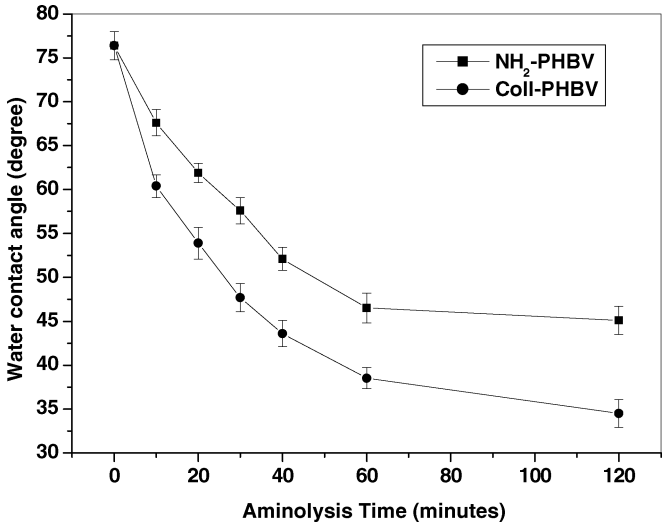


Fig. 2. The influence of the aminolysis time on the water contact angle of treated PHBV films (10 wt% 1,6-hexanediamine concentration).

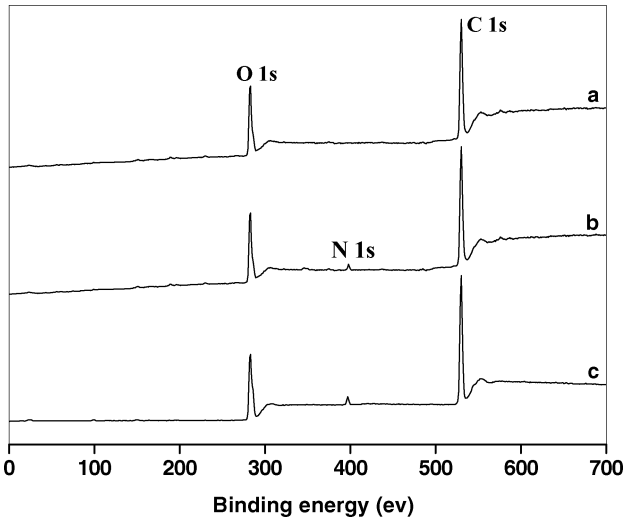


Fig. 3. XPS survey spectra of the surface of (a) untreated PHBV; (b) NH_2-PHBV and (c) coll-PHBV.

after collagen immobilization. For example, water contact angle values at 2, 4, 6, 8,10, 12 wt% were found to be 45.2 ± 1.3°, 38.3 ± 1.2°, 34.6 ± 1.5°, 31.2 ± 1.3°, 29.3 ± 1.6° and 27.6 ± 1.6°, respectively. The decrease in water contact angle of coll-PHBV surface is in general agreement with similar trend observed for type I atelocollagen grafted onto ozone treated polyurethane and poly(L-lactic acid) membranes (20, 28), which is 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 our coll-PHBV surface because collagen has the ability to adsorb water and behave as a hydrophilic membrane.

Figure 2 shows the effect of the aminolysis time on the water contact angle of treated PHBV films. For NH_2-PHBV, the data of contact angle within the first 10 min caused a decrease, from 76.4 ± 1.6° before treatment to 67.6 ± 1.5° after treatment. Thereafter, the contact angle reaches an equilibrium value after 60 min and remains constant after treatment time of 120 min (45.1 ± 1.6°). This is similar to contact angles measured for ED-treated PHBV samples, which were found to initially decrease with treatment time after they remained constant at longer treatment times (18). The reduction of the water contact angle suggests an obvious improvement of surface hydrophilicity, which may be attributed to the cleavage of hydrophobic groups and new formation of hydrophilic groups (amide bind, primary amine, and alcohol) on the PHBV surface. From Figure 2, it is also noticed that coll-PHBV surface shows relatively lower water contact angle than NH_2-PHBV film, suggesting more hydrophilic surface is formed.

3.3 XPS Analysis

XPS analysis has been employed to get information on the structure and chemical state of the surface of PHBV films before and after treatment. When PHBV is treated with 1,6-hexanediamine, some chemical bonds can be broken and new ones may form, thus the chemical environment of atoms should change. Survey spectra of the untreated and treated PHBV films are presented in Figure 3. As expected, PHBV presents two peaks separately corresponding to C 1s (binding energy, 285 eV) and O 1s (binding energy, 532 eV). After aminolysis and collagen-immobilization, evident N 1s peaks (binding energy, 400.1 eV) come forth in Figure 3 (b) and (c), respectively, which suggests that nitrogen has been bound onto the surface of PHBV films. The total amount of nitrogen introduced on the NH_2-PHBV films is at most 1.6 at %, which is lower as compared with that found on ED-treated PHBV films (4.0 at %) due to its harsher aminolysis conditions (40% ED, 50°C and pH 10) [18].

3.4 Chemical Assays

Recently, ninhydrin has been reported to detect qualitatively and quantitatively the existence of NH_2 groups on the polymer surface (21, 23). In this paper, the blue reaction product of ninhydrin with free NH_2 has a maximum absorbance at 558 nm. The absorbance varied along with variation of the aminolysis time within the studied range, while the control PHBV had not shown any absorbance under the same measuring conditions. The quantitative NH_2 amount on NH_2-PHBV film surface is shown in Figure 4. Whereas the NH_2 density increases to a aminolysis time 40 min (for a constant 1,6-hexanediamine of 10 wt%), the maximum NH_2 density of 3.8×10^{-7} mol/cm^2 is obtained at 40 min. However, the density of amino groups on the superlayer of PHBV film should be far more than 3.8×10^{-7} mol/cm^2 due to the existence of surface roughness and pores in solvent cast film (23), providing additional surface

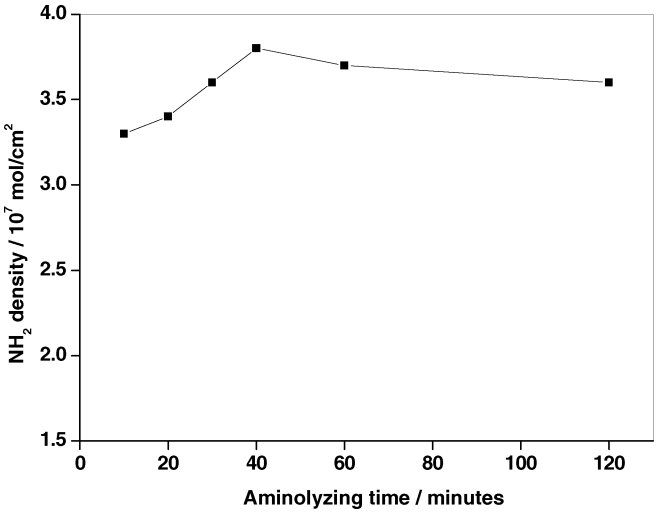


Fig. 4. The absorbance of the NH_2-PHBV membrane treated with ninhydrin as a function of aminolyzing time (10 wt% 1,6-hexanediamine/2-propanol solution).

for aminolysis to occur and leading to a higher concentration of amines than a smooth surface. From Figure 4, it indicates the NH_2 density on PHBV film can be regulated and controlled through controlling the aminolysis degree. From the data discussed above, it confirms that $-NH_2$ groups is successfully introduced to PHBV surface.

After collagen immobilizing by GA treatment and thoroughly washing to remove simply absorbed collagen, the coll-PHBV shows higher nitrogen content (2.8%) through XPS measurement, as shown in Figure 3(c). Since collagen is a molecule composed of repeated sequence Gly-X-Y, and has lots of nitrogen atom, the increase of nitrogen content on coll-PHBV surface can be attributed to immobilized collagen molecules on PHBV surface. This could indirectly support the collagen immobilizing on PHBV surface. To establish that collagen was indeed chemically immobilized, the surface density of collagen chemically immobilized on PHBV film was determined A collagen density of 1.46×10^{-12} mol/cm^2 was found (10 wt% 1,6-hexanediamine, 40 min). The density difference between NH_2 and immobilized collagen could be explained as followed. One mole of collagen is expected to cover a much larger area than one mole of amine groups. In other words, we expect that the molar areal density of collagen should be lower because the size of the collagen molecule is so much bigger than that of the amine groups. Assuming a collagen molecule is 300 nm long and 1.5 nm in diameter (29, 30) and lays down on the surface rather than standing up, one expects it to cover roughly 450 nm^2. In contrast, a single amine group should have an effective area on the order of perhaps 0.1 nm^2. As such, one would expect many thousands of amine groups to be covered by one collagen molecule anyway. Moreover, the differences of surface roughness and pores between NH_2-PHBV and Coll-PHBV films as well as reaction incompletion between NH_2 groups and collagen with GA as a chemical linker should also be considered. Therefore, the molecular concentration of immobilized collagen was less than that of the NH_2 groups.

3.5 SEM Analysis

SEM examination of the polymer surface morphology reveals that there are big differences between untreated and treated PHBV films, as shown in Figure 5. Small holes on untreated PHBV films are found due to the evaporation of the solvent of chloroform (Fig. 5(a) and (b)). Compared with the untreated surface, the NH_2-PHBV surface has more holes with larger sizes and rougher surfaces with more uniform pore distribution, as shown in Figure 5(c) and (d). The possible reason may be that when aminolysis begins, holes on the surface allow 1,6-hexanediamine molecules to come in contact with the film surface; thus the polymers around the holes involve to aminolysis, and the holes become bigger. Therefore, the surface roughening increases. When collagen is further immobilized on NH_2-PHBV film, the collagen-modified PHBV shown in Figures 5(e) and (f) show more porosity and roughness than NH_2-PHBV film, probably because amine-modified PHBV film possessing rough surface and pores continues to hydrolyze under collagen immobilization conditions. When compared Figure 5(d) with Figure 5(f), the change in topography suggests the presence of collagen on PHBV film. Accordingly, the evidence for anchoring of collagen on PHBV film is supported by contact angle data, XPS, chemical assay and SEM.

3.6 *In vitro* Cytocompatibility

The increase of the surface hydrophilicity after aminolysis and immobilization of collagen may provide the possibility of improving the cytocompatibility of PHBV. Morphologies of BMSc attaching and spreading on the untreated and treated PHBV films after 7 day culture are studied by SEM, as shown in Figure 6. Cells spread out and closely attach to the surface of the untreated PHBV film and retain their spherical morphology (Fig. 6(a)). On the surface of the NH_2-PHBV, cell process becomes visible and more ECM is secreted (Fig. 6(b)). This indicates that the introduction of NH_2 groups onto PHBV surface has a positive effect on improving cytocompatibility in a limited extent. The cells on the coll-PHBV spread with long filopodia extensions and attach to the farther cells (Fig. 6(c)) in the form of a confluent layer. These results suggest that coll-PHBV may provide more conducive environment for cell growth.

The cellular behaviors *in vitro*, such as cell adhesion and cell proliferation, are investigated. In general, hydrophobic polymers have been known as unfavorable for cell attachment unless modified to possess a hydrophilic surface with a higher surface energy and a correspondingly lower air-water contact angle. The effect of surface modification on the cell attachment is shown in Table 1. The order of

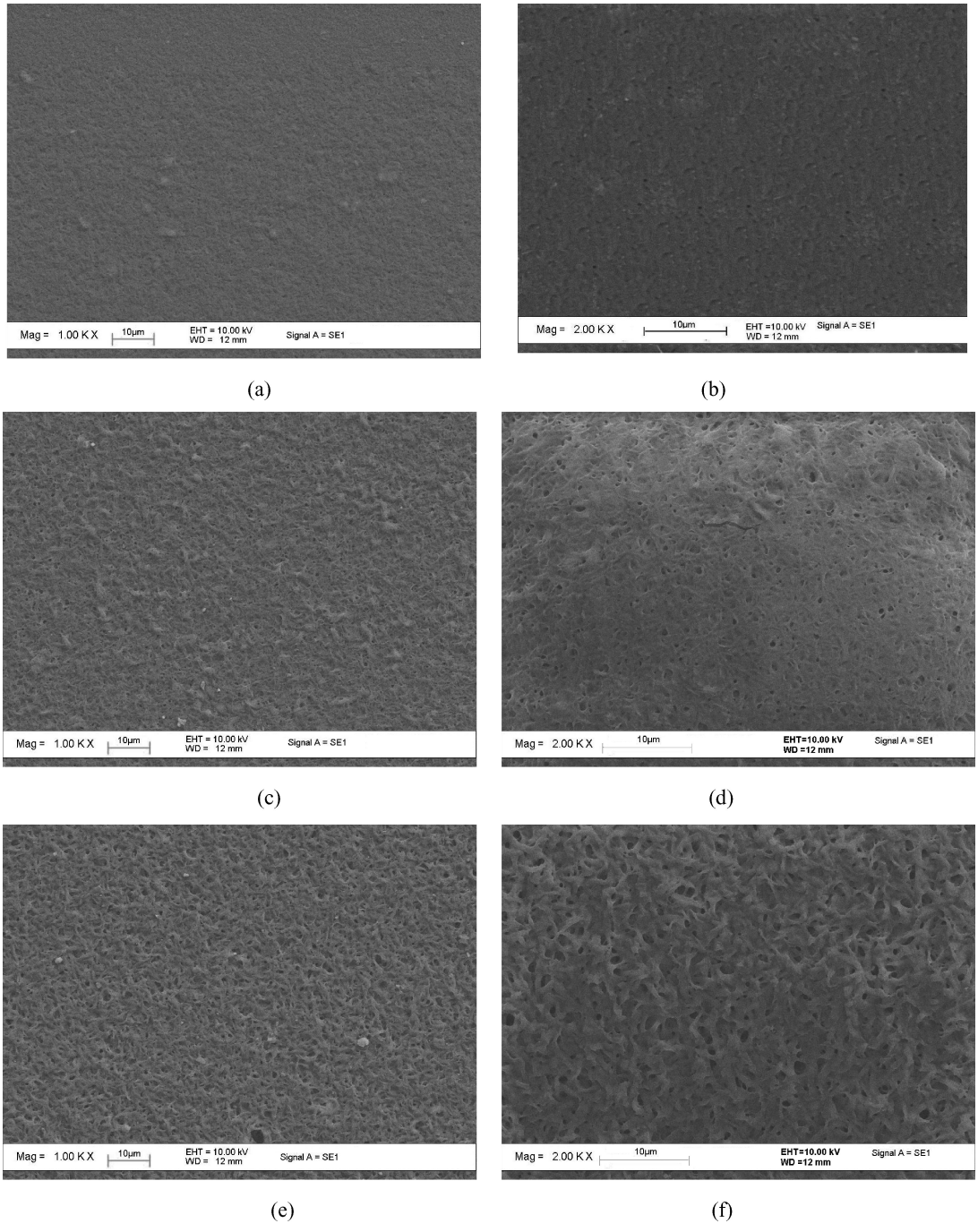


Fig. 5. Surface morphology of films examined by SEM: (a) and (b) for untreated PHBV; (c) and (d) for NH_2-PHBV; (e) and (f) for coll-PHBV.

attaching amount is Coll-PHBV > NH_2-PHBV > PHBV. The result suggests that NH_2-PHBV could improve the attachment of cell. Immobilization of collagen would result in most cell attachment. Being hydrophobic, cell attachment for PHBV was the least among three samples.

The effect of surface modification on the cell proliferation is also shown in Table 1. The order of cell proliferation is Coll-PHBV > NH_2-PHBV > PHBV. Many reported that collagen grafted to polymers show growth-promotion effect (21, 28). This agrees with our finding that collagen immobilization does improve the proliferation of BMSc. These trends follow observations of SEM and data of cell attachment.

It is generally agreed that the relationships between cell membrane and the biomaterial surface may directly influence their cellular activity and the ability of cells to migrate into biomaterials (31–33). Adhesion, proliferation, migration, and differentiation of cells at the surface

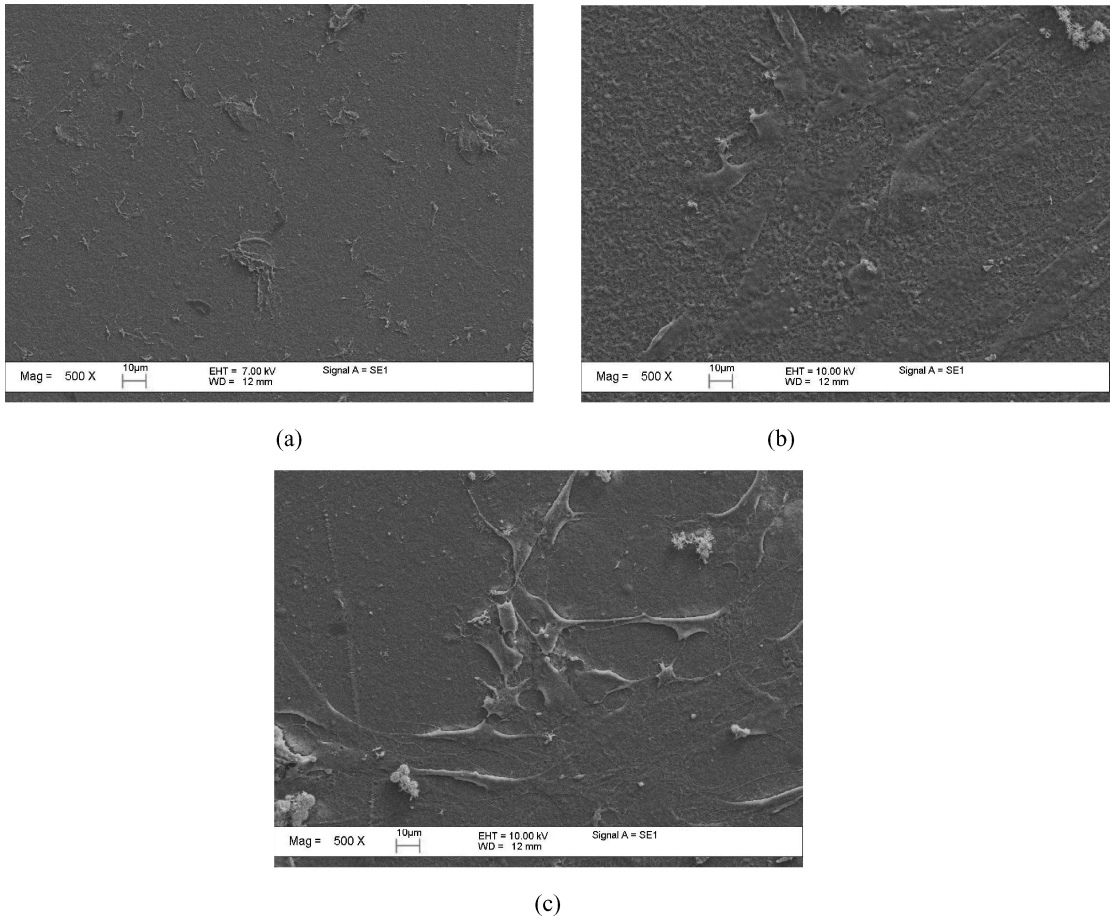


Fig. 6. SEM photographs of BMSc after 7 days of incubation on: (a) untreated PHBV film, (b) NH_2-PHBV, (c) coll-PHBV.

of immobilized materials are related to the ability of membrane receptors to bind molecules of ECM. The hydropholicity/hydrophilicity balance and chemical bioactive moieties of the surface can not only affect the amount and type of serum proteins adhered to the film surface, it may also affect the conformation of these proteins, and in turn the degree of cell adhesion (34). From our results, cell attachment is distinctly promoted on coll-PHBV, and then adhered cells proliferate to confluent layer rapidly in comparison with non-treated PHBV. Immobilized collagen matrix would play a useful role of matrix to support the initial attachment and to stimulate cell proliferation more efficiently. From above discussed results, it suggests that coll-PHBV film show best *in vitro* cytocompatibility comparing with both the control and NH_2-PHBV.

Table 1. Cell attachment and growth on the PHBV films (n = 5, ±SD)

Sample	*Relative cell attachment (%)*	*Relative cell viability (%)*
Control[a]	100 ± 2	58.5 ± 3
PHBV	121 ± 3	56.2 ± 2
NH_2-PHBV	286 ± 5	73.7 ± 4
Coll-PHBV	323 ± 4	92.5 ± 5

[a] Polystyrene Petri dish.

4 Conclusions

Amino groups are introduced onto the PHBV surface through aminolysis of 1,6-hexanediamine. The introduced NH_2 groups provide the opportunity to immobilize collagen onto the PHBV film surface. XPS analysis shows that amine functionalized surfaces are readily available for covalent binding of collagen using GA. Hydrophilicity of PHBV film is remarkably improved so that the water contact angle decreases notably after aminolysis and following immobilization of collagen. The preliminary cell culture results suggest that coll-PHBV provides a more favorable surface for cell adhesion, spread, and proliferation than NH_2-PHBV and untreated PHBV, thus demonstrating promising application for tissue engineering.

Acknowledgements

The study was financially supported by Guangdong Natural Science Foundation (07300602) and Postdoctoral Innovation Fund of South China University of Technology.

References

1. Pouton, C.W. and Akhtar, S. (1996) *Adv. Drug. Deliv. Rev.*, 18, 133.
2. Lee, S.D. Hsiue, G.H., Chang, P.C.C. and Kao, C.Y. (1996) *Biomaterials*, 17, 1599.
3. Sendil, N., Gursel, I., Wise, D.L. and Hasirci, V. (1999) *J. Control. Release*, 59, 207.
4. Köse, G.T., Ber, S., Korkusuz, F. and Hasirci, V. (2003) *J. Mater. Sci. Mater. Med.*, 14, 21.
5. Kose, G., Kenar, H., Hasirci, N. and Hasirci, V. (2003) *Biomaterials*, 24, 1949.
6. Hasirci, V., Tezcane, R.A., Hasirci, N. and Süzer, S. (2003) *J. Appl. Polym. Sci.*, 87, 1285.
7. Mas, A., Jaaba, H. and Schue, F. (1996) *Macromol. Chem. Phys.*, 197, 2331.
8. Flösch, D., Clarotti, G., Geckeler, K.E., Schue, F. and Göpel, W. (1992) *J. Memb. Sci.*, 73, 163.
9. Man, A., Jaaba, H. and Schue, F. (1997) *Macromol. Chem. Phys.*, 198, 3737.
10. Nitschke, M., Schmack, G., Janke, A., Simon, F., Pleul, D. and Werner, C. (2002) *J. Biomed. Mater. Res.*, 59, 632.
11. Mitomo, H., Enjôji, T., Watanabe, Y., Yoshii, F., Makuuchi K. and Saito, T. (1995) *Pure Appl. Chem., A* 32, 429.
12. Grøndahl, L., Chandler-Temple, A. and Trau, M. (2005) *Biomacromolecules*, 6, 2197.
13. Carswell-Pomerantz, T., Dong, L., Hill, D.J.T., O'Donnell, J.H. and Pomery, P.J. (1996) *ACS Symp. Ser.*, 620, 11.
14. Hu, S.G., Jou, C.H. and Yang, M.C. (2003) *J. Appl. Polym. Sci.*, 88, 2797.
15. Hu, S.G., Jou, C.H. and Yang, M.C. (2003) *Biomaterials*, 24, 2685.
16. Wang, Y.J., Lu, L., Zhen, Y.D. and Chen, X.F. (2006) *J. Biomed. Mater. Res.*, 76A, 589.
17. Wang, Y.J., Ke, Y., Ren, L., Wu, G., Chen, X.F. and Zhao, Q.C. (2009) *J. Biomed. Mater. Res.*, 88, 616.
18. Keen, I., Broota, P., Rintoul, L., Fredericks, P., Trau, M. and Grøndahl, L. (2006) *Biomacromolecules*, 7, 7427.
19. Mauch, C., Hatamochi, A., Scharffetter, K. and Krieg, T. (1988) *Exp. Cell. Res.*, 178, 1508.
20. Shu, H., Hwang, Y.S., Lee, J.E., Han, C.D. and Park, J.C. (2001) *Biomaterials*, 22, 219.
21. Zhu, Y.B., Gao, C.Y., Liu, X.Y. and Shen, J.C. (2002) *Biomacromolecules*, 3, 1312.
22. Croll, T.I., O'Connor, A.J., Stevens G.W. and Cooper-White, J.J. (2004) *Biomacromolecules*, 5, 463.
23. Zhu, Y.B., Gao, C.Y., He, T. and Shen, J.C. (2004) *Biomaterials*, 25, 423.
24. Bunnett, J.F. and Davis, G.T. (1960) *J. Am. Chem. Soc.*, 82, 665.
25. Chalmet, S., Harb, W. and Ruiz-Lopez, M.F. (2001) *J. Phys. Chem. A* 105, 11574.
26. Satterthwait, A.C. and Jencks, W.P. (1974) *J. Am. Chem. Soc.*, 96, 7018.
27. Talvik, A.T., Tuulmets, A. and Vaino, E. (1999) *J. Phys. Org. Chem.*, 12, 747.
28. Park, J.C., Hwang, Y.S., Lee, J.E., Park, K.D., Matsumura, K., Hyon, S.H. and Shu, H (2000) *J. Biomed. Mater. Res.*, 52, 669.
29. Riesle, J., Hollander, A.P., Langer, R., Freed, L.E. and Vunjak-Novakovic, G. (1998) *J. Cell. Biochem.*, 71, 313.
30. Lynn, A.K., Yannas, I.V. and Bonfield, W. (2004) *J. Biomed. Mater. Res., Part B: Appl. Biomater.*, 71B, 343.
31. Lampin, M., Waropuier-Clerout, L.C., Degrange, M. and Sigot-Luizard, M.F. (1997) *J. Biomed. Mater. Res.*, 36, 99.
32. Chang, G., Absolom, D.R., Strong, A.B., Stubley, G.D. and Zingg, W. (1998) *J. Biomed. Mater. Res.*, 22, 13.
33. Cai, K.Y., Yao, K.D., Lin, S.B., Yang, Z.M., Li, X.Q., Xie, H.Q., Qing T.W. and Gao, L.B. (2002) *Biomaterials*, 23, 1153.
34. Ishaug-Riley, S.L., Okun, L.E., Prado, G., Applegate, M.A. and Ratcliffe, A. (1999) *Biomaterials*, 20, 2245.