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Biomaterials 24 (2003) 2685–2693
Biomaterials
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Protein adsorption, fibroblast activity and antibacterial properties of poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) grafted with chitosan and chitooligosaccharide after immobilized with hyaluronic acid

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Received 14 October 2002; accepted 27 January 2003

Abstract

Poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) (PHBV) membrane was treated with ozone and grafted with acrylic acid. The resulting membranes were further grafted with chitosan (CS) or chitooligosaccharide (COS) via esterification. Afterward hyaluronic acid (HA) was immobilized onto CS- or COS-grafting membranes. The antibacterial activity of CS and COS against *Staphylococus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* was preserved after HA immobilization. Among them, CS-grafted PHBV membrane showed higher antibacterial activity than COS-grafted PHBV membrane. In addition, after CS- or COS-grafting, the L929 fibroblasts attachment and protein adsorption were improved, while the cell number was decrease. After immobilizing HA, the cell proliferation was promoted, the protein adsorption was decreased, and the cell attachment was slightly lower than CS- or COS-grafting PHBV.

Keywords: Poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid); Chitosan; Antibacterial activity; Protein adhesion; Hyaluronic acid

1. Introduction

Poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) (PHBV) is a biodegradable and biocompatible polyester polymerized by bacteria. PHBV can be used for biomedical applications due to their biocompatibility and non-toxicity to living tissues [1]. Because PHBV does not have functional groups available for covalent immobilization, several papers have reported that functional groups can be introduced to the surface of PHBV by plasma [2] or γ-ray radiation grafting of acrylic acid (AA) [3], or by oxidizing chemical grafting [4].

Chitosan and chitooligosaccharides (COS) are natural biocompatible cationic polysaccharides. COS, or D-glucosamine oligosaccharides, better known as chitosan oligomers, are prepared by hydrolyzing chitosan with either acid or chitosanase. When sticking to the bacterial cell wall [5,6], chitosan can suppress the metabolism of bacteria.

Hyaluronic acid (HA) is a linear polysaccharide composed of repeating disaccharide units of *N*-acetyl-glucosamine and D-glucuronic acid. Being a component of extracellular matrix (ECM), HA has the high capacity of lubrication, water-sorption, and water retention, especially, influence on several cellular functions such as attachment, migration, and proliferation [7,8]. Recent biomedical applications of HA include scaffolds for wound healing and tissue engineering, as well as surgery, arthritis treatment, and as a component of implant materials.

Ozone-induced grafting has been applied to a number of polymers such as silicone, polyurethane, poly(methyl methacrylate), polyethylene, poly(ethylene terephthalate), vinyl alcohol ethylene copolymer, and Teflon [9–11]. In our previous work, PHBV membranes were treated with ozone to graft AA, followed by the esterification of CS or COS [12]. In this work, HA was

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0142-9612/03/$ - see front matter
doi:10.1016/S0142-9612(03)00079-6

covalently bonded to CS- or COS-grafted PHBV membranes with glutaraldehyde (GA). The surface properties of functional group-grafted and HA-immobilized PHBV membranes were characterized using a water contact angle goniometer. The biocompatibility of these modified membranes was characterized including albumin and fibrinogen of adsorption, fibroblast activity and antibacterial activity of four clinically infectious bacteria.

2. Materials and methods

2.1. Materials

PHBV with 5 wt% HV was purchased from Aldrich Chemical Co., Inc. USA. Chitosan (CS) with molecular mass about 160 kDa and a degree of deacetylation of 85.3% was obtained from China Textile Institute, Taipei, Taiwan. COS (molecular mass 1170 Da) was purchased from Shin Era Technology Co., Taiwan. AA and 25% GA were purchased from Ferak Laborat Gmbh, Germany. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Acros Organics, New Jersey, USA. Hyaluronic acid, sodium salt (HA) of 757 kDa, was purchased Calbiochem, USA. Human plasma fibrinogen (HPF) of 341 kDa with clottable proteins more than 95% was purchased from Calbiochem, USA. Human serum albumin (HSA, type V) of 69 kDa was purchased from Sigma, USA.

2.2. Preparation of membrane

The PHBV powder was dissolved at 60°C in chloroform with a concentration of 50 mg/ml. The solution was cast on glass plates and dried in a hood. The thickness of the resulting membranes was about 0.3 mm. Afterwards, these membranes were treated by the Soxhlet extraction with methanol for 24 h to remove residual chloroform.

2.3. Ozone treatment

A piece of membrane of $2 \times 8\,cm^2$ was placed in a 250 ml Erlenmeyer flask and flushed with air containing $10.2\,g/m^3$ of ozone for a 20 min. The ozone was generated using an ozone generator (Kang I Ozone Enterprise Co., Ltd., Taiwan) and the ozone concentration was controlled by an Ozone UV Photometric Analyzer (SOZ-6000, Skei Electronics Co., Ltd., Tokyo, Japan). Afterwards, the sample was evacuated for 2 min to remove unreacted ozone, and then soaked at 65°C in an aqueous solution containing 10% of AA, 0.2 M H_2SO_4 and 1 mM $FeSO_4$. After 60 min, the sample was retrieved and rinsed with adequate double-distilled water for three times, followed by soaking in 150 ml of double-distilled water at 75°C for 24 h. In the first 10 h, the water was replenished every 2 h, and after 10 h, the water was replenished every 4 h. These procedures were carried out to remove unreacted AA and the homopolymer of AA.

2.4. Chitosan and chitooligosaccharides grafting

AA-grafted membranes were cut into pieces of $1 \times 1\,cm^2$, and placed in the reacting solution containing 1 N HCl and 0.25 mg/ml of CS (dissolved in 5 mM acetic acid) or 0.25 mg/ml of COS in pure water at 45°C to proceed the esterification for 5 min. Afterwards, the membrane was rinsed with PBS and double-distilled water three times to remove unreacted CS or COS. The reaction is given as scheme I in Fig. 1.

2.5. Hyaluronic acid immobilization on modified PHBV membranes

AA-grafted membranes were cut into pieces of $1 \times 1\,cm^2$, and placed in the reacting solution containing 0.2 mM EDC at pH 4.8 and 4°C for 24 h, and were washed three times with phosphate buffer solution (PBS) and double-distilled water, then the membrane was dried in an oven at 65°C. These EDC treated membranes were then placed in 0.25 mg/ml HA solution in double-distilled water, to react at 4°C for 24 h. Afterwards, these membranes were treated by the Soxhlet extraction with methanol for 24 h to remove

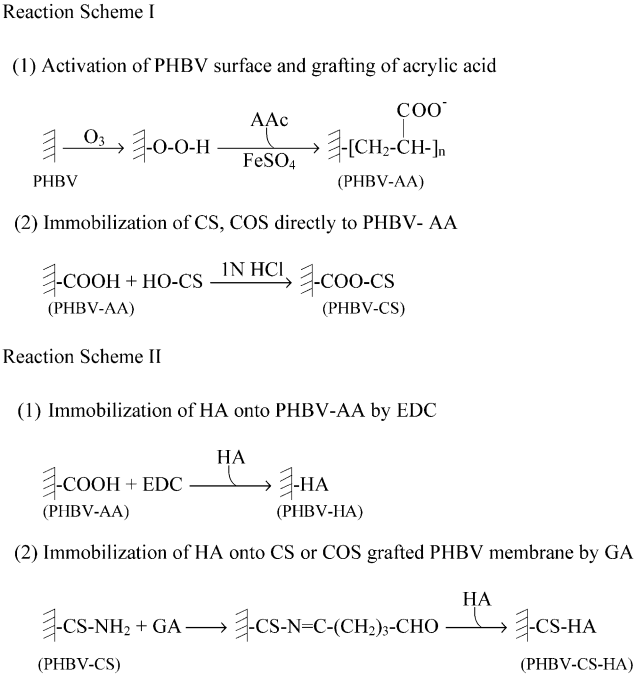

Fig. 1. Reaction schemes for grafting CS or COS with the carboxyl group and the immobilizing of HA onto the membrane surfaces.

residual EDC. The reaction is given as scheme II (1) in Fig. 1. The resulting samples were −40°C freeze-dried using freeze-dryer for 2 h.

CS- and COS-grafted membranes were first treated with 0.2 mM GA at 25°C for 30 min, and were washed three times with PBS and double-distilled water, then the membrane was dried in an oven at 65°C. These GA treated membranes were then placed in 0.25 mg/ml HA solution in double-distilled water, to react at 25°C for 30 min. Afterwards, these membranes were treated by the Soxhlet extraction with methanol for 24 h to remove residual GA and HA. The reaction is given as scheme II (2) in Fig. 1. The resulting samples were −40°C freeze-dried using freeze-dryer for 2 h.

2.6. Determination of surface grafting density

The surface density of carboxyl group for AA or HA was determined by dyeing with 0.01 g/ml of C.I. Basic Blue 17 (Chroma-Gesellschaft GmbH, Müster, Germany) at pH 10 and 30°C for 5 h. After dyeing, the membrane was rinsed with adequate double-distilled water, followed by soaking in 0.1 mM NaOH to remove adsorbed dye molecules. Finally, the associated dye molecules were desorbed in 50%(v/v) acetic acid. The dye concentration was determined at 633 nm using a spectrophotometer (UV 3101 PC, Shimadzu, Tokyo, Japan) and calculated from the calibration curve.

The surface density of amino groups of CS or COS on the membrane surface was determined by dyeing with 0.01 g/ml of C.I. Acid Orange 7 (Tokyo Kaseo Kogyo Co., Ltd., Japan) at pH 3 and 30°C for 5 h, and then rinsed with adequate double-distilled water, followed by 1 mM HCl to remove adsorbed dye molecules, and finally by 1 mM NaOH to desorb associated dye molecules. The dye concentration was determined at 485 nm and calculated from the calibration curve [10,12].

2.7. Measurement of water contact angle

The water contact angles of the surface-modified polyester were measured with a contact angle goniometer (DSA 100, Krüss GmbH, Germany). A piece of $1 \times 1\,cm^2$ membrane was stick on a glass slide and mounted on the goniometer. The drop size was 0.01 ml. After water dropping, the membrane was incubated in 65% RH and 20°C for 10 min before taking the value of contact angle. Each point was averaged from five measurements.

2.8. In vitro antibacterial test

Table 1 lists those four strains of bacteria used in this work. Frozen preserved stock was thawed at room temperature, and then 0.1 ml were pipetted and streaked into quadrant on sheep blood agar plate (Difco Laboratories, USA), and incubated at 37°C overnight. Afterwards, a single colony was scraped with a loop and swabbed to a 15° slant medium (10 ml of nutrient agar) and incubated at 37°C. After incubating for 18–24 h, 20 ml of PBS, which contains 72 ml of 0.2 M Na_2HPO_4, 28 ml of 0.2 M NaH_2PO_4, 0.5 g NaCl and 2 g Tween 80 in water (1 l) was added. After mixing, 1 ml of the solution was moved into 9 ml of nutrient broth (concentration = 8 g/l), and mixed with a vortex mixer. The solution was then diluted with PBS to $1.5 \pm 0.3 \times 10^5$ cells/ml, and placed in flasks (six samples of 0.4 g/sample for each group). After incubating at 37°C for 0–24 h, 20 ml of PBS were added and stirred for 30 s. Consecutive dilutions were prepared by taking 1 ml of the previous solution and mixed with 9 ml of PBS. From this solution, 1 ml was transferred to a 50-ml centrifugal tube, mixed with 15 ml of nutrient agar (at 45°C), poured into a 9-cm plate, and then incubated at 37°C for 0–24 h. The number of survival bacteria was then counted.

Table 1
Clinical source of bacteria used in this study

Bacteria	Source
Gram-positive bacteria	
Methicilin resistant *Staphylococus aureus* (MRSA; *S. aureus*-1)	Acute abscess infection culture
Staphylococus aureus strain-2 (*S. aureus*-2)	Wound infection culture due to suture
Gram-negative bacteria	
Escherichia coli O-157:H7	ATCC 43894
Pseudomonas aeruginosa	ATCC 10145

2.9. Protein adsorption

A piece of membrane of $1 \times 1\,cm^2$ was immersed in 5 ml of 0.5 mg/ml of HSA or HPF, and was shaken at 100 rpm and 37°C for 12 h. The membrane was gently taken out and rinsed five times with PBS. Then the membrane was placed in a glass bottle with 1 wt% aqueous solution of sodium dodecyl sulfate (SDS) and shaken for 60 min at room temperature to remove the protein adsorbed on the surface. A protein analysis kit (MicroBCA protein assay reagent kit, Pierce, Rockford, IL, USA) based on the bicinchoninic acid (BCA) was used to determine the concentration of the proteins in the SDS solution [13].

2.10. Activated partial thromboplastin time (APTT)

The sample membrane ($0.5 \times 2\,cm^2$) was put in a glass tube and 100 μl of actin activated cephaloplastin reagent (Dade Behring Inc., USA) was added, and then the tube

was incubated at 37°C for 1 min. Then 100 μl of PPP solution was added to the sample solution at 37°C, incubated for 3 min, and followed by adding 100 μl of 0.025 M $CaCl_2$ solution. The clotting time of the plasma solution was recorded at the first sign of fibrin formation with a hook [14].

2.11. Cell compatible properties

2.11.1. Cell culture

L929 Fibroblasts (obtained from Food Industry Research and Development Institute, Hsinchu, Taiwan) were grown in a medium supplemented with a minimum essential medium alpha medium (Gibco, Invitrogen Corporation, Newzeeland) with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 90% Earle's balanced salts (Sigma, USA), 10% horse serum in tissue culture flasks in a CO_2 incubator (5% CO_2 95%air) at 37°C. Confluent monolayers were propagated by trypsinization (0.25% trypsin, 0.02% EDTA) and replating at 1:2 dilution. The cells were maintained in a complete medium being replenished every day. After 3-day culture, the culture observation was carried out using an inverted light microscope (Axiovert 25 CFL, Carl Zeiss, Jena, Germemy). At confluency, cells were harvested and subcultivated in the same medium [15].

2.11.2. Cell attachment

Study of cell attachment was on two groups of six 3-cm polystyrene Petri dishes without biomaterial deposit: untreated PHBV, and modified membranes. Each specimen ($1 \times 1\ cm^2$) was thoroughly washed with PBS. Fibroblasts, 17,000 cells/cm^2, were seeded on top of each film, and cultured at 37°C for 3 h. Unattached cells were removed by washing with PBS, and the number of attached cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test [15].

2.11.3. Cell proliferation

Modified membranes were fixed with vacuum grease to the bottom of six 3-cm polystyrene Petri dishes without biomaterial deposit to prevent them from floating in the growth media. Fibroblasts, 350 cells/cm^2, 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 5 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 [15,16] on an inverted light microscope.

3. Results and discussion

3.1. Surfaces modification of membranes

The surface modification was based on the schemes described in Fig. 1. The surface density of grafted functional groups on each specimen in this work was summarized in Table 2. In our previous work [12], we found out that the optimal condition for treating PHBV was 20 min of ozone treatment followed by 1 h grafting at 65°C in 10 wt% AA. This will result in 0.26 nmol/cm^2 of carboxyl groups. After esterification for 5 min in 1 N HCl, the surface density of amino groups was 0.85 and 0.48 nmol/cm^2 for CS and COS, respectively. There are some carboxyl group were detected after grafting CS or COS. This indicates that the esterification did not use up all the carboxyl groups on the surface of PHBV-AA. In the third column of Table 2, the amino groups after immobilization of HA was from CS or COS, and contributed to the antibacterial activity of the modified PHBV. The surfaces of PHBV, PHBV-AA, and PHBV-HA were not grafted with either CS or COS, thus no amino groups were measured and no antibacterial activity was observed.

In Table 2, the surface density of amino group for PHBV-CS was higher than that of PHBV-COS. Although CS was much larger than COS (160 kDa vs. 1.17 kDa), the surface area available for grafting either CS or COS was the same; thus, the amount of amino groups on PHBV-CS was only 180% of that of PHBV-COS, considering the stereo hindrance of CS. The stereo hindrance of CS further reduced the immobilizing amount of HA on PHBV-CS-HA to be 130% of that on PHBV-COS-HA.

3.2. Hydrophilicity of surface-modified PHBV

The first event when biomaterials expose to blood is the adsorption of blood proteins such as globulin and fibrinogen. This may lead to the formation of thrombus. Adsorption of platelet-adhesive protein, such as fibrinogen, leads to the adhesion and activation of platelets. Since the thrombus formation begins with the

Table 2
Surface density of modified PHBV membranes ($n = 5$, ±SD)

Sample	Surface density (nmol/cm^2)	
	Carboxyl group	Amino group
PHBV	NA	NA
PHBV-AA	0.26±0.01	NA
PHBV-CS	0.06±0.01	0.85±0.05
PHBV-COS	0.06±0.01	0.48±0.02
PHBV-HA	0.30±0.02	NA
PHBV-CS-HA	0.46±0.02	0.14±0.01
PHBV-COS-HA	0.36±0.01	0.09±0.01

adsorption of protein and platelets, efforts have been on controlling protein adsorption and platelet adhesion [17]. It is established that hydrophilic surface can reduce the protein adhesion [11,18]. In this work, we employed CS, COS, and HA to improve the hydrophilicity of PHBV.

The effect of surface modification on the hydrophilicity was illustrated by the contact angle shown in Fig. 2. Based on the contact angle results, the order of hydrophilicity was in the following order: PHBV-COS-HA > PHBV-CS-HA > PHBV-HA > PHBV-COS > PHBV-CS > PHBV-AA > PHBV.

The hydrophobic nature of the unmodified PHBV surface showed relatively higher contact angle than other samples. The contact angle of polymer surface can be decreased by introducing hydrophilic groups, such as peroxides, hydroxyl group, carbonyl, and carboxyl group after ozone oxidation [10]. Although the surface density of PHBV-COS was only 56% of that of PHBV-CS, the former had a lower contact angle due to the water-dissolvable nature of COS. The hydrophilicity difference is further enhanced by the immobilization of HA, since HA is a water-soluble molecule. Although the surface density of carboxyl group on PHBV-AA was close to that on PHBV-HA, HA also had hydrophilic hydroxyl group; thus, the latter had a smaller contact angle.

3.3. Antibacterial activity

When using chitosan and its derivatives as biomaterials, it is important to prevent infection. The cationic amino group of CS and COS can associate with anions on the bacteria wall, suppress its biosynthesis, disrupt the mass transport across the wall, and accelerate the death of the bacteria [19].

The antibacterial activity was evaluated with those four clinical infectious bacteria listed in Table 1. Fig. 3 shows the effect of grafting on the growth curves of MRSA. When contacting with untreated PHBV, the bacteria grew from 1.5×10^5 to 3.2×10^7 CFU/ml after incubating at 37°C for 24 h. On the other hand, when contacting both PHBV-CS and PHBV-COS, the concentration of bacteria reduced rapidly and died out after incubating at 37°C for 24 h. Because PHBV-CS had more amino group (0.85 nmol/cm^2) than PHBV-COS (0.48 nmol/cm^2), the rate of reduction for PHBV-CS was faster than that of PHBV-COS. After HA immobilization, the reduction in the number of MRSA began to occur at 8 h, which was less than the 4 h of PHBV-CS and PHBV-COS. However, after 24 h of incubation, all the bacteria died out for all four samples (PHBV-CS, PHBV-COS, PHBV-CS-HA, PHBV-COS-HA). Similar results were observed for *Staphylococus aureus*-2, *Pseudomonas aeruginosa*, and *Escherichia coli* O-157:H7. This phenomenon is also observed for CS- and COS-grafted polyhydroxyalkanoates in our previous work [12].

Fig. 4 compares the antibacterial activity of these modified PHBV membranes based upon the survival ratio after incubating at 37°C for 12 h. Without grafting either CS or COS, bacteria would grow at least 140%. On the other hand, those membranes grafted with COS would reduce the bacterial survival ratio to less than 1%. The effect of CS grafting was even more pronounced. Therefore, we can conclude that membranes grafting with COS or CS can suppress the growth of all four bacteria used in this study. Our previous studies showed that the antibacterial activity of CS (or COS) is lower for *S. aureus* than for *E. coli* and

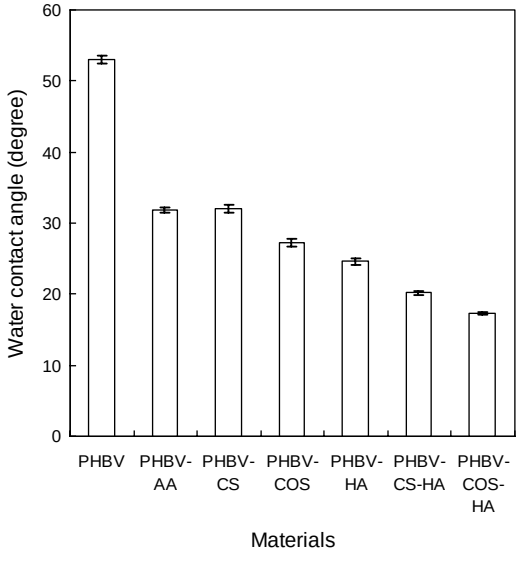

Fig. 2. Contact angle of native and modified PHBV membranes ($n = 5$, $\pm$SD).

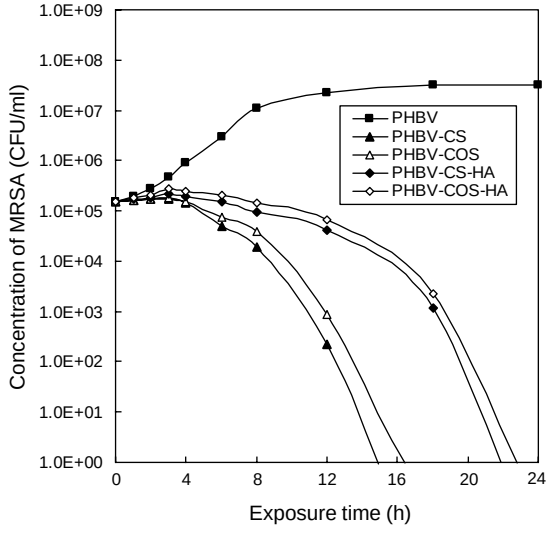

Fig. 3. Change in the viable cell number of MRSA with the time exposing to the modified membranes.

P. aeruginosa. Fig. 4 shows that the order of antibacterial activity of CS (or COS) is *E. coli* > *P aeruginosa* > *S. aureus-2* > MRSA. Although the antibacterial activity did drop after grafting with HA, the bacterial survival ratios of those two HA-grafting samples were still less than 1%.

The extracellular capsule of MRSA makes it more hydrophobic than capsule-less *S. aureus*-2. Bacterium with extracellular capsule carries less negative charges, and is less prone to be adsorbed on the positive-charged membrane surface. This makes MRSA less interactive with CS- (or COS-) grafted PHBV than *S. aureus-2*. For Gram-negative *E. coli* and *P. aeruginosa*, they have flagella on the structures external to the cell wall and thus have higher mobility. *P. aeruginosa* has less flagella than *E. coli*, thus is less mobile than *E. coli*. Furthermore, *E. coli* has fimbriae, which make the bacterium more adsorbable. Therefore, the grafting of CS and COS is more antibacterial to *E. coli* than other bacteria tested [12].

3.4. Protein adsorption

The effect of HA immobilization on the adsorption of proteins is shown in Fig. 5. From the results in Fig. 5, the order of the protein adsorption amount is PHBV-CS > PHBV-COS > PHBV > PHBV-AA > PHBV-CS-HV > PHBV-COS-HA > PHBV-HA. After grafting with AA, the adsorption amount was reduced, while the grafting of CS or COS, the adsorption amount was higher than the unmodified PHBV. On the other hand, after immobilized with HA, the adsorption amount was greatly reduced to about 5–6% of that of the precursor.

The isoelectric points of albumin and fibrinogen are 4.8 and 5.5, respectively [20,21]; thus, these proteins

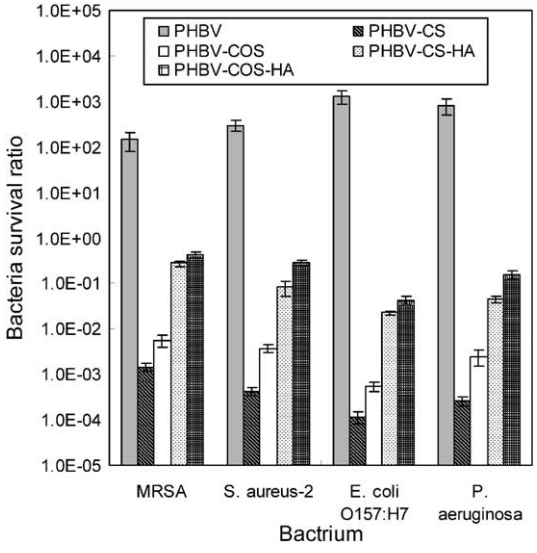

Fig. 4. Comparison of antibacterial activity of membranes for four pathogenic bacteria after 12 h of incubation ($n = 6$, $\pm$SD).

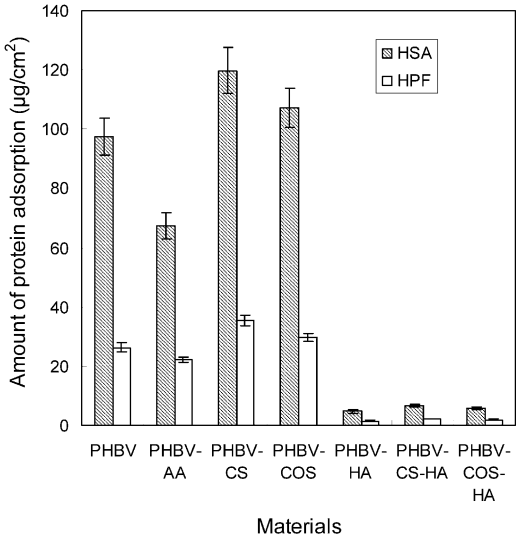

Fig. 5. HPF and HSA adsorbed to membrane surfaces ($n = 4$, $\pm$SD).

carries negative charge in the normal blood circumstance (pH 7.4). Both PHBV-CS and PHBV-COS were positively charged; thus, negatively charged HSA and HPF were attracted. On the contrary, PHBV-AA, PHBV-HA, PHBV-CS-HA, and PHBV-COS-HA were negatively charged; thus, negatively charged HSA and HPF were expelled.

In addition to electrostatic interaction, stereo hindrance also plays an important role in preventing protein adsorption. Although the surface densities of carboxyl group on those four negatively charged samples were close, those three HA-immobilizing samples adsorbed much less protein than PHBV-AA. In this work, the molecular masses of HA, HSA, HPF were 757, 69, and 341 kDa, respectively. Albumin has a heart-like shape with edges of $8.2 \times 7.0 \times 8.3$ nm and a thickness of 3.0 nm. Fibrinogen has a cylindrical shape with a diameter of 6.0 nm and a length of 45 nm [22]. Because HSA is much smaller than HPF, it can penetrate more easily into the HA layer; thus, the adsorbed amount of HSA was higher (about three times in terms of μg or 15 times in terms of mole) than that of HPF.

3.5. APTT

The blood compatibility of these samples is represented by APTT, and is summarized in Table 3. The APTT of PHBV-CS and PHBV-COS were 34 and 36 s, respectively. Both were shorter than that of the control, although not very significant. After HA immobilization, the APTT was extended to 42 and 41 s, respectively. These results indicate that HA does not improve much the blood compatibility of the substrate material. Magnani et al. [23] studied the anticoagulation of

Table 3
Blood compatibility evaluated by APTT ($n = 6$, $\pm$SD)

Membranes	APTT (s)
Negative plasma	38±3
PHBV	39±3
PHBV-AA	42±3
PHBV-CS	34±3
PHBV-COS	36±4
PHBV-HA	44±4
PHBV-CS-HA	42±4
PHBV-COS-HA	41±3

differently sulfated HA and reported unsulfated HA does not anticoagulate. This agrees with our results.

3.6. Cell attachment and cell proliferation

In general, hydrophobic polymers have been known as unfavorable for cell attachment unless modified to posses a hydrophilic surface with a higher surface energy and a correspondingly lower air–water contact angle [24]. The effect of surface modification on the cell attachment is shown in Fig. 6. The order of attaching amount is PHBV-CS > PHBV-COS > PHBV-CS-HA > PHBV-COS-HA > PHBV-HA > PHBV-AA > PHBV. The result suggests that positively charged CS and COS could improve the attachment of fibroblast. Chatelet et al. [25] reported that the attachment of fibroblast increases with the decrease of the degree of acetylation of CS. The degree of acetylation is similar to the amino group used up for immobilization of HA in our work. Thus, their result is similar to our finding that higher surface density of amino group leads to more cell attachment. Immobilization of HA would result in less attachment, although still higher than the control. This is probably due to the negative charge and the stereo hindrance of HA molecule. Being hydrophobic, the fibroblast attachment for PHBV was the least among all those samples.

The effect of surface modification on the proliferation of fibroblast is shown in Fig. 7. The order of cell proliferation is PHBV-HA > PHBV-COS-HA > PHBV-CS-HA > PHBV > PHBV-COS > PHBV-CS > PHBV-AA. Chen et al. reported that HA shows growth-promotion effect [26]. This agrees with our finding that HA immobilization did improve the proliferation of fibroblast. On the other hand, CS or COS grafting can reduce the number of cells. Chatelet et al. [25] suggested that chitosan seems to be cytostatic toward fibroblast: it is not cytotoxic, but inhibits cell proliferation. This is in agreement with our results of CS- or COS-grafting membranes.

Despite the difference in the molecular structures of CS and HA, we think the effect of the surface charge may play an important role in the cell attachment and

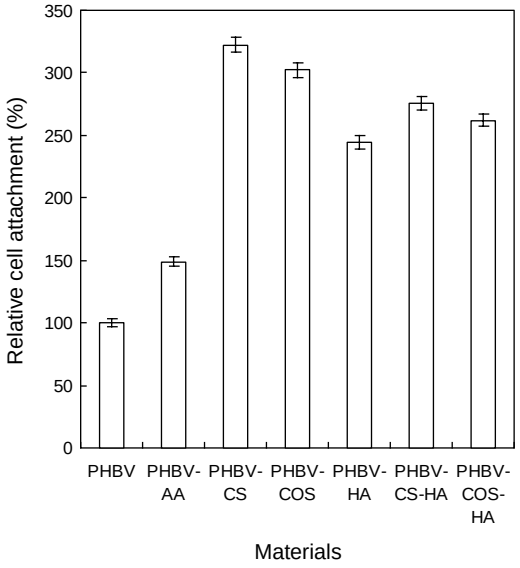

Fig. 6. Cell attachment to membrane surfaces. The initial concentration of fibroblast was 17,000 cells/cm^2, and incubated at 37°C for 3 h ($n = 6$, $\pm$SD).

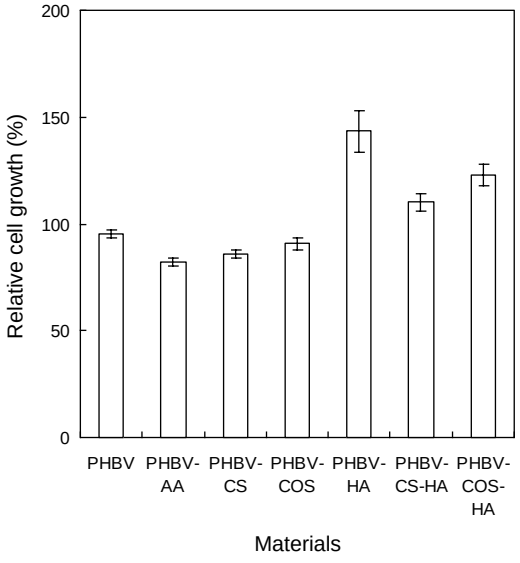

Fig. 7. Relative cell growth ratio of membranes. The initial concentration of fibroblast was 350 cells/cm^2, and incubated at 37°C for 5 days ($n = 6$, $\pm$SD).

cell proliferation of fibroblast. Fig. 8 shows that the cell attachment and cell proliferation depend on the surface density of amino group. The cell attachment increased with the surface density of amino group, while the cell proliferation decreased with the increase of the surface density of amino group. Both CS and HA are polysaccharides. The difference is that CS has one amino group for each saccharide unit, whereas HA has one carboxyl group and one amide group for each disaccharide unit. Among these samples, PHBV-HA has the highest cell proliferation and the lowest cell attachment, where as PHBV-CS has the lowest

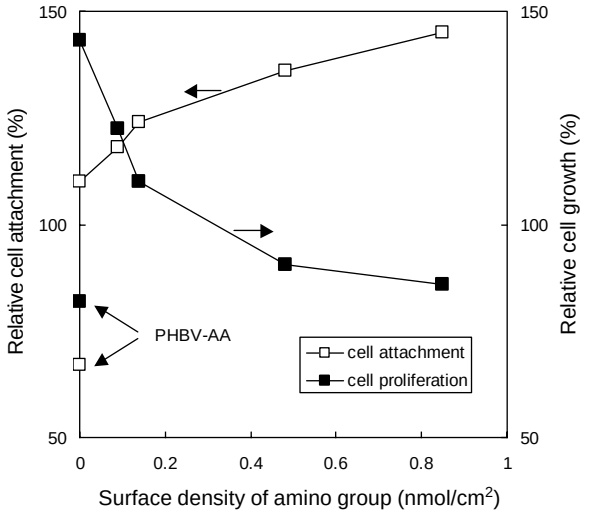

Fig. 8. Effect of surface density of amino group on the cell attachment and cell proliferation of fibroblast.

cell proliferation (in fact, reduction) and the highest cell attachment. For comparison, PHBV-AA, which bears only carboxyl groups on the surface, has even lower cell attachment and cell proliferation. It appears that other functional groups, for example, hydroxyl group in CS and HA, may also affect cell attachment and cell proliferation.

4. Conclusion

The hydrophilicity of PHBV films can be improved by grafting CS or COS. Due to the introduction of amino group, the grafting of CS or COS can also endow PHBV with antibacterial activity against four pathogenic bacteria. On the other hand, being positively charged, CS or COS grafting shows higher adsorption for HSA (120 and 107 $\mu g/cm^2$, respectively) and HPF (35 and 30 $\mu g/cm^2$, respectively). By the immobilization of HA, the surface becomes negative charged, and reduces the protein adsorption to about 5% of that of un-HA-immobilizing samples. However, HA immobilization shows no obvious effect on the thrombus formation.

Grafting CS or COS onto PHBV shows improvement in fibroblasts attachment, yet reduces the cell proliferation. Immobilization of HA without CS can promote the proliferation of fibroblast to 143%. Grafting only CS without HA can improve the attachment of fibroblast to 145%.

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