# Antibacterial and Biodegradable Properties of Polyhydroxyalkanoates Grafted With Chitosan and Chitooligosaccharides via Ozone Treatment

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Received 8 April 2002; accepted 12 August 2002

**ABSTRACT:** Acrylic acid was grafted to ozone-treated poly(3-hydroxybutyric acid) (PHB) and poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) (PHBV) membranes. The resulting membranes were further grafted with chitosan (CS) or chitooligosaccharide (COS) via esterification. These CS- or COS-grafted membranes showed antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, methicilin-resistant *Staphylococcus aureus* (MRSA), and *S. aureus*. The antibacterial activity to *E. coli* was the highest, whereas the antibacterial activity to MRSA was the lowest among these four bacteria tested. Acrylic acid grafting can increase the biodegradability with *Alcaligens faecalis*, whereas CS and COS grafting can reduce the biodegradability. In addition, CS-grafted PHBV membrane showed higher antibacterial activity and lower biodegradability than COS-grafted PHBV membrane. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 88: 2797–2803, 2003

**Key words:** biopolymers; modification; chitosan; antibacterial activity; biodegradability

## INTRODUCTION

Poly(3-hydroxybutyric acid) (PHB) and poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) (PHBV) are natural polyesters polymerized by bacteria. Both polyesters are biodegradable and biocompatible. They are often used as recyclable packing materials, kitchen films, diapers, and sanitary napkins. They can also be used for biomedical applications because of their biocompatibility and nontoxicity to living tissues. Their degradation product, *R*-$\beta$-hydroxybutyric acid, is a normal metabolite found in human blood. The biomedical applications mentioned in the literature include surgical suture, surgical swabs, wound dressings, vascular graft, blood vessel, scaffold for new tissue in growth, body parts, and syringe.[1]

The modification of PHB and PHBV can be achieved by plasma,[2] $\gamma$-ray radiation grafting of acrylic acid,[3] oxidizing chemical grafting,[4] as well as blending with poly(L-lactide), polycaprolactone, poly(ethylene adipate), chitin, and chitosan.[5–8]

The biodegradability of PHB and PHBV has been studied with bacteria, enzyme, buffer, and acid-hydrolysis.[9–12] They have been made into rods loaded with antibiotics and implanted into animals as a sustained drug-release system against infection of *Staphylococcus aureus.*[13]

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Journal of Applied Polymer Science, Vol. 88, 2797–2803 (2003)


In general, the content of hydroxyvalerate (HV) in PHBV is about 0–25%. The content of HV can affect the rate of biodegradation. The addition of HV can improve the flexibility and impact strength of the material, especially for making chiral building blocks.[1]

Infection often occurs with the implant of biomaterials and can cause death. Methicilin-resistant *S. aureus* (MRSA) of acute abscess infection and *Pseudomonas aeruginosa* are common infectious bacteria found in hospitals. *Escherichia coli* O157:H7 is a pathogenic infectious bacterium found in recent years. The symptoms range from mild to serious hemorrhage and sometimes may result in hemolytic uremic syndrome and thrombotic thrombocytopenic purpura and other complexes.[14]

The surface modification of polymer becomes important when polymeric material is in contact with a physiological component such as blood and living tissues. Chitosan (CS) and chitooligosaccharides (COS) are natural biocompatible cationic polysaccharides. When sticking to the bacterial cell wall,[15–16] chitosan can suppress the metabolism of bacteria. The antibacterial study of poly(ethylene terephthalate) (PET) grafted with chitosan and its derivatives has been performed on *S. aureus* and *E. coli.*[17]

Although ozone-induced grafting has been applied to a number of polymers such as silicone, polyurethane, poly(methyl methacrylate), polyethylene, PET, vinyl alcohol ethylene copolymer, and Teflon,[18] this technique has not yet been applied to PHB and PHBV. In this work, PHB and PHBV membranes were treated

with ozone to graft acrylic acid, followed by the esterification of CH or COS. The antibacterial activity of these modified membranes was demonstrated with four clinically infectious bacteria. The effect of grafting on the rate of biodegradation was also studied.

## EXPERIMENTAL

### Materials

PHB and PHBV with 5 wt % HV content were both purchased from Aldrich (St. Louis, MO). CS with an $M_w$ of about $1.6 \times 10^5$ and a degree of deacetylation of about 75% was obtained from China Textile Institute (Taipei, Taiwan). COS ($M_w$ = 1170) was purchased from Shin Era Technology Co. (Taipei, Taiwan). Acrylic acid (AA) was purchased from Ferak Laborat GmbH (Berlin, Germany).

### Preparation of membrane

The PHB powder was dissolved at 25°C in chloroform with a concentration of 160 mg/ml. The PHBV powder was dissolved at 60°C in chloroform with a concentration of 50 mg/ml. The solutions were cast on glass plates and dried in a hood. The thickness of the resulting membranes was about 0.3 mm. Afterward, these membranes were treated by the Soxhlet extraction method with methanol for 24 h to remove residual chloroform.

### Ozone treatment

A piece of membrane with dimensions 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 specified time. The ozone was generated using an ozone generator (Kang I Ozone Enterprise Co., Ltd., Kaohsiung, Taiwan) and the ozone concentration was controlled by an Ozone UV Photometric Analyzer (SOZ-6000, SKEI Electronics Co., Ltd., Osaka, Japan). Afterward, the sample was evacuated for 2 min to remove unreacted ozone and then soaked at 65°C in an aqueous solution containing either 5 or 10% AA, 0.2*M* $H_2SO_4$, and 1 mM $FeSO_4$. After a specified time, the sample was retrieved and rinsed with adequate double-distilled water 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; after 10 h, the water was replenished every 4 h. These procedures were carried out to remove unreacted AA and the homopolymer of AA.

### CS and COS grafting

AA-grafted membranes were cut into pieces of $1 \times 1$ cm$^2$ and placed in the reaction 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 continue the esterification for 1–20 min. Afterward, the membrane was rinsed with PBS and double-distilled water three times to remove unreacted CS or COS. The reaction schemes for grafting CS or COS are given in Figure 1.

Reaction Schemes

$$R \xrightarrow{O_3} R\text{-OOH} \xrightarrow{AA} R\text{-COOH}$$

$$R\text{-COOH} + \text{HO-Chitosan} \xrightarrow{1N\ HCl} R\text{-COO-Chitosan}$$

or

$$R\text{-COOH} + \text{HO-Chitooligosaccharide} \xrightarrow{1N\ HCl} R\text{-COO-Chitooligosaccharide}$$

where R = PHB or PHBV

**Figure 1** The reaction schemes for grafting CS or COS with the carboxyl group introduced onto the membrane surfaces by ozone treatment.

### Determination of surface peroxide

A piece of ozone-treated membrane measuring $1 \times 1$ cm$^2$ was placed in 20 mL of the benzene containing 25 $\mu$M of 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma, St. Louis, MO) and kept at 70°C for 24 h to react with free radicals on the surface. The absorbance of DPPH at 520 nm was measured before and after the reaction. The surface density of the peroxide can then be calculated from the absorbance.

### Determination of surface grafting density

The surface density of the carboxyl group 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 by using a spectrophotometer (UV 3101 PC, Shimadzu, Tokyo, Japan) and calculated from the calibration curve.

After the esterification, the surface density of the amino groups on the membrane surface was determined by dyeing with 0.01 g/mL of C.I. Acid Orange 7 (Tokyo Kaseo Kogyo Co., Ltd., Tokyo, 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.[19]

**TABLE I**
**Clinical Source of Bacteria Used in This Study**

| Bacteria | Source |
|---|---|
| Gram-positive bacteria | |
| Methicilin-resistant *Staphyloccocus 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 |

### 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, Hamburg, Germany). A piece of $1 \times 1$ cm$^2$ membrane was placed 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% relative humidity (RH) and 20°C for 10 min before taking the value of the contact angle. Each point was averaged from five measurements.

### Biodegradability

Three pieces of membranes, $1 \times 1$ cm$^2$ in size, were dried at 65°C for 48 h and weighed at 20 ± 1°C under 65 ± 3% RH using a balance (AE240, Mettler, Switzerland). After weighing, the membranes were placed in 5 mL of the medium containing $1 \times 10^5$ cells/mL of *Alcaligens faecalis* (ATCC 25094) and incubated at 37°C for 7 days. The membrane was flushed with sterile phosphate-buffered saline (PBS) six times to remove attaching bacteria and placed into a fresh bacterial culture. The concentration of the bacteria would grow to $1.43 \times 10^8$ cells/mL. The medium was replaced every 7 days. After a specified time, the membrane was flushed with water filtered with 45 $\mu$m cellulose triacetate filter (Type SC, Millipore, Billerica, MA). The membrane was then dried at 65°C for 48 h and weighed at 20°C under 65% RH. The weight loss of the membrane can then be calculated as

$$\text{Weight loss } (\%) = (W_0 - W)/W_0 \times 100\%$$

where $W_0$ is the original weight of the membrane, and $W$ is the weight after culturing with bacteria.

### Antibacterial activity

Table I lists the four strains of bacteria used in this work. Frozen preserved stock was thawed at room temperature and then 0.1 mL was pipetted and streaked into quadrant on sheep blood agar plate (Difco Laboratories, USA) and cultured at 37°C overnight. Afterward, 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 culturing for 18–24 h, 20 mL PBS, which contains 72 mL 0.2*M* $Na_2HPO_4$, 28 mL 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 $1.5 \pm 0.3 \times 10^5$ cell/mL and placed in flasks (six samples of 0.4 g/sample for each group). After incubating at 37°C for 0–18 h, 20 mL of PBS was added and stirred for 30 s. Consecutive dilute solutions were prepared by taking 1 mL of the previous solution and mixing with 9 mL PBS. From this solution, 1 mL was transferred to a 50-mL centrifugal tube, mixed with 15 mL of NA (at 45°C), poured into a 9-cm plate, cooled down, and incubated at 37°C for 18–24 h. The number of survival bacteria was then counted.

## RESULTS AND DISCUSSION

### Surface modification

Figure 2 shows that the surface density of peroxide on PHB and PHBV varies with the ozone treating time. Highest surface density of peroxide was attained after 20 min of ozone treatment, and the peroxide density of PHBV (7.2 ± 0.5 nmol/cm$^2$) was higher than that of

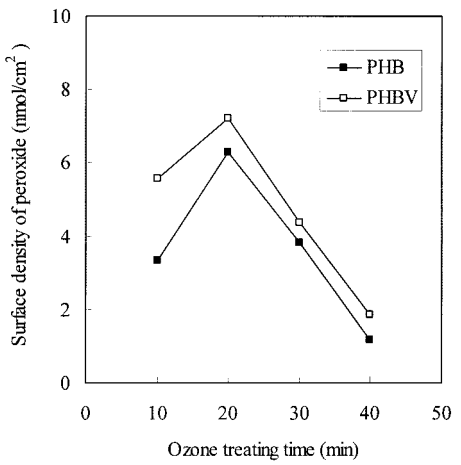


**Figure 2** Dependence of surface peroxide of the PHB and PHBV membranes on the ozone treating time.

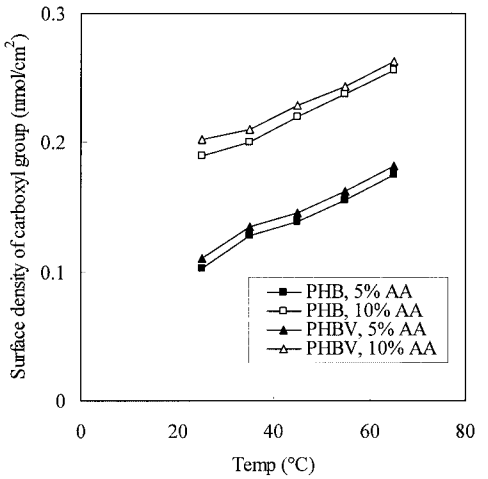


**Figure 3** Effect of the reacting temperature on the surface density of carboxyl group on PHB and PHBV membrane after reacting for 30 min.

PHB (6.9 ± 0.6 nmol/cm$^2$). Longer treatment would result in less peroxide on the surface. This is probably due to the degradation on the surface caused by ozone treatment. Therefore, extending the treating time cannot increase the surface density of the peroxide. The lattice of PHBV is larger than that of PHB due to the hydroxyvaleric acid segment. This makes ozone easier to attack the PHBV surface. Thus, the surface density of peroxide was higher than that of PHB. There is no ozone treatment of PHB or PHBV in the literature. However, for comparison, ozone treatment of PET membrane can produce peroxide at a concentration of 0.7 nmol/cm$^2$.[18]

Figure 3 shows the dependence of the surface density of carboxyl group on the PHB and PHBV membranes on the temperature after 30 min of grafting. The amount of AA grafted increased almost linearly with the temperature. The surface density of the carboxyl group on PHB was slightly less than that on PHBV by about 4%. In addition, the surface density of the carboxyl group also increased with the concentration of AA. At 25°C, the surface density grafted with 10% AA was higher than the surface density grafted with 5%.

Figure 4 shows the variation in the surface density of the amino group with respect to the esterification time. The surface density of amino group reached maximum at 5 min and then decreased with time. This is probably due to the acid hydrolysis of CS or COS because the reaction was carried out in 1*N* HCl. The maximum surface density of amino groups were as follows: for PHB-CS, 0.58 nmol/cm$^2$; for PHB-COS, 0.13 nmol/cm$^2$; for PHBV-CS, 0.85 nmol/cm$^2$; and for PHBV-COS, 0.48 nmol/cm$^2$. Both CS and COS were bonded to the same surface density of carboxylic group, but CS has higher molecular weight than that of COS; thus, CS can provide more unreacted amino groups than COS. In addition, PHBV-A had more carboxyl groups than PHB-A; thus, PHBV-CS and PHBV-COS has higher surface density of amino group than PHB-CS and PHB-COS.

Figure 5 shows the SEM micrographs (JEOL 5400, Japan) of untreated and modified PHB and PHBV. After grafting, the surface became smoother, especially when grafting with COS. The improvement in the hydrophilicity by the modification can be observed from the reduction in the water contact angles listed in Table II.

## Biodegradability

The degradation of PHB and PHBV membranes by *A. faecalis* after 28 days is shown in Figure 6. The weight loss of PHBV was only 44%, less than those of all other samples. The highest weight loss, 84%, was attained by PHB-AA. This can be explained in two aspects: the accessibility of bacterial attack and the number of bacteria. After grafting with AA, the surface was more hydrophilic, which made it more accessible for the bacterium to attack. In all the cases of PHB, PHBV, PHB-AA, and PHBV-AA, the bacteria grew in 7 days from $10^5$ to $1.43 \times 10^8$ colony forming unit (CFU)/mL. After grafting with CS and COS, although the surface was more hydrophilic than PHB-AA or PHBV-AA, the growth rate of bacteria was reduced owing to their antibacterial activities. In the 7-day cycle, the bacteria grew from $10^5$ to $4.4 \times 10^7$ CFU/mL, less than one-third of the other samples. This will surely reduce the rate of degradation. In addition, the micrographs shown in Figure 5 indicate that PHB-COS and PHBV-COS had smoother surfaces than other samples. This made them less accessible for the bacteria to attack.

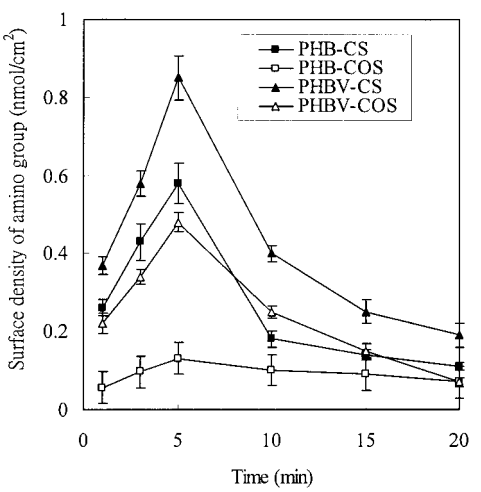


**Figure 4** Variation of the surface density of amino group on PHB and PHBV membrane with respect to the reaction time at 45°C.

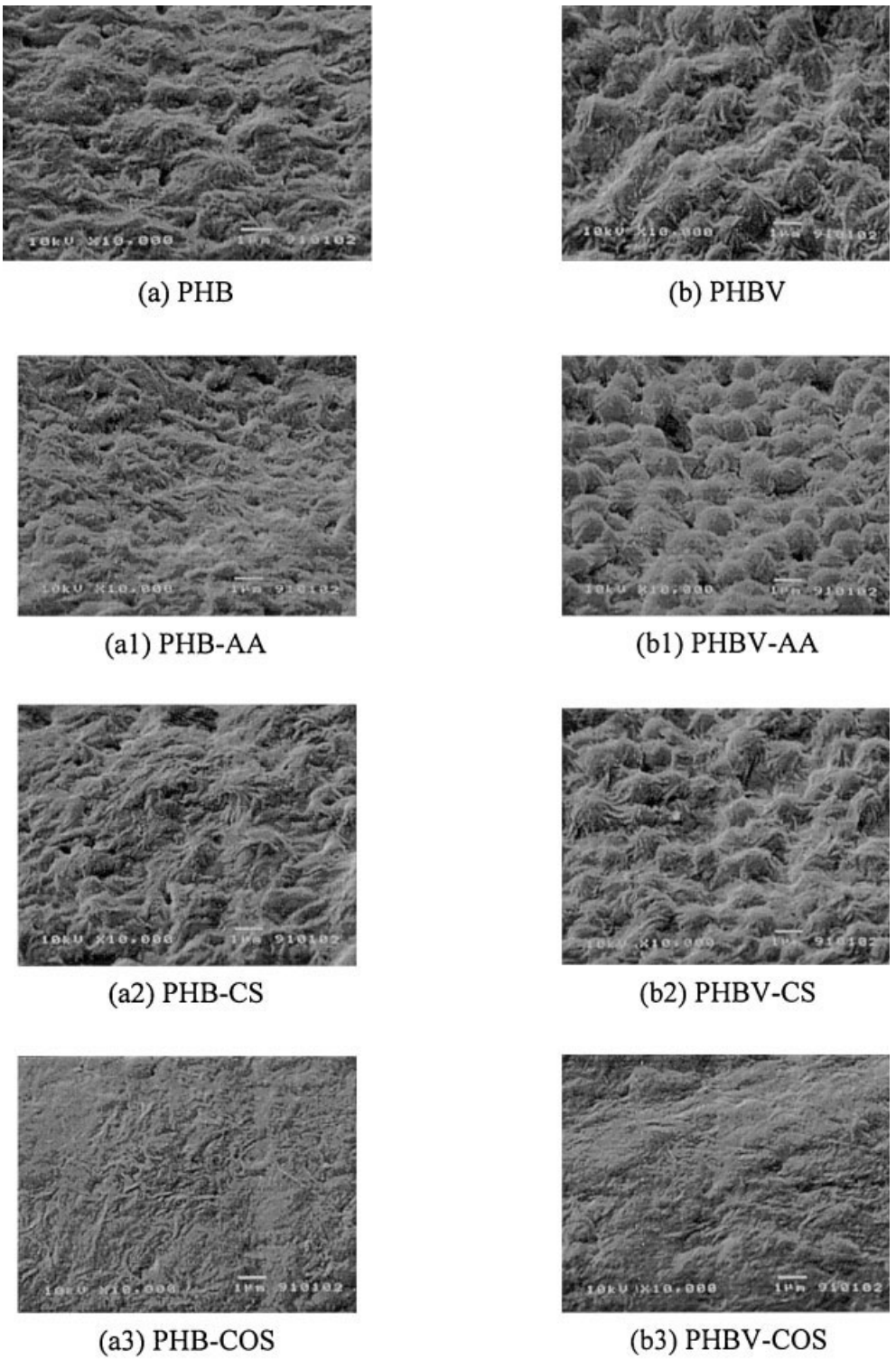


**Figure 5** The surface morphology of untreated, AA-grafted, CS-grafted, and COS-grafted PHB, PHBV film (SEM). (a) PHB, (a1) PHB-AA, (a2) PHB-CS, (a3) PHB-COS; (b) PHBV, (b1) PHBV-AA, (b2) PHBV-CS, (b3) PHBV-COS.

### Antibacterial activity

To study the antibacterial activity, four clinical infectious bacteria were employed, as listed in Table I. Because chitosan and its derivatives have been used for wound dressing, it is important to be able 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. The target site of the cationic biocide is the cytoplasmic membranes of bacteria, and the following elementary processes have been proposed for their

**TABLE II**
**Water Contact Angles of PHB and PHBV Membrane**

| Sample | $\theta_{water}$ (°) |
|---|---|
| PHB | 44.5 ± 0.3 |
| PHBV | 53.0 ± 0.6 |
| PHB-AA | 31.4 ± 0.4 |
| PHBV-AA | 31.9 ± 0.4 |
| PHB-CS | 27.1 ± 0.4 |
| PHBV-CS | 27.2 ± 0.6 |
| PHB-COS | 30.2 ± 0.4 |
| PHBV-COS | 32.0 ± 0.5 |

mode of action: (1) adsorption onto the bacterial cell surface; (2) diffusion through the cell wall; (3) binding to the cytoplasmic membrane; (4) disruption of the cytoplasmic membrane; (5) release of the cytoplasmic constituents, such as $K^+$ ions, DNA, and RNA; and(6) death of the cell.[20]

Figure 7 shows the effect of grafting on the growth curves of MRSA. When contacting with untreated PHBV, the bacteria grew from $1.5 \times 10^5$ to $3.2 \times 10^7$ CFU/mL after incubating at 37°C for 18 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 18 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. Similar results were observed for *S. aureus*-2, *P. aeruginosa*, and *E. coli* O-157:H7. This phenomenon is also observed for CS- and COS-grafted PET fiber in our previous work.[21]

Figure 8 compares the antibacterial activity of these CS- or COS-grafted PHBV membranes on the basis of 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.

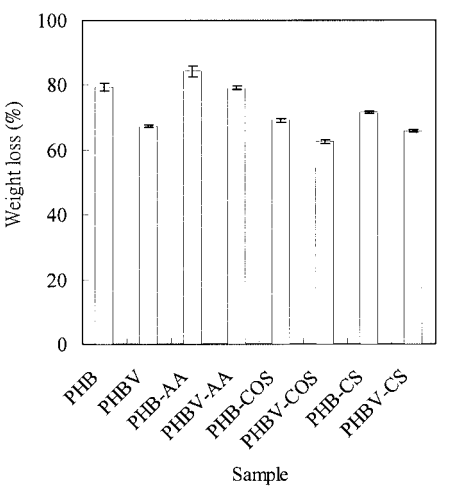


**Figure 6** Weight loss value of PHB and PHBV samples with grafted AA, CS, COS obtained by *Alcaligens faecalis* degradation test.

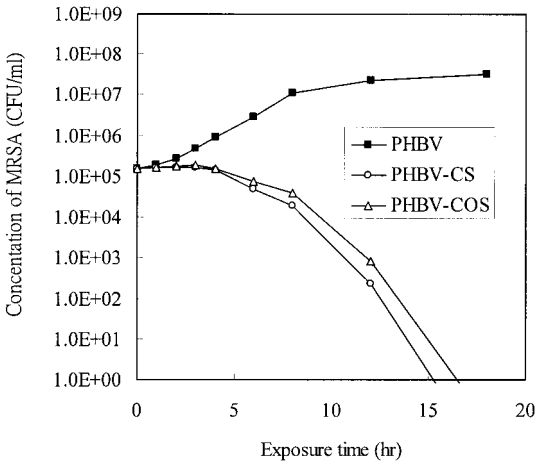


**Figure 7** Change in log plot of viable cell number of MRSA with the exposure time for the CS- and COS-grafted PHB and PHBV membrane surfaces.

Previous studies[16] showed that the antibacterial activity of CS (or COS) is lower for *S. aureus* than for *E. coli* and *P. aeruginosa*. Figure 8 shows that the order of antibacterial activity of CS (or COS) is *E. coli* > *P aeruginosa* > *S. aureus*-2 > MRSA. The extracellular capsule of MRSA makes it more hydrophobic than capsuleless *S. aureus*-2. Bacterium with an extracellular capsule carries less negative charges and is less prone to be adsorbed on the positive-charged membrane surface.[22] This makes MRSA less interactive with CS- (or COS-) grafted PHBV than *S. aureus*-2. For gram-negative *E. coli* and *P. aerugi-*

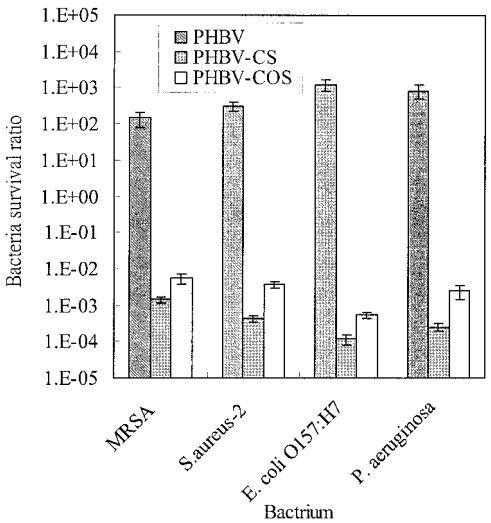


**Figure 8** Comparison of antibacterial activity of CS- and COS-grafted PHBV membranes for four pathogenic bacteria.

*nosa*, 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 absorbable. Therefore, the grafting of CS and COS is more antibacterial to *E. coli* than other bacteria tested.

## CONCLUSION

Grafting of carboxyl groups to PHB and PHBV was achieved by ozone treatment. However, longer treatment time caused the degradation of the membrane surface and reduced the surface density of peroxide. The grafting amount of AA increased with an increase in the temperature. The maximal amount of CS and COS grafted was achieved at 5 min in 1*N* HCl at 45°C. Longer reaction time caused the degradation of CS and COS, thus reducing the surface density of the amino group. Because of the antibacterial nature of CS and COS, the biodegradability with *A. faecalis* was reduced compared with AA-grafted membranes. Both PHBV-CS and PHBV-COS showed an antibacterial activity against four clinically infectious bacteria. Because of the higher surface density of the amino group, PHBV-CS showed higher antibacterial activity than PHBV-COS.

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