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Antibacterial Effect of the Surface-Modified Biomedical Polyurethane against *Staphylococcus aureus* and *Staphylococcus epidermidis*

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Abstract Staphylococcal infection still remains to be one of the most serious infections, having various complications in the clinical use of indwelling polymeric medical devices. However, there are a few promising systems showing a high antibacterial effect without causing any damage of polymer backbone under biological environments such as blood or body fluid. In order to resolve this problem, we have designed a new antibiotic releasing system via a hydrolysis mechanism. The surface of biomedical polyurethane (PU) was modified by using 1,6-diisocyanatohexane (HMDI) to immobilize the rifampicin. Also, the immobilized rifampicin was designed to be released by a selective cleavage of the unstable carbamate linkage that exists on the rifampicin-immobilized polyurethane (PHR). The immobilization of rifampicin on the surface of polyurethane was confirmed by the disappearance of the characteristic IR absorbance peak of the isocyanate (-NCO) group at 2,267 cm^{-1}. The PHR showed a continuous rifampicin release profile under an aqueous environment of 10 mM of PBS (phosphate-buffered saline) for over 6 days. The rifampicin molecules, which are released from PHR under an optimal bacterial infection environment, had a higher antibacterial activity against both *S. aureus* and *S. epidermidis* than rifampicin-incorporated polyurethane (RIP). In addition, the PHR maintained a stable antibacterial effect under a blood-mimic aqueous environment such as bovine calf serum.

Key words: Biomedical polyurethane, staphylococci, surface modification, rifampicin, carbamate linkage

A large number of synthetic polymers have been used for biomedical applications. Widely used synthetic polymers include medical-grade silicone rubber, plasticized polyvinyl chloride, polyethylene, polytetrafluoroethylene, and polyurethane. In particular, polyurethane (PU) has been used in many blood-contacting devices including vascular prosthesis, blood filters, catheters, heart valves, ventricular assist devices, and total artificial hearts due to their excellent mechanical properties and relatively better blood compatibility than others [19, 20, 22]. However, many blood-contacting synthetic polymers including PU have a risk of defecting biomaterial-associated infection and other foreign body reactions, in spite of the fact that they have good blood compatibility.

Thrombosis and bacterial infection have been widely known as two major complications in indwelling blood-contacting medical devices [3]. Furthermore, they induce an increased mortality, patient discomfort, prolonged hospitalization, and increased medical costs. In particular, staphylococcal infections on indwelling medical devices have been considered to be a serious problem, since the eradication of the microorganisms (e.g. *Staphylococcus aureus* and *Staphylococcus epidermidis*) is hardly possible without removal of the infected device from the patient [1, 29]. *Staphylococcus aureus* has been known as a major pathogen that is involved in the pathogenesis of natural endovascular infection as well as prosthetic implant endocarditis [5, 6, 7, 16]. *S. epidermidis* has also been considered as an etiological agent in an indwelling medical device infection [26]. It has been reported that adhesion, proliferation, and biofilm formation of staphylococci onto implants are associated with severe morbidity, amputation, or death, since most of staphylococci are highly resistant to antibiotics [7]. The difficulties in medical treatment of the infected implants are increased by antibiotic-resistant staphylococcal strains such as methicillin-resistant staphylococci. Therefore, a large number of β-lactam antibiotics (e.g. cephamandole, carbapenems) and antistaphylococcal substances (ASS) against these methicillin-resistant staphylococci have recently been isolated and synthesized [11]. However, rifampicin is still considered as

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an antibiotic that is the most widely used for preventing indwelling medical device infections.

There are two main approaches for protecting indwelling medical devices from being attacked by bacterial pathogens. One approach is to develop biomedical polymers with anti-adhesive surface properties using methods such as heparin or albumin coating [14, 21]. The other is the use of antibiotics with bactericidal or bacteriostatic activity in development of the biomedical polymers. For example, the latter includes techniques such as the incorporation of antibiotics into polymer matrix or the photochemical immobilization of antimicrobial peptides onto the polymeric surface [8, 9]. That is, the surface of biomedical polymers is mostly modified to render increased biocompatibility along with mechanical property [15]. However, there are a few promising surface modification methods showing a high antibacterial effect without any damage to the biomedical polymer backbone under biological environments such as human blood or body fluid. To resolve this problem, we have designed a new surface modification method for releasing the antibiotic from the polymer by a hydrolysis mechanism under biological environments. The surface of PU (Pellethane®2363) was chemically modified by 1,6-diisocyanatohexane (HMDI) to immobilize the rifampicin. The immobilized-rifampicin can be released by a selective cleaving of the carbamate linkage that exists on the surface-modified PU (PHR) under aqueous environments. We studied in the present investigation whether the rifampicin that was released from PHR by this antibiotic release mechanism showed an antibacterial effect against both *S. aureus* and *S. epidermidis*. The influence of blood component (e.g. serum) on its antibacterial effect was also investigated.

Materials and Methods

Bacterial Strains and Culture Conditions

Staphylococcus aureus (ATCC 27735) and *Staphylococcus epidermidis* RP12 (ATCC 35983) were used for the antibacterial evaluation of the surface-modified PU (PHR). They were grown in the nutrient broth (NB, Difco, Detroit, U.S.A.) or tryptic soy broth (TSB, Difco, U.S.A.) on a rotary shaker (LM-420, Labmate Co., Seoul, Korea) at 37°C for 3 h. After incubation, they were recovered from the broth by repeated centrifugation (12,000 rpm, 3 min). The bacterial pellets were suspended in a sterile phosphate-buffered saline (10 mM of PBS, pH 7.4), growth media (NB or TSB), and bovine calf serum (Hyclone Laboratories, Inc., Utah, U.S.A.), respectively. The final concentration of bacterial suspension was adjusted to approximately 1-2×10^6 cfu/ml.

Rifampicin-Susceptibility of Staphylococci

The susceptibility of two staphylococcal strains (*S. aureus* and *S. epidermidis*) to rifampicin was determined by MIC (minimal inhibitory concentration) measurement by using the microtiter twofold broth-dilution method [10]. *Escherichia coli* DH5α (rifampcin-insusceptible strain) was also used as a negative control for the rifampicin-susceptibility test. Rifampicin powder (MW=823, Jongkeundang, Seoul Korea) was dissolved in a small volume of undiluted methanol that was further diluted in the bacterial growth media. One-ml aliquots of rifampicin media with a final concentration range of 0-15 ng/ml were distributed into the tubes containing 1 ml of the log-phase bacterial cultures (2×10^6 cfu/ml), respectively. After an overnight incubation process, MICs of the three different bacterial strains to rifampicin were measured by observing the tube-turbidity. The MIC value was determined in a rifampicin concentration at the point above where the tube was clear.

Surface Modification of Biomedical Polyurethane

A biomedical grade poly(ether urethane), Pellethane®2363-80AE (Dow Chemical Co. Georgia, U.S.A.) sheet was prepared

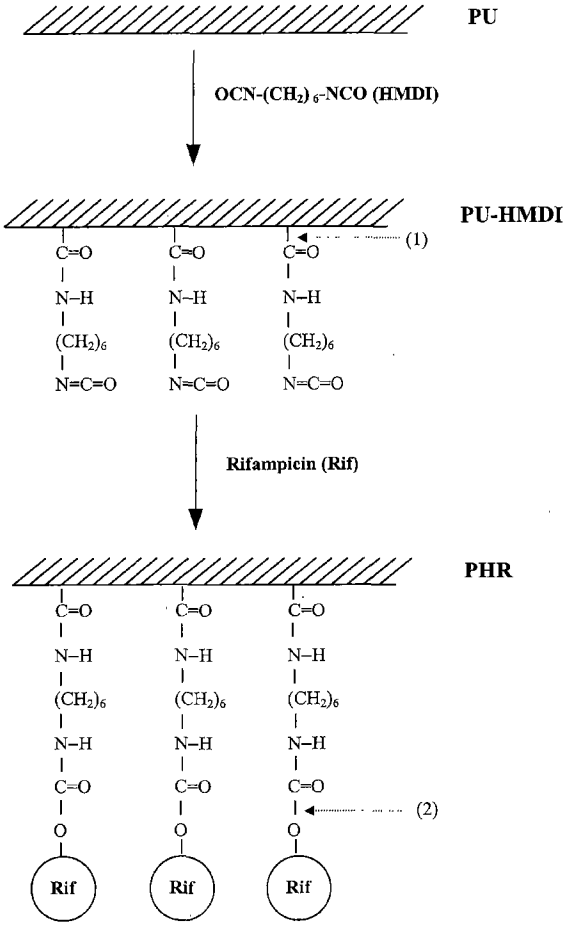

Fig. 1. Reaction scheme for the immobilization of rifampicin onto the polyurethane surface.
(1) Allophanate linkage; (2) carbamate linkage; PU (polyurethane); PU-HMDI (1,6-diisocyantohexane-linked polyurethane); PHR (rifampicin-immobilized polyurethane).

by using the solvent-cast method [19]. PU pellets were extracted with methanol and distilled water for 24 h to remove low molecular weight components or any other contaminants. After extraction, they were dried in the mechanical circulating drying oven for 48 h. In order to obtain a PU sheet, the dried pellets were dissolved in N,N-dimethylacetamide (DMAc, Shinyo Sangyo Co. Ltd., Hiroshima, Japan) to a concentration of 16% (w/v), poured onto the glass plate, and solidified in the drying oven at 44°C for 3 days. The PU fragments (10×20×1.0 mm, L×W×D) were prepared from the PU sheet. They were extracted with methanol for 1 day to remove a residual DMAc, washed with anhydrous toluene and 95% ethanol, and dried under a vacuum for 30 min.

The surface of PU was modified by two major chemical reactions as illustrated in Fig. 1. The PU fragments were swelled in an anhydrous toluene for 1 h. To prepare the HMDI-toluene mixture, 1,6-diisocyanatohexane (HMDI, Aldrich Chemical Co., Inc., Milwaukee, U.S.A.) was dissolved in an anhydrous toluene to the volume ratio of 1:10. The PU fragments were added into the HMDI-toluene mixture and maintained under N_2 gas at 40°C for 10 min. The allophanate reaction between the urethane proton of PU and the isocyanate group of HMDI was promoted by adding a catalyst (0.15 ml stannous 2-ethylhexanoate, Sigma, Chemical Co., St. Louis, U.S.A.). The reaction was continued under N_2 gas at 40°C for 30 min. The free isocyanate residues that remained on the surface of the isocyanate-derived PU (PU-HMDI) were removed by washing with anhydrous toluene. Rifampicin was immobilized onto the PU-HMDI surface by carbamate linkage. The PU-HMDI fragments were reacted with 100 ml of anhydrous toluene containing 205 mg of rifampicin for 24 h, then washed with anhydrous toluene and 95% ethanol and dried under a vacuum for 30 min. The chemical characteristics of the PU, PU-HMDI, and PHR surfaces were analyzed using an Attenuated Total Reflection-Fourier Transform Infrared spectroscopy (ATR-FTIR, Bio-Rad FTS-60, Bio-Rad Laboratories, CA, U.S.A.) [23].

Rifampicin-incorporated PU (RIP) was used as a counterpart of PHR. The PU fragments were swelled in anhydrous toluene for 1 h without HMDI or any catalyst. They were soaked in 100 ml of anhydrous toluene and 205 mg of rifampicin for 24 h. After soaking, the fragments were washed with anhydrous toluene and 95% ethanol, and were then dried under a vacuum for 30 min.

All specimens (PU, PU-HMDI, PHR, and RIP) were stored in the dark after sterilization by ethylene oxide (E. O.) gas.

Release of Rifampicin from the Surface-Modified Polyurethane

To compare the release profiles of rifampicin in PHR and RIP, the three polymer fragments were eluted in 5 ml of 10 mM phosphate-buffered saline (PBS, pH 7.4) at 37°C on a rotary shaker by constantly removing and replacing a fresh PBS for 6 days. The amounts of rifampicin released from PHR and RIP fragments at 1, 2, 3, 4, 5, and 6 days were measured from the optical density at 334 nm using a UV/visible spectrophotometer (Shimadzu UV/1600, Tokyo, Japan) [24]. The release curve of rifampicin was made from the cumulative amount of released antibiotic (ng) per total surface area (mm^2) of fragments as a function of release time (day).

Antibacterial Effect of PHR against Staphylococci

The antibacterial effect of the surface-modified polyurethane (PHR) against staphylococci was evaluated under two different aqueous environments. One was a bacterial growth environment and the other was a blood-mimic environment. The bacterial adhesion assay was used for evaluating the antibacterial effect of PHR under the bacterial growth condition. PU, PHR, and RIP fragments (10×20×1.0 mm, L×W×D) were exposed to bacterial suspension under constant agitation (120 rpm) at 37°C for 24 h. Bacterial suspension was prepared by inoculating the log-phase culture into a fresh broth. *S. aureus* was inoculated in the nutrient broth to a final concentration of 2.34×10^6 cfu/ml. The final concentration of *S. epidermidis* was adjusted to 8.67×10^5 cfu/ml with tryptic soy broth. After 24 h, the adherent bacteria were recovered from all fragments by the detachment method. The fragments were gently washed with PBS and then transferred into the test tubes containing a fresh PBS. The adherent bacteria were rapidly detached from the fragments using a vortex mixer (KMC-1300V, Vision Scientific Co., Kyunggido, Korea) at a maximum speed (3,000 rpm) for 2 min. The number of detached bacterial cells was counted by the plate count method, and the adhesion value was expressed as $\log_{10}$ cfu/mm^2 of the fragment. The antibacterial effects of PU, PHR, and RIP under the bacterial growth media were compared.

In order to estimate an antibacterial effect of PHR *in vivo*, bovine calf serum (BCS) was used as a blood-mimic liquid. PU, PHR, and RIP fragments were exposed to 10 mM of PBS or BCS (Hyclone Laboratories, Inc., Utah, U.S.A.) without any agitation at 37°C for 3 h. All treated fragments were incubated in a bacterial suspension containing *S. epidermidis* (inoculum size: 5×10^5 cfu/ml) for 24 h. The influence of serum on the antibacterial effect of PHR was evaluated by the bacterial adhesion assay and the plate count method. The adhesion value was expressed as $\log_{10}$ cfu/mm^2 of the fragment, and the antibacterial effects of PU, PHR, and RIP under BCS were compared.

RESULTS AND DISCUSSION

Rifampicin-Susceptibility of Staphylococci

Rifampicin is an antibiotic that blocks RNA synthesis by binding to and inhibiting the DNA-dependent RNA

Table 1. Minimum inhibitory concentration (MIC) values (ng/ml) of rifampicin against test microorganisms.

Microorganisms	Rifampicin (ng/ml)						MIC (ng/ml)
	0.00	0.47	0.94	1.88	3.75	7.50	
S. aureus	+	+	+	-	-	-	1.88
S. epidermidis	+	+	+	+	-	-	3.75
E. coli DH5α	+	+	+	+	+	+	>7.50

The MIC values of rifampicin for three microorganisms were defined as the lowest concentration level of the rifampicin solution that inhibited the bacterial growth after 24 h of incubation. +, microbial growth; -, no microbial growth.

polymerase of prokaryotes [17, 18, 27]. Also, the antibiotic has a wide antibacterial spectrum and is particularly active at low concentration levels against mycobacteria and Gram-positive organisms. In general, it has been reported that the Gram-positive staphylococci is very susceptible to rifampicin. Although the susceptibility to rifampicin greatly depends on bacterial strains, it is generally known that the range of MICs (minimum inhibitory concentrations) of both *S. aureus* and *S. epidermidis* to rifampicin is approximately 4-15 ng/ml [25]. However, most of the Gram-negative bacteria (e.g. *E. coli* and *P. aeruginosa*) are less susceptible to rifampicin than Gram-positive bacteria [25]. The ranges of MICs of *E. coli* and *P. aeruginosa* to rifampicin are 8,000-16,000 ng/ml and 32,000-64,000 ng/ml, respectively. Prior to the immobilization of rifampicin onto the PU surface, the antistaphylococcal activity of a free rifampicin molecule (not-immobilized) was evaluated by the MIC measurement, and the values for three different microorganisms are listed in Table 1. The MIC values for both *S. aureus* and *S. epidermidis* were 1.88 ng/ml and 3.75 ng/ml, respectively. Two different Gram-positive staphylococcal strains (*S. aureus* and *S. epidermidis*) were very susceptible to rifampicin while *E. coli* DH5α was very insusceptible to rifampicin. These results suggest that the rifampicin is an appropriate antibiotic in development of antistaphylococcal polymers.

Surface Modification of Biomedical Polyurethane using Diisocyanate

PU (Pellethane®2363) was selected as a raw material for preparing the antibacterial polymer, because it has a relatively excellent resistance to aqueous environments than other biomedical polymers (e.g., polycaprolactone, polyester) [20]. Therefore, the PU surface was modified via two major chemical reaction steps by using 1,6-diisocyanatohexane (HMDI); (1) Induction of the isocyanate (-NCO) group onto the PU surface by the allophanate reaction between -NCO groups of HMDI with -NH groups of PU; (2) immobilization of rifampicin onto the isocyanate-induced PU (PU-HMDI) surface by carbamate linkage (Fig. 1). It has generally been known that isocyanates containing unsaturated -N=C=O groups are highly reactive

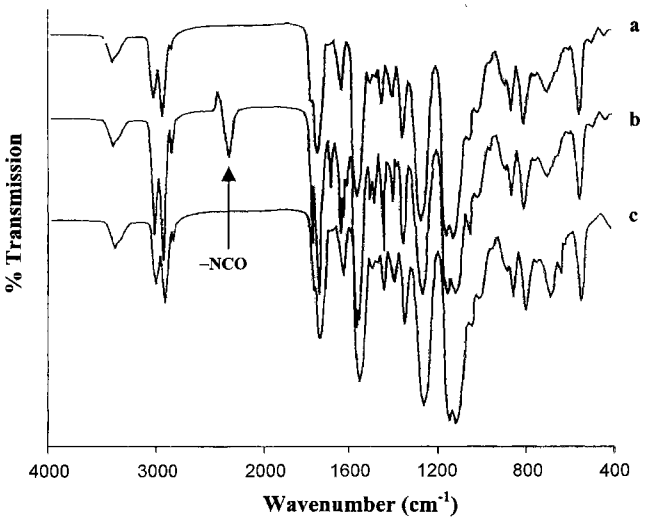

Fig. 2. ATR-FTIR spectra of polyurethane (PU, a), isocyanate-induced PU (PU-HMDI, b), and rifampicin-immobilized polyurethane (PHR, c).
A sharp peak of the isocyanate (NCO) group at 2,267 cm^{-1} was observed in (b).

to a large number of compounds such as alcohol, amines, carboxylic acid, water, urethane, urea, amide, and phenol, and they may also react among themselves [2]. In addition, the HMDI is a chemical reagent having diisocyanate groups which are capable of reacting with a urethane proton (-NH) of polyurethane (PU) as well as alcohol groups of rifampicin. Thus, 1,6-diisocyanatohexane (HMDI) was selected as a chemical reagent for the surface modification of PU.

The introduction of the isocyanate group on the surface of PU was confirmed by the characteristic IR absorbance peak of the isocyanate (NCO) group at 2,267 cm^{-1} (Fig. 2). The isocyanate groups introduced onto the PU-HMDI surface were able to form carbamate linkage with the alcohol group of rifampicin. Although the characteristic IR peak of rifampicin was not detected in rifampicin-immobilized polyurethane (PHR), the formation of a carbamate linkage was indirectly shown from the disappeared isocyanate peak of PHR. It is not easy to define an exact position of the hydroxyl group reacting with the isocyanate group of PU-HMDI by only the results obtained from ATR-FITR spectra. Rifampicin has five hydroxyl (-OH) groups at the positions of 5, 6, 9, 17, and 19 carbons. We presumed that the hydroxyl group at the position of carbon 9 (or 19) in the rifampicin structure would be a strong candidate in the formation of the carbamate linkage, because the position could potentially influence steric hindrance more than the other three positions (5, 6, and 17 carbons).

Release of Rifampicin from the Surface-Modified Polyurethane

The immobilized rifampicin should be isolated from the surface-modified PU (PHR) to exhibit its

antistaphylococcal activity because free rifampicin molecules could only be inserted into the prokaryotes. Fortunately, we have designed an antistaphylococcal polymer on the assumption that the formation of unstable linkage between polymer and antibiotic will release antibiotic *via* a hydrolytic mechanism. Two carbamate linkages exist on PHR. One is a carbamate linkage existing on the PU backbone and the other is a carbamate linkage existing between PU-HMDI and rifampicin. One of the two linkages has a possibility of linkage cleavage by hydrolytic attack. It has generally been known that the carbamate linkage formed by the reaction of isocyanate and allyl alcohol is stable, whereas the carbamate linkage formed by the reaction of aryl alcohol with isocyanate is unstable [12, 13, 28]. Therefore, the carbamate linkage on the PU backbone should be protected from the hydrolytic attack, because the PU was a polymer prepared by the reaction between the isocyanate of polyurethane prepolymer and the allyl alcohol of the 1,4 BP-chain extender [20]. On the other hand, the carbamate linkage formed between the isocyanate of PU-HMDI and the aryl group of rifampicin should be susceptible to the hydrolytic attack. If this hypothesis was correct, the rifampicin would be released from PHR via the hydrolysis mechanism without any damage to the PU backbone under biological environments.

In order to demonstrate that the rifampicin is released from PHR by the hydrolysis mechanism, the PHR was exposed to an aqueous environment such as PBS (phosphate-buffered saline, pH 7.4) for 6 days. Rifampicin-incorporated polyurethane (RIP) was also used as a counterpart of PHR. The reason was to clearly distinguish the release of immobilized rifampicin by the hydrolytic mechanism from the release of rifampicin residues incorporated into the PHR matrix. The release profile of rifampicin that was released from PHR differed remarkably from that of RIP. The continuous release of rifampicin in PHR was observed for more than 6 days. The amount of rifampicin released from PHR was more than 10-fold of that released from RIP (Fig. 3). After 2 h of exposure, the amount of rifampicin released from PHR was 0.26 ng per surface area (mm^2) of PU, whereas the amount of rifampicin released from RIP was 0.023 ng/mm^2. After 6 days of exposure, the cumulative amount of rifampicin that was released from PHR was 3.07 ng/mm^2. However, the RIP showed a lower cumulative release amount (0.38 ng/mm^2) than PHR. These results indicate that the antibiotic release mechanism of PHR differs from that of RIP. We found that most of the detected rifampicin was the antibiotic released by the hydrolysis of unstable carbamate linkage rather than the antibiotic residues which were incorporated into the PHR.

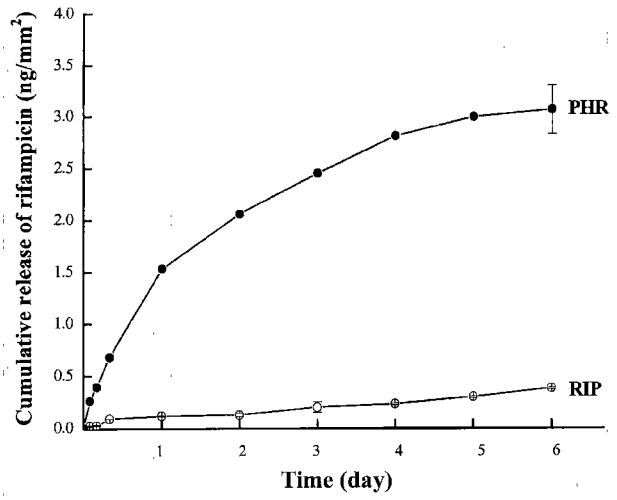

Fig. 3. The release profile of rifampicin in RIP (rifampicin-incorporated PU) and PHR (rifampicin-immobilized PU).

Antibacterial Effect of PHR against Staphylococci

The antibacterial effect of rifampicin released from the surface-modified polyurethane was evaluated under two different aqueous environments. In order to investigate the antibacterial effect of PHR under an optimal bacterial infection environment, the three different polymers (PU, RIP, and PHR) were exposed to the growth media

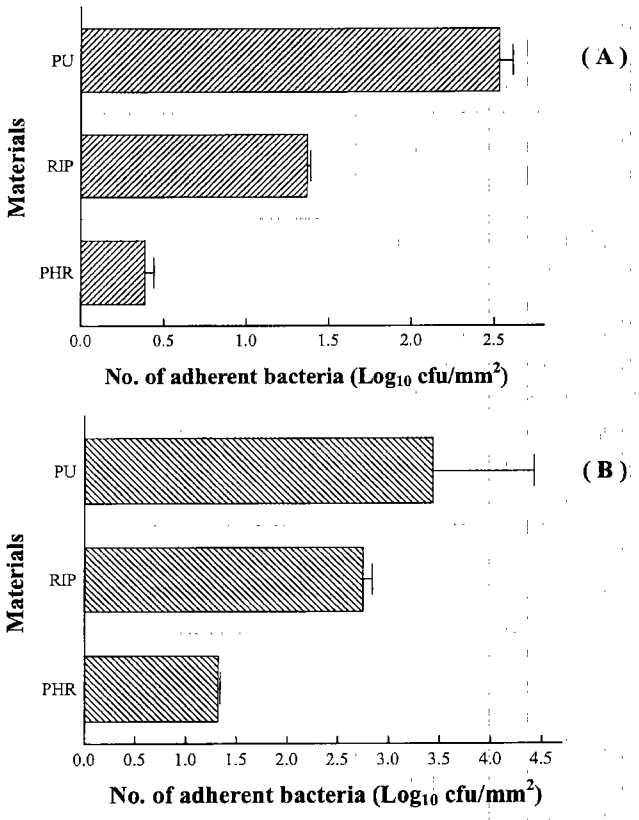

Fig. 4. Comparison of the antibacterial effects of RIP (rifampicin-incorporated PU) and PHR (rifampicin-immobilized PU) against staphylococci.
(A) *S. aureus*; (B) *S. epidermidis*. Error bars are the standard deviation to the averages calculated (mean±SD, n=3).

containing two different staphylococcal strains for 24 h, respectively.

Figure 4 shows the antibacterial effect of PU, RIP, and PHR in bacterial growth media against two different Gram-positive staphylococci. When PU was exposed to the nutrient broth (N. B.) containing *S. aureus*, the number of bacteria that survived onto the PU surface was 2.53±0.08 $\log_{10}$ cfu/mm^2 (Fig. 4A). The growth of *S. aureus* was inhibited by the rifampicin that was released from RIP and PHR. The number of bacteria that survived onto the RIP surface was 1.37±0.02 $\log_{10}$ cfu/mm^2. The number of *S. aureus* that survived onto the PHR surface was 0.39±0.05 $\log_{10}$ cfu/mm^2 and it was lower compared to that of PU or RIP. After 24 h of exposure to the tryptic soy broth (TSB) containing *S. epidermidis*, the number of bacteria that survived onto the polymeric surface (PU, RIP, and PHR) was 3.44±0.99 $\log_{10}$ cfu/mm^2, 2.75±0.09 $\log_{10}$ cfu/mm^2, and 1.32±0.02 $\log_{10}$ cfu/mm^2, respectively (Fig. 4B). Interestingly enough, the PHR showed a higher antibacterial effect against both *S. aureus* and *S. epidermidis* than RIP. In addition, as shown in Fig. 3, the amount of rifampicin that was released from PHR was more than that of RIP. These results indicate that the antistaphylococcal effect depends on the amount of rifampicin that was released from PHR or RIP.

The antibacterial effect of PHR should be maintained in human blood or body fluid, because it has generally been known that the biomaterial-associated infection in a circulating blood is mediated by the blood components that are pre-adsorbed onto the biomaterial surface [4]. In order to estimate the influence of blood components on the antibacterial effect of PHR *in vivo*, the three different polymers (PU, RIP, and PHR) were pre-exposed to blood-mimic environments such as bovine calf serum (BCS) before contacting with *S. epidermidis*. Ten mM of PBS (phosphate-buffered saline, pH 7.4) was also used as a counterpart of BCS. After 24 h of contacting with *S. epidermidis*, the number of adherent bacteria to the BCS-treated PU was similar to that of the PBS-treated PU (Fig. 5). The growth of *S. epidermidis* was remarkably inhibited by PHR that was pretreated with BCS. The number of bacteria adhered on the BCS-treated PHR was 0.22±0.04 $\log_{10}$ cfu/mm^2, whereas the number of bacteria adhered on the PBS-treated PHR was 0.51±0.02 $\log_{10}$ cfu/mm^2. In addition, the antibacterial effect of the BCS-treated PHR was higher than that of the RIP. The antibacterial effect of PHR against *S. epidermidis* was increased by bovine serum albumin. This result implies that the PHR maintained a stable antibacterial effect under the blood-mimic aqueous environments.

In conclusion, the surface-modification of biomedical polyurethane using diisocyanate was successfully achieved. The modified polyurethane showed a potent and stable antistaphylococcal effect under various aqueous environments.

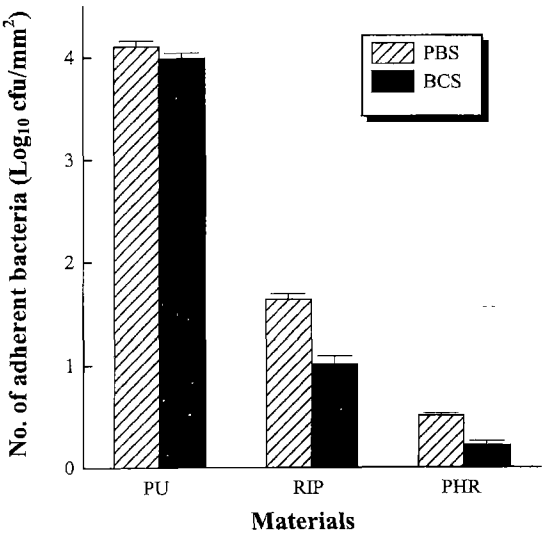

Fig. 5. The influence of BCS on the antibacterial effect of PHR against *S. epidermidis*.
PBS (phosphate-buffered saline); BCS (bovine calf serum); PU (polyurethane); RIP (rifampicin-incorporated polyurethane); PHR (rifampicin-immobilized polyurethane). The bars represent the standard deviation of three replicates.

This polymeric modification would be a useful tool for developing much more efficient antibacterial blood-contacting devices.

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