Polymeric Phosphonium Salts as a Novel Class of Cationic Biocides. VI. Antibacterial Activity of Fibers Surface-Treated with Phosphonium Salts Containing Trimethoxysilane Groups

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SYNOPSIS

Alkoxysilane with phosphonium biocides as coupling agents were covalently attached to cotton-fiber surfaces, and the antibacterial activity of the surface-treated fibers against *Staphylococcus aureus* and *Escherichia coli* was evaluated by the viable cell counting method in sterile distilled water. These fibers with phosphonium salts were found to exhibit high antibacterial activity against *S. aureus* and *E. coli*, particularly against *S. aureus*, and the activity increased as hydrophobicity of the substituents bonded directly to phosphonium ions increased. Furthermore, morphological changes of the cells of *S. aureus* and *E. coli* in contact with the fibers were evaluated by scanning electron microscopy. It was found that the fiber with the phosphonium biocides exhibited bacteriostatic as well as bactericidal activity against both strains, which was evident from observation of normal and deformed cells of these species in contact with the fibers. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

We have already reported that in order to provide fibers with surface antibacterial activity, the phosphonium biocides retained as counter ions of sodium sulfonate incorporated into polyesters are quite effective.[1] The resulting polyesters have been shown to act as fibers capable of controlled releasing of phosphonium biocides because biocides are released at their surfaces. Many trials have been conducted so far to provide polymeric materials with the surface antibacterial activity by incorporating antibacterial agents covalently onto the surfaces of the polymeric materials.[2-6] Merits of these immobilized biocides are evident. First, because of immobilization of the active groups, contamination of the environment with the biocides can be prevented. Second, owing to covalent bonding to the matrix media, the immobilized biocides can be regenerated by washing with appropriate solvents, so that they can be used for a long period. Recently, fibers are commercially available that are treated with alkoxysilane having quaternary ammonium salts as coupling agents.[7] Most of the immobilized antibacterial agents practically used or under investigation are those of quaternary ammonium salts. Although the immobilized biocides with quaternary ammonium salts have a broad antibacterial spectrum, they do not have a sufficient antibacterial activity for short periods of exposure to affect microorganisms.

In this study, we treated surfaces of cellulose fibers with 3-(trimethoxysilyl)propyltrialkyl phosphonium chlorides, coupling agents, and examined the surface antibacterial activity of the surface-treated fibers which retained the covalently bonded phosphonium salts with trimethoxysilane groups. Furthermore, morphological changes of the cells of *Staphylococcus aureus* and *Escherichia coli* in contact with the fibers having phosphonium salts were evaluated by means of scanning electron microscopy (SEM).

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EXPERIMENTAL

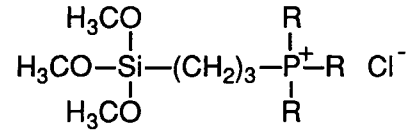
Structures of phosphonium salts with trimethoxysilane groups and a reference compound used in this study are shown in Figure 1.

Materials

Four compounds of 3-(trimethoxysilyl)propyltrialkyl phosphonium chlorides (**1**, **2**, **3**, and **4**) were synthesized by Nippon Kagaku Kogyo and used without further purification. 3-(Trimethoxysilyl)propyl chloride (**5**) used in this study was commercially available. Cotton fiber (approximately 4.2 m/g) was defatted with acetone and chloroform by ultrasonication, and then dried under reduced pressure.

Surface Treatment of Fiber

Preparation of fibers having phosphonium biocides is shown in Figure 2. To the mixture of methanol-water [200 mL, 95 : 5 (w/w), pH 4.5 (adjusted with acetic acid)], the organosilicon phosphonium chlorides were added with stirring to give the final concentration of 2 wt %. The resulting solutions were left with stirring at room temperature for 10 min to accelerate the hydrolysis of methoxy groups (steps 1 and 2 in Fig. 2). The cotton fibers (approximately 11.5 g) were then immersed in the slowly stirred solution and left at room temperature for 30 min. The cotton fibers removed from the solution were heated to 120°C and left for 30 min at this temperature to incorporate phosphonium salts onto the surface of the fibers (step 3 in Fig. 2). The treated fibers were washed several times with methanol by ultrasonication to remove untreated compounds, and dried under vacuum.



R	C_2H_5	C_4H_9	C_6H_{13}	C_8H_{17}
	1	2	3	4

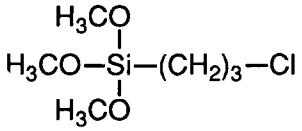


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Figure 1 Structures of silane coupling agents used in this study.

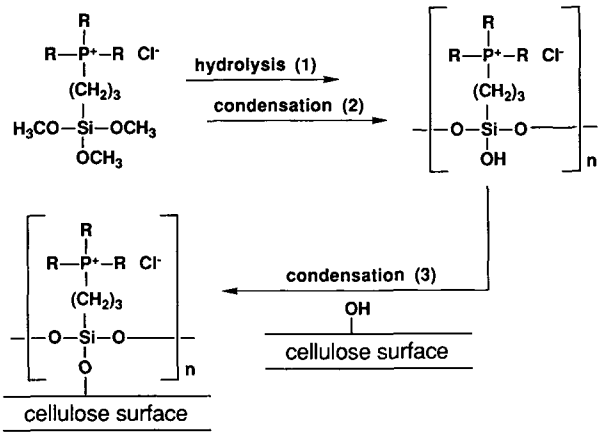

Figure 2 Preparation procedure of fibers containing phosphonium biocides.

Measurement of Amount of Phosphonium Salts Incorporated Into Fiber

Surface-treated fibers (1 g) were boiled in conc. HNO_3—H_2SO_4 [50 mL, 4 : 1 (v/v)] for 1 h to degrade the cellulose structure of the cotton fibers. The resulting homogeneous solution was subjected to inductively coupled plasma analysis to measure the amount of P atoms (see Table I).

Antibacterial Assessment

Cultures of *S. aureus* (IFO 12732) and *E. coli* (IFO 3806), each of which contained ca. 10^8 and ca. 10^9 cells/mL, respectively, were prepared by overnight

Table I Characterization of Fibers Containing Phosphonium Salts Used

Sample	Substituent[a] (R)	Amount of Phosphonium Salt[b] (μmol/g)
6	C_2H_5	51.2
7	C_4H_9	37.7
8	C_6H_{13}	40.8
9	C_8H_{17}	33.5

[a] See Figure 2.
[b] Determined by inductively coupled plasma analysis.

incubation at 37°C in nutrient broth (peptone, 10 g; NaCl, 5.0 g; beef extract, 5.0 g in 1,000 mL of sterile distilled water; pH 6.8). By diluting with sterile distilled water, culture containing ca. 10^8 cells/mL was prepared for each strain and used for antibacterial tests.

Compounds **1** and **2** were soluble in water. However, **3**, **4**, and **5** were not completely soluble in water, so that they were dissolved in methanol-water [1 : 9 (v/v)] at first and then diluted with sterile distilled water to give the correct final concentration when 18.0 mL of the biocide solution was combined with 2.0 mL of the bacterial culture. It was confirmed that methanol used for the preparation of 1% concentrate did not affect the result of the antibacterial tests. Exposure of bacterial cells to biocide was started when 2.0 mL of bacterial culture containing ca. 10^8 cells/mL was added to 18.0 mL of the biocide dispersion which was preequilibrated at 37°C. At the same time, 2.0 mL of the same culture was added to 18.0 mL of saline, decimal dilutions were prepared, and the starting cell concentration was enumerated by the spread plate method. At various contact times 1.0-mL portions were removed and decimal serial dilutions were prepared by taking 1 mL into 9 mL of saline and mixing. From these dilutions the surviving bacteria were counted by the spread plate method. After inoculation, the plates were incubated at 37°C, and the colonies were counted after 48 h. The counting was done in triplicate every time.

Surface-treated fibers (**6**, **7**, **8**, and **9**; see Table I) and untreated fiber (blank) were cut into small pieces and dispersed in 54 mL of sterile distilled water; and to this dispersion a 6.0-mL portion of bacterial culture containing ca. 10^8 cells/mL was added. After contact, the counting of the surviving

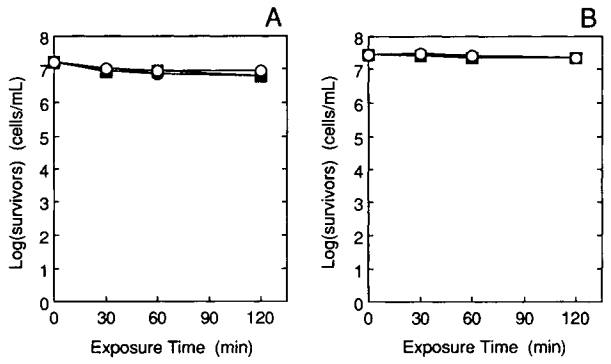

Figure 3 Plots of log (survivors) versus exposure time for **1** and **2** against *S. aureus* and *E. coli:* (A) against *S. aureus,* (B) against *E. coli;* (○) blank, (●) **1**, (■) **2**. Concentration, 250 μ*M* (100 μg/mL for 2).

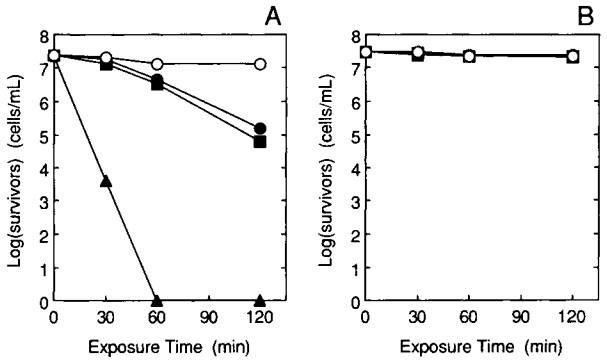

Figure 4 Plots of log (survivors) versus exposure time for **3** against *S. aureus* and *E. coli:* (A) against *S. aureus,* (B) against *E. coli.* Concentrations: (○) blank, (▲) 250 μ*M*, (■) 25 μ*M*, (●) 2.5 μ*M*.

cells was performed by the same method as described previously.

Observation of Bacterial Cells in Contact With Surface-Treated Fibers by SEM

After antibacterial test against *S. aureus* and *E. coli,* fiber **9** was removed from the bacterial suspension of each strain. The fibers were washed with sterile distilled water, and fixation and dehydration of samples were carried out by conventional methods. The fiber surfaces were observed with a TOPCON-DS130 SEM.

RESULTS AND DISCUSSION

Characterization of Fibers Containing Phosphonium Salts

The amount of phosphonium salts incorporated onto the surface of fibers is shown in Table I. The amount of phosphonium per unit weight of the surface-treated fiber did not change significantly for each sample. This means that the condensation reaction of silanol groups with hydroxyl groups of cellulose and self-condensation of organosilicon phosphonium salts were little affected by the alkyl chain of the substituents on P^+. Constancy of the values is favorable for comparison of the antibacterial activity. It has been reported that the rate of hydrolysis of trialkoxysilane increases as a number of carbon atoms of alkoxy groups decreases, so that the hydrolysis of trimethoxysilane proceeds most easily, and the hydrolysis products certainly give a condensed structure.[8] Therefore, the phosphonium salts having

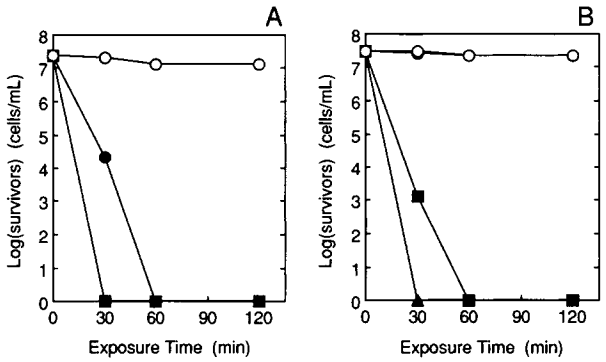

Figure 5 Plots of log (survivors) versus exposure time for **4** against *S. aureus* and *E. coli:* (A) against *S. aureus,* (B) against *E. coli.* Concentrations: (○) blank, (▲) 250 μM, (■) 25 μM, (●) 2.5 μM.

trimethoxysilane are expected to form a polymer network at the surface of the fibers.

Antibacterial Activity of Phosphonium Salts With Trimethoxysilane Groups

At first, we evaluated the antibacterial activity of the phosphonium salts with trimethoxysilane groups (**1**, **2**, **3**, and **4**) and reference compound (**5**) against *S. aureus* and *E. coli* by the viable cell counting method. Figure 3 shows plots of log (survivors) versus exposure time for phosphonium salts **1** and **2** against *S. aureus* (A) and *E. coli* (B). About 10^7 cells/mL of *S. aureus* and *E. coli* were exposed to 250 μM (100 μg/mL for **2**) of phosphonium salts **1** and **2** in saline. The phosphonium salts were inactive against both strains. Figure 4 shows plots of log (survivors) versus exposure time for phosphonium salt **3** against *S. aureus* (A) and *E. coli* (B). About 10^7 cells/mL of *S. aureus* and *E. coli* were exposed

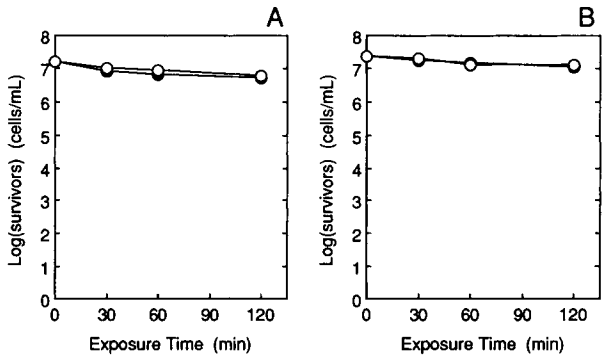

Figure 6 Plots of log (survivors) versus exposure time for **5** against *S. aureus* and *E. coli:* (A) against *S. aureus,* (B) against *E. coli.* Concentration, 250 μM.

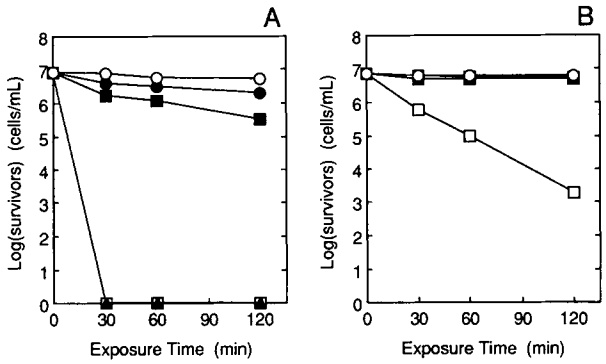

Figure 7 Plots of log (survivors) versus exposure time for the fibers containing phosphonium salts with different substituents against *S. aureus* and *E. coli:* (A) **6–9** against *S. aureus,* (B) **6–9** against *E. coli;* (○) blank, (●) **6,** (■) **7,** (▲) **8,** (□) **9.** Concentration, 3000 μM, based on the cations on the surface of the fibers.

to 250, 25, and 2.5 μM of the phosphonium salt with trihexyl groups in saline. Against *S. aureus,* 250 μM of **3** killed all bacterial cells within 60 min of contact [Fig. 4(A)]. At the concentrations of 25 and 2.5 μM, > 99% of *S. aureus* were killed within 120 min of contact. On the other hand, **3** was inactive against *E. coli* even at the highest concentration [Fig. 4(B)]. Antibacterial activity of phosphonium salt with the longest alkyl chain (trioctyl groups) **4** is shown in Figure 5. At concentrations of 250 and 25 μM, **4** was capable of killing all bacterial cells of *S. aureus* within 30 min of contact [Fig. 5(A)]. Even at the lowest concentration (2.5 μM), all bacterial cells were killed within 60 min of contact. Compound **4** also exhibited a high activity against *E. coli* [Fig.

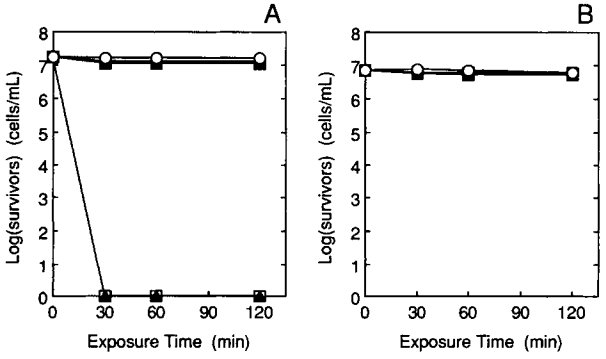

Figure 8 Plots of log (survivors) versus exposure time for the fibers containing phosphonium salts with different substituents against *S. aureus* and *E. coli:* (A) **6–9** against *S. aureus,* (B) **6–9** against *E. coli;* (○) blank, (●) **6,** (■) **7,** (▲) **8,** (□) **9.** Concentration, 300 μM, based on the cations on the surface of the fibers.

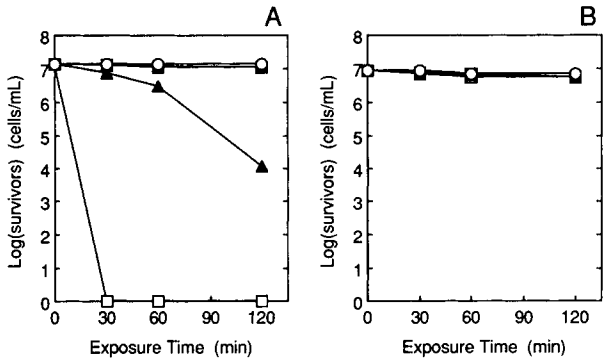

Figure 9 Plots of log (survivors) versus exposure time for the fibers containing phosphonium salts with different substituents against *S. aureus* and *E. coli:* (A) **6–9** against *S. aureus,* (B) **6–9** against *E. coli;* (○) blank, (●) **6,** (■) **7,** (▲) **8,** (□) **9.** Concentration, 30 μM, based on the cations on the surface of the fibers.

5(B)]. At the concentrations of 250 and 25 μM, all bacterial cells were killed within 30 and 60 min of contact, respectively, while at the lowest concentration (2.5 μM), **4** was inactive. These results demonstrate that the phosphonium salts with trimethoxysilane groups exhibited a high antibacterial activity, particularly against *S. aureus,* and the antibacterial activity increased with increasing chain length of the alkyl substituents. Such a trend has also been observed for other phosphonium salts.[1,9]

As a control experiment, we investigated the effect of the trimethoxysilane group on the antibacterial activity using 3-(trimethoxysilyl)propyl chloride **5,** which has no phosphonium salts, as a reference compound. Figure 6 shows plots of log (survivors) versus exposure time for **5** against *S. aureus* (A) and *E. coli* (B). About 10^7 cells/mL of *S. aureus* and *E. coli* were exposed to 250 μM of **5** in saline, and it was found that **5** was inactive against both strains. This result has revealed that the methoxysilane group itself does not affect the antibacterial activity of the alkoxysilane with phosphonium salts, in other words, the observed activity results from the $P^+(R)_3$ part.

Surface Antibacterial Activity of Fibers Containing Phosphonium Salts

Fibers surface treated with the alkoxysilane coupling agents were subjected to the viable cell counting

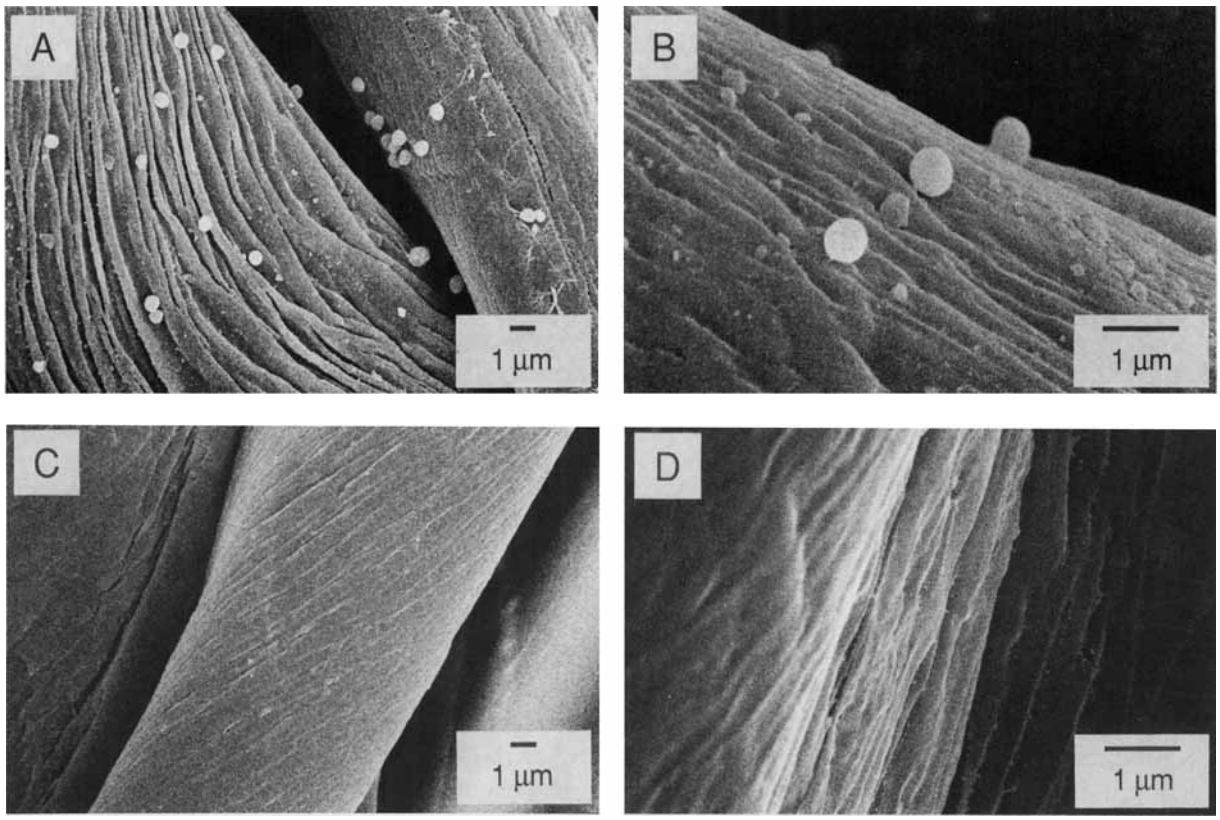

Figure 10 SEM photographs of the surfaces of the fibers exposed to *S. aureus:* (A) and (B) fiber **9,** (C) and (D) blank. Magnification: (A) and (C) ×5,000; (B) and (D) ×15,000.

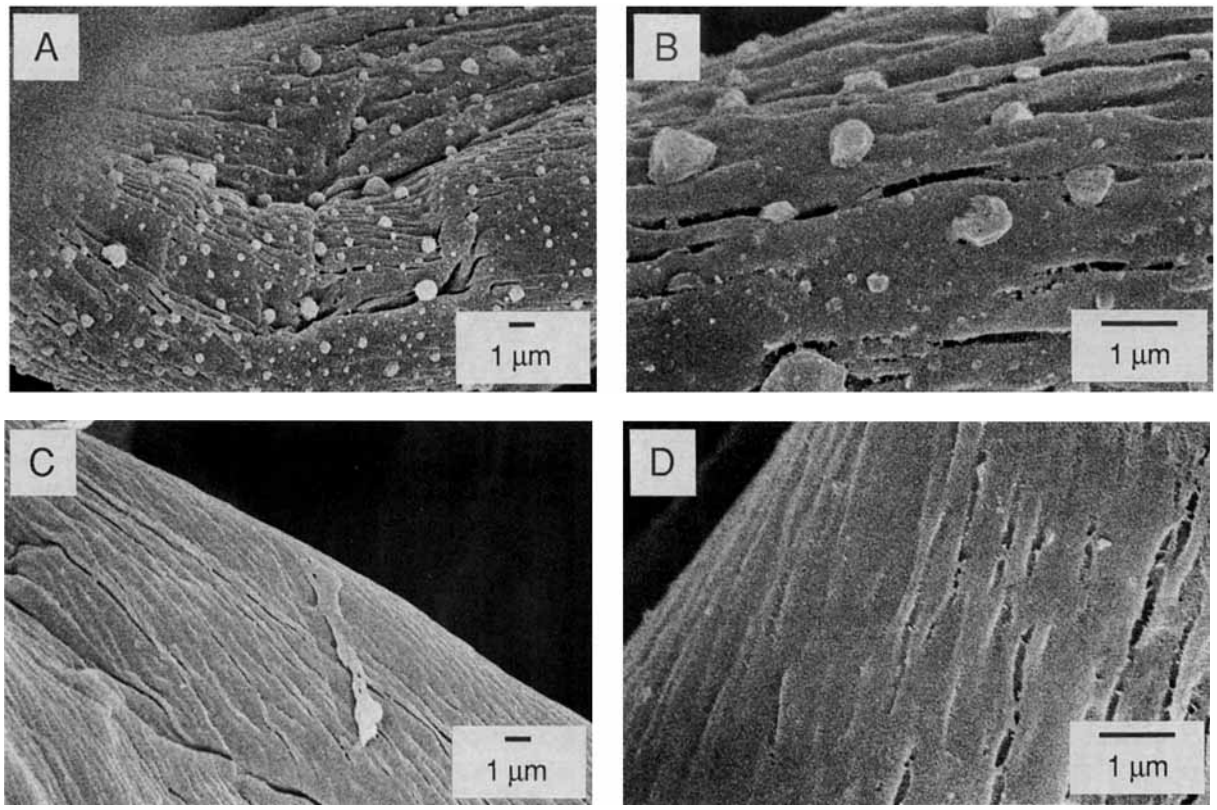

Figure 11 SEM photographs of the surfaces of the fibers exposed to *E. coli:* (A) and (B) fiber **9,** (C) and (D) blank. Magnification: (A) and (C) ×5,000; (B) and (D) ×15,000.

method described above. Figure 7 shows plots of log (survivors) versus exposure time for the surface-treated fibers **6, 7, 8,** and **9** against *S. aureus* (A) and *E. coli* (B). About 10^7 cells/mL of bacteria were exposed to the fibers (approximately 4.8 g) where the amount of the fibers was adjusted in such a way that the concentration of phosphonium salts in the bacterial suspension became 3000 μM (based on the P^+ units). All bacterial cells of *S. aureus* were killed within 30 min when exposed to fibers **8** and **9,** respectively, and > 90% of *S. aureus* was killed within 120 min of contact with fiber **7** [Fig. 7(A)]. Fiber **6** showed the activity to some extent, but in contact with the untreated cellulose fiber (blank), cells of *S. aureus* were intact even after 120 min. On the antibacterial activity against *E. coli,* reduction of the surviving cells was observed only for fiber **9** that killed > 99.9% of *E. coli,* while the other fibers were inactive [Fig. 7(B)]. From these results, it is clear that the fibers containing phosphonium salts show a high surface antibacterial activity and the fiber with phosphonium salts with longest alkyl chain exhibited the highest activity. The effect of substituent was similar to that of the free phosphonium salts with trimethoxysilane groups. Antibacterial activities of the fibers at 300 and 30 *μM* are shown in Figures 8 and 9, respectively (approximately 0.48 and 0.048 g). On the antibacterial activity against *S. aureus,* all the cells of *S. aureus* were killed within 30 min in contact with fibers **8** and **9** at the concentration of 300 *μM,* whereas fibers **6** and **7** were inactive [Fig. 8(A)]. At 30 *μM,* fiber **9** killed all bacterial cells within 30 min and fiber **8** killed about 99.9% of *S. aureus* within 120 min of contact. However, fibers **6** and **7** were again inactive [Fig. 9(A)]. On the antibacterial activity against *E. coli,* however, all the surface-treated fibers were inactive [Figs. 8(B) and 9(B)]. It was observed in this study that the sensitivity to the fibers containing phosphonium biocides was higher against *S. aureus* than *E. coli.* Similar strain dependence has been also reported for the immobilized biocides containing positively charged nitrogen atoms.[10] The ability for capture of bacterial cells onto their surface is strongly strain dependent. The adsorption behavior of bacterial cells by the immobilized biocides seems to be more affected by hydrophobicity of the cell surfaces than by the surface negative charges of the cells because

no correlation is recognized between the sensitivity and the surface charges of the cells. In fact, the cell surface of *S. aureus* is much more hydrophobic than that of *E. coli*.[10]

Observation of Bacterial Cells in Contact With Surface-Treated Fibers by SEM

Figures 10 shows the SEMs (magnification, ×5,000 and ×15,000) of the surfaces of the fibers in contact with *S. aureus*. When cells of *S. aureus* were exposed to fiber **9**, normal cells and shrunken cells were clearly observed as shown in plates (A) and (B). Plates (C) and (D) show the surface of the untreated fibers after exposure to the bacterial cells of *S. aureus*, demonstrating that the surfaces are free from cells. The SEM photographs of the *E. coli* exposed fibers are shown in Figure 11. Similar to *S. aureus*, shrunken and deformed cells could be clearly seen [plates (A) and (B)], whereas no cells were observed for blank fiber [plates (C) and (D)]. These results strongly suggest that the surface activity of the fibers having phosphonium biocides is bactericidal as well as bacteriostatic due to adsorption of bacterial cells.

In conclusion, the fibers containing phosphonium biocides were found to show high surface antibacterial activity against *S. aureus* and *E. coli*, particularly against *S. aureus*, and their activities were strongly affected by the alkyl chain length of substituents and the concentration of the phosphonium biocides incorporated onto the surface of fibers. The surface-treated fibers described here may act as self-sterilizing materials.

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