Polymeric Phosphonium Salts as a Novel Class of Cationic Biocides. X. Antibacterial Activity of Filters Incorporating Phosphonium Biocides

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SYNOPSIS

Phosphonium salts with different alkyl chains as substituents were incorporated covalently on the surfaces of cellulose filters with silane coupling agents containing phosphonium ions, and their surface antibacterial activities against *Staphylococcus aureus* and *Escherichia coli* were explored by the viable cell-counting method in sterile distilled water. The filters incorporating phosphonium biocides showed higher antibacterial activity, and its activity strongly depended on the structure of substituents. The surface-treated filters may be used to sterilize air and water, including microorganisms. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

Currently, many chemical materials such as ozone, liquid chlorine, sodium hypochlorite, and chloramine have been widely used to sterilize air and water. These materials require special equipment, and careless use may cause environmental pollution. In fact, the formation of trihalomethanes and carcinogens as a result of water disinfection with chlorine is currently a serious problem. Covalent incorporation of antimicrobial agents onto the substrate surfaces may be useful to solve the problems. Thus, contamination of the environment with biocides can be prevented because of covalent bonding, and continuous treatment of air and water containing microorganisms is possible.

Recently, many attempts have been performed to provide filters with surface antibacterial activity through immobilization of cationic biocides (quaternary ammonium salts) and inorganic materials containing metal ions (zeolites). However, they do not sufficiently disinfect air and water. This stimulated us to devise new approaches toward filters possessing the surface antibacterial activity. We have already reported that to provide fiber with the surface antibacterial activity, the phosphonium biocides incorporated onto fiber surfaces by silane coupling agents are quite effective, and the fibers exhibited a higher surface antibacterial activity against Gram-positive and Gram-negative strains.[1]

In this article, we describe the preparation of the cellulose filters incorporating phosphonium biocides on the surface and examine the surface antibacterial activity of the resulting filters against *Staphylococcus aureus* and *Escherichia coli.*

EXPERIMENTAL

Materials

The 3-(trimethoxysilyl)propyltrialkyl phosphonium chlorides used in this study were supplied by Nippon Kagaku Kogyo and used without further purification (see Fig. 1). Cellulose filter (approximately 0.23 g per 5.5-cm diameter) was defatted with acetone and chloroform by ultrasonication, and then dried under reduced pressure.

Incorporation of Phosphonium Biocides onto Filter Surfaces

The phosphonium salts were incorporated onto the filter surfaces according to the scheme shown in
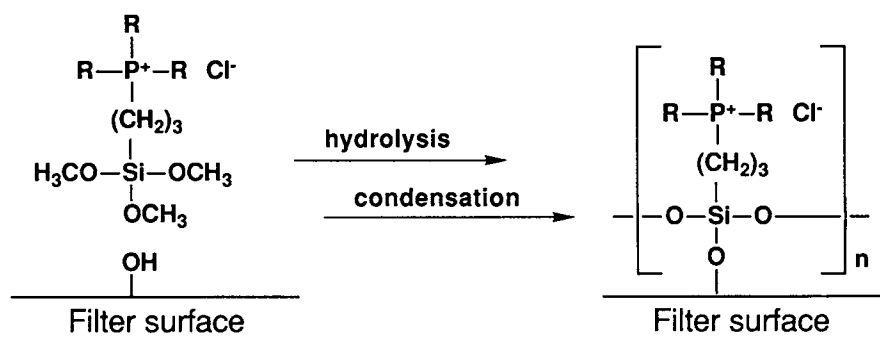

Figure 1   Scheme of incorporation of phosphonium biocides onto filter surfaces.

Figure 1. The samples were immersed at room temperature for 30 min in the mixture of methanol–water [95 : 5 (wt/wt), pH 4.5] containing 2 wt % silane coupling agents. Chemically treated samples removed from the solution were dried at 120°C for 30 min and washed several times with methanol by ultrasonication to remove untreated compounds, and again dried at 120°C under vacuum.

Amount of phosphonium salts at the surface of the filter was determined by inductively coupled plasma analysis. The surface-treated filters were degraded in acidic solution [HNO_3–H_2SO_4, 4 : 1 (vol/vol)] and subjected to the analysis. The amount of phosphonium salts was expressed as mol of bound phosphonium biocides per unit area of cellulose filter and listed in Table I.

Antibacterial Assessment

The antibacterial activity of the surface-treated filters was evaluated against *Staphylococcus aureus* (IFO 12732) and *Escherichia coli* (IFO 3806) by viable cell-counting method. The filters were cut into small pieces and exposed to the cell suspension (20 mL containing about 10^7 cells/mL), and then the

Table I   Amount of Phosphonium Biocides Incorporated onto the Surface of Filters

Sample	Substituent[a] (R)	Amount of Phosphonium Salt in Unit Area[b] (μmol/cm^2)
1	C_2H_5	0.214
2	C_4H_9	0.237
3	C_6H_{13}	0.181
4	C_8H_{17}	0.154

[a] See Figure 1.
[b] Determined by inductively coupled plasma analysis.

surviving cells were counted after being cultivated on agar plates. Details of this method have already been reported.[1]

RESULTS AND DISCUSSION

Characterization of Filters Incorporating Phosphonium Biocides

The amount of phosphonium salts per unit area of the surface-treated filters was nearly constant (0.154–0.237 μmol/cm^2; see Table I). In these estimations, both sides of the filters were regarded as active sites in the surface-treated filters because the filters were immersed in the methanolic solution containing silane coupling agents so that the phosphonium salts were covalently attached to both sides of the filters. Furthermore, the alkyl chain attached to phosphorus atom had no significant effect on hydrolysis and condensation of the organosilicon phosphonium chloride with reactive surfaces (Fig. 1). A similar result was previously obtained for the surface-treated fibers.[1]

It is worth mentioning here that we estimated the occupied area of the single phosphonium salt on the surface of the filter and found that the values (0.07–0.11 Å^2/molecule) were much smaller than those expected for a single molecule attached to the surface, which could be roughly estimated by CPK models of the phosphonium salts. These order-of-magnitude calculations allow us to evaluate the state of the phosphonium ions on the surfaces: namely, the phosphonium ions are closely packed, not in monolayers, but in stacked layers. This means that practically the whole surfaces of the filters (1–4) are covered with the phosphonium salts, and the different values of the amount of the phosphonium salts in unit area (Table I) have little meaning. Thus, when the antibacterial activity of the surface-treated

filters was evaluated, the area of each sample was kept constant.

Surface Antibacterial Activity of Filters Incorporating Phosphonium Biocides

The samples were cut into small pieces to facilitate the contact of surface-treated filters with bacterial cells in saline as described in the previous section.

Figure 2 shows plots of log (survivors) vs. exposure time for the samples 1 (A), 2 (B), 3 (C), and 4 (D) against *S. aureus*. The bacteria (about 10^7 cells/mL) were exposed to 237.5, 142.5, and 47.5 cm^2 of the surface-treated filters (2.5, 1.5, and 0.51 mM for 1, based on the phosphonium units) and 237.5 cm^2 of blank filter in 20 mL of saline. At the area of 237.5 cm^2, sample 1 with the shortest alkyl

chain (C_2) killed about 90% of *S. aureus* within 120 min of contact. At 142.5 cm^2, 1 exhibited an antibacterial activity to some extent, but 47.5 cm^2 of 1 was inactive [Fig. 2(A)]. Antibacterial activity of filter 2 with tributyl groups is shown in Figure 2(B). At the largest area (237.5 cm^2), about 99% of bacterial cells were killed within 120 min of contact, and even at the smallest area (47.5 cm^2), 2 showed the activity. It is reasonable that the antibacterial activity of the surface-treated filters is directly proportional to the area. For 3 and 4, all the bacterial cells were killed within the shortest time (30 min) of contact when the bacterial cells were exposed to each filter, and 3 and 4 showed the highest activity against *S. aureus* even at the smallest area [Fig. 2(C, D)]. On the surface antibacterial activity of the nontreated filters, no reduction of the surviving
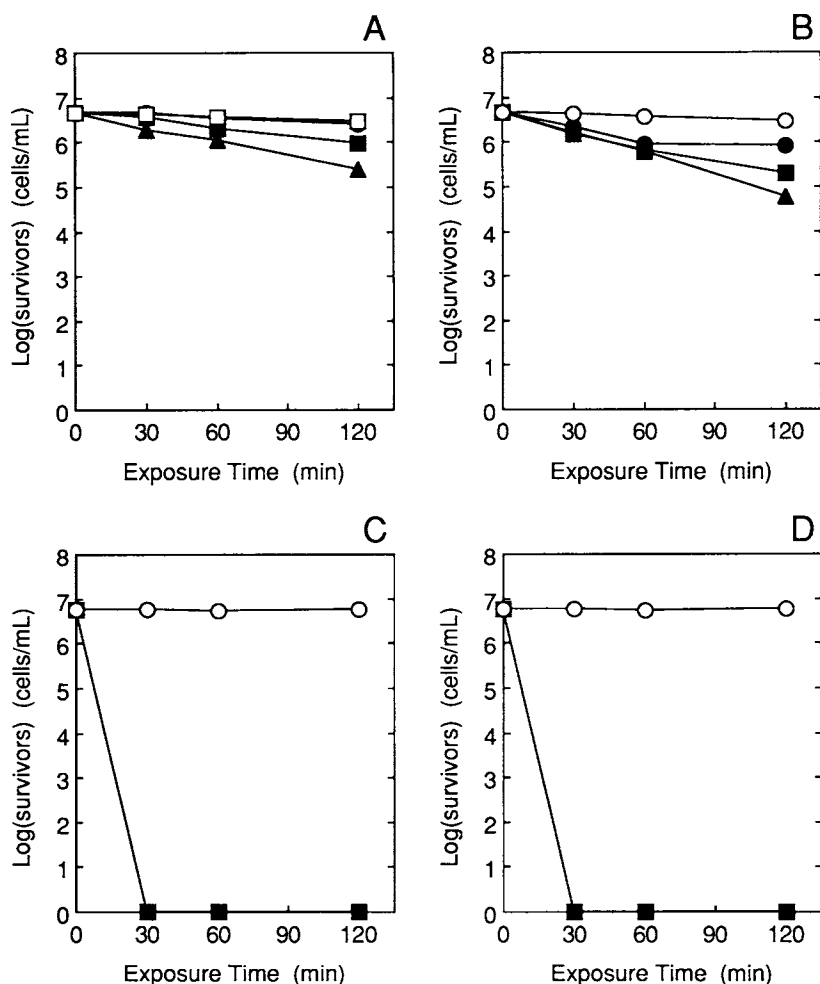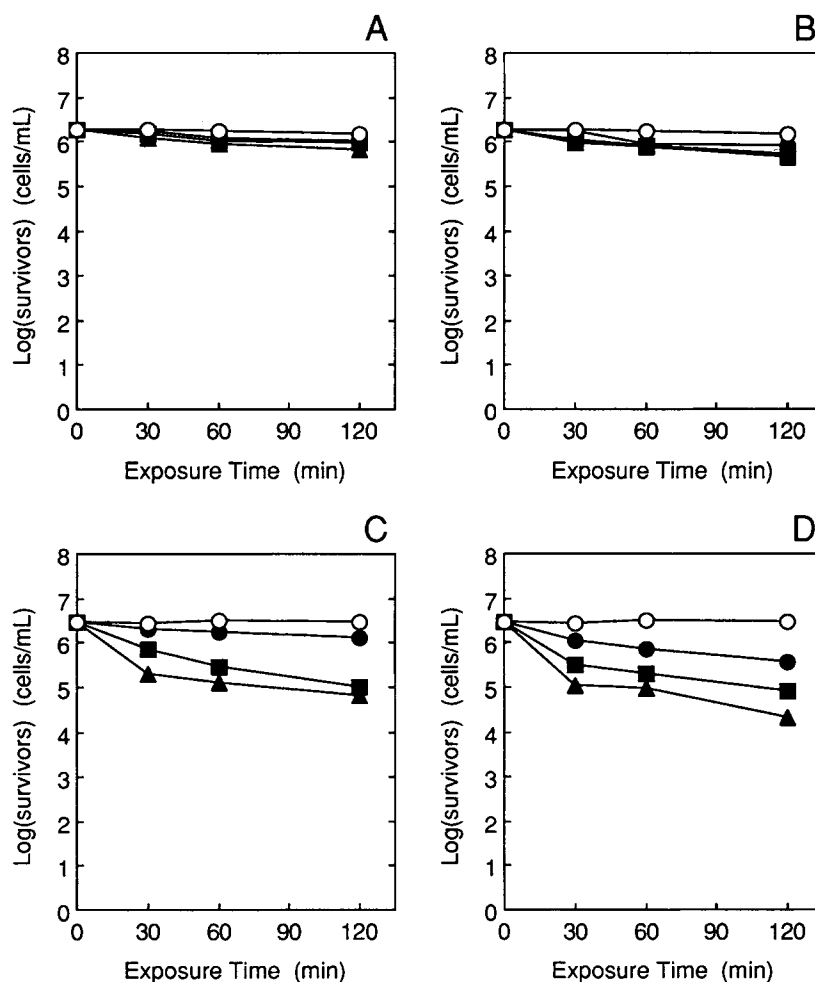

Figure 2   Plots of log (survivors) vs. exposure time for the filters incorporating phosphonium biocides with different substituents against *S. aureus*: (A) 1, (B) 2, (C) 3, (D) 4. Areas: (○) 237.5 cm^2 of nontreated sample (blank), (▲) 237.5 cm^2, (■) 142.5 cm^2, (●) 47.5 cm^2.

cells was observed; namely, the filters containing no phosphonium biocides were inactive. The high surface antibacterial activity of the surface-treated filters is not only of considerable scientific interest, but is of the greatest practical importance.

The surface antibacterial activity against *E. coli* is given in Figure 3. Filters 1 and 2 exhibited the activity to some extent [Fig. 3(A, B)]. With C_6 alkyl chains as substituents (filter 3), > 90% of the bacterial cells were killed within 120 min at the area of 237.5 cm^2 [Fig. 3(C)], but with the longest alkyl chains (C_8) as substituents > 99% of *E. coli* were killed [Fig. 3(D)]. The results indicate that the surface antibacterial activity of the surface-treated filters against *E. coli* is lower than against *S. aureus*. Thus, the filters used in this study may be advantageous to disinfection of Gram-positive strains over Gram-negative strains.

It is well-known that the cell envelopes (cytoplasmic membrane and cell wall) of bacteria are negatively charged at physiological pH as evidenced by the electrophoretic mobility measurements, which is ascribed to the dissociation of functional groups such as carboxylate and phosphate.[2] The surface negative charges of the bacterial cell play a significant role to produce the electrostatic interaction with positively charged surfaces of polymeric materials. The effect of negative charge density of the cell surfaces on the surface antibacterial activity was investigated for the crosslinked resin [poly(N-benzyl-4-vinylpyridinium salts)].[3] The surface negative charges of the bacterial cells were dependent on the strains; however, no correlation was recognized between the sensitivity and the surface charges of the cells. These results suggest that factors other than the surface charges of the cells may operate in

Figure 3 Plots of log (survivors) vs. exposure time for the filters incorporating phosphonium biocides with different substituents against *E. coli:* (A) 1, (B) 2, (C) 3, (D) 4. Areas: (○) 237.5 cm^2 of nontreated sample (blank), (▲) 237.5 cm^2, (■) 142.5 cm^2, (●) 47.5 cm^2.

the surface antibacterial activity of the polymeric materials incorporating cationic biocides. Another factor affecting the activity seems to be hydrophobicity of the cell surfaces, which is also strain-dependent.[3] Anyway, the surface antibacterial activity of the polymeric materials incorporating cationic biocides seems to be closely correlated to the properties of the bacterial cell surfaces.

Effect of Substituent on Surface Antibacterial Activity

It was found that the filters retaining C_2 (1) and C_4 (2) alkyl chains showed a lower activity, but those with C_6 (3) and C_8 (4) alkyl chains exhibited a high activity; namely, the alkyl chain length strongly affects the surface antibacterial activity. The adsorption of the cells to the filters is not only due to the electrostatic interaction between the positively charged filter surfaces and the negatively charged cell surfaces, but is also due to the hydrophobicity of substituents attached to hydrophilic parts of the phosphonium salts. Thus, the amphiphilic balance of the surfaces of immobilized biocides plays an important role in the ability of disinfection. In fact, systematic studies on the effect of the alkyl chain length were performed for crosslinked resins containing positively charged nitrogen atom, and it was revealed that the optimal chain length existed for the surface antibacterial activity.[4]

Capture of Bacterial Cells by Surface-Treated Filters

Ability of adsorption of the bacterial cells per unit area of the filters containing phosphonium biocides (cells/cm^2) is summarized in Table II. The ability of capture of filters in the unit area increased in the order of $1 < 2 < 3 < 4$, the order of increasing hydrophobicity of the substituent. In particular, filters

Table II Ability of Capture of Surface-Treated Filters

Sample	Substituent[a] (R)	Ability of Cell Capture (cells/cm^2)	
		S. aureus	E. coli
1	C_2H_5	3.76×10^5	1.07×10^5
2	C_4H_9	3.91×10^5	1.23×10^5
3	C_6H_{13}	$> 4.89 \times 10^5$	2.48×10^5
4	C_8H_{17}	$> 4.89 \times 10^5$	2.51×10^5

[a] See Figure 1.

with C_6 (3) and C_8 (4) alkyl chains showed a significant ability of adsorption for both Gram-positive and Gram-negative strains.

The mode of action is not well understood for the filters incorporating phosphonium biocides at the present stage of study. Many attempts have been conducted so far to provide substrates such as glass, fiber, and resin with surface antibacterial activity by incorporating quaternary ammonium salts covalently onto the surfaces of the substrates.[4-7] The bacterial cells captured at the surfaces of these materials seem to be alive; thus, the surfaces of these materials may not be bactericidal, but bacteriostatic. On the other hand, when phosphonium salts were covalently incorporated onto the surfaces of poly(propylene) by graft polymerization, shrinkage and deformation of bacterial cells were observed at the surfaces by scanning electron microscopy.[8] Therefore, the surfaces of the polymeric materials with phosphonium salts seem more bactericidal than bacteriostatic. Since the target site of free cationic disinfectants is a cytoplasmic membrane of bacteria, they must pass through a cell wall to reach their target site. At the present time, however, it is unclear whether the immobilized form of the biocides reaches their target sites, disorganizing the membrane structure, and causes the death of the cells. The cationic biocides may reach their target sites when they are immobilized through long, flexible spacers, as in the case of phosphonium ions immobilized by graft polymerization. When the cationic biocides are covalently attached to the surfaces with short spacers, it is unlikely that they penetrate through the cell walls of bacterial cells. The mode of action seems to be different.

Depression of biological activities of bacteria (*E. coli* and *Azotobacter agile*) and enzymes was observed when the cells were adsorbed on the materials.[9-11] The phenomena were interpreted in terms of depression of specific surface area of the cells because of adsorption and subsequent interference with substrate transport. Furthermore, in the case of *E. coli* adsorbed on a resin, morphological changes of the cells were observed.[9] The bacterial cells are generally insensitive to external stimulation because of the presence of the rigid layer (cell walls). If bacterial cells suffer from the partial breakdown of the cell walls resulting from the adsorption, it may be lethal. Therefore, the fate of the bacteria adsorbed on the immobilized surfaces of biocides may be affected by strength of adsorption. The higher surface antibacterial activity of the polymeric materials incorporating phosphonium biocides may originate from strong interaction with the cell surfaces and, consequently, lysis.

CONCLUSION

Filters incorporating phosphonium biocides are quite effective, particularly against Gram-positive bacteria. Effect of substituent was observed in which the surface antibacterial activity of the surface-treated filters increased with increasing hydrophobicity of the substituents. The filters incorporating phosphonium biocides can be used to sterilize media such as air and water, including microorganisms.

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