Antibacterial Activity and Characteristics of Modified Ferrite Powder Coated with a Gemini Pyridinium Salt Molecule

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This report describes the synthesis of an antibacterial material consisting of a gemini quaternary ammonium salt (gemini-QUAT) immobilized on ferrite powder, and its antibacterial activity. A gemini-QUAT containing two pyridinium residues per molecule, 4,4'-[1,3-(2,2-dihydroxymethyl-1,3-dithiapropane)]bis (1-octylpyridinium bromide), was immobilized on ferrite powder by a reaction between the hydroxyl group of the QUAT and trimethoxysilane. Immobilization of the gemini-QUAT on ferrite (F-gemini-QUAT) was confirmed when the dye, bromophenol blue, was released from F-gemini-QUAT-dye after contact between ferrite and the dye. Elemental analysis of the QUAT-ferrite determined the molar amount of QUAT on the ferrite. The antibacterial effect of the ferrite was investigated using a batch treatment system, and this effect was compared with that of another QUAT-ferrite (F-mono-QUAT) binding a mono-QUAT, which possesses one pyridinium residue, prepared by the same immobilization method as F-gemini-QUAT. Results indicated the F-gemini QUAT possessed a higher bactericidal potency and broader antibacterial spectrum compared to F-mono-QUAT. In addition, this study suggested that gemini-QUATs possessed high bactericidal potency without being influenced by immobilization to materials, and the antibacterial activity and characteristics of F-gemini-QUAT could be attributed to the unique structure of the immobilized gemini-QUAT.

Key words: Gemini pyridinium salt/Immobilization/Ferrite powder/Antibacterial activity/Antibacterial characteristic.

INTRODUCTION

Some antibacterial agents that are immobilized through a covalent chemical bond have been investigated and their antibacterial characteristics have been described. Quaternary ammonium salts (QUATs) bound to glass surfaces through silyl ether linkages act as a biocide to prevent microbial growth on those surfaces (Isquith et al., 1972). QUATs have been utilized extensively as immobilized biocides in food and health care applications because they provide wide antimicrobial activity and possess relatively low toxicity. In addition, QUATs are easily immobilized through a covalent bond between a terminal group and the surface of a material such as silicon rubber (Gottenbos et al., 2002), silica powder (Suhara et al., 1996), and cellulose fabric (Abel et al., 2002). Other polymers or textiles with a surface coated covalently by N-alkylated poly (4-vinylpyridine) (Tiller et al., 2002) or N-quaternarized polyethyleneimine (Lin et al., 2003) also have been...
investigated for antimicrobial activity. Treatment of these surfaces with QUATs produces materials that exhibit effective antibacterial activity.

Gemini quaternary ammonium compounds (gemini-QUATs) involve two groups of tertiary amine-quaternarized compounds (mono-QUATs) linked with hydrocarbon chains. The gemini-QUATs possess greater surface activity (Rosen et al., 1999) and antimicrobial potency (Diz et al., 1994; Menger and Keiper, 2000) compared to conventional mono-QUATs. The syntheses of gemini-QUATs, consisting of two cationic pyridinium salts, have been reported and their antimicrobial characteristics have been described (Kourai et al., 2006; Maeda et al., 1998 and 1999; Okazaki et al., 1997; Shirai et al., 2005 and 2006). These reports prove that the antimicrobial activity of gemini-QUATs is more potent than that of mono-QUATs against both Gram-negative and -positive bacteria and fungi. However, few studies have examined the antibacterial activity of materials modified with gemini-QUATs chemically bound to the surface. Gemini-QUATs were noncovalently bound to wool fabric by soaking the fabric in an aqueous solution of QUATs and heating it at 40°C for 1 h (Infante et al., 1996). Gemini-QUATs were also attached noncovalently to an amorphous silica surface by adsorption and heat-drying (Zaporozhets et al., 2000).

Here, we developed a novel gemini QUAT-immobilized material that possessed significant antibacterial activity due to the structural characteristics of the immobilized QUAT of gemini- or mono-structure. 4,4'-(1,3-(2,2-Dihydroxymethyl-1,3-dithiapropane)]bis(1-octylpyridinium bromide) (4HTBP-8), possessing two hydroxyl groups, was synthesized as described in the Japanese patent (Kourai, H, Nihon Funen Co. Ltd., Otsuka Chemical Holdings Co. Ltd., and Kataoka, D., Koukai Tokkyo Kohou, No. 2003-267953), and immobilized through a silyl ether bond (using trimethoxysilane) to a carrier. In the patent, this compound has been reported to possess strong antimicrobial activity against bacteria and fungi after suspension in distilled water. The activity of immobilized 4HTBP-8 was compared to that of the immobilized mono-QUAT 4-[1-(10-hydroxy-1-thiadecamethylene)]-1-octylpyridinium bromide (4HDP-8), which consists of one thio-pyridinium moiety corresponding to a half-structure of 4HTBP-8.

For this study, magnetic ferrite powder, Fe₃O₄, was selected as the carrier to immobilize these biocides. Many bioactive substances such as proteins (Koneracka et al., 2002), enzymes (Horst et al., 2006), and biocides (Lin et al., 2002) have been bound to magnetic particles without losing their bioactivity. The linking of bovine serum albumin, glucose oxidase, chymotripsin, streptokinase and dispase, directly to magnetic particles using 1-[3-(dimethylamino)propyl]-3-ethylcarbodimide hydrochloride as the coupling agent has been proposed (Koneracka et al., 2002). The binding is due to the presence of hydroxyl groups on the surface of the magnetic particles from Fe₃O₄. The use of magnetic powder as a support has certain advantages. For example, the materials can be easily separated from an aqueous system by using a magnetic field, a large surface area can be modified with the biocides, and there is a high dispersion efficiency due to the minute size of particles smaller than 1 μm. The antibacterial activity of the immobilized materials is related to the surface area and dispersion potency because the activity depends on the contact between the material's surface and the bacterial cell (Nakagawa et al., 1984b).

**MATERIALS AND METHODS**

**Materials**

Ferrite powder, consisting of minute particles smaller than 1 μm, was purchased from Kishida Chemical Co., Ltd., Japan (Fig. 1). Chemicals for the syntheses of QUATs and immobilization reactions on the ferrite powder were of commercial reagent grade and used without further purification. Materials used in biological experiments were obtained from commercial sources.

**Purity and structure of synthesized QUATs**

The purity of the synthesized compounds was examined by reversed phase thin-layer chromatography (TLC) (Merck RP-18 F₂₅₄₅ plate, thickness 0.25 mm,

**FIG. 1.** Scanning electron micrograph of ferrite powder. The white bar represents 2 μm.
Merck Japan Ltd., Japan). Elemental analysis was performed using a Yanagimoto MT-5 elemental analysis apparatus (Japan) and melting points were obtained with a trace amount melting point instrument (Mitamura Riken Kogyo Inc., Japan). 'H- and 'C-NMR spectra were obtained on a JEOL 400 MHz NMR spectrometer using CD3OD and tetramethylsilane as an internal standard.

**Syntheses of QUATs**

A gemini-QUAT, 4HTBP-8 was prepared as described in a Japanese patent (Kourai H, Nihon Funen Co. Ltd., Otsuka Chemical Holdings Co. Ltd., and Kataoka D., Koukai Tokkyo Kohou, No. 2003-267953), except that the quaternarization was conducted using 1-octyl bromide as shown in Fig. 2a. 4-Mercaptopyridine (0.2 mol) and 2,2-bis (bromomethyl)-1,3-propanediol (0.1 mol) in 200 ml of ethanol were refluxed for 24 h under nitrogen stream. Following the removal of the solvent with an evaporator and desalting of the residue with 350 ml of 0.56 mol/l NaOH, the precipitate was recrystallized from ethanol-water. The gemini-QUAT was synthesized by reaction of the crystals (0.01 mol) and 1-octyl bromide (0.02 mol) in 10 ml of absolute ethanol at 80 °C for 3 days under 80 MPa in a high-pressure reactor (YHP-92, Yamashita Giken, Japan). The crude white solid was precipitated from diethyl ether and recrystallized at least three times from an ethanol-tetrahydrofuran mixture until the purity of the compound was confirmed through TLC. The product, a white solid, was further characterized through NMR spectroscopy and elemental analysis.

4HDP-8 as a mono-QUAT was synthesized according to the following procedure (Fig. 2b). A mixture of 4-mercaptopyridine (0.1 mol) and 10-bromo-1-decanol (0.1 mol) was heated at 50 °C in acetone-methanol (400 ml-25 ml) for 9 h under a stream of nitrogen. A solid product, obtained by cooling and filtration, was desalted with 350 ml of 0.28 mol/l NaOH, and then purified by recrystallization from ethanol-water. Quaternization of the crystal (0.02 mol) was achieved by treatment with 1-octyl bromide (0.02 mol) in 10 ml of anhydrous ethanol at 80 °C for 24 h under 80 MPa using a high-pressure reactor. The reaction product was precipitated from diethyl ether and recrystallized from ethanol-hexane, ethanol-water, and methanol-water to obtain the desired compound as a white solid.

**Silicoating of ferrite powder**

Ferrite powder was soaked in 1 mol/l nitric acid for 24 h for surface cleaning, and then was washed with deionized water until the pH of the wash water was weakly acidic (pH 6.5), followed by drying at 40 °C for 24 h under reduced pressure. The ferrite (20 g) was washed repeatedly with methanol using a vortex mixer, and then was treated with 2 × 10⁻² mol of trimethoxysilane (Tokyo Chemical Industry Co., Ltd., Japan) and 50 ml of 99.5% (v/v) methanol for 1 h at 60 °C under stirring. The silicoated ferrite (F-Si) was isolated by removing the solvents under reduced pressure and drying at 40 °C for 24 h under reduced pressure. After rinsing the F-Si with methanol, F-Si was dried at 40 °C for 24 h in vacuo to give pure F-Si.

**Immobilization of QUATs on ferrite**

For binding 4HTBP-8 to the F-Si, 0.5 g of 4HTBP-8 was placed in a mixture of 10 g of F-Si and 30 ml of methanol. The suspension was warmed in a water bath at 60 °C for 3 min, and then a five-fold molar excess of trimethoxysilane based on the QUAT was added, followed by 0.15 ml of deionized water. After 3 h-stirring at 60 °C, the solvent was removed under reduced pressure and the residue dried at 100 °C for 24 h in an air oven. To obtain the purified F-gemini-QUAT, the treated ferrite sample was rinsed with methanol repeatedly, and then dried at 40 °C for 24 h under reduced pressure. The F-mono-QUAT immobilizing 4HDP-8 was derived according to the same procedure, except that immobilization was conducted using 0.2 g of the mono-QUAT at 80 °C in ethanol.

**Observation of QUAT immobilization on ferrite**

Samples of F-gemini-QUAT and F-mono-QUAT (0.1 g each) were made hydrophilic by treatment with 0.2 ml of ethanol in a glass test tube. Then, 5 ml of 50 × 10⁻³ mol/l sodium-potassium phosphate buffer (pH 7.0) containing 100 × 10⁻³ mol/l bromophenol
blue (BPB, Kanto Chemical Co., Inc.) was added. The mixtures were shaken at 120 strokes/min for 1 h at room temperature. The supernatant had no detectable BPB by optical density at 595.5 nm after thoroughly washing the ferrite material-BPB adducts with ethanol using a vortex mixer. The precipitate was dried under reduced pressure at 40°C, and then 5 ml of 10% (w/v) NaCl prepared with 50% (v/v) methanol was added and the mixture sonicated for 15 min at room temperature. The color of the supernatant was observed by visual inspection.

**Quantity of QUATs immobilized on ferrite**

The molar amount of QUATs immobilized on ferrite was calculated from a 50-mg sample of F-gemini-QUAT and F-mono-QUAT using the amount of nitrogen determined by elemental analysis. The amount of nitrogen also was measured in F-Si as a control.

**Microbes**

Microorganisms maintained in our laboratories, Klebsiella pneumoniae ATCC 4352, Proteus mirabilis IFO 3849, Pseudomonas aeruginosa ATCC 27583, Bacillus cereus IFO 3001, and Staphylococcus aureus IFO 12732, were used for the antibacterial tests. Except when noted, Escherichia coli IFO 12713 was used in the experiments.

**Cultivation and preparation of microbes**

Cultivation of the bacteria was performed as described previously (Okazaki et al., 1997). Each strain of the bacteria was inoculated in 5 ml of L-broth [tryptone 1% (w/v), yeast extract 0.5% (w/v), NaCl 0.5% (w/v), pH 7.2], and incubated at 37°C for 18 h. The bacterial suspension was harvested by centrifugation at 5000 x g for 10 min at 4°C, washed with sterilized physiological saline twice, and then resuspended in sterilized tap water.

**Antibacterial tests for immobilized-ferrite**

To determine the antibacterial activity of ferrite powders derivatized with gemini-QUAT and mono-QUAT, a batch treatment system was utilized (Nakagawa et al., 1984a). Mixtures of the two QUAT-ferrites (200 mg) were treated with 50 µl 80% (v/v) ethanol in a 100 ml flask to render their surfaces hydrophilic, followed by the addition of 10 ml sterilized tap water and incubation for 5 min at a predetermined temperature (10, 20, 30 or 40°C) with the addition of bacterial suspension (final concentration of bacteria 10^4 to 10^6 cfu/ml). After shaking for a predetermined time at 150 strokes/min, a 1 ml portion of the mixture was placed in a test tube. The remaining 0.5 ml portion of the suspension was separated from the ferrites by placing the tube on a permanent magnet for 30 seconds, followed by stepwise dilution with Soybean-Casein Digest Broth with Lecithin and Polysorbate 80 (SCDLP broth, Nihon Pharmaceutical Co. Ltd., Japan). 0.1 ml portions of the original suspension and the dilution series were spread on a SCDLP agar (Nihon Pharmaceutical Co. Ltd., Japan) plate, and the plates incubated at 37°C for 24 h to determine bacterial growth.

All measurements of bacterial growth were performed in triplicate; error bars indicate the S.D. from the mean values obtained.

**Scanning electron microscopy**

A suspension of E. coli was treated with the two immobilized-ferrites and F-Si at 30°C for 10 min with mild shaking, and then the solution was discarded by decantation. The solids were resuspended in physiological saline, followed by filtration through a polycarbonate membrane (pore size, 0.2 µm; membrane diameter, 25 mm; Whatman) coated with 0.1% (w/w) [3-(trimethoxysilyl)propyl]octadecyldimethylammonium chloride [72% (w/w) in methanol, Aldrich Chemical Co., Inc.]. The retentates were fixed chemically with 3% (w/w) glutaraldehyde [70% (w/w) solution, Wako Pure Chemical Industries, Ltd., Japan] and then dehydrated stepwise with ethanol for 1 h in the following order: 30, 50, 70, 80, 90, 95 and 100% (v/v). The solids obtained were dried further in a critical point dryer (HCP-2, Hitachi, Japan). The surface of the solids was coated with gold using an ion sputtering device (E-1020, Hitachi, Japan), followed by observation by scanning electron microscopy (SEM, S-4700, Hitachi, Japan).

**RESULTS**

**Syntheses of QUATs**

The data of elemental analysis, melting point, and yield for 4HTBP-8 as a gemini-QUAT and 4HDP-8 as a mono-QUAT are shown in Table 1, followed a summary of the 1H- and 13C-NMR spectra of the two compounds in Table 2. Elemental analysis and NMR spectroscopy data agreed well with the desired compound.

**Characteristics of antibacterial immobilized-ferrites**

Adsorption of BPB molecules to pyridinium cations through an ionic bond following contact between QUAT-immobilized ferrite and BPB dyestuff resulted in an adduct of immobilized-ferrite and BPB. A release of BPB molecules was confirmed by adding a
TABLE 1. Elemental analysis, m.p. and yield for synthesized 4HTBP-8 and 4HDP-8

<table>
<thead>
<tr>
<th>QUATs</th>
<th>Elemental analysis (%)</th>
<th>m.p. (°C)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calc.</td>
<td>Found</td>
<td>Calc.</td>
</tr>
<tr>
<td>4HTBP-8</td>
<td>52.54</td>
<td>52.35</td>
<td>7.40</td>
</tr>
<tr>
<td>4HDP-8</td>
<td>59.98</td>
<td>59.76</td>
<td>9.19</td>
</tr>
</tbody>
</table>

TABLE 2. Data of ¹H- and ¹³C-NMR spectroscopic analyses of synthesized 4HTBP-8 and 4HDP-8

<table>
<thead>
<tr>
<th>QUATs</th>
<th>¹H-NMR (400 MHz, CD₂OD, Me,Si)</th>
<th>¹³C-NMR (100 MHz, CD₂OD, Me,Si)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(6 H, t [J = 7.0 Hz], two CH₃)</td>
<td>(6 H, t [J = 7.3 Hz], two py)</td>
</tr>
<tr>
<td></td>
<td>1.30-1.37 [20 H, m, octyl group]</td>
<td>1.28 [4 H, m, octyl group]</td>
</tr>
<tr>
<td></td>
<td>two CH₂)</td>
<td>1.96-1.98 [4 H, m, octyl]</td>
</tr>
<tr>
<td>4HTBP-8</td>
<td>14.4, 23.7, 27.2, group-two CH₃</td>
<td>1.65 [H, t [J = 6.6 Hz], CH₃]</td>
</tr>
<tr>
<td></td>
<td>3.50 (4 H, 30.1, 30.2, 32.2, 35.0, 46.7, two CH₂C)</td>
<td>14.4, 23.7, 27.2, group-two CH₃</td>
</tr>
<tr>
<td></td>
<td>4.45 (4 H, t [J = 6.4 Hz], two CH₂C)</td>
<td>3.50 (4 H, 30.1, 30.2, 32.2, 35.0, 46.7, two CH₂C)</td>
</tr>
<tr>
<td>4HDP-8</td>
<td>7.99 (4 H, d [J = 7.3 Hz], two py</td>
<td>1.65 [H, t [J = 6.6 Hz], CH₃]</td>
</tr>
<tr>
<td></td>
<td>β-CH)</td>
<td>8.62 [4 H, d [J = 7.3 Hz], two py α-CH]</td>
</tr>
</tbody>
</table>

NaCl solution to the adducts. These results demonstrated that modified-ferrites can be coated with cationic QUATs. The calculation of amount of QUATs on the two ferrites was based on nitrogen elemental analysis, because all nitrogen is from immobilized QUATs. The resulting nitrogen ratios of immobilized-ferrites were 0.033% for F-gemini-QUAT and F-mono-QUAT, and 0% for F-Si (Table 3). Thus, the data indicate that both immobilized-ferrites contain 24× 10⁻⁶ mol nitrogen per gram ferrite, and the molar amounts of QUATs corresponding to 1 g of the F-gemini-QUAT and F-mono-QUAT are 12 and 24× 10⁻⁶ mol, respectively.

Effect of temperature on antibacterial activity

Figures 3a and b show antibacterial activity after treatment to an initial cell concentration of 1× 10⁸ cfu/ml with F-gemini-QUAT, F-mono-QUAT, and F-Si as a control at predetermined temperatures (10, 20, 30, and 40°C). For the control, the bacterial survival was 10⁵ cfu/ml after 2h-treatment of F-Si at 30°C (Fig. 3). At 10, 20, and 40°C, the change in bacterial survival was the same as that at 30°C (data not shown). F-Si did not reveal any bactericidal effect (i.e., no decrease in viable bacteria after over a 2-h period was observed). F-gemini-QUAT decreased the number of viable cells in the suspension to below 10² cfu/ml with a contact time of 0.5 h at temperatures of 30 and 40°C (Fig. 3). The rapid bactericidal rate of the QUAT-ferrite was lost upon a lowering in temperature from 30 to 10°C; however, the viable cell numbers at 20 and 10°C decreased below 10² cfu/ml upon treatment for 1 h and 2 h, respectively. Figure 3b shows that such a decrease in bacterial survival was not observed in the case of treating the cell sus-

![Figure 3a](image_url)

![Figure 3b](image_url)

FIG. 3. Effect of temperature on the antibacterial activity of F-gemini-QUAT (a) and F-mono-QUAT (b). Survival data represent surviving bacterial cells in suspension treated with the immobilized ferrites. Bactericidal temperatures: 10°C (diamonds), 20°C (triangles), 30°C (squares), and 40°C (circles). The cross indicates the surviving cells after treatment with F-Si as a control at 30°C. A log of surviving cells less than 1 indicates the survival of fewer than 10³ cfu/ml.
pension with F-mono-QUAT at 10 and 20 °C. Acceleration of bactericidal activity, which accompanied an increase in temperature from 20 to 40°C, indicated a reduction in bacterial survival from five to three orders of magnitude.

Effect of initial cell concentration

Cell suspensions (10⁴-10⁹ cfu/ml) were treated with F-gemini-QUAT and F-mono-QUAT, and bacterial survival after 2 h of treatment was plotted against the initial cell concentration as shown in Fig. 4. F-gemini-QUAT decreased the viable cell number of lower than 10⁷ cfu/ml to a level less than 10 cfu/ml.

SEM investigation of bacterial cells

SEM confirmed the antibacterial action of the two QUAT-modified ferrites against E. coli. Figure 5a shows intact bacterial cells treated with F-Si. Figures 5b and c show micrographs of bacterial cells incubated with immobilized ferrite, F-gemini-QUAT, and F-mono-QUAT. In contrast to F-Si, more damaged cells were observed after the contact between F-gemini-QUAT and intact cells, the surface of which was distinctly uneven, compared to cell surfaces treated with F-Si (Fig. 5b). In addition, intracellular materials appeared to leak from the surface. In contrast, the cell surface in case of the addition of F-mono-QUAT to intact cells was destroyed scarcely, compared with the damage to cells incubated with F-gemini QUAT (Fig. 5c).

Antibacterial spectrum

The activity of F-gemini-QUAT and F-mono-QUAT was tested against other bacteria, both four Gram-negative and two Gram-positive bacteria at an initial cell concentration of 1 × 10⁵ cfu/ml. Table 4 summarizes the data for bacterial survival after 0.5 h contact. F-gemini-QUAT decreased cell survival to a level less than 100 cfu/ml for both Gram-negative and positive bacteria, with the exception of Pr. mirabilis that de-
FERRITE POWDER HAVING GEMINI-PYRIDINIUM SALT

TABLE 4. Antibacterial spectra of F-gemini-QUAT and F-mono-QUAT

<table>
<thead>
<tr>
<th>Strain</th>
<th>Log survivor (cells/ml)*</th>
<th>F-gemini-QUAT</th>
<th>F-mono-QUAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>&lt;1</td>
<td>4.71 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>&lt;1</td>
<td>2.27 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>Pr. mirabilis</td>
<td>3.19 ± 0.42</td>
<td>3.43 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>1.16 ± 0.23</td>
<td>4.44 ± 0.043</td>
<td></td>
</tr>
<tr>
<td>B. cereus</td>
<td>1.49 ± 0.21</td>
<td>1.29 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>&lt;1</td>
<td>1.68 ± 0.40</td>
<td></td>
</tr>
</tbody>
</table>

* Survivors indicate cell concentrations after treating a cell suspension of 1 × 10^5 cfu/ml for 0.5 h at 30°C with shaking. Values, mean ± S.D.

creased to 10^3 cfu/ml. Two types of Gram-positive bacteria were killed by F-mono-QUAT to a level less than 100 cfu/ml, while the ferrite did not decrease the viable cell numbers of Gram-negative bacteria, particularly E. coli and Ps. aeruginosa, as well as two Gram-positive bacteria.

DISCUSSION

This study demonstrated that 4HTBP-8 as a gemini-QUAT and 4HDP-8 as a mono-QUAT could be successfully immobilized on magnetic ferrite powder, and F-gemini-QUAT possessed greater bactericidal activity than did F-mono-QUAT.

Immobilization of QUATs on ferrite particles was achieved through a reaction involving trimethoxysilane. This was confirmed by the observation of a release of BPB dye from immobilized-ferrite-BPB adducts. The molar amount of QUAT immobilized on ferrite was determined by elemental analysis.

We investigated the effect of temperature on antibacterial activity of F-gemini-QUAT and F-mono-QUAT. For these experiments, both QUAT-ferrites were prepared with equal amounts of ammonium cation, because the bactericidal action of QUATs is attributable to the hydrophilic ammonium head and the long alkyl chain as the hydrophobic group. The F-gemini-QUAT rapidly decreased viable cell numbers in a temperature range of 10 - 40°C, in contrast to the slow action of F-mono-QUAT at those temperatures, when the molar amount of ammonium residue was equal for the two immobilized-ferrites (Figs. 3a and b). These results confirmed that F-gemini-QUAT possesses antibacterial properties of gemini-QUATs dissolved in water, exhibiting higher activity than mono-QUATs (Kourai et al., 2006; Maeda et al., 1999; Okazaki et al., 1997). In particular, at low temperatures (10 and 20°C), F-gemini-QUATs effectively decreased viable cells in contrast to F-mono-QUAT at those temperatures, although the bactericidal rate was slower than that at 30 and 40°C. In general, incubation temperatures are closely related to the fluidity of the bacterial cell membrane, which influences the bactericidal activity of mono-QUATs (i.e., an increase in temperature tends to strengthen the activity) (Sumitomo et al., 2003). Therefore, the change in bactericidal rate was dependent on a change in fluidity due to temperature.

The high antibacterial activity of F-gemini-QUAT could also be seen in the relation between the numbers of initial treated cells and surviving cells. As shown in Fig. 4, F-gemini-QUAT caused a greater decrease in the number of surviving cells than did F-mono-QUAT, suggesting that immobilization of gemini-QUAT on the support is effective in causing the death of more viable cells than that of mono-QUAT, even though the bactericidal site of the immobilized-ferrites is the quaternary pyridinium salt in both cases.

The killing of bacterial cells by gemini-QUATs is possibly caused by the outflow of magnesium ion and ATP from the cells following the adsorption of QUATs on the bacterial surface, resulting in the physical disruption of the membrane rather than interference with a metabolic pathway (Maeda et al., 1998; Sumitomo et al., 2004). The high activity of F-gemini-QUAT also could be observed in the SEM pictures of bacterial cells destroyed after treatment with F-gemini-QUAT, similar to the surface of bacterial cells incubated with a gemini-QUAT as described in previous reports.

The antibacterial activity of the two immobilized QUAT-ferrites demonstrated that F-gemini-QUAT possessed activity against a broader spectrum of Gram-negative and -positive bacteria compared to F-mono-QUAT (Table 4). This result was consistent with the characteristics of gemini-QUAT, 4HTBP-8 reported in the Japanese patent (Kourai, H, Nihon Funen Co. Ltd., Otsuka Chemical Holdings Co. Ltd., and Kataoka, D., Koukai Tokkyo Kohou, No. 2003-26793), demonstrating high and broad antibacterial activity toward both Gram-negative and -positive bacteria. However, the low activity of F-gemini-QUAT against Pr. mirabilis compared with other strains was probably due to bacterial cell surface hydrophobicity; bacteria with low hydrophobicity are less susceptible to QUATs (Maeda et al., 1998).

These results indicate that F-gemini-QUAT possesses a much higher antibacterial activity compared to F-mono-QUAT, even when the molar content of nitrogen (indicating molar content of the n-octylpyridinium group) on the two immobilized
materials is identical. Thus, the high antibacterial activity of gemini-QUATs is preserved regardless of immobilization, and the characteristics of the immobilized gemini-QUATs are dependent on the specific gemini-structure. In particular, higher activity is presumed to be due to the distance between the pyridinium heads. At the air-water interface, a change in the length of spacer chain cross-linking two pyridinium moieties for gemini-QUATs influences surface tension and molecular area (Alami et al., 1993; Shirai et al., 2005), and the change affects biological activity (Shirai et al., 2005 and 2006). Consequently, the distance between the pyridinium groups of gemini-QUATs is assumed to be attributable to the spacer chain-length, so that the distance appears to be an important factor for antibacterial activity. It is suggested that the immobilized gemini-QUAT 4HTBP-8, possessing two pyridinium moieties cross-linked with dithiopropylene group, maintains a constant distance between the pyridinium heads, and the suitable distance for the gemini-QUAT to adsorb to and interact with the bacterial surface must give a high antibacterial activity to F-gemini-QUAT. In contrast, mono-QUAT contains only one pyridinium residue, and so the distance of the pyridinium moiety between the mono-QUAT molecules is not uniform, resulting from the random immobilization on the ferrous surface. Therefore, it is implied that the bacterial activity of gemini-QUATs is most heavily influenced by the distance between the pyridinium heads as described in the previous reports, considering that the molar content of the n-octylpyridinium group was identical for the two QUAT-ferrites, and so the distance of the pyridinium moiety resulting from the random immobilization on the ferrite surface. Consequently, the phosphorylating and disulfide bond between the mono-QUAT molecules is not uniform, and the characteristics of the immobilized gemini-QUATs are dependent on the specific gemini-structure.

REFERENCES


