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Polycationic Biocides with Pendant Active Groups: Molecular Weight Dependence of Antibacterial Activity

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Two types of polycations with pendant active groups were synthesized: one is polymethacrylate containing pendant biguanide units, and the other is poly(vinylbenzyl ammonium chloride). The two polycations were found to exhibit higher bactericidal activity against *Staphylococcus aureus* than the corresponding monomers. Fractionation of the polycations was successfully performed on gel filtration chromatography, and examination of the antibacterial activity against *S. aureus* of the well-characterized polymer samples with various molecular weights (MW) revealed that the activity was strongly dependent on the MW of the polycations and that there existed an optimal MW range for the cidal action of the polymeric biocides. Experiments on the lysis of protoplasts of *Bacillus subtilis* in contact with the polycations have shown that the target sites of the polycationic biocides are cytoplasmic membranes of bacteria.

In previous papers, we reported that in a clean system where there are no interfering materials such as negatively charged macromolecules, polycations having biguanide units or quaternary ammonium salts exhibit much higher antibacterial activity than the monomeric analogs (4, 5). In view of the fact that many properties of polymers depend strongly on their molecular weights (MW) and MW distributions, it is reasonable to expect that the antibacterial activity of such polycations is MW dependent. In fact, in the oligomers of in-chain quaternary ammonium salts ranging from monomer to tetramer, a strong MW dependence has been observed (T. Ikeda, H. Yamaguchi, and S. Tazuke, submitted for publication).

In this paper, we describe the preparation of well-characterized polycationic samples with various MW and narrow MW distributions. In addition, we report on the effect of MW of the polycations on bactericidal activity, focusing our attention on the high-MW polymers. Various physiological events were observed in intact cells and protoplasts when they were exposed to various polycations. The effects are discussed in terms of the mode of action of these polycationic biocides.

MATERIALS AND METHODS

Preparation. The synthetic route for the methacrylate monomer with a pendant biguanide is shown in Fig. 1. 2-Hydroxyethylphenyldicyandiamide (compound 1) was prepared as reported previously (5). To prepare 4-(2-methacryloyloxyethyl)phenyldicyandiamide (compound 2), a solution of compound 1 (14 g, 0.07 mol) dissolved in tetrahydrofuran-water (10:1, 93 ml) was cooled in an ice bath; to this solution, freshly distilled methacryloyl chloride (52 ml, 0.54 mol) was added dropwise with stirring over a period of 2 h; and the reaction mixture was left overnight at room temperature. It was then poured into a large excess of water, and the precipitate was collected and dried under vacuum (yield, 90%). The product was recrystallized from 2-propanol. mp 181–182°C; NMR (dimethyl sulfoxide-d_6, δ) 1.88 (3H, s, —CH_3), 2.88 (2H, t, —CH_2—), 4.28 (2H, s, dicyandiamide protons), 7.28 (4H, s, aromatic protons), 9.00 (1H, s, dicyandiamide proton); IR (KBr, cm^{-1}), 2180 (CN). Elemental analysis calculated (%): C, 61.76; H, 5.88; N, 20.58; elemental analysis found (%): C, 61.36; H, 5.98; N, 19.68. To prepare N^1-4-(2-methacryloyloxyethyl)phenyl-N^5-4-chlorophenylbiguanide hydrochloride (compound 3), an equimolar mixture of compound 2 (11 g, 0.04 mol) and 4-chloro-aniline hydrochloride (6.6 g, 0.04 mol) in 2-propanol (30 ml) was refluxed in the presence of a trace amount of phenothiazine for 15 min. The precipitate formed on cooling was collected, washed with 2-propanol, and dried under vacuum (yield, 50%). The product was recrystallized from 2-propanol. mp 228–229°C; NMR (dimethyl sulfoxide-d_6, δ), 1.85 (3H, s, —CH_3), 2.89 (2H, t, —CH_2—), 4.27 (2H, t, —CH_2O—), 5.63, 5.68 (2H, s, vinyl protons), 7.24 (4H, s, aromatic protons), 7.35 (4H, s, aromatic protons), 7.0–7.9 (broad, biguanide protons), 10.1–10.5 (broad, biguanide protons). Elemental analysis calculated (%): C, 55.05; H, 5.28; N, 16.06; Cl, 16.28; elemental analysis found (%): C, 54.78; H, 5.13; N, 15.47; Cl, 16.63.

Polymerization of (compound 3) was carried out at 60°C in *N*,*N*-dimethylformamide as reported previously (5). Dimethylbutyl(4-vinylbenzyl)ammonium chloride (compound 4; Fig. 1) and its polymer (poly 4) were prepared by the method previously reported (4).

Fractionation of polymers. Fractionation of the polymers was performed by gel filtration chromatography. To explore the effect of MW of the polymers on antibacterial activity, polymer samples with various MW and a narrow MW distribution were required. First, we tried the conventional fractional-precipitation method for several solvent-nonsolvent combinations such as *N*,*N*-dimethylformamide–acetone and methanol-acetone. However, this conventional technique did not give well-separated fractions. Consequently, we adopted the gel filtration. However, very few examples have been reported so far of the fractionation of synthetic polyelectrolytes by this method. We tried many gel media based on polystyrene-divinylbenzene and dextran and found that in these media, adsorption is predominant. Effec-

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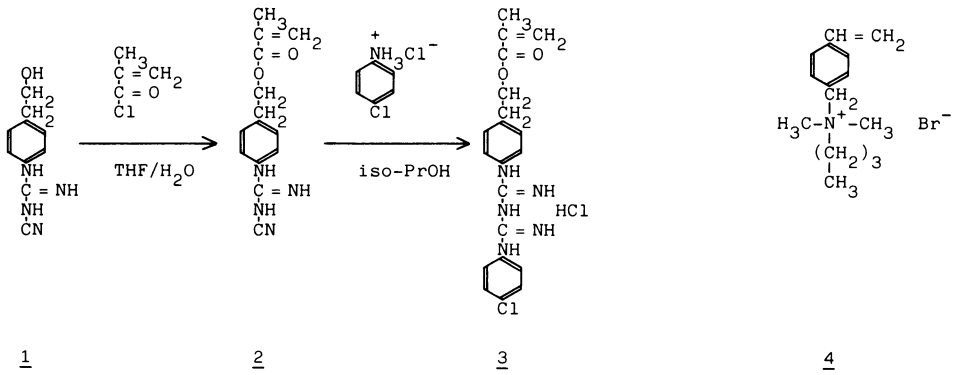

1 2 3 4

FIG. 1. Synthesis and structure of polycations with pendant active groups. For details, see Materials and Methods. THF, Tetrahydrofuran; iso-ProH, 2-propanol.

tive separation based on molecular size of the polycations was achieved only on Bio-Gel P gels.

Protoplast. Protoplasts of *Bacillus subtilis* were prepared as described previously (Ikeda et al., submitted). Lysis of the protoplasts was followed spectroscopically by measuring the amount of K^+ ions and of materials absorbing light at 260 nm that were released from the cells. To the protoplast suspension (~10^8 cells per ml) in hypertonic solution (0.5 M sucrose), the polycations, dissolved in the same solution, were added. After an appropriate time, the suspension was subjected to centrifugation (17,000 × *g*, 5 min). The amounts of K^+ ions and A_{260} in the supernatant were determined with a Varian AA-1000 atomic absorption spectrometer and a Shimazu UV-200S absorption spectrometer, respectively.

Measurements. The weight-average MW of the polycations were determined in methanol with a KMX-6 low-angle laser light scattering photometer. The details are reported in references 4 and 5. ^1H NMR spectra were recorded with a JEOL JNM-PM 60.

Antibacterial activity was evaluated by the viable cell-counting method already reported (5).

RESULTS AND DISCUSSION

Characterization of polycations. Figures 2 and 3 show gel filtration elution profiles of poly 3 and poly 4, respectively. Although poly 4 gave a normal MW distribution profile, i.e., a broad single peak, poly 3 gave two peaks. When the unfractionated poly 3 was subjected to ultrafiltration on a membrane filter (Millipore Corp.) with a pore size of 0.1 μm, the peak at around fraction 40 in Fig. 2 was greatly reduced. This fact indicates that this peak can be ascribed to a microgel. In preparative runs, some fractions were combined, and the fractionated polymer samples were recovered by removing the eluent under reduced pressure. A typical example of the procedure is shown in Fig. 3 and Table 1. In the fractionation of poly 3, the fractions corresponding to the first peak (microgels) were always combined.

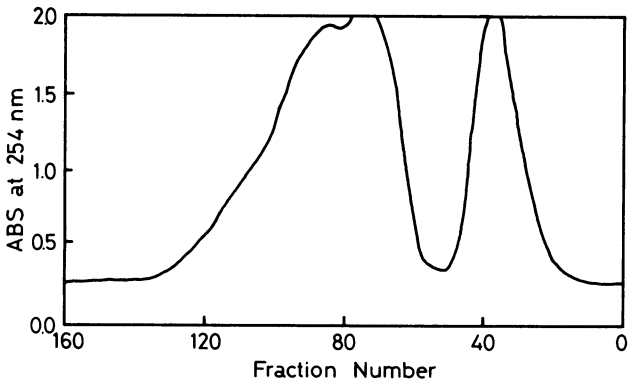

FIG. 2. Gel filtration elution profile of poly 3. Gel medium, Bio-Gel P-100; eluent, methanol-water (20:80, vol/vol); bed volume, 500 cm^3; flow rate, 0.2 ml/min; 6 ml per fraction; sample, 0.85 g. ABS, Absorbance.

Antibacterial activity. Typical plots of log(survivors) versus exposure time for the fractionated samples of poly 3 against *Staphylococcus aureus* at various concentrations are shown in Fig. 4. To evaluate quantitatively the effect of MW on antibacterial activity, log(% survivors) was plotted against MW of each fractionated sample (Fig. 5). The percentage of survivors was calculated by the following equation: 100 × (number of cells surviving at time *t*/initial number of cells).

The plots shown in Fig. 5 are those for 60-min exposures. Two series of plots for the polycations with different structures disclose an outstanding feature of MW dependence of the polycationic biocides. With respect to poly 3, the polymer samples with low MW as well as high MW exhibit lower bactericidal activity than those in the intermediate range; the optimal MW for cidal activity ranges between MW of 5 × 10^4 and 10 × 10^4. More interestingly, microgels of poly 3 (indicated by the black square in the figure) show some activity. Unfortunately, we were unable to prepare very high-MW samples of poly 4 because of its poor polymerizability. The highest MW that could be obtained was 77,000. With MW below 77,000, the bactericidal activity of poly 4 increases with increasing MW.

Release of cytoplasmic constituents. The amount of K^+ ions released from intact cells and protoplasts of *Bacillus subtilis* in contact with poly 4 and monomer 4 is shown in Table 2. It is clear from the table that the amount of K^+ ions released is higher in the fractionated samples than in the monomeric 4

TABLE 1. Fractionation of poly 4

Sample	Fractions combined	MW[a]
G1	44–64	32,300
G2	74–84	23,800
G3	94–104	16,100
G4	115–128	8,300

[a] Determined with a KMX-6 low-angle laser light-scattering photometer in methanol.

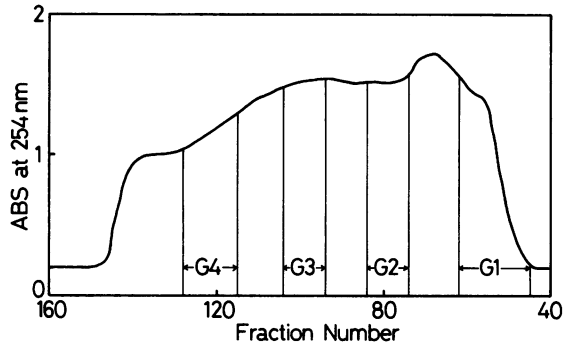

FIG. 3. Gel filtration elution profile of poly 4. Gel medium, Bio-Gel P-100; eluent, 1 M ammonium acetate buffer; bed volume, 2,000 cm^3; flow rate, 0.2 ml/min; 5 ml per fraction; sample, 2.0 g. A typical example for combining fractions is shown here. ABS, Absorbance. Abbreviations G1 through G4 indicate combined fractions used in this study. For details, see Table 1.

for both intact cells and protoplasts. However, no significant differences are seen among the fractionated polymer samples. This result is in sharp contrast to that obtained when intact cells and protoplasts of *B. subtilis* were exposed to oligomers of in-chain quaternary ammonium salts: the amount of the released K^+ ions increased in the order of monomer < tetramer < polymer (MW = 10,000) (Ikeda et al., submitted). It seems, therefore, that the K^+ ion loss depends on the MW of the organic cations only in the low-MW range.

Loss of the materials absorbing light at 260 nm (mainly

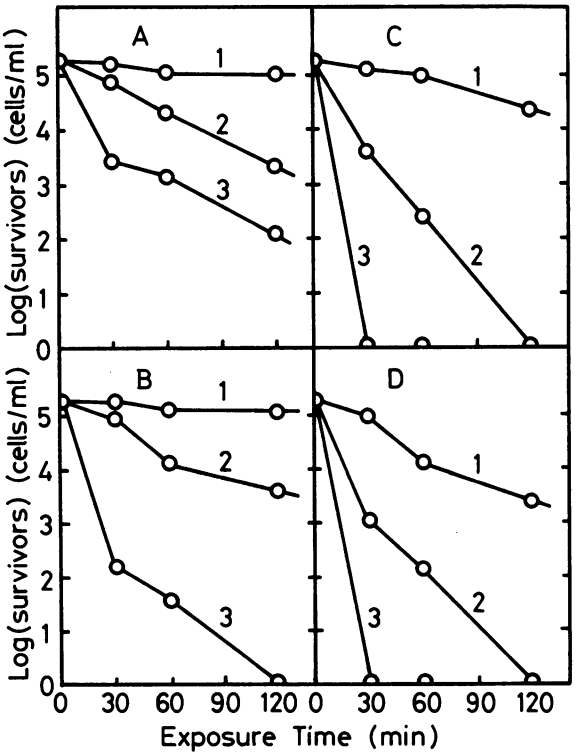

FIG. 4. Plots of log(survivors) versus exposure time for the fractionated samples of poly 3 against *S. aureus* at various concentrations. (A) Microgels; (B) $\bar{M}W = 1.3 \times 10^5$; (C) $MW = 1.2 \times 10^5$; (D) 4.2×10^4. Curve 1, 2.3 μM; curve 2, 23 μM; curve 3, 115 μM.

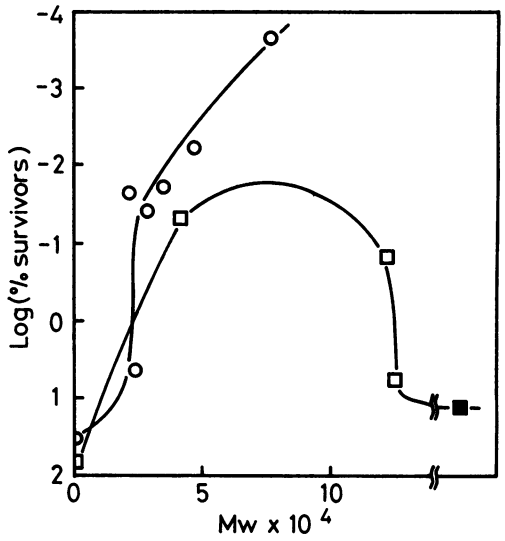

FIG. 5. Effect of MW on antibacterial activity against *S. aureus*. □, Poly 3, 23 μM; ○, poly 4, 2.0 μM; ■, plot for microgel of poly 3. The concentrations were calculated on the basis of monomer units.

DNA and RNA) may be a more direct measure of cell lysis. In Fig. 6 we show the amount of the materials absorbing light at 260 nm released from intact cells and protoplasts of *B. subtilis* in contact with 10 μg of a commercial cationic biocide, dimethyldodecylbenzyl ammonium chloride, per ml. In the absence of the biocide, we observed no release from the intact cells, but spontaneous leakage from the protoplasts. When the cells were exposed to the cation, the materials absorbing light at 260 nm were clearly released from both the intact cells and protoplasts, and the amount of the released materials was higher in the protoplasts. These results indicate that the loss of materials absorbing light at 260 nm can be used as a measure to evaluate the bactericidal activity of the cationic biocides. In the application of this method to poly 4, however, we found that the polycations formed insoluble complexes with the released materials. This result is not surprising, since DNA and RNA are

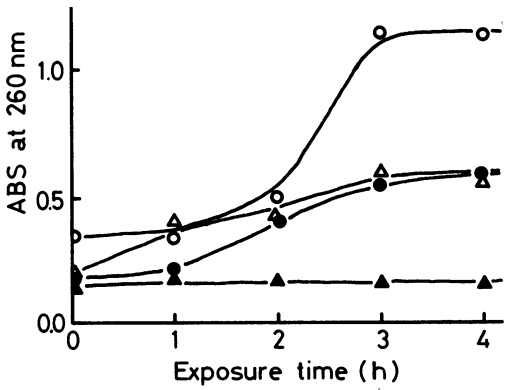

FIG. 6. Release of materials absorbing light at 260 nm from intact cells and protoplasts of *B. subtilis* in contact with dimethyldodecylbenzyl ammonium chloride (10 μg/ml) as a function of time. Release from protoplasts (○) or from intact cells (△). ● and ▲, Release from protoplasts and intact cells, respectively, in the absence of the cationic biocide. ABS, Absorbance.

TABLE 2. Amount of K^+ ions released from intact cells and protoplasts of *B. subtilis*[a]

Sample[b]	Amt of K^+[c] released from:	
	Intact cells	Protoplasts
G1	1.4	3.8
G2	1.4	3.8
G3	1.4	4.0
G4	1.3	4.1
4	1.0	1.0

[a] In contact with 100 μg of cations per ml for 60 min.
[b] Samples are those listed in Table 1.
[c] Values are normalized to those of monomer 4.

polyanions, and complexation between polycations and polyanions takes place quite easily.

We confirmed this behavior as follows. The cells of *B. subtilis* were destroyed by ultrasonication, and the cytoplasmic constituents were separated by ultracentrifugation. Insoluble complexes were formed when poly 4 was added to the supernatant containing the materials absorbing light at 260 nm. On the other hand, such insoluble complexes were not formed on the addition of dimethyldodecylbenzyl ammonium chloride to the same supernatant. The complex formation behavior was examined on the fractionated samples of poly 4. To the clear solutions containing the materials absorbing light at 260 nm, the fractionated samples were added (10 μg/ml), and after 90 min, the insoluble complexes were removed by ultracentrifugation, and the absorbance of the supernatant was measured. The absorbances thus measured were lower than the initial values (before addition) by 20%, but were nearly the same among the fractionated samples ranging in MW from 8,300 to 32,300. This result indicates that efficiency of the complex formation is rather insensitive to the MW of the polycations. This fact enabled us to determine reliably the amount of the released materials absorbing light at 260 nm in contact with the polycations having various MW. The actual amount released of the materials absorbing light at 260 nm can be calculated by the equation $(ABS_{obs} \times ABS_0)/ABS_{poly}$, where ABS_{obs} is the measured A_{260} of the supernatant which was obtained by ultracentrifugation after the cells were exposed to the polycations, ABS_0 is the absorbance of the initial solution containing the cytoplasmic constituents prepared by ultrasonication, and ABS_{poly} is the absorbance of the supernatant after the polycations were added to the clear solution and the insoluble part was removed. Figure 7 shows the amount of the materials absorbing light at 260 nm that was released from the intact cells and the protoplasts of *B. subtilis* thus estimated as a function of MW of the polycations. A bell-shaped release profile was obtained for the intact cells, while a monotonic increase was observed for the protoplasts. These results strongly suggest that the target sites of the polycationic biocides are cytoplasmic membranes and that exclusion at the cell walls operates for polymers with high MW.

Mode of action. At the present stage of study, the mode of action of cationic biocides with monomeric or dimeric forms may be summarized as follows (2): (i) adsorption onto the bacterial cell surface, (ii) diffusion through the cell wall, (iii) binding to the cytoplasmic membrane, (iv) disruption of the cytoplasmic membrane, (v) release of cytoplasmic constituents such as K^+, DNA, and RNA, and (vi) death of the cell. Since we observed the same physiological events as in process v, it is quite reasonable to assume that the mode of

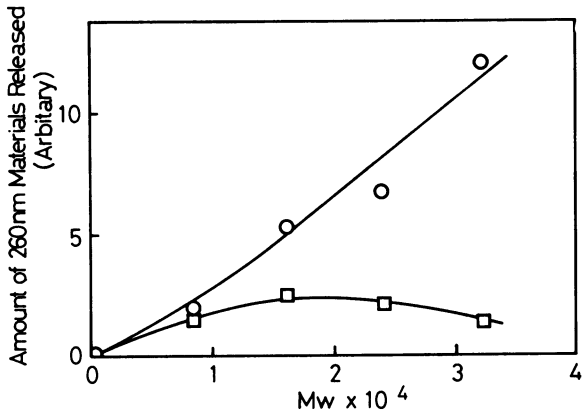

FIG. 7. Amount of materials absorbing light at 260 nm that were released from intact cells (□) and protoplasts (○) of *B. subtilis* in contact with poly 4 as a function of molecular weight. Concentration of poly 4, 10 μg/ml.

action of the polycationic biocides can be interpreted on the basis of each elementary process described above. It is well known that the bacterial cell surfaces are negatively charged. Then adsorption of the polycations onto the negatively charged cell surface (process i) is expected to be enhanced with increasing MW of the polymers due to the increasing charge density of the polycations (6). A similar situation can also be expected in process iii, since there are many negatively charged species present in the cytoplasmic membrane, such as acidic phospholipids and some membrane proteins (3). The disruption of the membrane (process iv) is a consequence of interaction of the bound polymers with the membrane and thus is expected to be facilitated with increasing amounts of the bound polymers. Process iv would be immediately followed by processes v and vi. Therefore, processes i, iii, and iv can be assumed to be enhanced with increasing MW of the polymers. On the other hand, process ii will undoubtedly be suppressed as the molecular size of the diffusing species increases, since the thick, rigid peptidoglycan layer of the gram-positive bacteria acts as a potential barrier against foreign molecules with high MW (1). The observed optimal-MW range for antibacterial activity against *S. aureus* can, thus, be interpreted in terms of a sum of two kinds of controlling factors, i.e., one that is positive with increasing MW (processes i, iii, and iv), and the other that is negative with increasing MW (process ii).

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