Removal of Bacteria from Water by Systems Based on Insoluble Polystyrene–Polyethyelnimine

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SYNOPSIS

A study was made of the removal of viable bacterial cells from sterilized physiological saline (saline) by insoluble polymer beads. The polymers (CMPS–PEI300 and CMPS–PEI600) were prepared by reactions of chloromethylated, divinylbenzene crosslinked polystyrene (CMPS) beads with polyethyleneimines (PEI) (MW = about 300 and 600). The bacterial strain cells used were *Escherichia coli (E. coli)*, *Staphylococcus aureus (S. aureus)*, and *Pseudomonas aeruginosa (P. aeruginosa)*. Decrease coefficients (D, which corresponds to adsorption rate constant) for the viable cell numbers of *E. coli* by CPMS–PEI300 and CMPS–PEI600 were 28 and 120 (mL/g h) in saline, respectively. These D's were less than those (72 and 270 mL/g h) in sterilized, distilled, and deionized water (sterilized water). The D's for *S. aureus* and *P. aeruginosa* by CMPS–PEI600 were 46 and 76 (mL/g h), respectively. The D for *E. coli* by CMPS–PEI600 was compared with R (removal coefficient) for that by pyridinium type polymers. Bactericidal activity of PEI600 was examined on *E. coli* and *P. aeruginosa* in saline. Also, that of poly(ethylene glycol) 600 was done on *E. coli* in saline.

INTRODUCTION

Water for domestic supply is usually disinfected or sterilized by using chlorine. However, this disinfection has problems in that the chlorine reacts with organic substances in the water to yield halomethane analogues and other carcinogens, and these remain in the water. The use of disinfectants also leads to the same problems. These problems can be solved by removal of microorganisms from water with insoluble substances.

From the viewpoints described above, the author studied adsorption behavior of *E. coli* cells onto the polymers by bringing the polymers into contact with viable cells with stirring in sterilized, distilled, and deionized water (hereafter referred to as sterilized water).[1] The polymers used were the reaction products (CMPS–PEG and CMPS–PEI) of chloromethylated, divinylbenzene crosslinked polystyrene (CMPS) with poly(ethylene glycol) (PEG) or polyethylenimines (PEI), and CMPS–ethylenediamine and CMPS–tetraethylenepentamine quaternized.

Although CMPS–PEG and its analogue caused the number of *E. coli* viable cells to decrease, the cells were not found on the surfaces of the polymer beads by observation with a scanning electron microscope. CMPS–PEI and CMPS–polyethylenepolyamine quaternized removed *E. coli* cells from water by adsorbing the cells onto their surfaces to cause the viable cell number to decrease, and CMPS–PEI600 (PEI of MW = ca. 600) was the most effective of the polymers.

It was clarified in the previous study that these polymers removed the bacterial cells from sterilized water by adsorption with electrostatic interaction. This interaction is sometimes influenced by salts coexisting in removal of anionic compounds from water with cationic polymers.[2,3] Coexisting salt should influence the adsorption of a bacterial cells onto these polymers. Therefore, we carried out the contacts of these polymers with bacterial cells in sterilized physiological saline (hereafter referred to as saline) to study the influence of the salt. Some studies have been made on antimicrobial activity of

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agents and removal of bacterial strain cells with polymers in saline.[4-6]

While *E. coli* viable cells were stirred for 4 h without a polymer in sterilized water, the viable cell number scarcely decreased. In the case of *Staphylococcus aureus (S. aureus),* when the viable cells were stirred without a polymer in sterilized water and in saline, the cell number decreased significantly in the water, but scarcely at all in saline. Therefore, the decrease coefficient (D) for *S. aureus* viable cell number by polymers could not be determined in sterilized water.

It is interesting to study the effect of salt on electrostatic interaction, and compare removal behaviors for CMPS–PEI polymers with those for pyridinium type polymers,[6] which were used for removal of bacterial strain cells from saline. From the two viewpoints described above and for the reason that *S. aureus* cells are easily killed in sterilized water, contacts of polymers with bacterial cells were carried out in saline.

EXPERIMENTAL

Materials

Polymers used in the present study were CMPS–PEI300 (PEI with MW about 300) and CMPS–PEI600 (average diameters of both are about 0.09 mm), which were the same as those used in the previous study.[1]

Organisms and Growth Conditions

Bacterial strain cells used here were *E. coli* IFO 12734, *S. aureus* IFO 12732, and *P. aeruginosa* IFO 13275, which were obtained commercially from the Institute for Fermentation, Osaka.

Growth conditions of microorganisms and preparation of bacterial cell suspensions made of physiological saline (sterilized 8.5 g/L sodium chloride aqueous solution using distilled and deionized water) for contact procedure were carried out as in the previous study.[1] A volume of 6.5 mL of the cultivated cell suspension was collected by centrifugation at $2200 \times g$ for 12 min in a centrifuge refrigerated below 4°C, washed twice with 6.5 mL of fresh saline. The *S. aureus* cell suspension was further diluted to the concentration of 1/10.

Contacts of Polymers, PEG600 or PEI600, with Bacterial Cells

One-tenth gram of each polymer was placed in a round-bottomed flask of 50 mL, and then 18 or 19 mL of water were poured into it. The polymer was completely wetted, and then stirred at about 300 rpm using a magnetic stirrer. Two or 1 mL of the cell suspension were added into the flask kept in a thermostatted bath at 37°C, thus bringing the total volume to 20 mL. The polymer was brought into contact with the cells by stirring.

Bactericidal action of PEI600 or PEG600 on bacterial cells was examined as follows: 2 mL of cell suspension were added to a round-bottomed flask of 50 mL containing $(18 - x)$ mL of saline and x mL of a 1000 μg/mL solution of PEI600 or PEG600, which were dissolved in saline and subsequently sterilized. (At this time, the concentration of PEI600 or PEG600 is $50 \cdot x$ μg/mL.) The mixture was stirred like the contact of insoluble polymers with bacterial cells.

Others

Measurements of viable cell numbers in the contact suspensions and scanning electron microscopy were carried out by the procedure described in the previous paper.[1]

RESULTS AND DISCUSSION

Adsorption of Viable *E. coli* Cells by CMPS–PEI600 and CMPS–PEI300

CMPS–PEI300 or CMPS–PEI600 was brought into contact with *E. coli* cells in saline. Figure 1 illustrates the plots of logarithm of viable cell number vs. contact time. A linear relation was observed in the initial stage of contact. D's for viable cell numbers were calculated by the following equation[6,1]:

$$D = \frac{V}{W} \cdot \frac{1}{t} \log \frac{N_0}{N_t} \qquad (1)$$

where V is the volume (mL) of viable cell suspension, W the dry weight (g) of the polymer, t contact time (h), N_0 the initial viable cell number, and N_t the viable cell numbers (cells/mL) at contact time t.

Table I lists D and the optical densities of 660 nm (OD_{660}) of cell suspensions before and after contact with CMPS–PEI600 and CMPS–PEI300. Even if initial cell numbers are different, D's for *E. coli* viable cells by CMPS–PEI300 and CMPS–PEI600 are approximately equal, respectively. The average of D for *E. coli* by CMPS–PEI300 was 28 (mL/g h) and that by CMPS–PEI600 was 120. The

ratios of D's obtained in saline to those in sterilized water are 28/72 (=0.39) for CMPS-PEI300 and 120/270 (=0.44) for CMPS-PEI600. In this way, salt in the cell suspension slowed the rate of adsorption of *E. coli* viable cells onto the polymers.

Organic, cationic polymers can remove organic, anionic species such as benzenesulfonate, *p*-toluenesulfonate, and dodecylbenzenesulfonate solutes from water by adsorption with electrostatic interaction.[2] In this removal, salt causes the removal capacity to decrease.[2] CMPS-PEI600 can also remove methyl orange (acidic dye)[3] and dodecylbenzenesulfonate solutes from water.[7] In the removal of methyl orange with CMPS-PEI600, the coexistence of salt caused the removal rate to decrease greatly and the removal capacity to decrease a little.[3]

OD_{660}s of cell suspensions were less after contact for 4 h than before contact (Table I). For example, in the contact of CMPS-PEI300 with *E. coli* cell suspension, OD_{660} of the suspension with 7.4×10^7 cells/mL was 0.055 before contact and 0.031 after contact for 4 h. This demonstrates clearly a decrease in the cell number. This decrease could be caused by adhesion of the cells to the polymer surfaces. This speculation can be deduced from the observation of the surfaces with a scanning electron microscope carried out in the previous study.[1] This shows that both the polymers adsorbed *E. coli* cells on their surfaces, even in physiological saline.

Bacterial cell surface is usually negatively charged at physiological pH.[8,9] PEI is protonated to a considerable extent in water at pH 7.[10] Accordingly, nitrogen atoms in CMPS-PEI should be protonated in water. Therefore, CMPS-PEI can adsorb bacterial cells from water by electrostatic interaction.[1] It has been reported that poly(*N*-lauryl-4-vinylpyridinium iodide-*co*-divinylbenzene)[11] adsorbs bacterial cells in sterilized water and poly(*N*-benzyl-4-pyridinium halide-*co*-divinylbenzene)s[12] adsorb the cells in physiological saline. D (270 mL/g h) for *E. coli* by CMPS-PEI600 in sterilized water was greater than that (120) in saline. This indicates that the salt coexisting in the cell suspension lessened D in a way similar to the removal of anionic, organic compounds by cationic polymers. (D corresponds to the rate constant for adsorption of viable bacterial cells onto polymer.)

Comparison of *D* for *E. coli* by CMPS-PEI with *R* for That by Pyridinium Type Polymer

Kawabata et al. reported that the removal coefficients [R, which were defined as eq. (1) on the basis of the initial rate for decrease in viable cell counts]

Table I Decrease Coefficients (D)[a] of Viable Cell Number in Contact of Bacterial Cells with CMPS-PEI300 or CMPS-PEI600

Polymer	Combined[b] PEI Groups (mmol/g)	Bacterium	Initial Viable Cells (cells/mL)	D[a] (mL/g h)	OD_{660} of Cell Suspension	
					Before Contact	After[c] Contact
CMPS-PEI300[d]	0.843	*E. coli*	7.4×10^7	30	0.055	0.031
			5.7×10^7	26	0.046	0.040
			4.0×10^7	27	0.046	0.037
CMPS-PEI600[e]	0.640	*E. coli*	9.5×10^7 [f]	112	0.106	0.046
			4.1×10^7	138	0.044	0.016
			1.0×10^8 [f]	111	0.103	0.055
CMPS-PEI600[e]	0.640	*S. aureus*	6.3×10^6	44	0.006	0.003
			7.4×10^6	42	0.007	0.003
			5.0×10^6	53	0.006	0.003
		P. aeruginosa	9.1×10^7 [f]	76	0.082	0.045
			8.7×10^7 [f]	72	0.052	0.028

[a] Determined at 37°C by the contact of the insoluble polymer 0.100 g with 20 mL of viable bacterial cell suspension.
[b] Molar number of PEI300 or PEI600 contained in 1 g of each polymer. These were calculated on the basis of the elemental analysis results.
[c] These were measured after the contact for 4 h.
[d] Elemental analysis results, H: 8.35%, C: 74.36%, N: 8.99%.
[e] Elemental analysis results, H: 8.87%, C: 74.29%, N: 12.97%.
[f] The added amount of original cell suspension, 2 mL; the others, 1 mL.

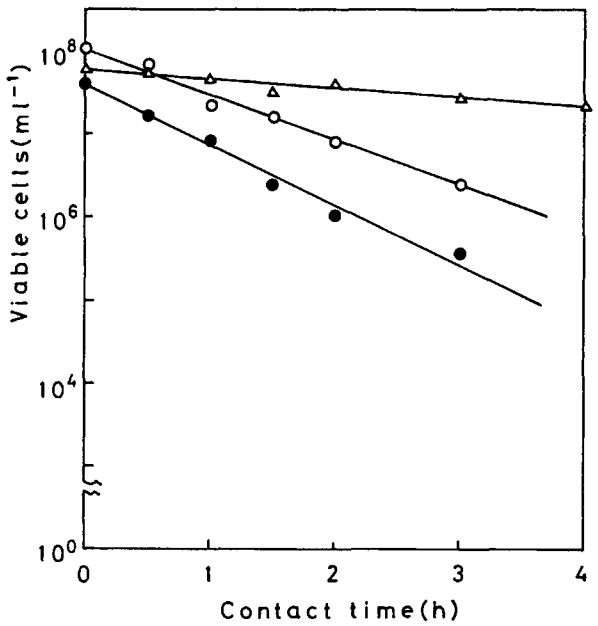


Figure 1 Decrease in the viable cell numbers with time in the contact of *E. coli* cells with CMPS–PEI300 and CMPS–PEI600 in sterilized physiological saline at 37°C: (△) CMPS–PEI300, 0.100 g, D = 26; (○) CMPS–PEI600, 0.100 g, D = 111; (●) CMPS–PEI600, 0.100 g, D = 138.

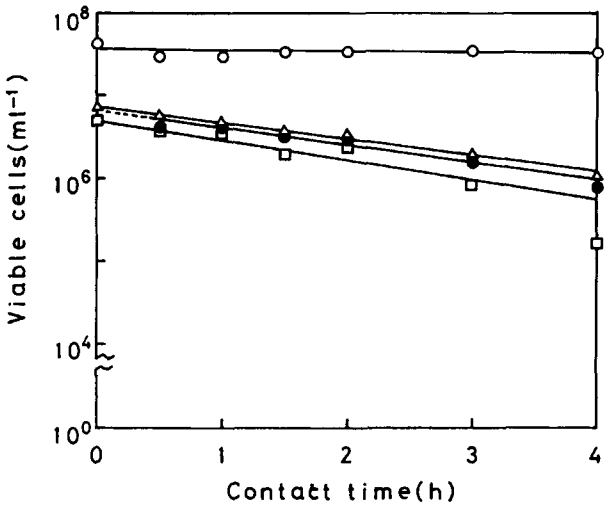
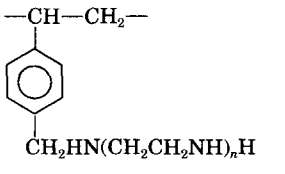
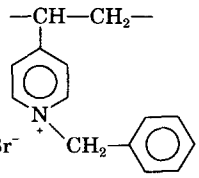


Figure 2 Decreases in the viable cell numbers with time in the contact of *S. aureus* cells with CMPS–PEI600 in sterilized physiological saline: (○) CMPS–PEI600, 0 g; (△); 0.100 g, D = 42; (●) 0.100 g, D = 44; (□) 0.100 g, D = 53.

of *E. coli* viable cells by insoluble poly(*N*-benzyl–4-vinylpyridinium bromide-*co*-divinylbenzene)s are 4.3[6] and 7.1.[12] The sizes of these polymer granules are 0.30 mm on the average, respectively.[6,13] The diameters of CMPS–PEI600 beads in the present study are 0.09 mm on the average, measured from a scanning electron microscopic photograph of about 30 beads. Since it can be considered that the rate for adhesion of *E. coli* onto polymer is proportional to the surface area of polymer, D for *E. coli* viable cells by this CMPS–PEI600 was compared with R for the cells by poly(*N*-benzyl–4-vinylpyridinium bromide-*co*-divinylbenzene)s (Table II). Assuming that specific gravities of these polymers are not significantly different, the ratio of surface area per unit mass of these polymers is 3.33, for the CMPS–

Table II. Comparison of D of CMPS–PEI600 with R of Pyridinium Type Polymer

		
Average Diameter (mm)	0.09	0.30
Ratio of total surface area	0.30/0.09 = 3.33	0.30/0.30 = 1
D or R (in saline)	120	4.3[a], 7.1[b]
D of CMPS–PEI600 and R of pyridinium type polymer of diameter 0.30 mm	120/3.33 = 36	4.3/1 = 4.3, 7.1/1 = 7.1
Ratio of D/R	36/4.3 or 36/7.1 = 8.4 or 5.1	4.3/4.3 = 1 7.1/7.1 = 1

[a]4-Vinylpyridine 72 and divinylbenzene 28 mol %, pyridinium group 2.9 mmol/g. See Ref. 6.
[b]4-Vinylpyridine 72, divinylbenzene 10, and styrene 18 mol %, pyridinium group 2.9 mmol/g. See Ref. 12.

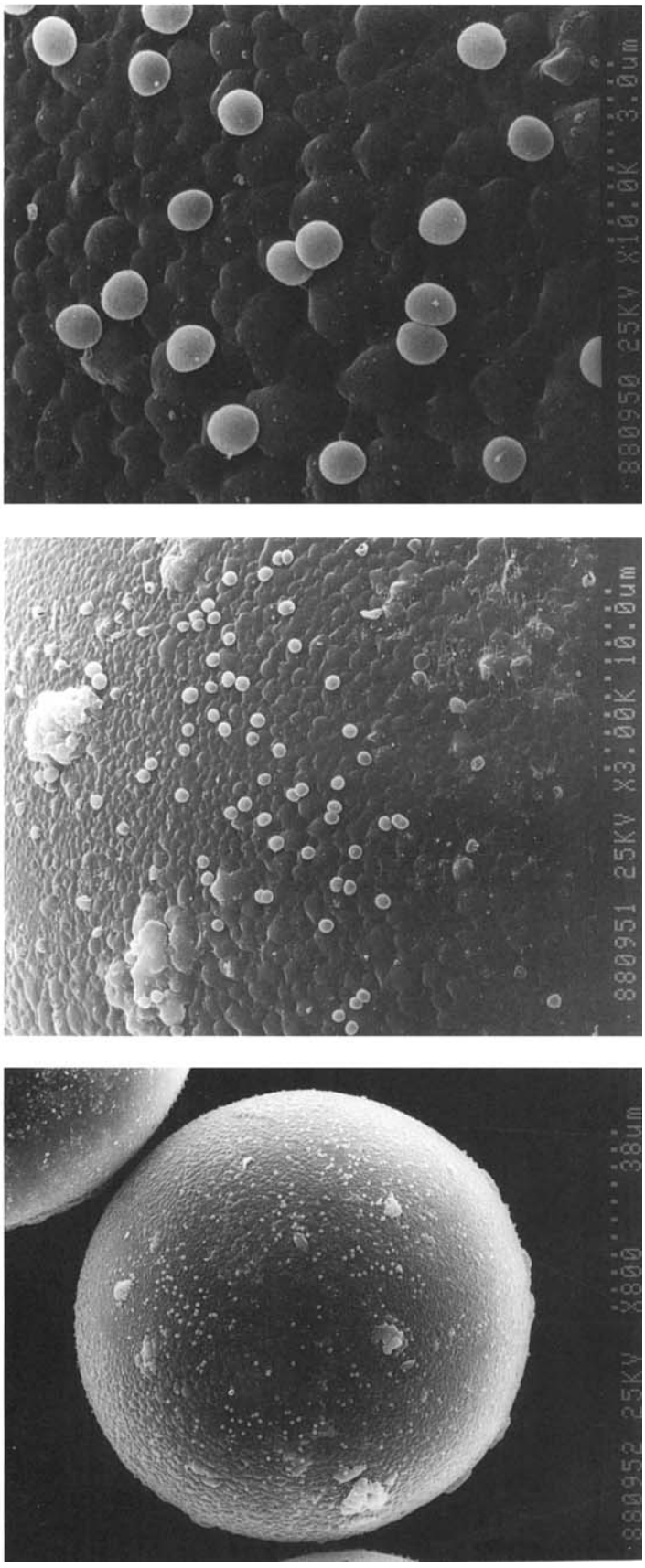


Figure 3 Scanning electron micrographs of the surface of a polymer (CMPS–PEI600) bead brought into contact with *S. aureus* cells in sterilized physiological saline.

PEI600, to 1 for these poly(*N*-benzyl-4-vinylpyridinium bromide-*co*-divinylbenzene)s.

When the average diameter of this CMPS-PEI600 is 0.30 mm, since D is calculated to be about 36, this is 5.1–8.6 times greater than R for the 4-vinylpyridinium polymers. This comparison is only for reference, because it has been assumed that the specific gravities of these polymers are approximately equal in this comparison, and also D and R may vary with different kinds of *E. coli* strains. (The kind of *E. coli* cells used in the present study may not be the same as that used in Kawabata et al.'s studies.[6,12] They used the cells which have been stored in their laboratory.)

R for 4-vinylpyridinium polymers with and without styrene as polymer component are reported as 7.1[12] and 4.3[6] for *E. coli* cells, respectively. From these results and the results obtained in the present study, it can be presumed that polymers containing styrene as a component remove bacterial cells more effectively than those not containing styrene.

In the removal of bacterial cells, the important factors are not only D, but also the saturation amount of adsorption. In the removal of nonionic, anionic surfactants,[7] and methyl orange[3] with CMPS-PEI type polymers, one which had a large removal rate also had a large saturation amount of adsorption.

Adsorption of Other Bacterial Cells to CMPS-PEI600

S. aureus cells were brought into contact with CMPS-PEI600 in saline. When CMPS-PEI600 was not present in a contact suspension, the number of viable *S. aureus* cells scarcely decreased. When the cell suspensions were stirred together with the polymer, the viable cell number clearly decreased (Fig. 2). Linear relations held between the logarithm of viable cell number and contact time. The average value of D was about 46 (Table I), being less than that for *E. coli* cells.

The OD_{660}s of *S. aureus* cell suspensions before the contact were scarcely different from those after contact for 4 h. However, it was confirmed by observation with a scanning electron microscope that *S. aureus* cells had been present on the surface of these CMPS-PEI600 beads (Fig. 3). From this result, *S. aureus* cells were found to adhere to the surfaces of CMPS-PEI600 beads.

When a *P. aeruginosa* viable cell suspension was stirred without CMPS-PEI600 in saline, the viable cell number scarcely decreased in the early stage, but began to decrease a little after about 2 h (Fig. 4). In contacts of *P. aeruginosa* viable cell suspensions with CMPS-PEI600, viable cell number decreased with time. In the contacts of CMPS-PEI600, 0.100 g, with suspensions containing initial viable cells 9.1×10^7 and 8.7×10^7 cells/mL, since the plots for decreasing viability of the two cell numbers were almost superimposed, only one of the two was illustrated in Figure 4.

The OD_{660} of the cell suspension containing 9.1×10^7 cells was 0.082 before the contact, and decreased to 0.045 after 4 h contact (Table I). Observation with a scanning electron microscope showed also that the bacterial cells were present on the surface of the polymer bead (Fig. 5). From these facts, it was confirmed that *P. aeruginosa* cells were also adsorbed on the surfaces of the polymer beads. The average of D for *P. aeruginosa* by CMPS-PEI600 was about 74 (mL/g h).

The magnitude order of D for viable cell numbers of the three bacterial strain cells by CMPS-PEI600 from saline, is as follows:

E. coli (120 mL/g h) > *P. aeruginosa* (74) > *S. aureus* (46)

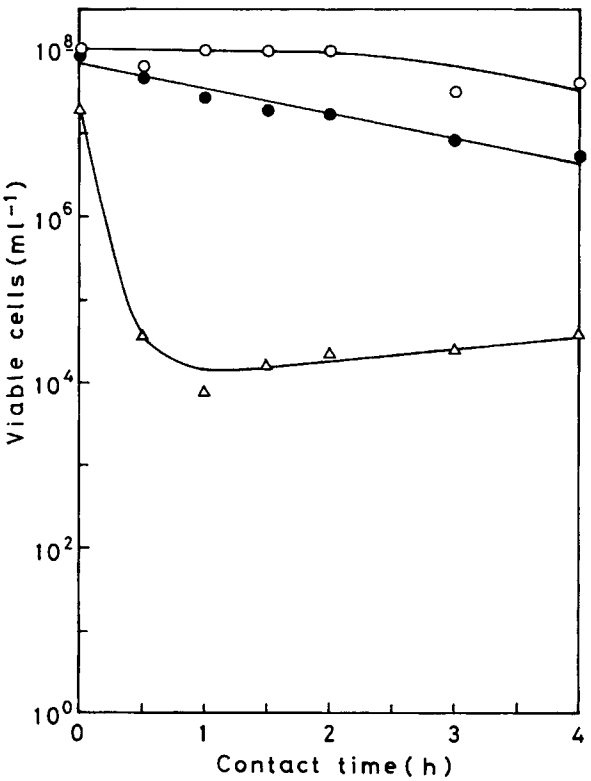


Figure 4 Variations in viable cell numbers with time in the contact of *P. aeruginosa* cells with CMPS-PEI600 and PEI600 in sterilized physiological saline at 37°C: (○) control; (●) CMPS-PEI600, 0.100 g; (△) PEI600, 25 μg/mL.

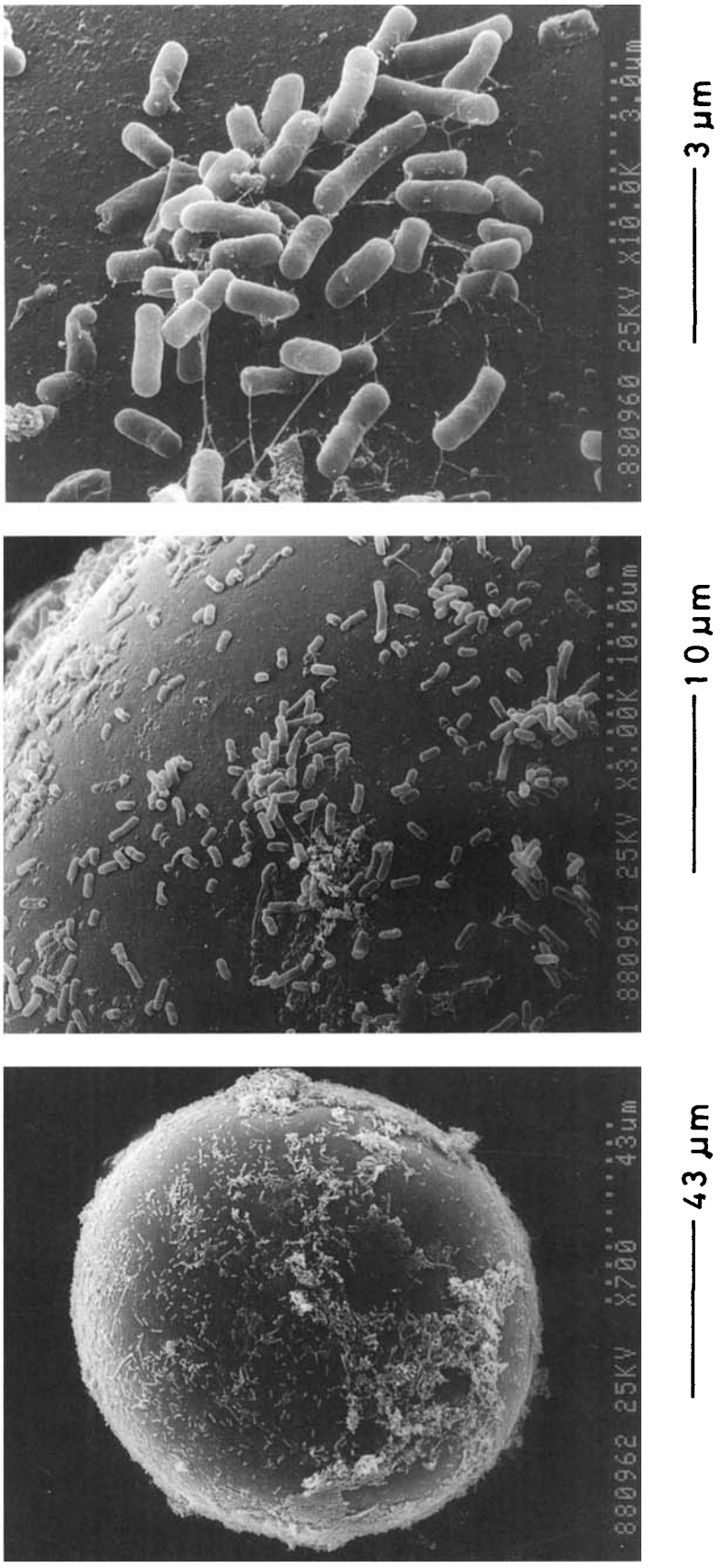


Figure 5 Scanning electron micrographs of the surface of a polymer (CMPS–PEI600) bead brought into contact with *P. aeruginosa* cells in sterilized physiological saline.

The magnitude order of R for these cells by poly(N-benzyl-4-vinylpyridinium bromide-*co*-divinylbenzene) is as follows:
S. aureus (4.4) > *E. coli* (4.3) > *P. aeuginosa* (4.2)[6]

Bactericidal Activity of PEI600

In the previous study,[1] it was observed that certain components (PEI600, styrene, hydroxystyrene, etc.) had leached from CMPS–PEI beads during stirring of the beads in a cell suspension. The bactericidal action of PEI600 on *E. coli* cells was examined in sterilized water. When a cell suspension was stirred for 4 h, viable cell number decreased greatly with an increase in the concentration of PEI600 (Fig. 5 in Ref. 1). It was found that PEI600 had bactericidal activity on *E. coli* cells.

In the present study, bactericidal activity of PEI600 on *E. coli* cells was also examined in saline (Fig. 6). The decrease in *E. coli* viable cell number was less than that in sterilized water (Fig. 6 in Ref. 1). The presence of salt restrained a decrease in *E. coli* viable cell number in the aqueous solution of PEI600. This cause has to be studied further.

The decrease behavior showed approximately linear relations between the logarithm of viable cell number and contact (stirring) time. In the concentrations of 5, 25, 50, and 100 μg/mL of PEI600, the slopes of the decrease lines, which were calculated from the relation of $-\log(N_0/N_t)$ vs. time, were 0.09, 0.42, 0.52, and 0.64 (h^{-1}), respectively. The slope increased approximately in proportion to the concentration of PEI in concentrations less than 25 μg/mL, but the slope increased only a little in larger concentrations than 25 μg/mL.

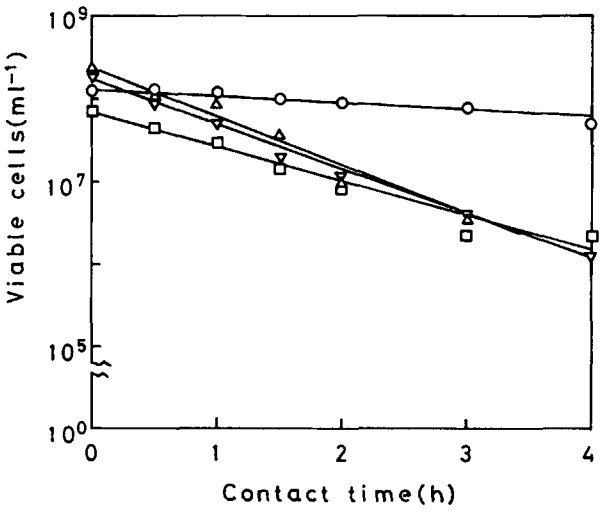


Figure 6 Decreases in viable cell numbers with time in the contacts of *E. coli* cells with PEI600 in sterilized physiological saline. PEI600: (○) 5; (□) 25; (▽) 50; (△) 100 μg/mL.

Action of PEI600 on *P. aeruginosa* cells was examined in saline. When the cells were stirred in saline containing 25 μg/mL of PEI600, the viable cell number decreased rapidly during about the first 30 min after the start, and then it began to increase gradually after about 1 h (Fig. 4). Inorganic carbon was detected in the cell suspension. When the suspension was allowed to stand at room temperature, its amount increased with the lapse of days. *P. aeruginosa* cells reduce nitrate to yield nitrogen gas.[14] When *P. aeruginosa* cells were stirred in a saline containing 25 μg/mL of PEI600, although they were mostly killed by the PEI of this concentration, a part of them which were strong seemed to be able to accommodate themselves to this environment and to reduce PEI by consuming it to yield carbon dioxide.

Bactericidal Activity of Poly(ethylene Glycol) 600 (PEG600) on *E. coli*

In the previous study,[1] it was also observed that certain components were leached from CMPS–PEG600 beads while the beads were stirred in a bacterial suspension. The bactericidal activity of PEG600 was also examined. The decrease in viable cell number was small in contacts of *E. coli* with PEG600 in sterilized water (see Fig. 5 in Ref. 1). The degree of the decrease was approximately equal in sterilized water and in saline.

SUMMARY

CMPS–PEI300 and CMPS–PEI600 removed *E. coli* cells by adsorption from saline as well as from sterilized water. However, salt slowed the adsorption rates of the cells onto these polymers. D's for *E. coli* by CMPS–PEI600 and CMPS–PEI300 were 120 and 28 (mL/g h) in saline, respectively. The D's for *S. aureus* and *P. aeruginosa* by CMPS–PEI600 were 120, 46, and 76 (mL/g h), respectively. Adsorption rates of the three bacterial strain cells onto CMPS–PEI600 were seemingly greater than by 4-vinylpyridine type polymers.[6,12]

Bactericidal activity of PEI600 on *E. coli* cells was less in saline than in sterilized water. Salt restrained the bactericidal activity on *E. coli* cells. In saline, the activity did not significantly increase with an increase in a PEI600 concentration in the region above 50 μg/mL. This is very different from the activity in sterilized water. The bactericidal activity

of PEI600 on *E. coli* was large, and less in saline than in sterilized water. In saline the activity on *P. aereginosa* was less than that on *E. coli.* Although viable cell number of *P. aeruginosa* at first decreased rapidly during stirring of the cells in a saline containing PEI600 of 25 $\mu g/mL$, the viable cell number began to increase gradually after about 1 h from the start of contact.

Bactericidal activity of PEG600 on *E. coli* was very small, and nearly equal in sterilized water and in saline.

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