A Study of Pyridinium-Type Functional Polymers. IV. Behavioral Features of the Antibacterial Activity of Insoluble Pyridinium-Type Polymers

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ABSTRACT: The antibacterial activity of insoluble pyridinium-type polymers with different structures against *Escherichia coli* suspended in sterilized and distilled water was investigated by a colony count method. The results show that the antibacterial activity of insoluble pyridinium-type polymers, except for one containing I^-, is characterized by an ability to capture bacterial cells in a living state by adsorption or adhesion, with the process of capturing bacterial cells being at least partially irreversible. This feature differs from the antibacterial activity of the corresponding soluble polymers, which is characterized by the ability to kill bacterial cells in water. In addition, insoluble pyridinium-type polymers can also capture dead bacterial cells. This implies that insoluble pyridinium-type polymers possess broad prospects for development in new water treatment techniques and whole-cell immobilization techniques.
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Key words: 4-vinylpyridine; insoluble pyridinium-type polymer; functional polymer; antibacterial activity; antibacterial behavior

INTRODUCTION

It is well-known that conventional disinfectants or antibacterial agents are liquids or gases of low molecular weight. With the use of these disinfectants or antibacterial agents, the problem of residues cannot be avoided, bringing about more serious consequences. For example, in the case of water treatment for the domestic water supply, the most popular treatment method is to use chlorine and other related chemicals to disinfect and sterilize water. However, their residues can become concentrated in the food chain in the environment. In addition, because chlorine and other related chemicals can react with organic substances in the water to yield halomethane analogues that are suspected of being carcinogenic,[1] their use should be avoided. These drawbacks can be solved by the removal of microorganisms from the water with insoluble substances. In light of this idea, researchers in many countries have been attempting to find effective alternatives to conventional water disinfection processes. One approach is the use of insoluble contact disinfectants that can inactivate, kill, or remove target microorganisms by mere contact without releasing any reactive agents to the bulk phase to be disinfected. Among the disinfectants possessing this characteristic are crosslinked polymer materials called *insoluble polymeric contact disinfectants* (IPCD).[2,3] Because bacteria bear negative charges on their cell surfaces under usual conditions, insoluble polymers containing cations or

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bearing positive charges on their surfaces can be candidates for IPCDs. These polymers are mainly crosslinked anion-exchange resins, including quaternary ammonium-type resins,[2,4,5] macroporous and macroreticular resins,[6] polyiodide resins,[7–11] and insoluble polyelectrolytes.[12] Their preparation and disinfective or purifying effect have been studied. The research undoubtedly provides a basis for developing a new method of water treatment.

As a kind of crosslinked quaternary ammonium-type resin, insoluble pyridinium-type polymers have positive charges on their macromolecular networks and so can interact with negatively charged species. Therefore, insoluble pyridinium-type polymers may exhibit many unique properties in the capture or isolation of bacteria and viruses[13–18] and the removal of organic pollutants from aquatic environments.[19–23]

In our previous research work, the preparation, characterization, solution properties, and antibacterial activity of soluble (linear) pyridinium-type polymers were investigated.[24,25] It was demonstrated that the antibacterial activity of soluble pyridinium-type polymers against bacteria suspended in sterilized and distilled water is characterized by the ability to kill them. As an extension of this research, we attempted to clarify the behavioral features of the antibacterial activity of the corresponding polymers with a three-dimensional network structure. For this purpose, we designed and performed some antibacterial experiments tests using *Escherichia coli* (*E. coli*) as a test bacterium and sterilized and distilled water as the suspending medium of the bacterial cells. In this article, the results of these experiments are analyzed and discussed, and the behavioral features of the antibacterial activity of insoluble (crosslinked) pyridinium-type polymers are summarized.

EXPERIMENTAL

Materials

Insoluble Pyridinium-Type Polymers

With a procedure described elsewhere,[26] a copolymer of 4-vinylpyridine (4VP), styrene (St), and divinylbenzene (DVB), P(4VP-St-DVB), was first synthesized by suspension polymerization. The amounts of 4VP, St, and DVB in the feed were 61.67, 35.98, and 2.35 mol %, respectively. The synthesized copolymer P(4VP-St-DVB), in which the 4VP content measured by nonaqueous titration was 5.41 mmol/g, was quaternized with an excess of halohydrocarbons (RX) to prepare a series of insoluble pyridinium-type polymers, Q-P(4VP-St-DVB)-RX. Halohydrocarbons such as benzyl bromide (BzBr), C_4H_9Cl, C_4H_9Br, and C_4H_9I were used as quaternizing agents in the quaternization of P(4VP-St-DVB). Each P(4VP-St-DVB)-RX sample was purified by Soxhlet extraction with ethanol as an extractant for 24 h to thoroughly remove oligomers and other impurities.

Table I Composition Analysis of the Insoluble Pyridinium-Type Polymers

Sample No.	C_q (mmol/g)	DQ (%)
Q-P(4VP-St-DVB)-BzBr	2.47	78.82
Q-P(4VP-St-DVB)-C_4H_9Cl	0.075	1.40
Q-P(4VP-St-DVB)-C_4H_9Br	2.48	69.60
Q-P(4VP-St-DVB)-C_4H_9I	2.54	88.44

The composition analysis results of the prepared insoluble pyridinium-type polymers are given in Table I. C_q and DQ stand for the pyridinium group content and the degree of quaternization in each Q-P(4VP-St)-RX sample, respectively. C_q was determined via back titration in argentometry. From C_q and the mole fraction of 4VP in the corresponding precursor copolymer, DQ was calculated.[26] DQ is the percentage of the quaternized 4VP to the total 4VP in each Q-P(4VP-St)-RX sample.

Bacteria

The Gram-negative bacterium *E. coli* 44113, provided by the University of the First Military Medicine, was used as a test bacterium in the experiments on the antibacterial activity of an insoluble pyridinium-type polymer. *E. coli* was incubated at 37°C for 24 h on a nutrient agar plate before use.

Antibacterial Tests for Insoluble Pyridinium-Type Polymers

All procedures in the antibacterial tests for insoluble pyridinium-type polymers were performed under aseptic conditions. Sterilized distilled water was used as a suspension medium for the *E. coli* cells.

Preparation of the Bacterial Cell Suspension

One loopful of fresh bacteria was suspended in an appropriate amount of sterilized distilled water, forming a bacterial cell suspension. The suspension was fully shaken to make its turbidity uniform. The viable cell number in the suspension was controlled via the comparison turbidity method. Then this suspension was diluted to a prescribed cell concentration with sterilized distilled water, thus preparing a bacterial cell suspension that was directly used for the antibacterial tests for insoluble pyridinium-type polymers. The initial viable cell number of the bacterial suspension, $N(0)$, was usually controlled over a range of about 10^6 cells/mL.

Batch Treatment

In each test, 20.0 mL of the bacterial suspension and 0.5 g (dry weight) of a polymer sample were placed in a sterilized glass container with a cotton stopper. The container was continually shaken. At prescribed time intervals, 0.2 mL of the supernatant of the polymer/bacterial suspension system was pipetted out from the container and quickly mixed with 1.8 mL of sterilized physiological saline; then decimal serial dilutions were prepared from this by 0.5 mL being added to 4.5 mL of sterilized physiological saline and mixed. The viable cell number in each of the polymer/bacterial suspension systems at the contact time t, $N(t)$, was determined by a colony count method, including the spread-plate method and the pour-plate method. The colonies were counted after the inoculated plates were incubated at 35–37°C for 24 h. The counting was done in triplicate every time.

Contact of an Insoluble Pyridinium-Type Polymer with Dead Bacterial Cells

An experiment was made to clarify whether or not an insoluble pyridinium-type polymer can adsorb or adhere dead bacterial cells. The *E. coli* cell suspension only containing dead cells was prepared by an *E. coli* cell suspension, in which the initial viable cell number was around 10^7 cells/mL, being heated at 70°C for 50 min. The contact study of an insoluble pyridinium-type polymer with dead bacterial cells was performed with the *E. coli* cell suspension only containing dead cells. After the cells were in contact with an insoluble pyridinium-type polymer sample for a prescribed time, an appropriate amount of the supernatant was pipetted out, and its optical density at 660 nm (OD_{660}) was measured with a spectrophotometer. The OD_{660} value of a bacterial suspension indirectly reflects its cell concentration.[5] The lower the OD_{660} value is, the lower the cell concentration in the bacterial suspension is, and vice versa.

In addition, a control test was also made in the same manner with an *E. coli* cell suspension in which the initial viable cell number was around 10^7 cells/mL.

Examination of the Viability of Bacteria Adsorbed or Adhered on the Surface of a Sample

An insoluble pyridinium-type polymer sample was brought into contact with a bacterial suspension in which the initial viable cell number was around 10^6 cells/mL. After 4 h, the sample was taken out and washed extensively with sterilized physiological saline. Then the moisture on the sample surface was sucked up with sterilized filter paper. The treated sample was inoculated into a nutrient broth. The turbidity of the nutrient broth was observed upon being cultured at 37°C for 12–18 h. If the nutrient broth became turbid, a multiplication of *E. coli* was proven to have occurred in it. For the sake of convenient observation, the cultured nutrient broth could be inoculated on the surface of a nutrient agar plate. After the inoculated plates were incubated at 37°C for 12–18 h, it could be clearly observed whether *E. coli* colonies appeared on the plates or not.

This experiment is helpful for elucidating whether the adsorbed or adhered bacteria on the surface of an insoluble pyridinium-type polymer can be maintained alive or killed.

Scanning Electron Microscopy (SEM) Observations

The sample that was brought into contact with a *E. coli* cell suspension in a batch system was collected and treated by the same method as that described in our previous article.[25] Then the treated sample was observed with a scanning microscope (JEOL Ltd. JSM-T300).

RESULTS AND DISCUSSION

Interaction between an Insoluble Pyridinium-Type Polymer and Living Bacterial Cells

Figure 1 shows the time course of the viable cell number, $N(t)$, during the contact process of repre-

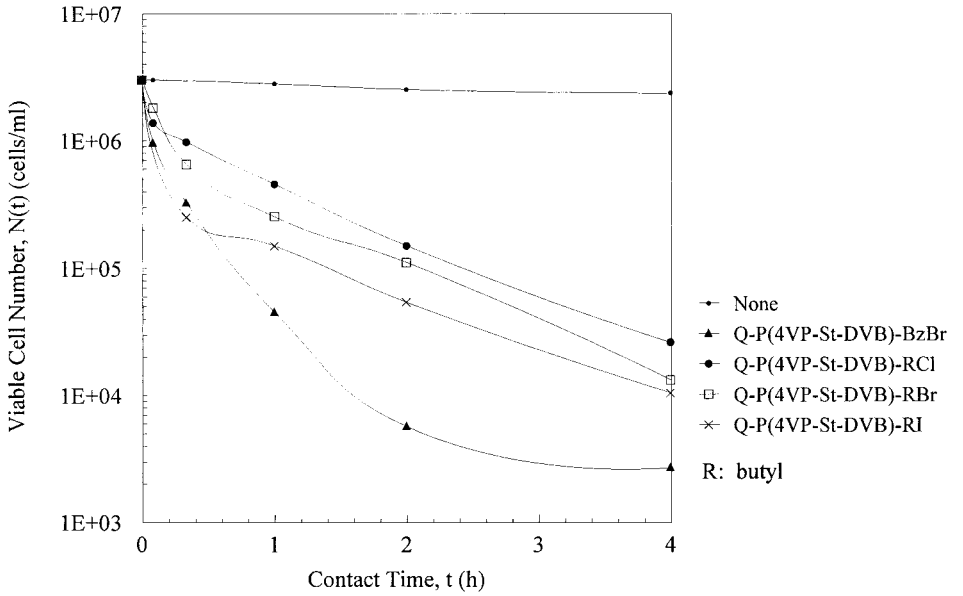

Figure 1 Decrease in the viable cell number, $N(t)$, with the lapse of time in the contact of *E. coli* cells with Q-P(4VP-St-DVB)-RX samples in sterilized and distilled water.

sentative insoluble pyridinium-type polymer samples with a bacterial suspension and the change in $N(t)$ with the contact time t in the absence of the polymer. $N(t)$ obviously decreases in the presence of the polymer sample, and this decrease cannot be explained by the normal death of bacterial cells. A reduction of $N(t)$ in the bacterial suspension can only be caused in two ways. First, the presence of an insoluble pyridinium-type polymer may change or destroy the living surroundings of bacterial cells to cause them to be killed or inactivated. Second, the adsorptive or adhesive interaction between the polymer and the bacterial cells occurs in the contact process, leading to cell capture onto the surface of polymer particles. As a result, the number of viable cells that are found in the bacterial suspension decreases rapidly.

To clarify the reason for the decrease in $N(t)$, the surface of the polymer sample that was brought into contact with a bacterial suspension was observed by SEM. SEM photographs are shown in Figure 2. The arrows in the photographs are used to indicate the captured cells.

From these two SEM photographs, the *E. coli* cells captured onto the surfaces of the sample particles can be clearly seen. This indicates that an insoluble pyridinium-type polymer can adsorb or adhere bacterial cells, causing the viable cell number in the bacterial suspension to decrease, and that the interaction between the polymer and the bacterial cells is very firm, for otherwise the cells captured on the sample surface would be expected to come off when the sample was prepared (e.g., washing, fixing, dehydrating, and drying) for the SEM observations. Moreover, the biological activity of the bacterial cells adhering to the surface of an insoluble pyridinium-type polymer was further examined via the aforementioned experiment. The results show that the bacterial cells captured on the surface of each polymer whose quaternizing agent is BzBr, RCl, or RBr can exhibit the ability of proliferation, indicating they are still alive, whereas those captured on the surface of the polymer quaternized with RI do not have the ability of proliferate, so they are dead. Thus, the insoluble pyridinium-type polymers in the bromide or chloride form can capture the bacterial cells in a bacterial suspension onto the surface of their particles, leading to the removal of viable bacteria from the bacterial suspension. Although we cannot judge whether there is the possibility that a portion of the bacteria captured on the polymer surface retains physiological activity and another portion of them loses this activity, we have good reason to say that the insoluble pyridinium-type polymers in the bromide or chloride form can at least partially maintain the viability of the bacterial cells captured on the surface of their particles. As far as the polymer in the iodine form is concerned, it can also capture the bacterial cells, but it causes them to

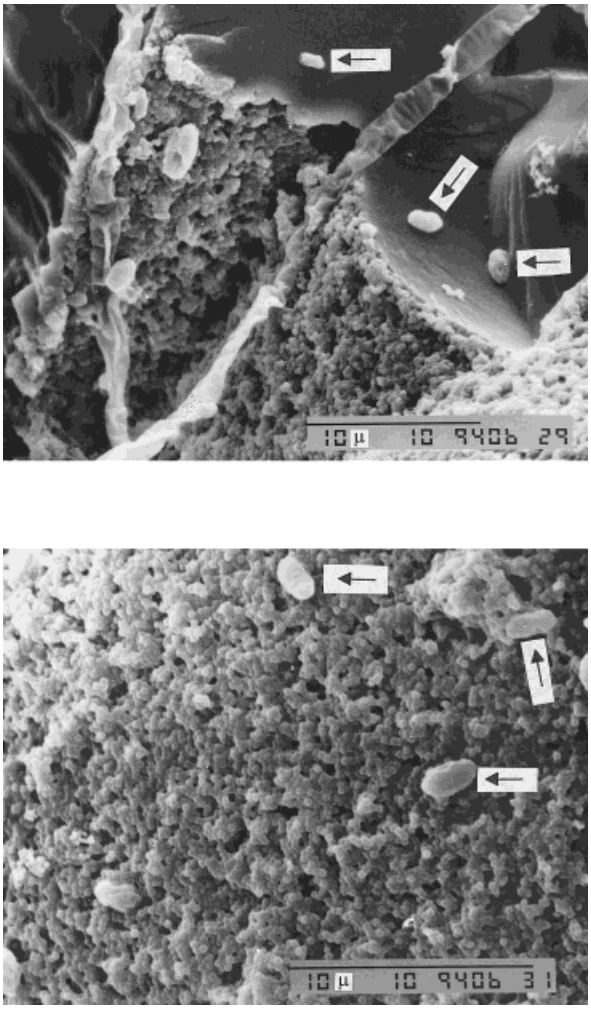

Figure 2 SEM photographs of the surface of Q-P(4VP-St-DVB)-BzBr brought into contact with the *E. coli* cell suspension. The captured cells are marked with arrows.

die at the same time. This feature differs from the behavioral feature of the antibacterial activity of a soluble pyridinium-type polymer, which is characterized by its ability to kill bacteria in water.[25,27]

Pyridinium-type polymers are macromolecular quaternary ammonium salts belonging to the class of cationic polyelectrolytes. There are positive charges on their macromolecular chains or their particle surface. Quaternary ammonium salts of low mass are widely used as effective antibacterial agents because of their strong ability to kill bacteria. The antibacterial mechanism of these cationic disinfectants can be summarized in the following six steps:[27]

1. Adsorption onto the bacterial cell surface.
2. Diffusion through the cell wall.
3. Binding to the cytoplasmic membrane.
4. Disruption of the cytoplasmic membrane.
5. Release of K^+ ion and other cytoplasmic constituents.
6. Precipitation of the cell contents and the death of the cell.

Because it possesses a three-dimensional network structure and the activity of its segments is inevitably subjected to restraint, the segments of an insoluble pyridinium-type polymer are unable to diffuse through the cell wall. Thus, the interaction between the polymer and the bacterial cells will come to a halt at step 1, the stage of adsorption. This mechanism can explain why insoluble pyridinium-type polymers containing no I^- capture bacterial cells on their surfaces in a living state.

As for the lethal action of the insoluble pyridinium-type polymer Q-P(4VP-St-DVB)-C_4H_9I, which contains I^- on the bacteria, it is related to the properties of the leaving group I^- in this polymeric quaternary ammonium salt. Among the leaving groups I^-, Br^- and Cl^-, the tendency to leave decreases in the order I^-, Br^-, and Cl^-. Therefore, it is very likely that the polymer Q-P(4VP-St-DVB)-C_4H_9I will release I^- under the action of a solvent or in the course of being in contact with a bacterial suspension. Investigations into polymers containing I^- by many researchers[10,11,28] have given evidence for it. The released I^- is in itself an effective disinfectant, so the polymer Q-P(4VP-St-DVB)-C_4H_9I is able to kill bacterial cells upon capturing them.

Interaction between an Insoluble Pyridinium-Type Polymer and Killed Bacterial Cells

In a study of the antibacterial activity of an insoluble pyridinium-type polymer, it was found that the bacteria concentration determined by the surface-plate method or by the pour-plate method only represent the viable bacterial cell number in a bacterial suspension. However, in a bacterial suspension there always exist more or less dead bacterial cells. Thus, the bacteria concentration of a bacterial suspension prepared by turbidimetry is always greater than its viable cell number.

Because there are not only living bacteria but also dead bacteria in the prepared bacterial suspension, there are two problems with a study of the antibacterial activity of a polymer. One is whether an interaction between the polymer and

Table II Determination of the OD_{660} in Living and Heat-Killed *E. coli* Cell Suspensions by Contact of *E. coli* with Q-P(4VP-St-DVB)-BzBr

E. coli Cell Suspension	OD_{660} at the Following Contact Times, t (h)			$N(t)$ (cells/mL) in a Cell Suspension at the Following Contact Times, t (h)		
	0	2	4	0	2	4
Heat-killed	0.051	0.014	0.024	7.2×10^{7}[a]	—	—
Living	0.041	0.021	0.024	7.2×10^{7}	5.7×10^{7}	3.5×10^{4}

[a] This number indicates that before it was made into the heat-killed cell suspension, the cell suspension contained the number of viable cells.

dead cells can occur, and the other is whether the presence of dead cells in the bacterial suspension can have an effect on the experimental results when the colony count method, which can merely determine the number of viable bacterial cells, is used to investigate the antibacterial activity of a polymer. For this reason, it is desirable to do further research into the interaction between an insoluble pyridinium-type polymer and dead *E. coli* cells.

The change in the OD_{660} value of the heat-killed-cell suspension that was in contact with the polymer Q-P(4VP-St-DVB)-BzBr was measured at different prescribed contact times. For the sake of convenient comparison and analysis, a control test was made with the living-cell suspension, the same suspension used to prepare the heat-killed-cell suspension. The results are compiled in Table II.

Table II indicates that after Q-P(4VP-St-DVB)-BzBr is brought into contact with the living-cell suspension and the heat-killed-cell suspension, the $N(t)$ in the living-cell suspension and the corresponding OD_{660}, respectively, decrease distinctly, and so does the OD_{660} value of the heat-killed-cell suspension. This fact reflects the reduction in the dead cell number in the heat-killed-cell suspension.

The SEM photograph in Figure 3 shows the surface of Q-P(4VP-St-DVB)-BzBr that was brought into contact with the heat-killed *E. coli* cell suspension. *E. coli* cells can be clearly seen from this photograph, and on the polymer surface there is evidence of fragments smaller than a complete cell that look like cell fragments. These may be due to the destruction of bacterial cells caused by a bacterial suspension being heated to prepare the suspension containing only dead cells.

It is concluded that an insoluble pyridinium-type polymer is also able to capture dead bacterial cells. Kawabata et al.[15] examined the change in the amount of total organic carbon during the contact of crosslinked poly(vinylpyridinum halide) with killed bacteria suspended in sterilized physiological saline with a column test. Although the method and conditions used in our experiment are different from those used in their experiment, the same conclusion can be reasoned out.

According to this conclusion, the presence of dead cells in a bacterial suspension can have an effect on the experimental results when the colony count method is used to investigate the antibacterial activity of a pyridinium-type polymer. If there is a great difference in the proportion of living cells to dead cells in the bacterial suspension used in an antibacterial test for each polymer, the experimental result can be affected to some extent. However, the proportion of living cells to dead cells in a bacterial suspension may vary with the method of culturing bacteria (e.g., the culture medium and time may differ). Thus, to

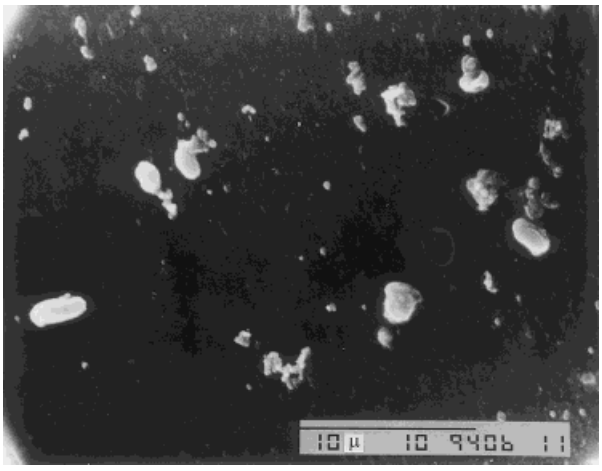

Figure 3 SEM photograph of the surface of Q-P(4VP-St-DVB)-BzBr brought into contact with the heat-killed *E. coli* cell suspension.

diminish the effect of a difference in the composition (i.e., the proportion of living cells to dead cells) of a bacterial suspension on the experimental results, it is necessary to use a fresh bacterium cultured by the same method to prepare a bacterial suspension used for antibacterial tests.

The dead bacterial cells can be also removed from water by the polymer in the same way as the viable cells: the physicochemical interaction between the polymer and the bacterial cells appears to be more important than the physiological action in this process, although the physiological activity of the cells cannot be ignored. This is a subject for further investigation and analysis.

Study of the Reversibility of Capturing Interactions between an Insoluble Pyridinium-Type Polymer and Bacterial Cells

After being in contact with an *E. coli* cell suspension, an insoluble pyridinium-type polymer captures the *E. coli* cells on the surface of the polymer particles. Some attempts were made to clarify whether the bacterial cells on the polymer surface could be washed away from the surface. Evidently, the fact that bacterial cells can be observed from the SEM photographs shown in Figures 2 and 3 proves that the cells captured on the polymer surface cannot be completely washed away from the surface, at least by sterilized distilled water and sterilized physiological saline.

E. coli is classified among Gram-negative bacteria, whose lengths are usually 2–3 μm and whose diameters are 0.5–1.0 μm. It has no gemmae, but it has flagella and can move about. It also possesses physicochemical properties common to general bacteria. One of the most important features among these properties is a charged phenomenon of bacterial cells. In a bacterial suspension, an amino acid constituting a bacterial protein on the cell wall may dissociate into positively charged amino groups (NH_3^+) and negatively charged carboxyl groups (—COO^-). This phenomenon has much to do with the pH of the medium. In an acid medium with a pH value lower than the isoelectric point of the bacteria, there are more amino groups dissociated than carboxyl groups, so the bacterial cells bear positive charges. Conversely, in a basic medium with a pH value higher than the isoelectric point of the bacteria, the dissociation of amino groups is partially inhibited with a relative increase in the dissociated carboxyl groups, so the bacterial cells bear negative charges. When the pH value of a medium equals the isoelectric point of the bacteria, on the surface of the bacterial cells in the medium there are as many positive charges as negative charges, making the cells exhibit electrical neutrality. Because the isoelectric point of Gram-negative bacteria is pH 4–5, *E. coli* cells have negative charges on their surface in the usual neutral environment. However, on the particle surface of an insoluble pyridinium-type polymer, there are positive charges. In this case, if the capturing interaction between the polymer and the bacterial cells is caused only by the electrostatic interaction between them, a change in the charged state of the cell surface will probably make the bacterial cells on the polymer surface come off. On the basis of this inference, the bacterial cells captured on the polymer surface should be washed away from the surface with an acid solution, which can cause the cells to be positively charged and thereby produce a repelling force against the polymer surface with like charges. Thus, the following experiment was performed.

The polymer Q-P(4VP-St-DVB)-BzBr, treated with a bacterial suspension, first was repeatedly washed with sterilized physiological saline, then was washed with aqueous hydrochloric acid (pH 2–3), and again was washed extensively with sterilized physiological saline. The washed Q-P(4VP-St-DVB)-BzBr sample was observed with SEM. The results of the observation are shown in the SEM photographs of Figure 4.

Obviously, there were bacterial cells on the polymer surface. This observation qualitatively indicates that a strong interaction between the bacterial cells and the polymer occurred, thus making the cells firmly adhere to the polymer surface and come off with difficulty, even though the charged state of their surfaces was changed. There is the possibility that a portion of the bacterial cells captured on the polymer surface came off. However, the capturing interaction between an insoluble pyridinium-type polymer and bacterial cells is at least partially irreversible. The strong interaction between them is affected not only by the electrostatic interaction between the positive charges on the polymer surface and the negative charges on the cell surface but also by other factors.

These experimental results strongly suggest that an insoluble pyridinium-type polymer can capture bacterial cells via irreversible adhesion, acting as a polymeric binder for them. The probable reason for this is that an insoluble pyri-

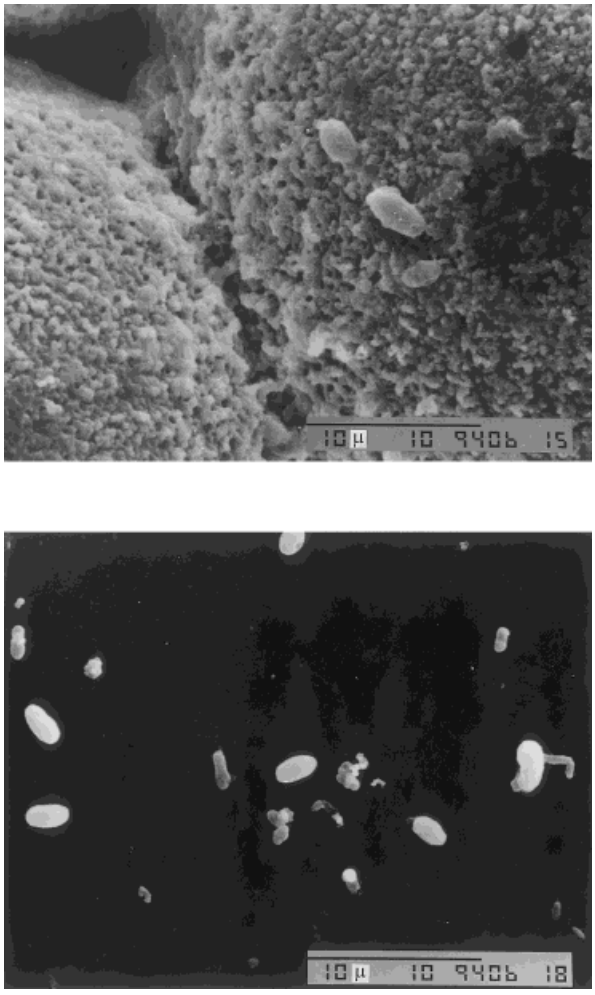

Figure 4 SEM photographs of the surface of Q-P(4VP-St-DVB)-BzBr brought into contact with the *E. coli* cell suspension, washed with aqueous hydrochloric acid (pH 2–3), and extensively washed with sterilized physiological saline.

dinium-type polymer can come into contact with bacterial cells in the polymer/bacterial suspension system via adsorption for a time long enough to create favorable conditions for the adhesion of bacterial cells to the polymer surface.

CONCLUSIONS

On the basis of this investigation into the behavioral features of the antibacterial activity of insoluble pyridinium-type polymers, several important conclusions can be drawn:

1. Insoluble pyridinium-type polymers, except for polymers containing I^-, can capture bacterial cells via adsorption or adhesion and can at least partially maintain the viability of the bacteria captured on the polymer surface.
2. The process of capturing bacterial cells by an insoluble pyridinium-type polymer is at least partially irreversible.
3. An insoluble pyridinium-type polymer is also able to capture dead bacterial cells via adsorption or adhesion.

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