Synthesis and Antibacterial Activity of Copolymers Having a Quaternary Ammonium Salt Side Group

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Synopsis

Copolymers with a quaternary ammonium salt side group have been prepared from vinylbenzyl-cetyldimethylammonium chloride and acrylonitrile and their antibacterial activity has been examined with *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*. Growth inhibitory effect has been found to increase with the increase in the quaternary ammonium salt concentration in the copolymer. The effect is small on gram-negative bacteria and large on gram-positive bacteria.

INTRODUCTION

Recently, much attention has been directed to specialty polymers, of which chloromethylstyrene is one of the useful raw materials. We have been continuing the synthesis and the examination of poly(chloromethyl styrene) derivatives. The reaction products of chloromethylated divinylbenzene–crosslinked polystyrene with poly(ethylene glycol) were effective for removal of nonionic surfactant, polyethylene glycol mono(*p*-nonyl-phenyl ether) solutes in water.[1] The chloromethylated polystyrene beads grafted with polyethylenepolyamines or polyethyleneimines efficiently removed the nonionic surfactant and dodecylbenzene sulfonate from dilute aqueous solutions.[2] Polymer complexes from copolymers of acrylonitrile and ionic vinyl benzyl compounds could be cast into a film with the property of enhanced dyeability and low electrical resistivity.[3,4]

Ikeda et al. synthesized polymers of quaternary ammonium salt surfactants, i.e., poly(trialkyl-3(and 4)-vinylbenzylammonium chloride and observed their antibacterial activity.[5] These cationic surfactants are widely used as disinfectants in hospitals and other places.[6] It is interesting from a practical point of view whether or not these surfactants, when incorporated in copolymers with good processability, would work as antibacterial agents usable in the medical field and waste water treatment. In this connection, we have investigated copolymerization of acrylonitrile and substituted styrenes which are derived from chloromethylstyrene and tertiary amines and have examined the antibacterial activities of the copolymers.

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EXPERIMENTAL

Reagents

Chloromethylstyrene (CMS) (Seimi Chem. Corp., 95% purity) was washed with 3% aqueous NaOH, then with deionized water, and distilled under reduced pressure. Cetyldimethylamine (CDA) (40% aqueous solution) was used as supplied. Acrylonitrile was distilled in inert atmosphere after adding small amount of 2,4,6-tris(dimethylamino)methylphenol. *N,N*-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were dried with molecular shieves 4 A and distilled under reduced pressure. α,α'-Azobisisobutyronitrile (AIBN) was recrystallized from alcohol and dried *in vacuo*.

Synthesis of Vinylbenzyl–Cetyldimethylammonium Chloride

Chloromethylstyrene (73.3 g) (0.48 mol), CDA aq solution (161.7 g) (0.60 mol), and ether (470 g) were swirled in a three-necked flask for 8 h at 20°C under nitrogen atmosphere.[6] From the resulting viscous white solution, ether was removed to give a waxy product, which was dissolved in water and extracted with chloroform. Unreacted amine and chloromethylstyrene were removed by the repeated extraction with chloroform. Residual chloroform was removed from the aqueous solution by evaporation under reduced pressure and vinylbenzyl–cetyldimetylammonium chloride (VB16) was obtained in 76% yield. VB16 was diluted to 50% aqueous solution with deionized water.

Copolymerization of Acrylonitrile and Vinylbenzyl–Cetyldimethylammonium Chloride

Acrylonitrile and VB16 were radically copolymerized in aqueous DMF and an example is given below.

A 29.9 g mixture of 50 mol % acrylonitrile and 50 mol % VB16 and 65.1 g of DMF (total water content of 14.1 g) were put in a 200-mL four-necked flask. While swirling under nitrogen atmosphere, the mixture was added with AIBN 0.095 g and heated at 55°C for 26 h. DMF was distilled off under reduced pressure, and the mixture was poured into ethyl acetate to precipitate produced copolymer. The crude copolymer was extracted with methanol, vacuum-dried to give white powdery poly(acrylonitrile–vinylbenzyl–cetyldimethylammonium chloride) (PANVB16). Yield: 12.7 g (80%). η_{sp}/C at 25°C:

TABLE I
Elemental Analysis of Polymers

Feed AN : VB16		Polymers C	H	N	Cl
90 : 10	Observed	72.03	8.91	14.26	5.08
	Calcd for 88 : 12	72.55	8.77	14.39	4.37
70 : 30	Observed	73.97	10.60	6.61	7.71
	Calcd for 60 : 40	75.41	10.55	6.98	7.07
50 : 50	Observed	73.69	10.77	4.51	8.04
	Calcd for 37 : 63	76.21	11.07	4.90	7.82

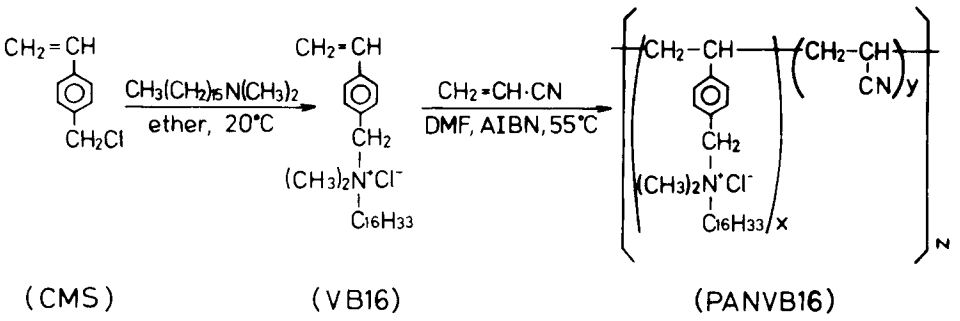

Fig. 1. Reaction scheme.

TABLE II
Synthesis of Copolymers Having Quaternary Ammonium Salt Side Groups[a]

Expt no.	Copolymer composition (mol %) AN : VB16	Solvent (DMF/H_2O %)	Initiator (AIBN %)	Temp. (°C)	Time (h)	Yield (%)	Viscosity[b]
1	95 5	6	0.5	60	30	67	0.64
2	90 10	9.4	0.6	55	24	30	0.59
3	70 30	15.5	0.6	60	24	73	0.80
4	50 50	17.8	0.6	55	26	80	0.41
5	0 100	20	0.6	55	24	70	0.36

[a] Monomer concentration was 20%.

[b] η_{sp}/C measured at the concentration 0.05 g polymer in 25 mL DMF containing 2.0 mmol NaI at 25°C.

0.41 (C = 0.2 g/100 mL, with 0.02 mol NaI). IR: 2250 cm^{-1} (—CN), 1450 cm^{-1} ($-C_6H_5$), 1390 cm^{-1} ($-NH_3Cl$).

Elemental analysis values for three copolymers of different initial charge composition are shown in Table I. Reaction scheme is shown in Figure 1 and polymerization conditions and the viscosities of products are given in Table II.

Antibacterial Property

Antibacterial property was tested on VB16 aqueous solution and powdery PANVB16 samples by the agar dilution method using *Bacillus subtilis* (*B.subtilis*) MI 112 and IFO 3134, *Staphylococcus aureus* (*S. aureus*) IFO 12732, and *Escherichia coli* (*E. coli*) IFO 12734. For the cultivation of *B. subtilis* MI 112, an agar medium consisted of Bacto-Antibiotic Medium-3 (8.75 g) and Bacto-Agar (7.5 g) was dissolved in water 500 mL. *B. subtilis* IFO 12732, *S. aureus*, and *E. coli* were grown on agar medium consisting of Bacto-Nutrient Agar (11.5 g) and water (500 mL).

VB16 monomer was diluted to 0.2 g/mL and VB16 homopolymer (PVB16) was dissolved in 1 : 1 mixture of DMF and ethanol to make 0.1 g/mL original solution. PANVB16 was dissolved in 1 : 1 mixture of DMF and DMSO to make 0.1 g/mL original solution. All original solutions were diluted with the

same mixed solvent to the concentration of 10^3–10^{-1} μg/mL, of which 0.1 mL was added to 10 mL agar medium.

Cultivation media for innoculating bacteria were Antibiotic Medium-3 for *B. subtilis* MI 112 and Nutrient Broth for other bacterial strains. The cultivation was continued for 18 h at 37°C. This bacterial suspension was diluted to the concentration of 10^3, 10^4, and 10^5 cells/mL and from there 0.1 mL was spread on agar plates containing VB16 or PANVB16. After cultivating for 24 h at 37°C, the number of the colonies was counted, and the growth inhibition effect was evaluated as ratio of surviving cell number.

RESULTS AND DISCUSSION

Polymer Synthesis

In aqueous suspension polymerization, the reaction is heterogeneous because the monomers are insoluble in water.[7] In the DMF–water system, monomers are soluble and accordingly the polymerization proceeded smoothly.[3] Elemental analysis values for homopolymers were in good agreement with the calculated values.

Copolymerization reaction was carried out with the initial charges of 90 : 10, 70 : 30, and 50 : 50 AN : VB16 ratios. From the nitrogen content, compositions of the obtained copolymers were calculated respectively 88 : 12, 60 : 40, and 37 : 63.

Antibacterial activity

Growth inhibitory tests for three types of bacteria, *B. subtilis*, *S. aureus*, and *E. coli*, were made on VB16, VB16 homopolymer and PANVB16 copolymers of different compositions. To determine the effects of the solvent for dissolving polymers on bacterial growth, the number (C) of the bacterial colonies formed on the organic solvent-free medium and those (CS) on the media containing organic solvents (DMF : DMSO and DMF : ethanol) were measured for the three types of bacteria. For every bacterial strain, 10^2, 10^3, and 10^4 cells were innoculated on the solvent-free medium and on the media containing the solvents. But there was no appreciable difference in C and CS. Therefore, in these experimental conditions, the organic solvents used for dissolving polymers had no influence on the growth of bacteria.

To clearly show the effect of antibacterial activity, the ratio (M/C) of the surviving cell number (M) on the medium containing VB16 to that (C) without VB16, and the ratio (P/CS) of the surviving cell number (P) on the medium containing polymers to that (CS) containing only solvents were determined. The growth inhibitory effect was quantitatively evaluated by these ratios. Figure 2 shows the M/C values for the three bacterial strains as a function of VB16 monomer concentrations when 10^2 cells were innoculated. Similar results were obtained when 10^3 and 10^4 cells were innoculated. As shown in the figure, the growth inhibitory effects of VB16 monomer differed among these bacterial strains. To clarify the difference, the 50% inhibitory concentration (C_{50}) of VB16 was estimated from Figure 2. The C_{50} values for *B. subtilis*, *S. aureus*, and *E. coli* were 0.65, 1.5, and 330 μg/mL, respectively. The growth inhibition, namely, antibacterial activity, of VB16 was

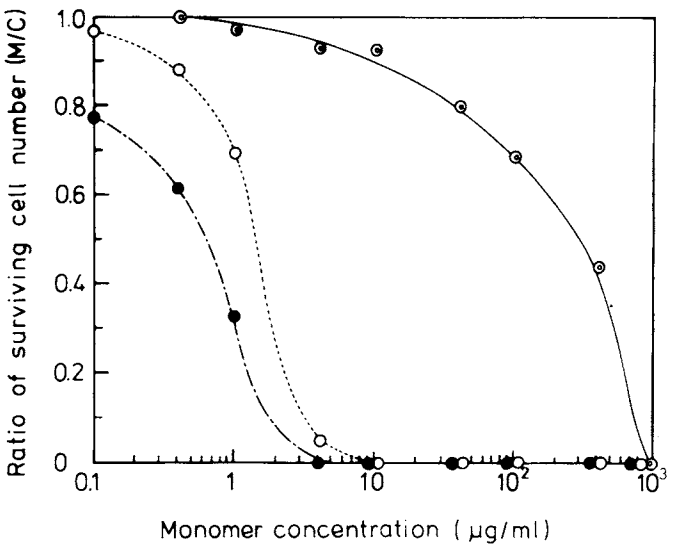

Fig. 2. Ratio of surviving cell number vs. monomer concentration. Inoculation: 10^2 cells; (— ◎ —) *E. coli*; (---○---) *S. aureus*; (- - -●- - -) *B. subtilis*.

most effective for *B. subtilis*, considerably effective for *S. aureus*, and almost ineffective for *E. coli*. Thus, the specificity of the antibacterial agent on the bacterial strains was quantitatively defined.

Ikeda et al. have reported the antibacterial activity of the quaternary ammonium salt against various bacterial strains.[5] Our results were similar to theirs. The antibacterial mechanism has already been discussed by them. They used the "minimum inhibitory concentration (MIC)" to express the antibacterial activity, and therefore their results were presented only semi-quantitatively. In order to develop versatile antibacterial substances, it is favorable to use better measures to compare their antibacterial effect as well as to prepare new agents. Our results suggest that the C_{50} value is suitable for indicating the antibacterial activity and demonstrate that VB16 has an obvious antibacterial activity, although it greatly changes among bacterial strains.

It is important to examine whether or not the antibacterial activity of VB16 is retained in polymeric substances. To determine the antibacterial activity of VB16 homopolymer and PANVB16 copolymer, similar experiments as shown in Figure 2 were carried out using these polymers. Figure 3 shows the *P/MC* values of the polymers as a function of polymer concentrations for the three types of bacterial strains when 10^2 cells were used for inoculation. The AN homopolymer showed no antibacterial activity even at the concentration exceeding 10^3 μg/mL. Similar results were also obtained when 10^3 and 10^4 cells were inoculated. From the figure, the C_{50} values were determined at various experimental conditions and plotted versus molar content of VB16 in copolymers as shown in Figure 4. Two important conclusions were derived from this figure: (1) The antibacterial activity increased with increasing content of VB16 in copolymers, suggesting that the antibacterial function was due to the VB16 moiety in copolymers, and therefore, that the antibacterial

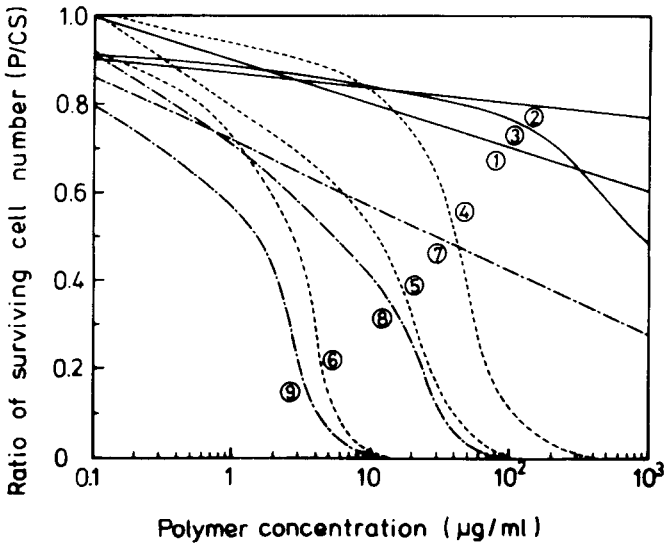

Fig. 3. Ratio of surviving cell number vs. polymer concentration. Inoculation: 10^2 cells. Bacteria: (①, ②, ③) *E. coli*; (④, ⑤, ⑥) *S. aureus*; (⑦, ⑧, ⑨) *B. subtilis*. Polymer composition: (①, ④, ⑦) AN : VB16 = 88 : 12; (②, ⑤, ⑧) AN : VB16 = 37 : 63; (③, ⑥, ⑨) AN : VB16 = 0 : 100.

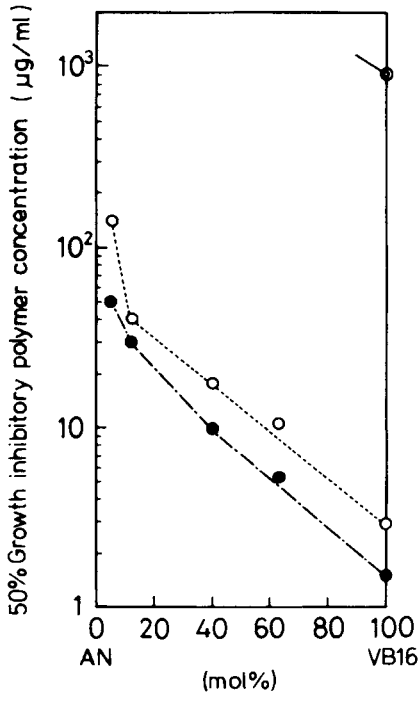

Fig. 4. 50% growth inhibitory concentration vs. copolymer. Inoculation: 10^2 cells; (— ◎ —) *E. coli*; (---○---) *S. aureus*; (- - -●- - -) *B. subtilis*.

ability of VB16 was retained in the copolymers. (2) The nature of the growth inhibitory activity seems to be dependent on the bacterial species: *B. subtilis* was most sensitive to the polymers, *S. aureus* next, and *E. coli* was most resistant, similarly as shown for the monomer in Figure 2. This suggest that the antibacterial spectrum of the agent does not change by copolymerization.

Since the antibacterial agents incorporated in polymers retained their effect on bacteria, it would be possible to prepare polymeric antibacterial agents having good mechanical properties. Indeed, tough films were prepared by casting the solution of the present copolymers. Further studies on the preparation of antibacterial polymers out of chloromethylstyrene are in progress.

CONCLUSION

By the radical copolymerization of vinylbenzyl–cetyldimethylammonium chloride and acrylonitrile, copolymers with a quaternary ammonium salt side group have been prepared. They have been characterized by elemental analysis, viscosity, and IR spectra.

Antibacterial activity of the copolymers has been examined by dissolving them in organic mixed solvent and inoculating *B. subtilis*, *S. aureus*, and *E. coli* by means of agar dilution method. Growth inhibitory effect has been found to increase with the increase in VB16 concentration in the copolymer. The inhibition is effective even in low concentration of VB16. The effect is small on gram-negative bacteria such as *E. coli* but it is large on gram-positive bacteria such as *B. subtilis* and *S. aureus*.

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