Coagulation and Sedimentation of Bacteria Using a Highly Biodegradable Polymeric Coagulant

Nariyoshi Kawabata, Tomoaki Fuse

Department of Materials Science, Faculty of Engineering, University of Shiga Prefecture, Hassaka-cho, Hikone, Shiga 522-8533, Japan

Received 8 April 2005; accepted 30 September 2005
DOI 10.1002/app.23669
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Copolymer of acrylamide with *N*-benzyl-4-vinylpyridinium chloride (PAAM-*co*-BVP) produced coagulation and sedimentation of *Escherichia coli, Bacillus subtillus, Pseudomonas aeruginosa,* and *Staphylococcus aureus.* Addition of more than 50 mg L^{-1} of PAAM-*co*-BVP produced bacterial flocks that precipitated at a rate of around 200 cm h^{-1}. Supernatant population reduced in the range 1/30,000–1/25,000,000. Reduction of supernatant population was most effective when about 200 mg L^{-1} of PAAM-*co*-BVP was added. PAAM-*co*-BVP was highly biodegradable and the half-life estimated was 2.4 days when treated with activated sludge. The ratio of biochemical oxygen demand for 5 days (BOD_5) to total organic carbon (TOC) was 0.607. Coagulation and sedimentation of bacteria using PAAM-*co*-BVP is expected to improve the water disinfection processes by saving chlorine and other hazardous chemical fungicides and by reducing the formation of trihalomethanes and other toxic chemical materials. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 100: 1618–1623, 2006

Key words: functionalization of polymers; biodegradable; separation techniques

INTRODUCTION

Water disinfection processes involve the removal and destruction of microorganisms by both physical and chemical means. The most common process involves treatment with chlorine and other related chemicals as fungicides, but the formation of trihalomethanes and other toxic chemical materials is a serious defect in this procedure, since they are suspected of being carcinogenic.[1] Generally, these materials do not undergo biological degradation, and can become concentrated in the food chain in the environment.

During the course of studies to develop an alternative method, we found remarkable ability of crosslinked poly(*N*-benzyl-4-vinylpyridinium chloride) (insoluble PBVP) to capture bacterial cells.[2] In a previous study,[3] we described removal of microorganisms by filtration using nonwoven cloth coated with a copolymer of *N*-benzyl-4-vinylpyridinium chloride with styrene (PBVP-*co*-ST). However, removal of suspended solids by coagulation would be more suitable than the filtration for a large scale water treatment.

Although removal of microbial cells by coagulation using cationic polymers in the presence of suspended solids was available in the literature, we reported coagulation of microbial cells using not-*cross*-linked poly(*N*-benzyl-4-vinylpyridinium chloride) (soluble PBVP).[4] In the removal of suspended solids by coagulation, the coagulant remains in the treated water. Therefore, biodegradability is desirable for polymeric coagulant. However, biodegradation of soluble PBVP appeared difficult because of its bactericidal activity.[5]

In this work, therefore, we tried to develop a biodegradable coagulant for bacteria. We used polyacrylamide as base polymer, which is widely used for industrial water treatment,[6] and incorporated *N*-benzyl-4-vinylpyridinium chloride to invest affinity with microbial cells. We used a copolymer of acrylamide with *N*-benzyl-4-vinylpyridinium chloride (PAAM-*co*-BVP), but did not incorporate too much amount of the pyridinium group, because soluble PBVP showed bactericidal activity. We expected PAAM-*co*-BVP to be highly biodegradable because poly(methyl methacrylate) turned biodegradable when *N*-benzyl-4-vinylpyridinium chloride was incorporated into the main chain.[7]

EXPERIMENTAL

Materials

Reagent grade acrylonitrile was purchased from Nacalai Tesque (Kyoto, Japan) and was purified by distillation under atmospheric pressure. Reagent grade 4-vinylpyridine was purchased from Nacalai Tesque and was purified by distillation under a reduced pressure of around 8 mm Hg. Reagent grade benzyl chloride, 2,2′-azobisisobutyronitrile (AIBN), dimethylfor-

Correspondence to: N. Kawabata (nkawabat@mat.usp.ac.jp).

mamide (DMF), ethyl acetate, $MgCl_2 \cdot 6H_2O$, and other chemicals were purchased from Nacalai Tesque and were used without further purification. High pressure nitrogen provided by Izumi Sangyo (Kyoto, Japan) was used without further purification.

Polymerization

Polymerizations were performed using a 500-mL round-bottomed three-necked flask equipped with a nitrogen inlet, a reflux condenser, and a mechanical stirrer. We added AIBN (0.49 g, 3.0 mmol) in ethyl acetate (10 g) to a mixture of acrylonitrile (47.8 g, 0.90 mol), 4-vinylpyridine (10.5 g, 0.10 mol) and ethyl acetate (70 g), and stirred under a nitrogen atmosphere at 65°C for 90 min. After the reaction, we separated copolymer of acrylonitrile with 4-vinylpyridine (PAN-*co*-VP) by filtration, and dried to constant weight at room temperature under a reduced pressure. Intrinsic viscosity of PAN-*co*-VP determined in DMF at 25°C was 3.24 dL g^{-1}. Based on the relationship between molecular weight and intrinsic viscosity for polyacrylonitrile,[8] degree of polymerization of PAN-*co*-VP was estimated to be 5380.

We performed the following synthetic reactions using a 50-mL three-necked round-bottom flask equipped with a mechanical stirrer, a nitrogen inlet, and a reflux condenser. We prepared solution of PAN-*co*-VP (1.25 g) in DMF (25 g) by stirring the mixture at room temperature for 24 h. We added 40% aqueous sodium hydroxide solution (0.75 mL) to the earlier mentioned solution with vigorous stirring, and raised the temperature to 85°C. We allowed the mixture to react for 24 h with stirring. After the reaction, we isolated PAAM-*co*-VP by removal of solvents from the mixture using a rotary evaporator, and dried to constant weight under a reduced pressure at room temperature.

We dissolved dried PAAM-*co*-VP (1.00 g) in DMF (25 g), and then added benzyl chloride (43 mg, 0.34 mmol) to the solution, and allowed the mixture to react at 80°C with stirring for 24 h. After the reaction, we isolated PAAM-*co*-BVP by removal of residual benzyl chloride and solvent using a rotary evaporator, followed by washing with ethyl acetate, and dried to constant weight under a reduced pressure at room temperature.

We recorded infrared spectrum of PAAM-*co*-BVP on a Nihon Bunko model FTIR 430 spectrophotometer. The spectrum showed almost absence of C≡N and presence of a small amount of COOH. Elemental analyses performed at Elementary Analyses Center of Kyoto University indicated that PAAM-*co*-BVP contained 82.1 mol % of acrylamide, 11.0 mol % *N*-benzyl-4-vinylpyridinium chloride, and 7.0 mol % acrylic acid.

We prepared polyacrylamide that did not contain *N*-benzyl-4-vinylpyridinium chloride in a similar manner for comparison. Intrinsic viscosity of polyacrylonitrile determined in DMF at 25°C was 2.56 dL g^{-1}, and degree of polymerization was 4350. Thus, obtained polyacrylonitrile was converted to polyacrylamide in a similar manner. Infrared spectrum showed almost absence of C≡N and presence of a small amount of COOH. Elementary analyses showed that the polyacrylamide contained 95.6 mol % acrylamide and 4.4 mol % acrylic acid.

Biochemical oxygen demand and total organic carbon

We performed the measurement of biochemical oxygen demand for 5 days (BOD_5) at 20°C, according to the procedure prescribed by Japanese Industrial Standard (JIS) K 0102. We obtained activated sludge from Sewage Works of Hikone City immediately before the BOD measurement, and washed three times with sterilized physiological saline. We measured concentration of dissolved oxygen using Hach model *sension™8* laboratory dissolved oxygen meter, and that of total organic carbon (TOC) using a Shimadzu model TOC-500 total organic carbon analyzer.

Biological degradation

Specially prepared reagents of meat extract and tryptone were purchased from Nacali Tesque and were used without further purification. Reagent grade yeast extract and polypeptone were purchased from Wako Pure Chemical Industries (Osaka, Japan), and were used without further purification. Reagent grade peptone, urea, sodium chloride, potassium chloride, calcium chloride, magnesium sulfate, and sodium hydrogenphosphate were purchased from Nakalai Tesque and were used without further purification. An artificial sewage prepared according to a literature recipe[9] was used to assist the biodegradation. The artificial sewage was prepared by the addition of peptone (6.0 g), meat extract (4.0 g), urea (1.0 g), sodium chloride (0.30 g), potassium chloride (0.14 g), calcium chloride (0.14 g), magnesium sulfate (0.10 g), and sodium hydrogenphosphate (1.0 g) to 1.0 L of deionized water, and pH was adjusted to 8.5. The chemical oxygen demand (COD) of this undiluted solution was reported to be about 10,000 mg L^{-1}. This solution was diluted with an appropriate amount of deionized water to prepare artificial sewage of prescribed COD concentration.

We obtained activated sludge from Sewage Works of Hikone City immediately before the biological degradation, and washed with sterilized physiological saline as described earlier.

We performed degradation of PAAM-*co*-BVP by the treatment with activated sludge using 200-mL shaking flasks at 25°C. Total amount of the medium was set to be 50 mL. After washing with sterilized physiological saline, activated sludge was suspended in sterilized physiological saline, and the suspension was allowed to stand for 1 h at room temperature. After the standing, 2.5 mL of the supernatant layer of the suspension was taken out and was added to sterilized physiological saline that contained a prescribed amount of polymer sample and artificial sewage. We shook the flask at 120 rpm at 25°C. After a prescribed time, we took out samples and sterilized at 121°C for 20 min, and measured TOC of the sample solution.

Bacteria

We incubated *Escherichia coli* strain *B, Pseudomonas aeruginosa* IFO 3080, and *Staphylococcus aureus* IFO 3060 in nutrient broth prepared by dissolving polypeptone (5.0 g), and meat extract (3.0 g) in 1.0 L of sterilized distilled water at 37°C for 24 h. We incubated *Bacillus subtillus* IFO 3037 in nutrient broth prepared by dissolving tryptone (10.0 g), yeast extract (5.0 g), and sodium chloride (5.0 g) in 1.0 L of sterilized distilled water at 37°C for 24 h. We harvested these cells by centrifugation and washed three times with sterilized physiological saline.

Coagulation and sedimentation of bacteria

We performed coagulation and sedimentation of bacterial cells under aseptic conditions using a glass tube of 30 mm diameter and 20 cm length, connected with a silicone-rubber. We placed suspensions of bacteria in sterilized physiological saline in the glass tube, and added aqueous solution of PAAM-*co*-BVP. We settled total amount of the suspension to about 40 mL. We stirred the mixture using a mixer for several minutes, and allowed to stand in a cold chamber kept at 2°C. During this period, formation of bacterial flocks was visually observed. We evaluated efficiency of the coagulation and sedimentation based on the rate of precipitation of the bacterial flocks.

We also evaluated efficiency of coagulation and sedimentation based on the rate of reduction of bacterial population in the supernatant layer. After a prescribed time, we removed 0.1-mL portion of the suspensions and quickly mixed it with 0.9 mL of sterilized physiological saline, and then prepared decimal serial dilutions from this, by mixing 0.1 mL into 0.9 mL of sterilized physiological saline and mixing. From these dilutions, we counted the surviving bacteria on nutrient media by the spread-plate method. After inoculation, we incubated the plates of bacteria at 37°C, and counted the colonies after 24 h, except for *E. coli.* In this case, we counted the colonies after incubation for 72 h at 30°C. These countings were done in quintuplicate every time.

RESULTS AND DISCUSSION

Biodegradability of PAAM-*co*-BVP

The ratio of BOD to TOC is an index of biodegradability of chemical materials, because TOC reflects substantial concentration of organic materials, whereas BOD is a measure of concentration of biologically degradable materials, in sample solutions.

We found that BOD_5/TOC of PAAM-*co*-BVP (average molecular weight, 470,000) was 0.607. Poly(vinyl alcohol) is well-known as a biodegradable synthetic polymer,[10–13] but BOD_5/TOC of a commercial product of poly(vinyl alcohol) (100% hydrolyzed; average molecular weight, 14,000) was 0.033. PAAM-*co*-BVP was much more biodegradable than poly(vinyl alcohol) in spite of the fact that average molecular weight of the polymer sample was much higher.

In the absence of *N*-benzyl-4-vinylpyridinium chloride, however, polyacrylamide did not exhibit oxygen consumption under the conditions prescribed by JIS K0102, and BOD_5/TOC was substantially zero. The result is not surprising, because most of synthetic polymers are poorly biodegradable. Biodegradability of PAAM-*co*-BVP was attributed to BVP incorporated into the polymer chain, as the case of poly(methyl methacrylate).[7]

Degradation of PAAM-*co*-BVP by the treatment with activated sludge

We performed degradation of PAAM-*co*-BVP by the treatment with activated sludge in water using a 200-mL shaking flask at 25°C. Time course of TOC during the treatment is shown in Figure 1. Initial amount of TOC was 38.0 mg L^{-1} that consisted of PAAM-*co*-BVP (30.0 mg L^{-1}), artificial sewage[9] added to assist the biodegradation (1.2 mg L^{-1}), and activated sludge that performed the biological degradation (6.8 mg L^{-1}). After 7 days, TOC of the sample mixture was 7.1 mg L^{-1}, i.e., it reduced to 1/5.4 of the initial concentration. We have investigated the total amount of organic carbon during the biological treatment, and did not pursue the real amount of PAAM-*co*-BVP. However, Figure 1 indicates that PAAM-*co*-BVP is highly biodegradable. Amount of TOC that remained after 7 days of the treatment was nearly equal to the initial amount of TOC due to activated sludge. This result suggests that most of PAAM-*co*-BVP was consumed by activated sludge within 7 days, and the half-life estimated was around 2.4 days based on the time course of the decrease of TOC shown in Figure 1.

Coagulation and sedimentation of *E. coli*

We placed suspension of *E. coli* in sterilized physiological saline in a glass tube and added aqueous solution of PAAM-*co*-BVP, followed by stirring using a mixer for several minutes, and allowed to stand in a cold chamber at 2°C. In the case where concentration of PAAM-*co*-BVP was in the range 100–700 mg L^{-1} and initial population of *E. coli* was in the order of 10^8 cfu mL^{-1}, we observed formation of bacterial flocks immediately after the mixture was allowed to stand. The bacterial flocks gradually grew and precipitated. Rate of precipitation of the bacterial flocks was not very sensitive to the concentration of PAAM-*co*-BVP, and in the range 190–210 cm h^{-1}.

PAAM-*co*-BVP was much more effective than soluble PBVP for the coagulation and sedimentation of *E. coli*. When soluble PBVP was used under the most appropriate conditions,[4] the rate of precipitation of the bacterial flocks was 22 cm h^{-1}. PAAM-*co*-BVP appeared to fasten the bacterial cells together more strongly than soluble PBVP.

Figure 2 shows time course of population of *E. coli* in the supernatant layer. Cross marks and the dotted line show the results of control experiment performed in the absence of PAAM-*co*-BVP. Open triangle and open circles were obtained in the presence of 40 and 100 mg L^{-1} of PAAM-*co*-BVP, respectively. Supernatant population immediately reduced to 1/20 and 1/200, respectively. After 15 min, however, the supernatant population reduced to 1/1500 and 1/30,000, respectively.

In the absence of *N*-benzyl-4-vinylpyridinium chloride, polyacrylamide did not exhibit coagulation and sedimentation of *E. coli*. Closed circles in Figure 2 show the results obtained by the addition of 100 mg L^{-1} of polyacrylamide that did not contain *N*-benzyl-4-vinylpyridinium chloride. Coagulation of the bacterial cells by PAAM-*co*-BVP appeared ascribable to the strong affinity of *N*-benzyl-4-vinylpyridinium chloride contained in PAAM-*co*-BVP with bacterial cells.[2,3]

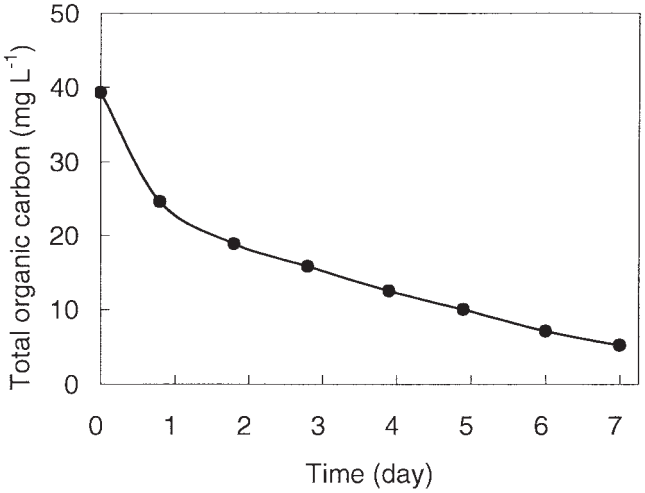

Figure 1 Time course of TOC during the treatment of PAAM-*co*-BVP with activated sludge, performed in water at 25°C using a shaking flask. Initial amounts of TOC due to PAAM-*co*-BVP, artificial sewage added to assist the biodegradation, and activated sludge that performed the biodegradation were 30.0, 1.2, and 6.8 mg L^{-1}, respectively.

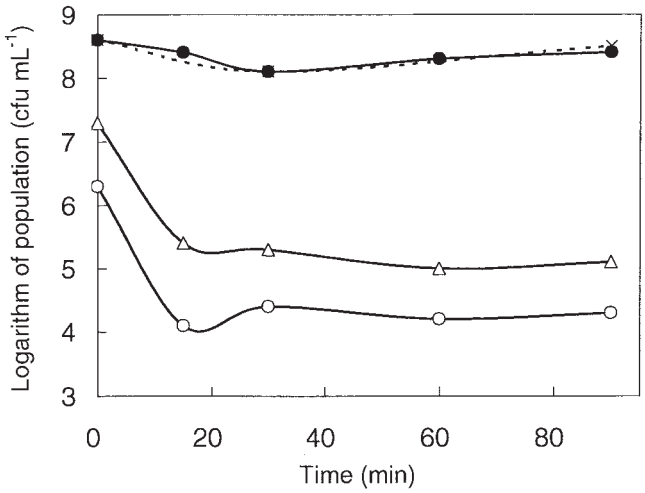

Figure 2 Time course of supernatant population of *E. coli*. (×) Control experiment performed in the absence of polymer; (●) reference experiment using 100 mg L^{-1} of polyacrylamide that did not contain *N*-benzyl-4-vinylpyridinium chloride; (Δ) coagulation and sedimentation using 40 mg L^{-1} of PAAM-*co*-BVP; (○) coagulation and sedimentation using 100 mg L^{-1} of PAAM-*co*-BVP. Initial population, 3.7×10^8 cfu mL^{-1}.

Figure 3 shows influence of concentration of PAAM-*co*-BVP on the rate of reduction of supernatant population after 90 min of the contact time. Most effective concentration of PAAM-*co*-BVP was 200 mg L^{-1} when population was 10^8 cfu mL^{-1}, and the supernatant population reduced to 1/1850,000 under these conditions.

Coagulation and sedimentation of other bacteria

In the case of *P. aeruginosa*, we found it difficult to recognize formation of visually detectable bacterial flocks, similar to the case of bacterial coagulation by soluble PBVP.[4] However, the supernatant population was markedly reduced by the addition of PAAM-*co*-BVP (Fig. 4, open circles). Supernatant population reduced to 1/5300 immediately after the mixture was allowed to stand when 200 mg L^{-1} of PAAM-*co*-BVP was added, and reduced to 1/10,000 after 15 min under the conditions. Addition of 200 mg L^{-1} of PAAM-*co*-BVP was most effective (Fig. 5, open circles). Based on the results, we concluded that PAAM-*co*-BVP produced coagulation and sedimentation of *P. aeruginosa*, but visual recognition of the bacterial flocks was rather difficult in this case.

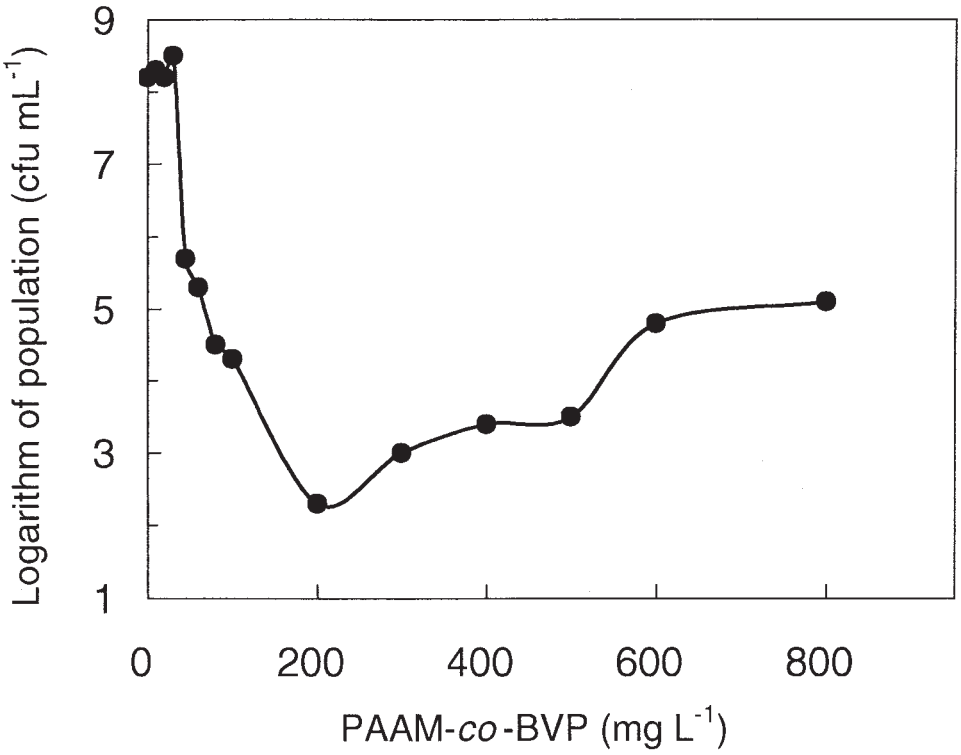

Figure 3 Influence of the concentration of PAAM-*co*-BVP on the supernatant population of *E. coli* after 90 min of contact. Initial population, 1.6×10^8 cfu mL^{-1}.

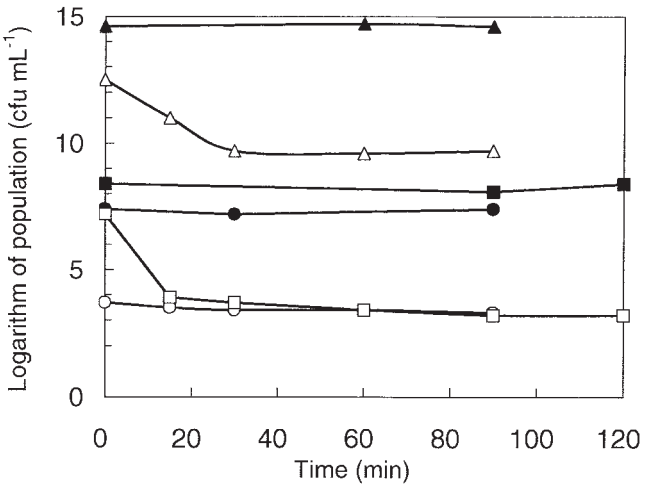

Figure 4 Time course of supernatant population of *P. aeruginosa*, *S. aureus*, and *B. subtillus.* Initial populations are given in parentheses (cfu mL^{-1}). (●) Control experiments for *P. aeruginosa* performed in the absence of polymer (2.8×10^7); (○) coagulation and sedimentation of *P. aeruginiosa* using 200 mg L^{-1} of PAAM-*co*-BVP (2.8×10^7); (▲) control experiments for *S. aureus* performed in the absence of polymer (3.6×10^{14}); (Δ) coagulation and sedimentation of *S. aureus* using 100 mg L^{-1} of PAAM-*co*-BVP (3.6×10^{14}); (■) control experiments for *B. subtillus* performed in the absence of polymer (2×10^8); and (□) coagulation and sedimentation of *B. subtillus* using 100 mg L^{-1} of PAAM-*co*-BVP (2×10^8).

In the case of *S. aureus,* formation of bacterial flocks was observed immediately after the mixture was allowed to stand. Rate of precipitation of the bacterial flocks was around 200 cm h^{-1}. Supernatant population immediately reduced to 1/130 and reduced to 1/80,000 after 30 min when 100 mg L^{-1} of PAAM-*co*-

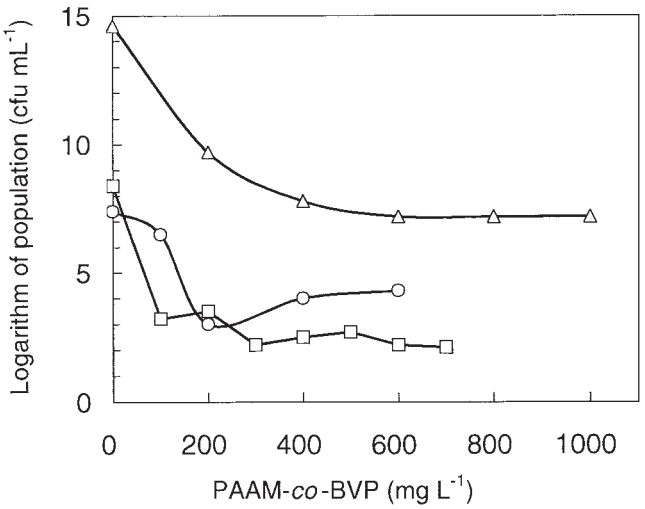

Figure 5 Influence of the concentration of PAAM-*co*-BVP on the supernatant populations of (○) *P. aeruginosa,* (Δ) *S. aureus*, and (□) *B. subtillus* after 90 min of contact. Initial populations of *P. aeruginosa, S. aureus*, and *B. subtillus* were 2.7×10^7, 4.2×10^{14}, and 1.3×10^8 cfu mL^{-1}, respectively.

BVP was added (Fig. 4, open triangles). Addition of more than 500 mg L^{-1} appeared necessary for most effective coagulation (Fig. 5, open triangles), and the supernatant population reduced to 1/25,000,000 when 600 mg L^{-1} of PAAM-*co*-BVP was added. The rate of reduction of supernatant population was exceedingly large among the bacteria used in this study, probably due to the strong interaction between cells of *S. aureus* and PAAM-*co*-BVP, similarly to the case of capture of bacterial cells on the surface of crosslinked PBVP.[14]

In the case of *B. subtillus,* formation of the bacterial flocks was observed immediately after the mixture was allowed to stand. Rate of precipitation of the bacterial flocks was 200 to 270 cm h^{-1} in the cases where the concentration of PAAM-*co*-BVP was 100–700 mg L^{-1}. In coagulation and sedimentation of *B. subtillus* by soluble PBVP,[4] the rate of precipitation of the bacterial flocks was up to 26 cm h^{-1}. Supernatant population immediately reduced to 1/20 and reduced to 1/30,000 after 15 min when 100 mg L^{-1} of PAAM-*co*-BVP was added (Fig. 4, open squares). Addition of 300 mg L^{-1} of PAAM-*co*-BVP was necessary for most effective coagulation (Fig. 5, open squares). Under the conditions, supernatant population reduced to 1/800,000. Coagulation and sedimentation of *B. subtillus* appeared easier than *E. coli.*

CONCLUSIONS

PAAM-*co*-BVP produced coagulation and sedimentation of bacterial cells suspended in water. Addition of more than 50 mg L^{-1} of PAAM-*co*-BVP produced bacterial flocks, and the flocks precipitated at a rate of around 200 cm h^{-1}. PAAM-*co*-BVP exerted the bacterial coagulation more effectively than soluble PBVP. When soluble PBVP was used under the most appropriate conditions,[4] the rate of precipitation of the bacterial flocks was around 20 cm h^{-1}. PAAM-*co*-BVP appeared to fasten the bacterial cells together more strongly than soluble PBVP.

Bacterial population in the supernatant layer reduced to 1/30,000–1/25,000,000 during the treatment. Addition of about 200 mg L^{-1} of PAAM-*co*-BVP was most effective for the flocculation and the reduction of supernatant population.

PAAM-*co*-BVP was highly biodegradable and the half-life was about 2.4 days when treated with activated sludge. The ratio of BOD_5 for 5 days to TOC was 0.607. This result means that PAAM-*co*-BVP is much more biodegradable than poly(vinyl alcohol). The ratio of BOD_5 to TOC for poly(vinyl alcohol) was 0.033. On the other hand, soluble PBVP exhibited bactericidal activity[5] and we could not measure its BOD because of the strong bactericidal activity.

Coagulation and sedimentation of bacterial cells using PAAM-*co*-BVP was expected to improve the water disinfection processes by saving chlorine and other hazardous chemical fungicides and by reducing the formation of trihalomethanes and other toxic chemical materials.

References

1. Hogan, M. D.; Chi, P. Y.; Hoel, D. G.; Mitchel, T. J. J Environ Pathol Toxicol 1979, 2, 873.
2. Kawabata, N.; Hayashi, T.; Matsumoto, T. Appl Environ Microbiol 1983, 46, 203.
3. Kawabata, N.; Inoue, T.; Tomita, H. Epidemiol Infect 1992, 108, 123.
4. Kawabata, N.; Takagishi, K.; Nishiguchi, M. React Polym 1989, 10, 269.
5. Kawabata, N.; Nishiguchi, M. Appl Environ Microbiol 1988, 54, 2532.
6. Yang, W. Y.; Qian, J. W.; Shen, Z. Q. J Colloid Interface Sci 2004, 273, 400.
7. Kawabata, N.; Utihori, D.; Fukuda, S.; Funahashi, H. J Appl Polym Sci 1994, 51, 33.
8. Cleland, R. L.; Stockmayer, W. H. J Polym Sci 1955, 17, 473.
9. Maeda, Y. Hakko Kogaku Kaishi 1979, 57, 114.
10. Suzuki, T.; Ichihara, Y.; Yamada, M.; Tonomura, K. Agric Biol Chem 1973, 37, 747.
11. Morita, M.; Hamada, N.; Sakai, K.; Watanabe, Y. Agric Biol Chem 1979, 43, 1225.
12. Sakai, K.; Morita, M.; Hamada, N.; Watanabe, Y. Agric Biol Chem 1981, 45, 63.
13. Sakazawa, C.; Shimao, M.; Taniguchi, Y.; Kato, N. Appl Environ Microbiol 1981, 41, 261.
14. Kawabata, N.; Ueno, Y.; Torii, K.; Matsumoto, T. Agric Biol Chem 1987, 51, 1085.