Antibacterial Property and Mechanism of Copolymer of Acrylamide and Quaternary Salt of 4-Vinyl Pyridine

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ABSTRACT: The copolymers P(AM-*co*-4VP) of acrylamide (AM) and 4- vinyl pyridine (4-VP) were synthesized. They had serial varied composition and molecular weight and then were quaternarized by using dimethyl sulfate. Two series of cationic polyacrylamide of quaternary pyridine salt-type(Quaternary P(AM-*co*-4VP)'QPAV) had been synthesized, which have different cationic degree and molecular weight respectively. In this paper, the antibacterial property of QPAV was mainly investigated using *Escherichia coli* (*E. coli*) as model bacterium. Influences of various factors, such as cationic degree, molecular weight, and pH value on the antibacterial property of QPAV were examined with the method of the plate counting, and the antibacterial mechanism of QPAV was studied using the method of measuring the activity of β-D-galactosidase and TTC-dehydrogenase. The experimental results show that QPAV possesses excellent antibacterial property, and the antibacterial ratio of QPAV for bacterium suspension of 10^9CFU/mL reached 100% under the conditions of concentration of 20mg/L and contact time of 5 min. The higher cationic degree of QPAV, the stronger is the antibacterial property. In a certain range of molecular weight, the higher the molecular weight of QPAV, the better is the antibacterial activity. The isoelectric point of protein of *E. coli* cell is probably pH = 4.4. When pH > 4.4, the antibacterial activity of QPAV increases as the pH increases, and antibacterial ratio reaches to 100% and remain nearly constant when pH > 5.3. When pH < 4.4, however, the apparent antibacterial ratio increases with the decrease of pH value, and this phenomenon may result from combined effects of QPAV and H^+ ions on microbe. The experimental results reveal that the antibacterial effect of QPAV is not only based on a restraining action but also on a sterilization action. QPAV is believed to cause cell death by disrupting cell membranes, which allows release of the intracellular contents. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 100: 1531–1537, 2006

Key words: biological application of polymers; biomaterials; polyelectrolytes

INTRODUCTION

Among water-soluble polymers, copolymers and homopolymers based on acrylamide are widely used in many fields. Recently, the study of copolymerization of acrylamide with functional monomers has attracted increasing attention. Making polycrylamide cationic and multiple-functional is an important research goal for many scholars.[1–4] It has been reported that cationic polymers of quaternary ammonium salt-type possess excellent flocculation, corrosion inhibition, and sterilization functions, that is, they have multiple functions.[5–7] In view of the above consideration, we have designed molecular devices and prepared a cationic polyacrylamide of quaternary pyridine salt-type in the previous study.[8,9] The synthesis process involved two-step reactions. First, acrylamide and 4-vinyl pyridine were copolymerized, and second, the copolymer was quaternarized using cream reagent. In this way, not only polyacrylamide was cationizated, but also pyridine quaternary ammonium salt was introduced into the copolymer chain. After a detailed study of the copolymerization of AM and 4-vinyl pyridine and quaternarization of the copolymer, the flocculation and corrosion inhibition functions and mechanism of the cationic polyacrylamide of quaternary pyridine salt-type were deeply investigated,[8,9] and in this article, its antibacterial property and mechanism were emphatically studied. A great deal of experimental results showed that the quaternary salt of the copolymer of AM and 4-vinyl pyridine (QPAV) not only has good flocculation and corrosion inhibition properties, but also has strong antibacterial property, so the cationic polyacrylamide of quaternary pyridine salt-type is a kind of multiple-functional polymers. The experimental result of this study shows that the antibacterial property of QPAV not only is based on restraining bacterium, but also is strongly based on the sterilizing. This result is entirely different from some conclusions that although antibacterial property of quaternary pyridine salt species has strong adsorption for bacterium cells, these cells still remain alive.[10,11] Besides, concerning the effect of molecular weight of macromolecules on antibacterial property, some literature described that diffusing and penetrating resistance of antibacterial macromolecules increases with the in-

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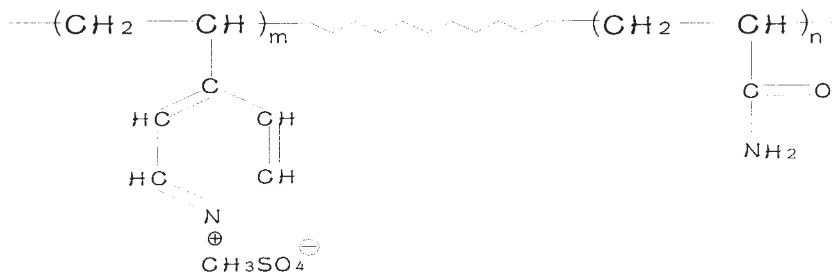

Scheme 1 Structure formula of QPAV.

crease of their molecular weights, so the molecular weight of macromolecules can bring a negative effect on their antibacterial property.[12] However, the experimental result of this study indicates that in certain molecular weight range the antibacterial property of cationic polyacrylamide of quaternary pyridine salt-type QPAV is very obviously enhanced with the increase of the molecular weight. So generally speaking, the antibacterial property of macromolecular antibacterial is predominant over that small molecular antibacterial with similar chemical structure.[13]

EXPERIMENTAL

Materials and instruments

Acrylamide (Merck) was twice recrystallized from chloroform. 4-Vinylpyridine (Aldrich,98%) was dried over calcium hydride overnight and fractionally distilled under reduced pressure (15 mmHg) at 68–69°C, discarding the first and last 10% of the monomer. Potassium persulphate (Aldrich) was used as the initiator, without further purification. Water used here was deionized and then distilled. Dimethyl sulfate and all the solvents (such as methanol, *N,N*-dimethylamide, glycol, and tetrahydrofurane) were of analytical grade. *Escherichia Coli* (*E. coli*) was supplied by a microorganism institute of Shanxi Province of China. The culture medium contained beef cream, peptone, agar, and NaCl that were all commercial. *O*-nitraphenyl,β-D-galactopyranoside (ONPG, Aldrich), 2,3,5-triphenyl-2H-tetrazolium chloride (TTC, Shanghai Reagent Factory), Tri-hydroxymethyl aminomethane (Tris, Beijing Chemical Factory), and Glucose were all of analytical grade. Phosphate buffered solution (PBS) was prepared.

Used instruments were as follows: Unic -2602 UV spectrometer (American Unic Company), PHS-2 acidimeter (The Second Analytical Instrument Factory of Shanghai). 250B biochemical culture box, pressure stainless steel sterilizing boiler, and THZ-82 constant temperature shaker equipped with water bath were all made in China.

Preparing of QPAV

Copolymers of AM and 4-VP, with serial varied composition and molecular weight were prepared according to Ref. 8, and cationic polyacrylamide of quaternary pyridine salt-type (QPAV) was prepared according to Ref. 9, respectively. The main steps of quaternarization were as follows: a given amount of P(AM-*co*-4VP) was dissolved in a mixture of methanol and glycol. The cationization reaction was carried out at room temperature under magnetic stirring. Dimethyl sulfate as the quaternarizing reagent, whose amount was in 5 times excess of pyridine in copolymer to ensure quaternarization reaction complete, was slowly added into the copolymer solution. The reaction was run for 11 h at room temperature. The cationic copolymer (QPAV) then was precipitated by THF, and washed thoroughly with THF. After filtration, the product was dried under vacuum at room temperature for 24 h, and then stored in a desiccator. The structure formula of QPAV is shown in Scheme 1.

The content of 4VP in copolymer P(AM-*co*-4VP) was determined with Ultraviolet spectroscopy photometry. The pyridine unit in copolymer shows an intense absorption at 258 nm, whereas acrylamide unit has no absorption at this wavelength region, so we can determined the composition of copolymer by measuring the content of pyridine unit in the copolymer chain. Because the quaternarizing reagent was excessive and the pyridine ring in the copolymer was quaternarized completely (the fact have been confirmed by ^{1}H-NMR spectra), the molar percentage of the pyridine unit in the copolymer P(AM-*co*-4VP) was equal to the cationic degree of the cationic copolymer QPAV.[8]

Determining antibacterial property of QPAV for *E. coli*

Determining concentration of original cell suspension

Activated *E. coli* was taken and inoculated into liquid culture. The culture was incubated at 37°C while being shaken at 300 rpm for 14 h, and the concentration of the cell suspension was about 10^9CFU/mL. Several clean tubes were prepared, in which 9 mL of sterile water had been added. One milliliter of original cell suspension was added into one of these tubes, and then one by one, the system was diluted to form serial dilutions in these tubes. One fifth of cell suspension of

grads of 10^{-8} and 10^{-9} was taken and plated on plates, respectively. The solid culture was incubated at 37°C for 24 h, the number of viable cells was counted for the plates on which colony forming units were about 10, and the concentration of original cell suspension was obtained as colony forming units (CFU).

Determining antibacterial activity of QPAV under different contact time

One milliliter of original cell suspension of *E. coli* with bacterium age of 14 h and concentration of about 10^9 CFU/mL was added into several clean tubes, respectively, in which 8 mL of water had been added, then 1 mL of QPAV solution with concentration of 0.5 mg/mL was added into these tubes. These mixtures were shaken, and after contacting under different time they were diluted with standard serial dilution method, respectively. For different sample mixture with different contacting time, the plate counting was carried out at different grade, so that the concentration of viable cell for the different sample mixture was determined as CFU. At the same time, the concentration of original cell suspension was determined with the same method as a parallel experiment. The antibacterial ratio was calculated according to the following equation.

$$\text{Antibacterial ratio} = \frac{\text{Number of original cell} - \text{Number of viable cell}}{\text{Number of original cell}} \times 100\% \quad (1)$$

Determining antibacterial activity of QPAV under different dosage

One milliliter of original cell suspension of *E. coli* with bacterium age of 14 h and concentration of about 10^9 CFU/mL was added into several clean tubes, different volume of water was added, and different volume of QPAV solution with concentration of 0.5 mg/mL was also added, so that the final volume of these mixtures all reached to 10 mL. These mixtures with different concentration of QPAV were shaken. After 5 min of the same contacting time, they were diluted with standard serial dilution method. For different sample with different concentration of QPAV, the plate counting was carried out at different grade, so that the concentration of viable cell for the different sample was determined as CFU. At the same time, the concentration of original cell suspension was determined, as described earlier, and the antibacterial ratio was calculated according to eq. (1).

Determining antibacterial activity of QPAV of A series with different cationic degree

QPAV of A series were all prepared as aqueous solution with concentration of 0.5 mg/mL. Each solution of QPAV of 0.2 mL was added into several clean tubes in which 8.8 mL of water had been added, then 1 mL of original cell suspension of *E. coli* with bacterium age of 14 h and concentration of about 10^9 CFU/mL was added into these tubes. These mixtures were shaken, and after 5 min of the same contacting time, the same procedure described in earlier was followed.

Determining antibacterial activity of QPAV of B series with different molecular weight

QPAV of B series were all prepared as aqueous solution with concentration of 0.5 mg/mL. Each solution of QPAV of 0.2 mL was taken and added into several clean tubes in which 8.8 mL of water had been added. Then 1 mL of original cell suspension of *E. coli* with bacterium age of 14 h and concentration of about 10^9 CFU/mL was added into these tubes. These mixtures were shaken, and after 5 min the same procedure described earlier was followed.

Determining antibacterial activity of QPAV under different pH value

First, 44 mL of distilled water was added into several clean beakers, then 5 mL of original cell suspension of *E. coli* with bacterium age of 14 h and concentration of about 10^9 CFU/mL was added into these beakers, finally, 1 mL of QPAV solution with concentration of 0.5 mg/mL was added. The pH value of various mixtures was adjusted by adding dilute solution of HCl and NaOH, so that they had different pH value. These mixtures were shaken for 5 min, and the same procedure described earlier was followed.

Determining bacterial survival probability under different pH value

First, 45 mL of water was added into several clean beakers, then 5 mL of original cell suspension of *E. coli* with bacterium age of 14 h and concentration of about 10^9 CFU/mL was added into these beakers. The pH value of various mixtures was adjusted by adding dilute solution of HCl and NaOH, so that they had different pH value. These mixtures were shaken for 10 min, and the same procedure described in earlier was followed. The survival probability of *E. coli* under different pH value was calculated according to eq. (2).

$$\text{Survival probability} = \frac{\text{Number of viable cell}}{\text{Number of original cell}} \times 100\% \quad (2)$$

Measuring activity of β-D-galactosidase

The substrate of ONPG can be hydrolyzed to form *O*-nitro phenol (ONP) under catalysis of β-D-galac-

TABLE I
Cationic Degree and Intrinsic Viscosity of QPAV

QPAV	Content of 4-VP in monomers (mol %)	Cationic degree (mol %)	Intrinsic viscosity [η] (mL/g)
A-1	75.0	81.03	73.63
A-2	66.7	75.03	75.52
A-3	50.0	64.30	74.25
A-4	33.3	56.50	76.83
A-5	25.0	48.47	75.02
B-1	33.3	54.80	45.10
B-2	33.3	57.30	53.34
B-3	33.3	55.32	66.75
B-4	33.3	56.50	76.83
B-5	33.3	55.87	84.16

tosidase, which is produced by alive cell. So it is possible to estimate whether the cell membrane of *E. coli* is disrupted through examining the situation of the above enzyme-accelerating reaction. The cell suspension of 0.75 mL with concentration of 10^8CFU/mL and 0.75 mL of ONPG solution with concentration of 25 m*M* were added simultaneously into a couple of 5.5 mL PBS buffer solution (pH = 7.4). After shaking for 15 min, different volume of QPAV solution with concentration of 0.5 mg/mL was added into the two mixtures, and their volumes were adjusted up to 10 mL with buffer solution. The changes of absorption of the two mixtures at 420 nm with reaction time were recorded on a spectrophotometer.

Measuring activity of TTC-dehydrogenase

TTC as an acceptor of hydrogen can be reduced to form triphenylformazane (TF) under the catalysis of dehydrogenase, so the sterilizing situation of QPAV for *E. coli* can be estimated by examining the amount of product TF.[14,15] First, 4 mL of water was added into several tubes, then 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 mL of QPAV solution with concentration of 0.5 mg/mL was added into these tubes with an injector, finally 1 mL of cell suspension was added. After contacting for 10 min, 2 mL of Tris-HCl buffer solution (pH = 8.5) and 2 mL of TTC-glucose standard solution were added into these tubes. These mixtures were placed in culture box which temperature remained constant at 37 ± 1°C. After 2 h, when these mixtures cooled, 5 mL of toluene was added to extract the product TF from water phase. Statically placed, these mixtures were demixed, and the absorptions of various organic phases at 485 nm were measured by using toluene as blank liquid. The curve of absorption A against volume of QPAV solution was plotted.

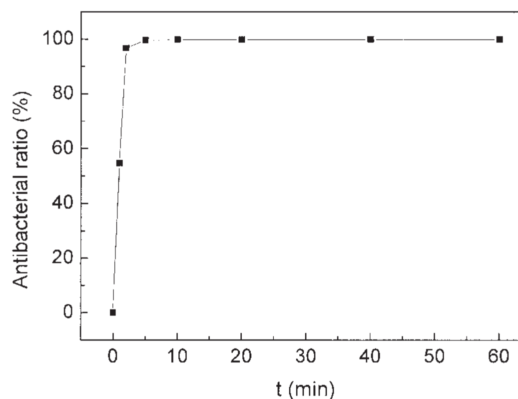

Figure 1 Antibacterial ability of QPAV under different contact time. Sample A-1 and concentration of 50 mg/L.

RESULTS AND DISCUSSION

The composition of cationic polyacrylamide of pyridine salt-type

As described earlier, two series copolymers AM and 4-VP were prepared, which had serial varied composition and molecular weight, respectively. At a fixed amount of initiator, the copolymers with varying content of 4-VP were prepared by varying the feed ratio of two monomers. At a fixed feed ratio, the copolymers with varying molecular weight were prepared by varying the added amount of initiator. After quaternarization, two series of cationic copolymers were obtained. A series had different cationic degree and the same molecular weight (it was characterized with intrinsic viscosity [η]), and B series had different molecular weight and the same cationic degree. The related data about the cationic copolymers are listed in Table I.

The antibacterial activity of QPAV for *E. coli*

Figures 1 and 2 show the varying curves of antibacterial ratio of QPAV for *E. coli* with contact time and dosage.

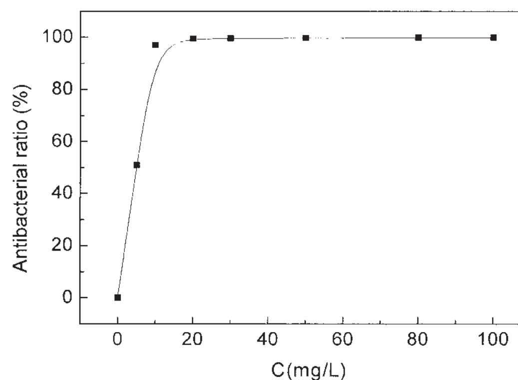

Figure 2 Antibacterial ability of QPAV at different concentration. Sample A-1 and contact time of 5 min.

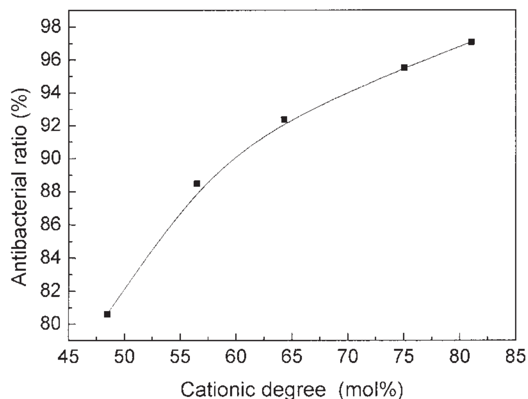

Figure 3 Effect of cationic degree on bactericidal ratio. Concentration of 10.mg/L for QPAV and contact time of 5 min.

Obviously, QPAV enable the number of viable cells of *E. coli* to decrease rapidly in a short time (about 3 min) and under a small dosage (about 10 mg/L). For sample A-1, which has the highest cationic degree, the antibacterial ratio reaches to 100% under the conditions of contact time of 5 min and concentration of 20 mg/L. The experiment results show clearly that the cationic polyacrylamide of quaternary pyridine salt-type possesses very strong antibacterial ability.

Effect of cationic degree on antibacterial activity of QPAV

Figure 3 shows the curve of antibacterial activity for A series copolymers. As described earlier, QPAV possesses very strong antibacterial activity, so to display more distinctly the effect of cationic degree on antibacterial activity of QPAV, the experiment was carried out under the condition of a lower concentration (10 mg/L) and shorter contact time (5 min).

It is seen from Figure 3 that the antibacterial ratio of QPAV increases distinctly with its cationic degree. As quaternary ammonium salt relying on electostatic interaction, quaternary pyridine salt will adsorb strongly bacterial cells that are charged negatively. The higher the cationic degree of QPAV is, the stronger its adsorption for bacterial cells is, so the antibacterial ratio of QPAV enhances as its cationic degree increases.

Effect of molecular weight on antibacterial activity of QPAV

Figure 4 shows the curve of antibacterial activity for B series copolymers. Similarly, to display more distinctly the effect of molecular weight on antibacterial activity of QPAV, the experiment was carried out under the condition of lower concentration (10 mg/L) and shorter contact time (5 min). It is seen from Figure 4 that the antibacterial ratio of QPAV increases obviously with its molecular weight (represented by [η]).

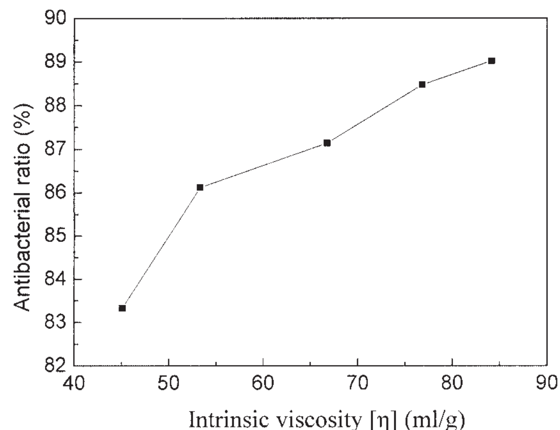

Figure 4 Effect of molecular weight of QPAV on bactericidal ratio. Concentration of 10 mg/L for QPAV and contact time of 5 min.

This article considers that macromolecular antibacterial has higher antibacterial activity relative to smaller molecular antibacterial owing to higher density of antibacterial groups on molecular chain. The "collection effect" will enhance with the increase of molecular weight, so the rule in Figure 4 appears, and it is consistent with Ref. 14. Ref. 12 analyzes that diffusing and penetrating resistance of macromolecular antibacterial in the step of penetrating cell wall will increase with molecular weight, so the penetrating step will be restrained and molecular weight will produce a negative effect on antibacterial property for macromolecular antibacterial. However, the result of this investigation displays that the negative effect do not appear in a certain molecular weight range, and the "collection effect" of macromolecular antibacterial is more predominant.

Survival probability of *E. coli* under different pH value in the absence of QPAV

Figure 5 shows the survival probability curve of *E. coli* under different pH value.

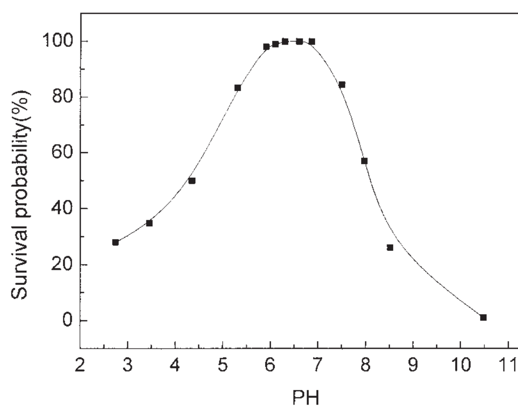

Figure 5 Effect of pH value on bacterium survival probability without QPVA.Operation time 10 min.

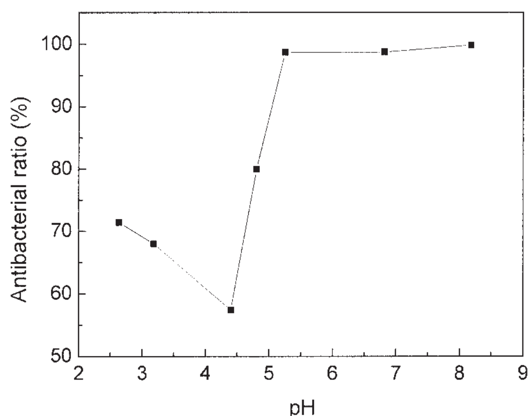

Figure 6 Effect of pH value on bactericidal activity of QVAP. Sample A-1, concentration of 10 mg/L and contact time of 10 min.

It can be seen that the survival probability of *E. coli* is different as the original cell suspension is added into water with different pH value. The survival probability of *E. coli* decreases with falling of pH value at pH < 6.1; the survival probability decreases with boosting of pH value at pH > 6.8; the survival probability is nearly 100% at pH = 6.1–6.8. Earlier mentioned experimental results show that H^+ ions in acidic solution and OH^- ions in basic solution are all deleterious to *E. coli*.

Effect of pH value on antibacterial activity of QPAV

The antibacterial activity of QPAV under different pH value was tested, and Figure 6 shows the changes of antibacterial activity of QPAV with change in pH value. It can be seen that there is a lowest point on the curve, and the corresponding pH value is 4.4. This investigation considers that the lowest point is probably corresponding to the isoelectric point of the protein of *E. coli* cells, namely, the cells of *E. coli* are not charged at pH = 4.4. So, adsorption force between QPAV and cells does not exist any longer, and the antibacterial ability is the weakest. Over the isoelectric point, the neutralized degree of the carboxylic groups of amino acid, which is the main component of cell protein, generally increases with enhancing of pH value, negative electricity on surface of cells increases, and the adsorption interaction between QPAV and cells increases, so the antibacterial activity of QPAV increases with enhancing of pH value, and the antibacterial activity reaches to 100% as pH increases up to 5.3; to the left of the isoelectric point, the cells are charged positively, and mutual repulsion of electricity of the same nature is disadvantageous to the adsorption interaction between QPAV and cells, so the antibacterial activity of QPAV should be very low. However, the apparent antibacterial activity of QPAV increases with the decrease of pH value. How is the phenomena explained? Combined with Figure 5, there is mainly the antibacterial effect of ion H^+ at pH < 4.4, and the effect of ion H^+ on the animalcule increases with the decrease of pH value to the left of the isoelectric point. So the action of QPAV at pH < 4.4 may be negligible.

Antibacterial mechanism of QPAV

β-D-galactosidase has special hydrolyzing activity for β-D-galactose glycoside bond in sugar, fat, and lactose.[16] Especially, β-D-galactosidase from *E. coli* has substrate specificity for the hydrolysis of ONPG.[17] If the cells of *E. coli* break, β-D-galactosidase will leak out of the cells, and further it will catalyze the hydrolysis of ONPG in the solution. Because ONP, the product of ONPG hydrolysis, has a characteristic absorption at 420 nm, with spectroscopy method it can be determined that whether the cells of *E. coli* are disrupted. In this article, two different volume amounts of QPAV solution were added into the cell suspension containing ONPG. The changes of absorption with time were measured, and the absorption of blank system into which QPAV solution was not added was also measured at the same time. In Figure 7 the measuring results are shown. From Figure 7 it can be seen that the absorption of the blank system is nearly zero and it does not vary with time. The fact displays that the membrane of *E. coli* cell is intact and no β-D-galactosidase is released as QPAV solution is not added. However, for the cell suspension into which QPAV solution was added, the absorption at 420 nm increases with time, and for the cell suspension into which more volume of QPAV solution was added, the absorption increases more rapidly. The experimental fact indicates clearly that added QPAV solution disrupts cell membrane of *E. coli* and the intracellular contents including β-D-galactosidase are released. Moreover, the more the added amount of QPAV so-

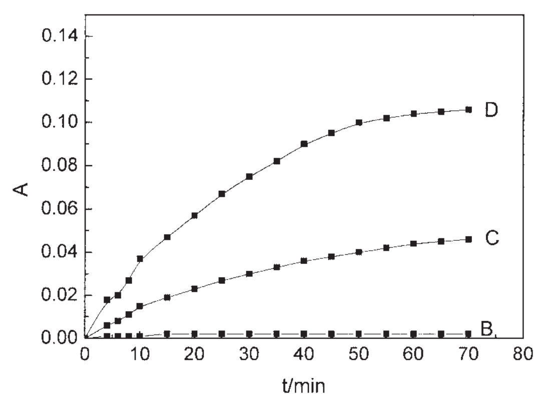

Figure 7 Varying of absorption of ONP at 420 nm with time with different amounts of QPVA solution. B 0 C 1 mL D 2.5 mL.

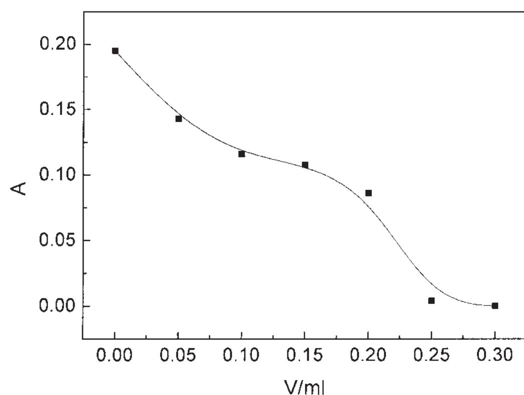

Figure 8 Absorption of TF versus added volume of QPAV solution. Extracting time 1 h.

lution is, the higher is the destroying action to the cell membrane, and stronger is the activity of β-D-galactosidase. The breaking of cell membrane means the death of bacteria, so it can be concluded that the antibacterial effect of quaternary pyridine salt is not only based on restraining bacterial, but also based on killing bacteria. Thereby, the conclusion that after action of quaternary pyridine salt the adsorbed bacterial cells still remain alive[10,11] needs to be argued further.

This article examined further the antibacterial mechanism of QPAV by adopting TTC-color method. TTC is a small substance; it can pass through cell wall and membrane and be incepted by live bacteria. Furthermore, under the action of dehydrogenase from live cells, it will be reduced into the product TF, which is red. The more the number of live cells, the more vigorous is the metabolism, the stronger is the ability of transforming TTC, and more is the produced amount of TF. If the bacterium is killed partly, the transforming ability will weaken, and the produced amount of TF will decrease. So the survival amount of live bacteria can be estimated by measuring the produced amount of TF. Figure 8 shows the relationship between the absorption of TF at 485 nm and the amount of QPAV solution added into the cell suspension. It can be obviously seen from Figure 8 that absorption of TF gradually decreases with the increase of the added volume of QPAV solution. The experiment fact illuminates fully that QPAV exerts a killing effect on *E. coli*.

Now the antibacterial mechanism of QPAV can be explained as follows. First, relying on electrostatic interaction, QPAV molecule charged positively is absorbed onto the surface of bacterial cells, further penetrates through cell wall, combines with the protein layer and analogous fat layer of cell membrane, and blocks normal exchange of ions and substance between bacteria and environment. Furthermore, the combination of QPAV and cell membrane destroys the protoplasmic membrane, causes leakage of intracellular contents, and leads the bacteria to death. So the antibacterial mechanism of cationic polyacrylamide of quaternary pyridine salt-type is not only based on a process of restraining bacterium, but also is strongly based on the sterilizing process.

CONCLUSIONS

In conclusion, we have investigated the antibacterial property of the cationic polyacrylamide of quaternary pyridine salt-type in detail, and have seriously probed into its antibacterial mechanism. The experimental results show distinctly that QPAV possesses excellent antibacterial activity, and the antibacterial property is based not only on restraining but also on sterilizing for bacteria. When the concentration of QPAV is 20 mg/L, the sterilization ratio can reach to 100% in 5 min for the cell suspension of 10^9CFU/L. The antibacterial mechanism of QPAV have been revealed by measuring the activity of β-D-galactosidase and TTC-dehydrogenase. The action of QPAV can be thought to be as follows: first, its molecule is absorbed on surface of cells due to electrostatic interaction, then penetrates the cell wall and combines with cell membrane, finally disrupts the protoplasmic membrane and causes intracellular components to leak out of the cells, and leads the bacteria to death. Combined with the previous study, it can be concluded that the cationic polyacrylamide of quaternary pyridine salt-type is a soluble copolymer with multiple functions, it has flocculation, corrosion inhibition, and antibacterial functions concurrently, and will have wide application foreground in water purification treatment field.

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