Study on Antibacterial Behavior of Insoluble Quaternary Ammonium

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Received 15 September 2004; accepted 13 May 2005
DOI 10.1002/app.22810
Published online 15 December 2005 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Insoluble crosslinked polystyrenes (PS) grafted with different quaternary ammonium groups were synthesized as antibacterial agents. Their antibacterial activities against *Staphylococcus aureus* were investigated by a colony count method. It was found that when the concentration of the insoluble antibacterial agent was low, the antibacterial activity was different from that of the typical soluble antibacterial agent. The antibacterial activity was bacteriostatic rather than bactericidal. A model for explanation of the antibacterial activity of the insoluble ammonium salt was set up. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 99: 2389–2394, 2006

Key words: antibacterial agent; polystyrene; quaternary ammonium; structure-property relations; synthesis

INTRODUCTION

Generally, conventional antibacterial agents are liquids or gases with low molecular weight. The use of these disinfectants may bring about recontamination problems.[1] For example, in the case of water treatment for domestic water supply, the most popular method is to use chlorine and other related chemicals to sterilize water. But their residues will concentrate in the food chain in the environment. In addition, chlorine and other related chemicals may react with organic substances in the water to yield halomethane analogues that are suspected of being carcinogenic.[2] However, these drawbacks can be solved by using insoluble antibiotic agents, which do not release small molecular chemicals.[3] In light of this idea, insoluble cationic polymers that can inactivate, kill, or remove target microorganisms by mere contact without releasing any reactive agents were investigated. These polymers are mainly crosslinked anion-exchange resins, including quaternary ammonium-type resins,[4] polyiodide resins,[5] and pyridium-type resins.[6] The polymers exhibit many unique properties in the capture or isolation of bacteria and viruses and removal of organic pollutants from aquatic environment.[7] Their disinfective or purifying effect has been studied. The research undoubtedly provides a basis for developing a new method of antibiotic treatment.

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Journal of Applied Polymer Science, Vol. 99, 2389–2394 (2006)

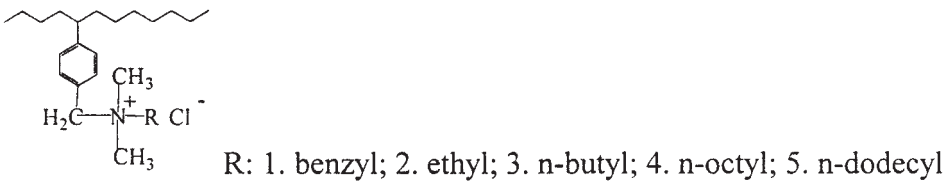
Ron *et al*. reported an easy way of synthesizing a tertiary amine quaternized with a water insoluble carrier to provide a quaternary ammonium salt.[4] Ikeda and Tazuke investigated antibacterial activity of soluble polymeric quaternary ammonium salts by improving the conventional spread plate method and the viable cell counting method.[8,9] They found that the polymeric salts are more active than the corresponding monomer, and compounds with the longest alkyl chain exhibited particularly high activity. Li *et al*. synthesized and investigated the soluble and insoluble pyridium-type functional polymers as antibacterial agents.[10,11] They found that the antibacterial activity of insoluble pyridium-type polymers is characterized by an ability to capture bacterial cells in a living state, with the process that is partially irreversible.

In this study, we synthesized a family of five insoluble polymers with different ternary ammonium groups. By investigating their antibacterial activity, the mechanism for the action of antibacterial agent and the relationship between the ammonium structure and the antibacterial activity were proposed. It was found that the insoluble structure and the adsorption of bacteria on the antibacterial agent play an important role in the antibacterial behavior. A novel model for antibacterial activity of insoluble ammonium salt was set up.

EXPERIMENTAL

Ternary amine and polymer carrier

Ternary amine (Alderich Co.) was used as received. Macroporous crosslinked chloromethylated polysty-



Scheme 1 The structures of the insoluble quaternary ammonium salts.

rene (PS) resin (Chemical Plant of Nankai University) with the pearl size of 0.3–1.25 mm and porosity of 65–70% was washed thoroughly by cycles of aqueous hydrochloric acid (2*M*, 50 mL) and aqueous sodium hydroxide (0.1*M*, 50 mL), thereafter with dilute saline (2*M*, 25 mL) and with deionized water until the effluent was free of chloride. The resultant resin was dried at 50°C under vacuum until constant weight.

Preparation of broth for bacterium incubation and of plate for spread-plate method

Peptone (10 g), NaCl (5 g), and beef extract (3 g) were dissolved in 1000 mL sterilized water, and the pH was adjusted to 1–1.2 by adding NaOH solution. The obtained broth was sterilized in a pressure cooker at 121°C for 15 min. The broth was later mixed with the melting agar, and then cooled for the use of spread-plate method.

Bacteria

The gram-positive bacterium *Staphylococcus aureus*, provided by the Department of Food Science and Nutrition of Zhejiang University, was used as a test bacterium in the experiment of the antibacterial activity. *S. aureus* was incubated at 30°C for 24 h in broth before use.

Preparation of insoluble quaternary ammonium PS

The dried, chloromethylated crosslinked PS (5.0 g) was immersed in dioxane (100 mL) for 48 h at room temperature (~20°C). The ternary amine was fed into the mixture and reacted for 48 h at room temperature with intermittent stirring. The mixture was filtered and the filtrate was collected and used for later titration. The residual resin was washed thoroughly by cycles of aqueous hydrochloric acid (2*M*, 50 mL) and aqueous sodium hydroxide (0.1*M*, 50 mL), thereafter with dilute saline (2*M*, 25 mL) and with deionized water till the effluent was free of chloride. The resultant quaternary ammonium salt in resin form was dried at 50°C under vacuum.[4] Scheme 1 shows the different structures of the prepared insoluble quaternary ammonium polymer resin.

Characterization of the insoluble ammonium PS

The amine content in the filtrate of each sample was determined by nonaqueous titration developed by Burleigh *et al.*[12] Therefore, the amine grafted onto the polymer carrier can be calculated by subtracting the residue from the initial amount.

Antibacterial test for insoluble ammonium PS

All procedures in the antibacterial tests for insoluble ammonium-type polymers were performed under aseptic conditions. Everything in contact was sterilized in pressure cooker by vapor. Sterilized distilled water was used as a suspension medium for the *S. aureus* cells.

Preparation of the bacterial cell suspension

One loopful of fresh bacteria was suspended in an appropriate amount of sterilized water, forming a bacterial cell suspension. The viable cell number in the suspension was controlled via the turbidity comparison method. This suspension was diluted to a prescribed cell concentration with sterilized distilled water, and then the diluted bacterial cell suspension was used directly for the antibacterial tests of the insoluble ammonium polymer salt.

Batch treatment

In each test, 20.0 mL of the bacterial suspension and certain amount (dry weight) of a polymer sample were placed in a sterilized glass container with a cotton stopper. The container was shaken 200 rpm. At prescribed time intervals, 0.5 mL of the suspension system was pipetted out from the container and quickly mixed with 49.5 mL of sterilized physiological saline, and then decimal serial dilutions were prepared from this by adding 0.5 mL of it to 4.5 mL of sterilized physiological saline and by mixing. The viable cell number in each of the polymer/bacterial suspension systems at the contact time was determined by conventional spread-plate method. The colonies were counted after the inoculated plates were incubated at 30°C for 24 h. The counting was done in triplicate each time.

TABLE I
The Reactivity of Quaternization of Different Ternary Amines

Sample no.	Ternary amines	Ammonium content[a] (10^3 mol ammonium/g product)
1	*N,N*-Dimethylbenzylamine	3.22
2	*N,N*-Dimethylethylamine	5.75
3	*N,N*-Dimethylbutylamine	4.89
4	*N,N*-Dimethyloctylamine	3.19
5	*N,N*-Dimethyldodecylamine	2.11

[a] Crosslinked PS (5 g) and 1mol ternary were reacted in 100 ml dioxane.

RESULTS AND DISCUSSION

Activity of different ternary amine reacting with crosslinked PS

Table I shows the quaternization ability of different ternary amines. The activity decreases as the length of the substitute alkyl group increases. This can be explained by the increasing steric hindrance of the amine. Because the reaction mechanism is a typical S_N2 reaction, when the length of the substitute increases, it is more difficult for the N atom in the amine to attack the C atom in the polymer carrier.

Antibacterial activity of insoluble ammonium salt

Table II shows that the insoluble quaternary ammonium salts have good ability to remove bacteria in suspension. Then, we decreased the concentration of antibacterial agent and in the same time increased the initial viable cell concentration. The antibacterial activities became quite different. Figure 1 shows that the viable cell number ($N(t)$) decreases within 30 min of contact. Samples 3 and 5 cut down the cell number less than 10^3 cell/mL, and kept this level afterwards. But for samples 1, 2, and 4, the behaviors were more complex. $N(t)$ increases drastically from 30 to 120 min, and then reaches level even higher than the blank test.

TABLE II
The Removal for *S. aureus* in Suspension of Insoluble Ammonium Salts

Quaternization agent	Viable cell concentration[a] ($N(t)$) (cells/mL) 30 min	60 min	120 min
N,N-Dimethylbenzylamine	$<10^3$	$<10^3$	$<10^3$
N,N-Dimethylethylamine	$<10^3$	$<10^3$	$<10^3$
N,N-Dimethyloctylamine	$<10^3$	$<10^3$	$<10^3$
N,N-Dimethyldodecylamine	$<10^3$	$<10^3$	$<10^3$

[a] Initial viable cell concentration is 2.00×10^7 cells/mL; 0.400 g insoluble agent was added.

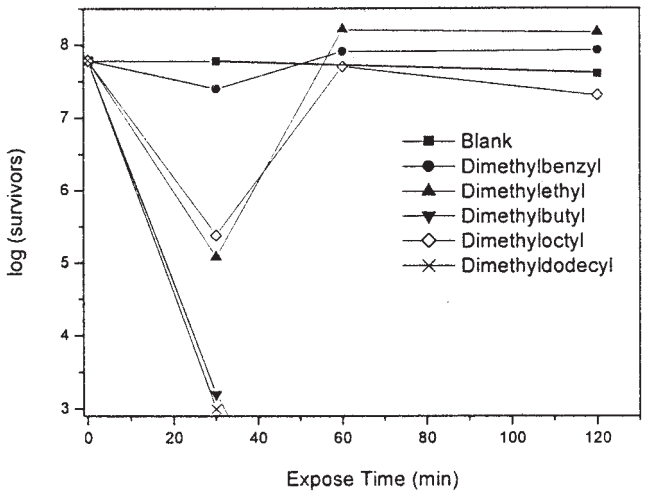


Figure 1 Extent of removal for *S. aureus* of insoluble ammonium salts versus exposure time; Initial viable cell concentration is 6.10×10^7 cells/mL; 0.200 g insoluble agents was added.

To confirm this phenomenon, we repeated the test under higher initial bacterial concentration and obtained Figure 2. Figure 2 shows the same phenomenon as in Figure 1 that $N(t)$ increases drastically from 30 to 120 min, and then reaches a level even higher than the blank test.

The antibacterial mechanism for the insoluble quaternary ammonium salts

Generally believed mechanism for small molecule antibacterial agent

The antibacterial mechanism for ammonium salts, especially the low molecular or soluble ones, has long been studied.[13] Their antibacterial activity is characterized by the ability to kill bacteria in water. It is generally believed that the process can be summarized in terms of the following steps: (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 cytoplasmic constituents such as K^+ ions, DNA and RNA; (6) death of the cell.

Possible mechanism for insoluble ammonium polymer antibacterial agent

But when the cationic group was riveted on the polymer backbone that was insoluble, the situation is quite different. The segments of an insoluble polymer agent may be unable to diffuse through the cell wall. Therefore, the interaction between the polymer and the bacterial cells will come to a halt at the stage of absorption. The bacterial cells captured on the surface can still remain alive, and the antibacterial activity of

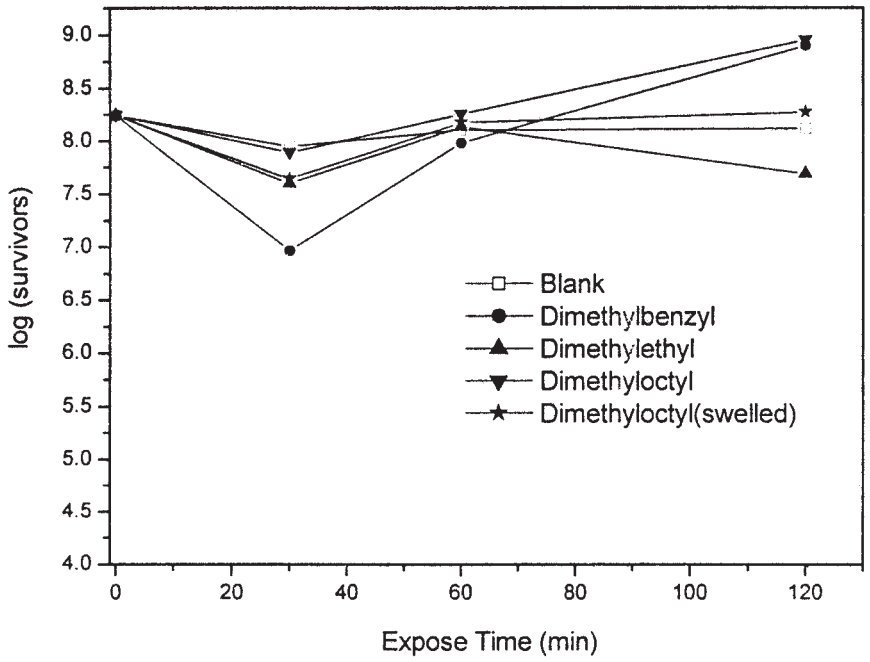


Figure 2 Extent of removal for *S. aureus* of insoluble ammonium salts versus exposure time; Initial viable cell concentration is 1.76×10^8 cells/mL; 0.200 g insoluble agents were added.

this material may be bacteriostatic rather than bactericidal.[14,15]

Li and Shen studied the antibacterial activity of insoluble pyridium-type polymers with different structures against *S. aureus*.[11] They found that the antibacterial activity of insoluble pyridinium-type polymers 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. In addition, they found that insoluble pyridinium-type polymers can capture dead bacteria, and the capture strength was very strong.

We can summarize the antibacterial process as chemical reactions in the following way:

$$L + P \leftrightarrow LP \text{ physical adsorption of viable cells} \quad (1)$$

$$D + P \rightarrow DP \text{ physical adsorption of dead cells} \quad (2)$$

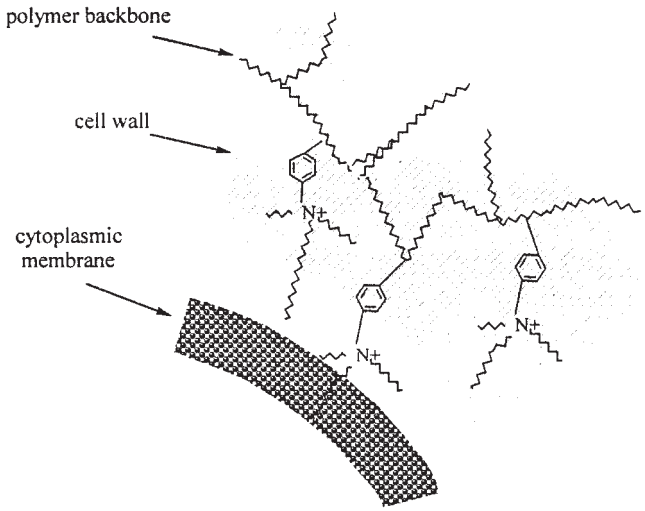
$$LP \rightarrow DP \text{ killing of the viable cells} \quad (3)$$

L is the viable cells, P is the insoluble ammonium polymers, D is the dead cells, LP is the living cells adsorbed on polymers, and DP is the dead cells adsorbed on polymers. eq. (1) directly decreases the number of viable cell in suspension, while eq. (3) is the key step in killing the bacteria. It is assumed that the adsorption of viable cells is a reversible process, but the adsorption of the dead cells is an irreversible one. Because dead cells loose their mobility and the interaction between dead cell and ammonium group is strong.

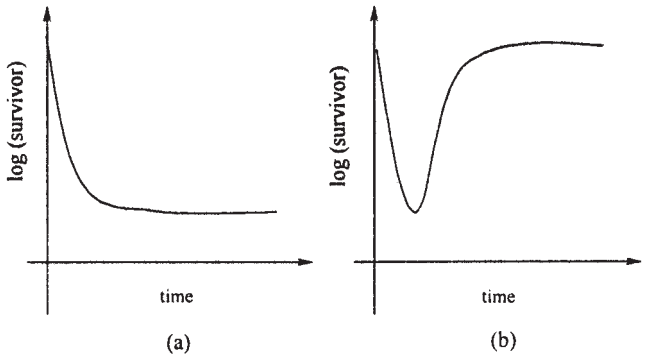
Scheme 2 shows that the penetration of the insoluble ammonium polymers into the viable cells is intensively affected by the flexibility of the polymer backbone and mobility of the functional group. With more flexible backbone, the ammonium group is easier to reach the cytoplasmic membrane.

Explanation for the antibacterial activity of insoluble ammonium salts

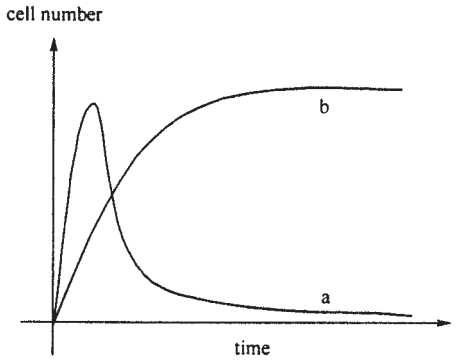
Scheme 3 shows the profile of antibacterial activity of typical soluble ammonium salts and the insoluble



Scheme 2 The penetration of ammonium group into the bacterial cell.



Scheme 3 The profile of antibacterial activity of ammonium salts with structure:(a) soluble; (b) insoluble.



Scheme 5 Estimated profile of cell absorption of insoluble ammonium salt versus time:(a) for viable cells and (b) for dead cells.

ammonium salt studied in this article. The profile can be seen as the kinetic profile of assumptive reactions shown as eqs. (1)–(3)

For insoluble quaternary ammonium salts, $N(t)$ first decreases, and then recovers to the initial level, or even exceeds the initial level. To the best of our knowledge, this phenomenon has never been reported before. Because the bacteria cannot incubate under a low nutrient concentration or within such short period of time, the recovery of $N(t)$ may be due to the release of the viable bacterial cells from the polymer surface.

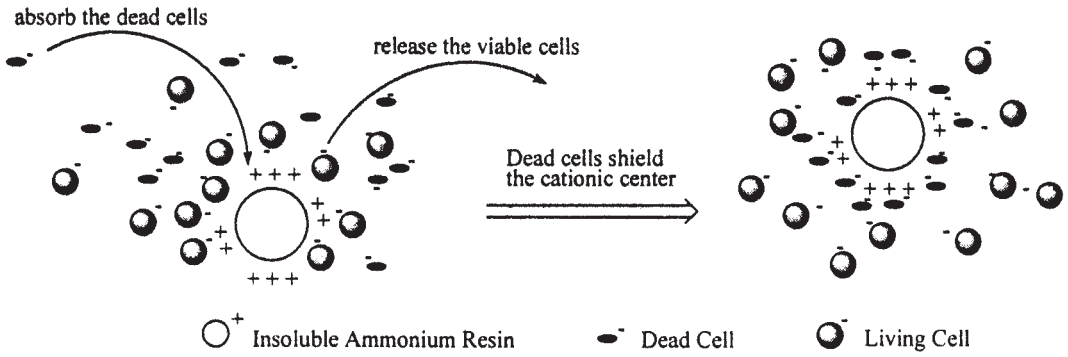
A possible process was proposed. The insoluble ammonium salt may first absorb the living bacterial cells, rather than kill them, but keep them alive. As shown in reaction (1), the absorption and release of the viable cells is a reversible process. The agent may also absorb the dead cells, as shown in reaction (2); the process is supposed to be an irreversible one. When the dead cells accumulate on the surface of the agent and shield the cationic center, the ammonium salt cannot absorb the living cells any more, and the previous absorbed viable cells are released from the insoluble ammonium salt, as shown in Schemes 4 and 5.

There are always dead bacterial cells, even in the newly prepared bacterial suspension. When the ammonium group concentration is low or the initial bacterial concentration is high, the dead cells may totally screen the cationic center on the insoluble ammonium salt; therefore, the agent cannot show any ability to remove the viable cells in the suspension.

But the supposed model and mechanism cannot explain why $N(t)$ even exceeds the blank test after 60 min of contact. It seems that the low concentration of insoluble ammonium salt will stimulate the growth of the bacteria.

CONCLUSIONS

The result of antibacterial activity of insoluble ammonium salts in low concentration shows that the removal of bacteria in the suspension is by the way of capturing the bacterial in a living mode, rather than biocidal. The adsorption process plays an important role in the antibacterial behavior. Especially, the adsorption of the dead cells may greatly affect the antibacterial activity of the insoluble ammonium salt. However, the reason why the viable cell number even exceeds the blank test after a period of contact is still a matter for discussion.



Scheme 4 Possible process of the antibacterial activity of insoluble ammonium salt.

Professor Yuanmin Yin is gratefully acknowledged for his effort in helping the antibacterial experiment.

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