Water-Soluble Cationic Polymers and Their Polymer–Metal Complexes with Biocidal Activity: A Genotoxicity Study

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ABSTRACT: Water-soluble polyelectrolytes containing ammonium and sulfonic groups, their polymer–Ag(I) complexes, and silver nitrate were investigated as bactericidal compounds for *Staphylococcus aureus* (Collection No. ATCC 28922) and *Escherichia coli* (Collection No. 6538P) according to the National Committee for Clinical Laboratory Standards (NCCL) method. All the compounds, except Ag(I), showed bactericidal activity only for *S. aureus*. Ag(I) showed high bactericidal activity for both bacteria. No important effect of the molecular weight or macromolecular size on the maximum bactericidal concentration value was observed. The genotoxicity was studied using the *rec* assay. None of the macromolecular compounds showed genotoxicity, except silver ions, whose value was borderline. © 2002 Wiley Periodicals, Inc. J Appl Polym Sci 87: 452–457, 2003

Key words: water-soluble polymers; polymer–Ag^+ complexes; biocides; genotoxicity

INTRODUCTION

In the last few years, synthetic water-soluble polymers have become of great interest in the biological field.[1–7] Some polymers have been investigated as biocides because of the advantages inrespect to the monomeric analogues.[8,9] In general, antibacterial agents like bactericides or disinfectants are low-molecular-weight compounds.[10–13] Phenols and cationic compounds are two groups of compounds used almost exclusively as disinfectants. The latter covers many kind of compounds that differ considerably in chemical structure. The most common groups present in this type of compound are quaternary ammonium salts and phosphonium salts.[14]

Moreover, polyelectrolytes, basically cationic, containing ammonium and phosphonic groups at the main and side chains show a strong bactericidal activity against gram-positive and gram-negative bacteria.[15,16] In this sense, the electrostatic interaction of the positive charges on the molecules of the antibacterial agents with the negatively charged species present in the cytoplasm membranes (i.e., acidic phospholipids and membrane proteins) is the first step for the biocidal action of the compounds. It is followed by a series of events that cause the death of the bacteria.[16]

Advantages of polymeric-based antibacterial agents over conventional antibacterial agents composed of low-molecular-weight compounds include being nonvolatilizable, chemically stable, and difficult to permeate through the skin of human or animal. So, they can reduce the loss associated with volatilization, photolic decomposition, and transportation. On the other hand, many metal ions and metal complexes that have biological activity are being used and studied as biocidal agents.[17–18]

For this reason investigating the antibacterial activity of polymers and polymer–metal complexes represents a new, developing direction in the field of antibacterial agents. In line with that, in this article the synthesis and study of water-soluble cationic polymers, copolymers, and polymer–metal complexes that have potential bactericidal activity for gram-positive and gram-negative bacteria is reported.

EXPERIMENTAL

Obtaining homo- and copolymers

Poly(4-vinylpyridine) was obtained by bulk radical polymerization. The reaction was maintained for 24 h at 70°C by using 2,2′-azo-*bis*-isobutyronitrile (AIBN) as an initiator (0.5 mol %). Subsequently, poly(4-vinylpyridine) (PVPy) was N-alkylated by a heterogeneous reaction with methyl iodide, yielding PVPyMe.

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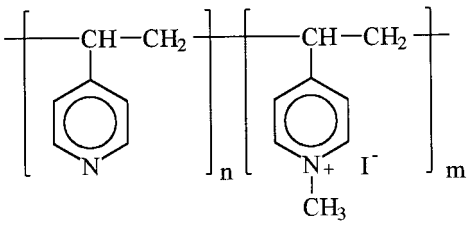
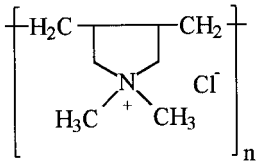
Purification and characterization

PVPyMe (Structure 1) and the commercial polymers poly(diallyl dimethylammonium) chloride (PDDA) (Structure 2), and poly(2-acrylamido-2-methyl-1-propane sulfonic acid) (PAPSA) (Structure 3) were purified and fractionated through ultrafiltration membranes with molecular mass cutoffs of 3000, 10,000, 30,000, and 100,000 Da. Fractions less than 3000 Da were discarded. Then the polymers were lyophilized, obtaining polymer fractions with different molecular weights, which were characterized by FTIR, ^{1}H-NMR, and ^{13}C-NMR spectroscopy.



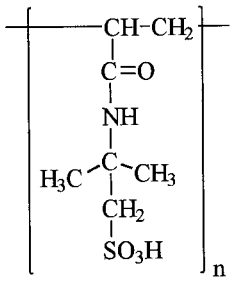
PVPyMe

Structure 1



PDDA

Structure 2



PAPSA

Structure 3

Obtaining polymer–metal complexes

Metal ion retention using the liquid-phase polymer-based retention (LPR) technique[19] was carried out prior to synthesizing the polymer–metal complexes. Retained were polymers that had a polymer fraction greater than 100,000 Da for the following metal ions: Ag(I), Hg(II), Cd(II), Cu(II), Ni(II), Zn(II), and Co(II) at pH 1, 3, 5, and 7. The metal ion Ag^{+} and pH = 5 as the optimum retention pH were selected according to the retention profiles. The Ag^{+} ion was also selected because of its known biological activity and low toxicity and because it is the only one that forms a complex with PVPyMe. Subsequently, polymer–metal complexes were obtained in an aqueous solution at different polymer–metal ion ratios.

Study of bactericidal activity

The antibacterial activity of polymers, the polymer–Ag(I) complex, and metal ions was investigated for *Escherichia coli* (6538P), a gram-negative bacteria, and *Staphylococcus aureus* (ATCC 28922), a gram-positive bacteria. Antibacterial activity was evaluated by the NCCL method.[20] According to that method, different aqueous solutions of the compounds were prepared. The concentrations of these solutions were 1, 2, 4, 8, 16, 32, 64, and 128 μg/mL. These solutions were inoculated with those corresponding to the bacteria and then incubated for 24 h at 37°C using a nutrient solution of tripticase soya. With this experiment it was possible to determine the minimum inhibitory concentration (MIC), that is, the minimum concentration of a compound that stops the growth of bacteria but does not necessarily kill them. From the tubes corresponding to dilutions that did not show bacterial development, a sample was taken and spread in agar-blood medium and incubated at 37°C for 24 h. From this experiment the minimum bactericidal concentration (MBC), that is, the minimum concentration of a compound necessary to kill the bacteria, was obtained. If the MIC had the same value as that of the MBC, the compound was designated a bactericide.

The effect of the molecular weight and the polymer–metal ion ratio on biological activity was also investigated. Moreover, for those compounds with antibacterial activity, a kinetics study on bacteria death was performed.

Root mean square and molecular weight

The root means square (RMS) and molecular weight were obtained in a Dawn DSP-F Laser Photometer (Wyatt Technology Co., Santa Barbara, CA) and the increment of the refraction index in a WYATT/OPTI-LAB 903 interferometric refractometer. The measurements were done in a salt-free aqueous solution and in an aqueous solution with added salt.

Transmission electron microscopy

The *S. aureus* bacteria that were put into contact with the different compounds for antibacterial study were collected and repeatedly rinsed with sterilized physiological saline. The rinsed sample was fixed in a grid for transmission electronic microscopy and then observed with a Jeol JEM 1200EX II electron microscope.

TABLE I
Molecular Mass Cutoff of PVPyMe and Polymer/Ag^+ Ion Ratio Used to Prepare Complexes at pH 5

Polymer fraction (Da)	Polymer/metal ion ratio (mol)
100,000–1,000.000	21.6
100,000–1,000.000	10.8
100,000–1,000.000	5.4

Toxicity

To study toxicity, the *rec* assay was used. This is a simple repair test that uses the *Bacillus subtilis* strains. Although the *rec* assay is not a mutation assay, it is very useful, in addition to a mutagenic assay, for preliminary results.

Culture and test media

Difco nutrient broth with 0.5% glucose added (NBG) was used for overnight cultures. Plates containing 25 mL of nutrient agar (2% agar) were used for different *rec* assay procedures. Soft agar (2 parts nutrient broth and 1 part nutrient agar) was maintained at 45°C. Minimal medium was used to select transformants and to check strain genotype.

Inhibition halo assay

A 0.1 mL portion of an overnight culture of the tester strain [*rec*(+) and rec(−)] in NBG medium grown at 37°C under stirring was added to the tubes containing 2 mL of soft agar maintained at 45°C. The tubes were mixed, and the soft agar was distributed over the surface of a dried nutrient agar plate. When the soft agar was solidified, three 0.5-cm holes were made in each plate. Then 100 μL of 800, 400, 200, 100, and 50 μ/mL polymer, polymer–Ag complexes, and Ag^+ solution were placed into the holes. The plates were maintained at 4°C for 24 h, then incubated at 37°C for another 24 h, and then the inhibition halo was measured. The genotoxicity was calculated as the ratio of the diameter of inhibition measured as *rec*(−) over *rec*(+).[21]

RESULTS AND DISCUSSION

The degree of N-alkylation of PVPyMe was 8.8%, which was determined by ^{1}H-NMR spectrum.[22] The retention profiles for the polymers PVPyMe, PDDA, and PAPSA were obtained as described. PVPyMe retained significant Ag(I) and Hg(II) metal cations. PDDA only retained significant Ag(I). However, during the ultrafiltration runs a precipitate of AgCl was observed. Because of that, the retention cannot be attributed only to the complex formation between Ag(I) and PDDA. In general, cationic polymers did not show interactions with metal ions because of the electrostatic repulsion between the charges. On the other hand, PAPSA, which contains a strong acid moiety, showed an important interaction with almost all metal ions. Polymer–metal ion retention behavior is basically a result of electrostatic interaction type between the sulfonate groups of the polymer and the metal ions. In this study this interaction increased as the pH and the ion charge were increased.

According to the metal ion retention results, only the silver(I)–polymer complexes were prepared. The selected pH was 5. Hence, minimum bactericidal concentration and genotoxic activities were investigated only for polymer–Ag^+ complex. The conditions and results are summarized in Table I, and the results for bactericidal activity (MIC, MBC) are summarized in Table II. All the compounds showed bactericidal activity only for *S. aureus*. Ag(I) ions showed high bac-

TABLE II
Minimum Inhibitory Concentration (MIC) and Minumum Bactericide Concentration (MBC) of Polymers, Ag(I), and Polymer–Ag(I) Complex

Compound	RMS[a] (nm)	M_w[a] (g/mol) $\times 10^{-5}$	MIC (μg/mL) *S. aureus* 6538P	MIC (μg/mL) *E. Coli* ATCC28922	MBC (μg/mL) *S. aureus* 6538P	MBC (μg/mL) *E. coli* ATCC28922
PVPyMe (100,000–1,000.000 Da)	51.4	1.48	64	>128	128	—
PVPyMe (30,000–100,000 Da)	41.6	0.85	64	>128	64	—
PDDA (10,000–30,000 Da)	24.1	0.59	16	>128	16	—
PDDA (30,000–100,000 Da)	28.0	1.03	32	>128	32	—
PDDA (>00,000 Da)	36.4	2.44	32	>128	32	—
PAPSA (>100,000 Da)	42.3	2.43	>128	>128	—	—
PVPyMe–Ag (21.6)[b] (100,000–1,000.000 Da)	—	—	64	>128	64	—
PVPyMe–Ag (10.8)[b] (100,000–1,000.000 Da)	—	—	32	>128	64	—
PVPyMe–Ag (5.4)[b] (100,000–1,000.000 Da)	—	—	32	>128	64	—
Ag^+ (NO_3^-)	—	—	2.0	2.0	2.0	2.0

[a] Obtained by light scattering.
[b] Polymer–Ag^+ mol ratio.
Dash means not detected by bactericide activity.

tericidal activity either for *S. aureus* or for *E. coli*. It is well known that the structural difference between gram-positive and gram-negative bacteria is the cell wall. Hence, it is possible to conclude that the different bactericidal actions of these compounds can be explained by this fact.

The first interaction between a compound and bacteria is at the cell-wall level. The basis for this interaction is the strong attraction on positively charged compounds such as polycations. This was corroborated in our study because the polyanion PAPSA (at pH 5) did not show bactericidal activity. Having positively charges is one of its important characteristics. If the compounds can form bond complexes or interact electrostatically with the cell wall, it is very probable the compounds will show biocidal activity.

The anterior phenomena occurs most easily with gram-positive bacteria, which is attributed to the cell wall of these microorganisms being constituted basically of a network of peptidoglycan whose holes are not efficient barriers for solutes of determined size. This fact motivated us to determine the macromolecular dimensions of the polymers, which ranged between 23 nm and 50 nm in salt solution. The ionic strength of the medium in which the experiments were carried out was influenced by the presence of the nutrient medium formed by sugars (tripticase soya) and mineral salts, which can be considered as a high-ionic-strength medium similar to those conditions used to determine the macromolecular dimensions. Hence, these will be not significantly affected.

The bacteria size varied between 500 and 3000 nm. This value was 20–60 times higher than the size of the

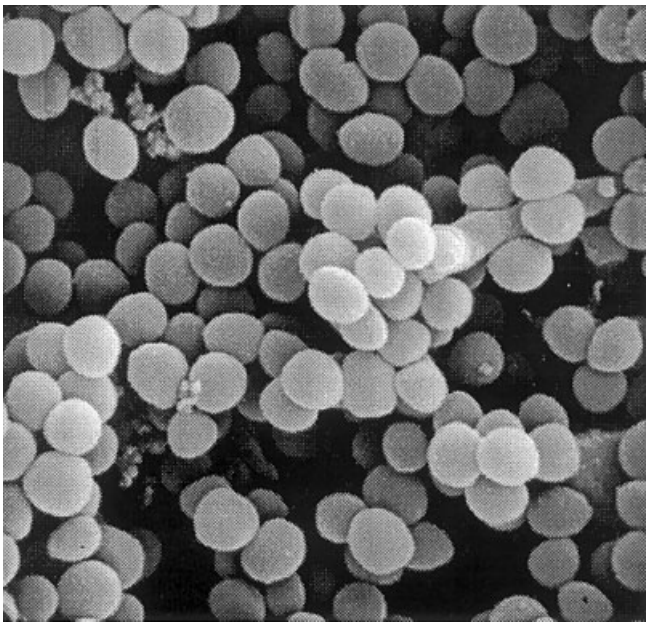


Figure 1 Electron micrograph of *S. aureus* after application of PVPyMe antibacterial agent.

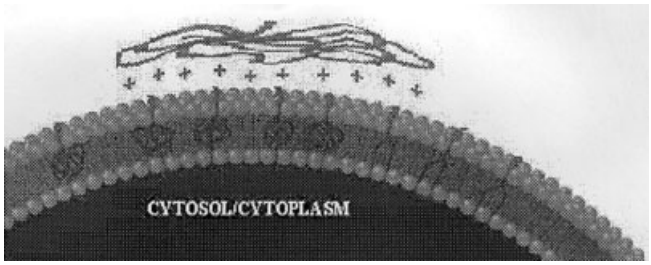


Figure 2 Suggested action mechanism of bactericidal compounds toward gram-positive bacteria.

macromolecules, which were in the size range of the peptidoglycan network holes. These compounds could pass through the membrane and interact with the cell membrane, producing changes that would finally cause the death of the bacteria. It is possible that these compounds achieve cytoplasm bonding with important compounds such as DNA and proteins, avoiding replication of the bacteria. However, another probable mechanism is the interaction with teichoic acid at the cell wall, which contains phosphate bridges (negative charges) that could interact electrostatically with the positive charges of the polycations. This would permit that the polymer to stay around the bacteria cell, blocking the ion exchange channels, inhibiting growth, and producing cell death. Another possibility is the interaction with peptidoglycan, producing degradation and finally cell death. However, this possible mechanism can be discarded as morphological changes were not observed with electron microscopy (see Fig. 1).

The nonbactericidal activity of these compounds, except Ag(I) ions, toward gram-positive bacteria can be attributed to this type of bacteria, which has an additional membrane at the cell wall whose main components are proteins, lipids, and lipopolysaccharides (LPS). LPS provides negative charges at the cell surface cell that in principle could allow interactions with the polycations PDDA and PVPyMe. However, LPS repels hydrophobic molecules. Nevertheless, the polymers have positive charges and hydrophobicity because of their large carbon backbones and side chains. The lower MBC value corresponded to the Ag^+ ion, which demonstrated the highest antibacterial activity.

No important effect of molecular weight or macromolecular size on the MBC value was observed. One important aspect of the bactericidal compounds Ag^+ ions, PVPyMe (3000–10,000 Da, 100,000–1,000,000 Da), PDDA (all fractions), and PVPyMe–Ag^+ is the presence of a positive charge close to the backbone, which increased the charge density and hence also increased their bactericidal action. The polycation may be placed near the cell wall or to penetrate the cell wall of the *S. aureus* bacteria, blocking the ion channels,

producing a total blocking of ionic transport, and causing the death of the cell (see Fig. 2).

The Ag^+-complexes showed MBC values similar to those of the polymers. Increasing the content of Ag^+ in the polymer did not increase its bactericidal activity. That means that the Ag^+ ions of the complex were not available to interact with the cell wall and increase its bactericidal activity.

Our interest was also focused on the study of the kinetics of bacterial death from the presence of the bactericidal compound. For this, the polymers PVPyMe and PVPyMe–Ag^+ (polymer–Ag^+; mole: metal ion ratio of 21.6) were selected and investigated with respect to *S. aureus*. The results are shown in Figure 3. Both compounds showed a similar behavior, with a reduction in the percentage of bacteria that was greater than 90% before 60 min had elapsed.

Because compounds with bactericidal activity toward gram-positive bacteria may be used as a bactericides or antiseptic agents, with all the potential advantages, it is very important to carry out genotoxicity studies. For this reason the *rec* assay[22] was performed with *B. subtilis* bacteria. This test allowed, by comparing the size of the inhibition halo, the determination of the DNA damage that was produced for the different compounds. In this case *B. subtilis* [*rec*(+)] with reparation capability and *B. subtilis* [*rec*(−)] without reparation capability were included. In this situation, the ratio between the inhibition halo of rec(−) bacteria over *rec*(+) bacteria indicates the possible genotoxicity of the investigated compounds. For the macromolecular compounds PVPyMe and PVPyMe–Ag the presence of halo for either bacteria [*rec*(+) or *rec*(−)] was not observed. Silver nitrate showed inhibition halos for both bacteria (see Table III).

According to the data shown in Table III, a possible genotoxic effect can be postulated, but it is not concludive, as the *rec*(−)/*rec*(+) ratio value is close to the limit, 1.2–1.3. However, it is very important to incorporate a probable genotoxic agent such as $Ag^+(NO_3^-)$ to a polymer–metal complex because it allows a decrease in or elimination of their genotoxic activity.

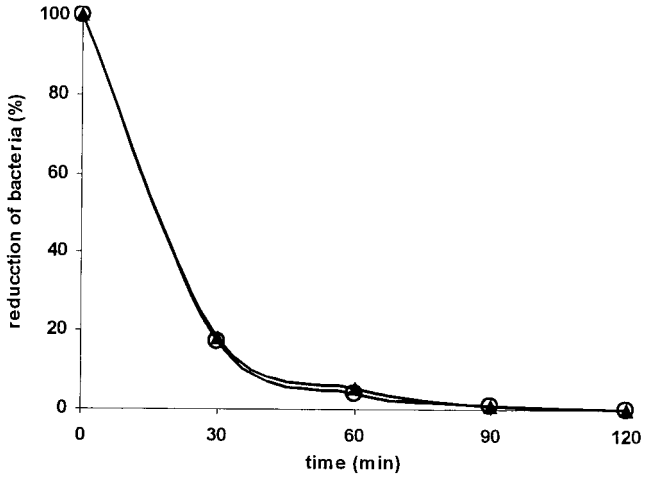


Figure 3 Kinetics of bacterial death for (▲) PVPyMe (100,000–1,000.000 Da) and (○) PVPyMe–Ag(I) (polymer/metal ion ratio: 21.6).

TABLE III
Genotoxicity Activity of Ag^+ Ion Against B. Subtilis

Sample concentration (μg/mL)	Halo diameter (cm)		Diameter ratio *rec*(−)/*rec*(+)
	B. subtilis *rec*(−)	*B. Subtilis* *rec*(+)	
0	—	—	—
50	1.6	1.5	1.07
100	1.8	1.5	1.20
200	1.9	1.6	1.19
400	2.0	1.7	1.18
800	2.2	1.9	1.16

CONCLUSIONS

The bactericidal and genotoxic activities of water-soluble functional polymers containing cationic groups and their Ag complexes were investigated. These results were compared with those for silver nitrate. All the compounds showed low bactericidal activity against gram-positive and gram-negative bacteria such as *S. aureus* and *E. coli*, respectively. No important effect was observed on minimum bactericidal concentration and minimum inhibitory concentration values by the presence of silver ions in the polymer matrix. However, the results for genotoxicity for *B. subtilis* according to the *rec* assay were very interesting. Of all the compounds only silver ions showed genotoxicity, which was borderline. That means that the polymer backbone plays an important role in the incorporation of silver ion, a potential genotoxic agent, in this water-functional polymer, noteworthily decreasing the genotoxicity.

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