Antimicrobial Poly(*N*-vinyl-2-pyrrolidone-*alt*-maleic anhydride)/Poly(ethylene imine) Macrocomplexes

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ABSTRACT: The antimicrobial polymer/polymer macrocomplexes were synthesized by radical alternating copolymerization of *N*-vinyl-2-pyrrolidone with maleic anhydride [poly(VP-*alt*-MA)] with 2,2′-azobis-isobutyronitrile as an initiator at 65°C in dioxane solutions under nitrogen atmosphere, and interaction of prepared copolymer with poly(ethylene imine) (PEI) in aqueous solutions. The susceptibility of some Gram-negative (*Salmonella enteritidis* and *Escherichia coli*) and Gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes*) bacteria to the alternating copolymer and its PEI macrocomplexes with different compositions in microbiological medium was studied using pour-plate technique. All the studied polymers, containing biologically active moieties in the form of ionized cyclic amide, and macrobranched aliphatic amine groups and acid/amine complexed fragments, were more effective against *L. monocytogenes* than those for Gram-positive *S. aureus* bacterium. This fact was explained by different surface layer structural architectures of biomacromolecules of tested bacteria. The resulting polymeric antimicrobial materials are expected to be used in various areas of medicine and food industry. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 5841–5847, 2006

Key words: hydrophilic polymers; copolymerization; water-soluble polymers; antimicrobial activity

INTRODUCTION

Microbial contamination of polymeric materials is of great concern in several areas, such as medical devices, healthcare products, water purification systems, hospital and dental equipment, food packaging and storage materials. After microbial contamination, these materials may become new sources of further contamination. Microorganisms can be transmitted from these materials to people by direct or indirect ways and may cause serious infectious diseases and intoxications. On the other hand, microorganisms, which are transferred to food from packaging materials, may also cause different kinds of food spoilages. Gram-positive *Listeria monocytogenes* and *Staphylococcus aureus* and Gram-negative *Salmonella spp*. are the predominant infecting pathogenic bacteria. Gram-negative *Escherichia coli*, an important indicator of fecal contamination in foods, is another predominant infecting bacterium. Although *E. coli* is principally nonpathogenic, certain strains of *E. coli* are pathogenic, and there have been reported the outbreaks caused by these enteropathogenic *E. coli*.[1–3] Outbreaks in diseases have increased the awareness of these health-hazards and the need for protection against them.[4]

One possible effective way to avoid microbial contamination is to develop the polymeric materials possessing antimicrobial properties.[5–11] These polymeric biocides can significantly reduce the loss of antimicrobial activity associated with volatilization, photholytic decomposition, dissolution, and permeation-migration. Moreover, increased efficiency, selectivity, and handling safety are additional benefits that may be realized.[11,12] For this purpose, polymeric antimicrobial agents were synthesized by reaction of poly(maleic anhydride-*co*-styrene) with two antimicrobial agents, 4-aminobenzoic acid (ABA) and 4-hydroxybenzoic acid (HBA), through amidation and esterification of succinic anhydride units in copolymer, respectively.[13] It was observed that antimicrobial activity of the resulting copolymers was lower than that of the corresponding free bioactive molecules. Authors attributed this to the slow release rate of the bioactive agents from the polymer side-chains via hydrolysis. Hydrolysis of the amide linkage should take place slower than that of the ester linkage at the chosen experimental conditions. Copolymer with ABA ester units were far less active against microorganisms than copolymer with HBA amide units.

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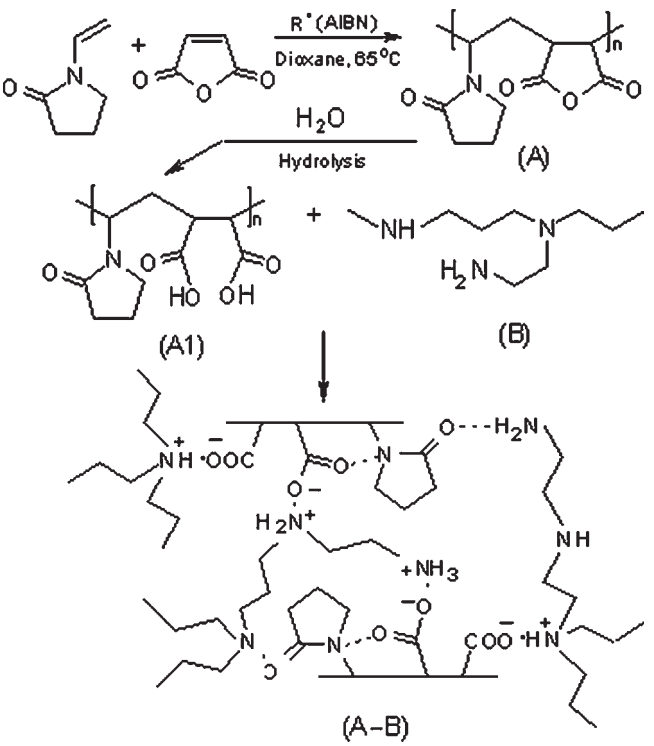
Antimicrobial activity of some linear copolymers containing quaternary ammonium and phosphonium salts have been reported by Kenawy et al.[4,6,14] Patel et al. found that homo- and copolymers of *N*-vinylpyrrolidone (VP) and 2,4-dichlorophenyl methacrylate (2,4-DMA) were effective in inhibiting selective microorganisms.[5] It has been reported that polymers prepared using 2,4-DMA showed strong inhibitory effect toward such tested microrganisms as bacterial strains (*S. aureus*, *S. citreus,* and *E. coli*), molds, and yeasts, while poly(VP) has been shown to have relatively lower antimicrobial activity.

Quaternary ammonium compounds (QACs) are widely used in food industry as chemical sanitizers. It is pointed out that QACs are effective sanitizers for *L. monocytogenes*. In these QAC molecules, the organic radical is the cation, and chlorine is usually the anion.[15] The mechanism of germicidal action is not fully understood, but is associated with enzyme inhibition and leakage of cell constituents. After being applied to surfaces in food plants, they form a bacteriostatic film. It is indicated that limited effectiveness is an important disadvantage of these compounds. QACs are typically more potent against Gram-positive bacteria such as *S. aureus* and are less active against Gram-negative bacteria such as *E. coli*.

The cell membrane in bacteria is a phospholipid-protein bilayer similar to the one present in eukaryotic cells.[16] It is pointed out that QAC molecules in solution can interact with lipid bilayer structures of microbial cell membranes. Gottenbos et al.[17] reported that quaternary ammonium silane-coated silicone rubber showed antimicrobial properties against adhering bacteria, both Gram-positive *Staphylococcus aureus, S. epidermidis*, and Gram-negative *E. coli and Pseudomonas aeruginosa*. They concluded that immobilized this QAC molecule still interacts with the cell membranes of adhering bacteria, presumably causing membrane leakage and cell death. Kenawy and Mahmoud[4] reported that the phosphonium containing polycationic biocides are more effective than the quaternary ammonium salt polymers. Examining the yeast *Candida albicans* and *S. aureus* polymer-treated cells by electron microscopy indicated disruption of the cell membrane and release of potassium ion as shown by the assay of potassium leakage. There are some contradictory results about antimicrobial activity of QAC toward Gram-negative bacteria. Although antimicrobial activity of quaternary ammonium silane toward *E. coli* was reported,[18] the results of another study showed that Gram-negative bacilli were not affected by this QAC.[19] Recently, synthesis and antitumor activity of the anhydride- and pyran-containing copolymers have been also reported.[20]

In the present study, synthesis and antimicrobial behavior poly(VP-*alt*-MA)] and its PEI macrocomplexes (their ways of synthesis are presented in Scheme 1) in aqueous solutions with given concentrations against some Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and Gram-negative (*Salmonella enteritidis* and *Escherichia coli*) bacteria have been described and discussed. Special attention is paid to the complexing effect on the antimicrobial properties of studied polymer systems in microbiological medium.



Scheme 1 General scheme of the copolymerization–hydrolysis-complexing reactions: (A) poly(VP-*co*-MA), (A1) hydrolyzed copolymer, (B) poly(ethylene imine) (PEI) and (A-B) poly(VP-*co*-MA)/PEI macrocomplex.

EXPERIMENTAL

Materials

Bacterial strains

Staphylococcus aureus ATCC 29213, *Listeria monocytogenes* ATCC 1462, *Salmonella enteritidis* ATCC 13076 and *Escherichia coli* E 1.3.3 isolated from poultry[21] were used as test bacteria. First, these bacteria were cultured in tryptic soy broth (TSB) (Merck, Germany) at 37°C for 24 h as their pure cultures. Then each pure culture was subcultured in TSB to obtain the studied culture. The viable cell number of each studied culture as colony forming units (cfu) was determined by pour-plate count method.[22] using tryptic soy agar (TSA) (Merck, Germany) before the test day.

Chemicals

N-Vinyl-2-pyrrolidone (VP) (Fluka, Germany) was purified before use by distillation under moderate vacuum.

Maleic anhydride (MA) (Aldrich, Germany) was purified before use by recrystallization from anhydrous benzene solution and sublimation in vacuum. α,α′-Azobisisobutyronitrile (AIBN) (Fluka, Germany) was recrystallized twice from methanol. Poly(ethylene imine) (PEI) (Aldrich, Germany) had molecular weight of $M_n = 2000$ g/mol. The solvents (dioxane, benzene, and diethyl ether), used as copolymerization medium and for precipitation and extraction, were all analytical grade.

Polymer synthesis

Alternating copolymer of VP with MA was prepared by radical-initiated copolymerization in 1,4-dioxane at 65°C in the presence of AIBN as an initiator in glass tube type of microreactors under nitrogen atmosphere using equimolar (1 : 1) monomer mixture. The time of reaction was about 48 h. Copolymer was isolated from reaction mixture and purified by two precipitation procedures from 1,4-dioxane solution to diethyl ether, and washing with benzene. After last extraction by diethyl ether, the copolymer was then isolated by centrifugation and dried at 40°C under moderated vacuum to constant weight. Synthesized poly(VP-*co*-MA) (A) had the following average characteristics: acid number 460 mg KOH/g (by alkaline titration); content of nitrogen N = 7.15 wt % (by elemental analysis); molar monomer unit ratio m_1 (VP)/m_2 (MA) = 53.65 : 46.35. Intrinsic viscosity $[\eta]_{in} = 0.79$ dL/g [in deionized water at (25 ± 0.1)°C]; temperature sensitiveness $T_s = 43.2$°C; and glass transition temperature $T_g = 159.8$°C (by DSC). ^{1}H-NMR spectra (in $CHCl_3$-d_1 at 27°C), δ (ppm): 1.30–1.83 CH_2 (backbone), 3.56–3.95 CH (CH—N backbone), 1.83–2.33 CH_2 and 2.95–3.54 $2CH_2$ (pyrrolidone ring) for VP unit and 4.08–4.45 CH (backbone) for anhydride unit. Poly(VP-*co*-MA)/PEI (A-B)s macrocomplexes were prepared by copolymer/polymer interaction in aqueous solution at 40°C using given molar copolymer/PEI feed ratio. For the antimicrobial testing, the following molar concentration ratios of the copolymer (A), PEI (B) and complexed copolymers (A-B)s were used: copolymer/PEI = 1 : 1 or COOH/N molar ratio 1 : 1.5 (A-B)-1 and 1 : 0.67 and 1 : 1 (A−B)-2, respectively, in aqueous solutions with constant total concentration of 1.4 g/dL; where (A−B)-1 and (A−B)-2 are macrocomplexes containing partially and fully incorporated carboxylic/amine groups, respectively. The aqueous solutions of polymer samples were adjusted to the appropriate concentration in sterile distilled water before adding the microbiological medium for antimicrobial testing.

Analytical techniques

Fourier transform infrared (FTIR) spectra of the copolymers (KBr pellet) were recorded with FTIR Nicolet 510 spectrometer in the 4500–400 cm^{-1} range, where 30 scans were taken at 4 cm^{-1} resolution. Proton NMR spectra were recorded on a JEOL 6X-400 (400 MHz) spectrometer. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) of copolymers were performed on a DuPont TA 2000 calorimeter and Setaram Labsys TG-TGA 12 thermal analyzer, respectively, under nitrogen atmosphere at a heating rate of 10°C/min. Aleco CHNS-932 elemental analyzer was used for the determination of nitrogen content in copolymer. Acid number of the copolymer was determined by a standard alkaline titration method with a Consort P901 pH-meter. Intrinsic viscosity of the copolymer was measured in aqueous solution at (25 ± 0.1)°C in the concentration range of 0.1–1.5 g/dL with an Ubbelohde viscometer.

Antimicrobial activity test

The poly(VP-*co*-MA)] and its poly(ethylene imine) (PEI) macrocomplexes, as well as PEI as a model polymer were tested against two Gram-positive bacteria (*S. aureus* and *L. monocytogenes*) and two Gram-negative bacteria (*S. enteritidis* and *E. coli*), which are important food pathogens and indicators. The studied cultures were used for the determination of antimicrobial activity of the copolymers and their macrocomplexes. First, the studied culture was diluted in sterile physiological saline solution to give the final viable cell numbers of the test bacterium (about 10^3 cfu/mL). At the same time, 1 mL of liquid was withdrawn from this diluted culture (inoculation culture), decimal dilutions were prepared, and the viable cell number of the diluted culture was determined by pour-plate count method on TSA agar. Two milliliters of inoculation culture was inoculated into 98 mL of control medium (TSB without polymer) and 98 mL of test medium (TSB containing 50 mg polymer sample). For preparation of the test medium, the polymer solution prepared before was added to the TSB medium to give the final concentration of 50 mg/98 mL as recommended by Patel et al.[5] Inoculated media in flasks were incubated in a shaking water bath at 100 rpm and 37°C for 24 h, 1 mL of liquid was withdrawn from the media at the end of incubation, decimal dilutions were prepared, and the viable cell number was determined by pour-plate count method on TSA agar. All experiments were carried out twice using separately cultured bacteria.

RESULTS AND DISCUSSION

Synthesis of copolymer and its hydrolysis and complexation

Homo- and copolymers of *N*-vinyl-2-pyrrolidone (VP) are of considerable academic and industrial in-

terest due to their unique properties, allowing the use of these polymer systems in lithography as light sensitive thin coatings for printing plates, for the preparation of separating membranes for ultrafiltration, biocompatible polymers with low toxicity and carriers of biologically active compounds, sorbents, coagulants, and flocculants.[23–27] The use of MA copolymers in medicine or pharmacy were described as antitumor agents,[28–30] drug carriers, supports for enzymes, or protein modifiers.[31–34] Antitumor functional polymers were synthesized by reaction of poly(MA-*co*-VP) with hydroxy- and amino-containing physiologically active compounds.[35–37] Synthesis and characterization of maleic acid or anhydride copolymers, their interactions with various functional molecules, macromolecules, particles and surfaces,[38,39] especially with biomacromolecules[40–42] have been reported. By the intermolecular reactions of poly (MA-*co*-styrene)[43] and poly(MA-*co*-methyl methacrylate)[44] with 3,6-diamino-10-methylacridinium chloride (acriflavine as an antiseptic agent) in DMF using triethylamine as catalyst, new derivatives of these copolymers with antimicrobial properties were also synthesized.[45]

Synthesis route of copolymer and its hydrolyzed and macrocomplexed derivatives includes (1) radical-initiated copolymerization of VP with MA (A), (2) hydrolysis of synthesized poly(VP-*co*-MA), and (3) complexation with PEI (B). General scheme of synthesis of this polymer system can be presented as follows (Scheme 1).

The poly(VP-*co*-MA) is easily dissolved in water. This solution process is accompanied by full hydrolysis of anhydride units and formation of strong hydrogen-bonding intermolecular fragments (Scheme, A1). Hydrolyzed copolymer again accepts an initial form by anhydration of dicarboxylic units through thermotreatment of its thin coating or film at 110–120°C during 15 min. It is interesting that anhydration via dehydration reaction does not undergo crosslinking (intermolecular anhydration) and proceeds selectively having only intramolecular character, which is confirmed by its solubility and identical structure as initial VP-MA copolymer. In FTIR spectra of this copolymer, the characteristic bands for anhydride units (1836 and 1766 cm^{-1}) appeared as 1945 and 1630 cm^{-1} bands relating to anhydride units disappeared and 2575 cm^{-1} is shifted to 2545 cm^{-1} field relating to acid units.

Complexation of copolymer with PEI is carried out in aqueous medium using two different ratios of copolymer/PEI. The formation of macrocomplexes proceeds through incorporation of free carboxylic groups with amine fragments of copolymer and PEI, respectively. Similar macrocomplexes in the poly(*N*-isopropyl acrylamide-*co*-MA)/PEI system were described in our previous publications.[42,46]

Antimicrobial properties of copolymer and its macrocomplexes

The conventional viable cell count methods have been widely used in the evaluation of biocide efficiency. Pour-plate method enables counting of the number of living organisms or clumps of organisms (i.e., colony forming units) in a sample. However, turbidimetric methods depend on the microorganisms in a suspension blocking a light beam by scattering or absorption, causing the suspension to appear turbid. Greater is the concentration of organisms, less light can penetrate the suspension and more light is scattered. Therefore, turbidimetric methods can be used only for estimating concentrations of microorganisms that are suspended in liquids that have a low innate turbidity.[22]

It is not possible to distinguish between living and dead organisms using turbidimetric techniques. In this study, pour-plate method was used to evaluate the antimicrobial activity of the polymers. We believe that pour-plate method or other viable cell count methods will give meaningful results about the antimicrobial effect of the chemicals tested. On the other hand, clear correlation between bacterial bioluminescence and viability would suggest universal applicability of the bioluminescence method in evaluating biocide efficiency. This method has proven to be fast, convenient, and efficient.[47]

The antimicrobial effect of poly(VP-*alt*-MA) (A1), poly(ethylene imine) (PEI) (B) and the copolymer/PEI macrocomplexes (A−B)s on the bacterial strains tested are presented in Figure 1, 2, 3 and 4, respectively, while the resulting reduction in log viable cell numbers of the bacterial strains are summarized in Table I. Taking into consideration the Gram reaction of bacteria, *L. monocytogenes* (a Gram-positive bacterium) is more responsive to all the studied polymer systems than *S. aureus* (another Gram-positive bacterium). All polymer samples showed high antimicrobial activity against *L. monocytogenes*. However, A1 and its PEI macrocomplexes (A-B)s had relatively higher antimicrobial activ-

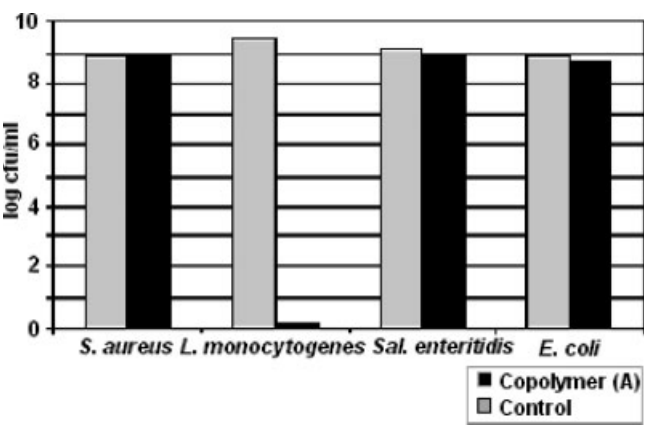


Figure 1 Antimicrobial activity of copolymer (A) on the bacterial strains tested.

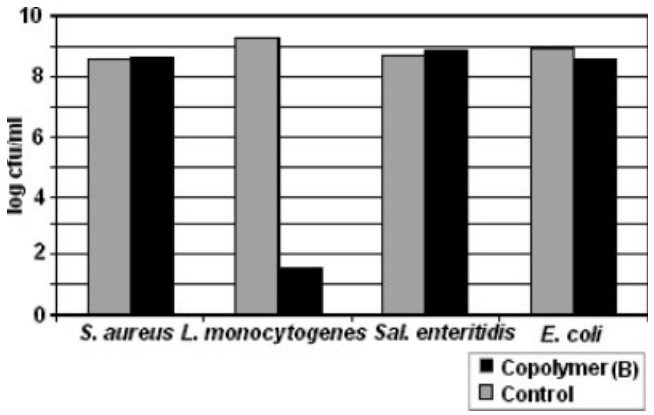


Figure 2 Antimicrobial activity of PEI (B) on the bacterial strains tested.

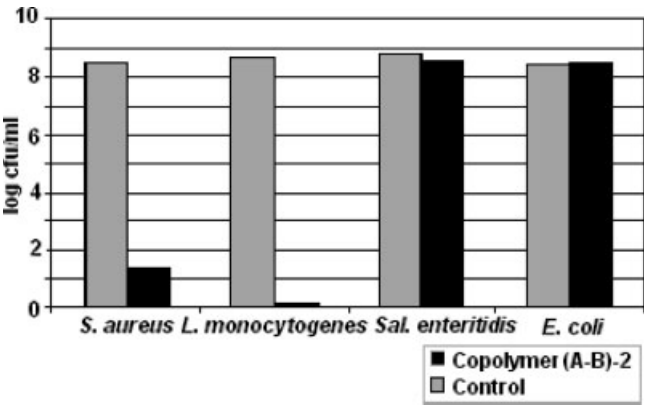


Figure 4 Antimicrobial activity of copolymer (A-B)-2 on the bacterial strains tested.

ity on *L. monocytogenes* than B. Copolymer (A1), PEI (B) and macrocomplex (A−B)-1 were not active against *S. aureus*. Sensitivity of *S. aureus* was only limited toward macrocomplex (A−B)-2 which caused about 7 log reduction in the viable cell number of *S. aureus*. The hydrolyzed copolymers with strong hydrogen bonding structure easily form assembled macrocomplexes with PEI through —COO^-. $^+$NH— noncovalent interaction between free carboxylic groups of MA units and PEI amine (tertiary, secondary, and primary amine fragments) groups (Scheme 1). As the ratio of COOH/N increases (i.e., the amount of —COOH groups increases) in copolymers, the limited antimicrobial activity of macrocomplex toward *S. aureus* increases. It is concluded that increased number of COOH groups would cause disruption and disintegration of the *S. aureus* cell membrane due to the electrostatic interactions. The study of Can et al.[47] has also shown that polymer biocides on the base of quaternary ammonium functionalized poly(propylene imine) dendrimers are more capable of disrupting and disintegrating cell membrane than small biocides due to the enhancement of interactions through polyvalency. According to authors, these dendric cationic biocides have one more advantage in that they can displace calcium and magnesium ions bound on the membranes easily by competition. These ions are proven to stabilize the negatively charged phospholipid membrane structure through electrostatic interactions. Recently, synthesis and antimicrobial activities of new water-soluble bisquaternary ammonium methacrylate polymers have been reported by Dizman et al.[48] It was found that these polymers with relatively long alkylene side-chain containing pendant heterocyclic quaternary ammonium moiety show higher antimicrobial activity against *S. auereus* and *E. coli*.

In this study, it was clearly demonstrated that poly (VP-*co*-MA) and its PEI macrocomplexes did not affected by Gram-negative *S. enteritidis* and *E. coli* (Figs. 1–4). This fact can be explained by the different structural peculiarities of biomacromolecular architectures of the studied bacteria. It is known that the bacterial cell wall is the structure that immediately surrounds the cell membrane. The most

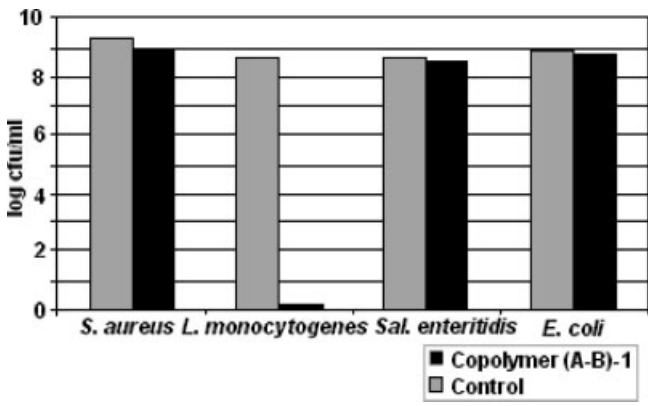


Figure 3 Antimicrobial activity of macrocomplex (A-B)-1 on the bacterial strains tested.

TABLE I
Reduction in Log Viable Cell Numbers of the Bacterial Strains by the Polymer Samples

Polymer Sample	*S. aureus*	*L. monocytogenes*	*S. enteritidis*	*E. coli*
(A)				
Control	8.87	0.05	8.87	8.72
Inoculation	8.85	9.48	9.11	8.94
Culture	3.73	3.61	4.03	3.85
(B)				
Control	8.59	1.53	8.87	8.54
Inoculation	8.56	9.29	8.66	8.91
Culture	3.92	3.12	3.65	3.50
(A−B)-1				
Control	8.92	0.05	8.57	8.78
Inoculation	9.30	8.68	8.66	8.91
Culture	3.92	3.12	3.65	3.50
(A−B)-2				
Control	1.41	0.05	8.57	8.50
Inoculation	8.54	8.68	8.84	8.47
Culture	3.45	3.12	3.45	3.59

important function of the cell wall is to protect the cell physically. This protection is necessary because of the sensitivity of the cell membrane to physical or osmotic disruption.[16] The cell wall of Gram-positive bacteria contains primarily several layers of peptidoglycan, to which biomolecules of teichoic acids, polysaccharides, and proteins are covalently linked.[11,16] Teichoic acids give the cell surface a negative charge. Outer two layers, a lipoprotein, and a lipopolysaccharide surround a thin peptidoglycan layer of Gram-negative bacterial cell wall. This outer membrane structure is an additional barrier for foreign molecules.[16,47] Therefore, in many cases, Gram-negative bacteria are more resistant to antimicrobial agents than Gram-positive ones.[11]

Incorporation of PEI into the hydrolyzed copolymers significantly increases biological activity of these copolymers, which can be compared with activity of the well known polymeric QACs.[49] The antibacterial mechanism of the cationic disinfectants, such as QACs, can be summarized in the following six steps: (i) adsorption onto the bacterial cell surface, (ii) diffussion through the cell wall, (iii) binding to the cell membrane, (iv) disruption of the cell membrane, (v) release of K^+ ion and other cytoplasmic constituents, and (vi) precipitation of the cell contents and death of the cell.[4] We proposed that interactions between macromolecules of the studied polymers and the outer two layers of Gram-negative bacteria prevent the membrane leakage, presumably causing difficulty in the diffusion of the polymers through the cell wall. It can be assumed that these outer layers may protect Gram-negative bacteria from the antimicrobial effect of such polymers by making their diffusion difficult through the cell wall. Besides Can et al.[47] also pointed out that QACs are not very effective on Gram-negative bacteria such as *E. coli* because these cells have very sophisticated outer membrane structures that effectively keep out antibacterial agents.

CONCLUSIONS

Antimicrobial activity of poly(VP-*alt*-MA) (A1), PEI (B), and their copolymer/PEI (A−B) macrocomplexes was determined using 1.4 g/dL of copolymer solutions in water and the viable cell count method using pour-plate technique. It was demonstrated that *L. monocytogenes* is more susceptible Gram-positive bacterium to the studied copolymer systems than *S. aureus*. Sensitivity of *S. aureus* is only limited toward the copolymer/PEI macrocomplex (A-B)-2 containing full incorporated carboxylic/amine groups. With an increasing number of COOH groups in the macrocomplex of A-B, the antimicrobial activity of the macrocomplex of A-B on a variety of Gram-positive bacteria was found to increase, presumably causing membrane leakage and cell death. Both Gram-negative bacteria (*S. enteritidis* and *E. coli*) were not affected by all the studied polymer systems. This fact can be explained by the different responsive behavior of surface layer structures of these two types of bacteria. With the combination of the functional groups and the increased permeabilitiy due to the polycationic structure of used polymer systems (Scheme 1), the macrocomplex may become effective on Gram-negative bacteria. This new polymeric biocide system can be recommended for the biomedical and food industry applications.

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