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Complexes of Water-Soluble Polymers with Cu^{2+} and Ag^{+} as Antibacterial Agents

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Summary: The potential application of polymers in the biomedical area is huge, especially as antimicrobial agent. We studied the recovery of metal ions (silver and copper) with a known antibacterial activity. Using the liquid-phase polymer-based retention, LPR, technique it was generated the polymer-metal complexes from these metal ions. Their antibacterial activity was studied for gram-positive and gram-negative bacteria. The results indicated that the anionic polymers easily yield polymer-metal complexes with increased antibacterial activity with respect to their polymers. Cationic polymers demonstrated efficient antibacterial activity, and the polymer metal-complexes, PMC, showed similar antibacterial results. Neither system presented a genotoxic effect.

Keywords: antibacterial; silver and copper metal ions; water-soluble polymers

Introduction

Ag(I) and Cu(II) ions are highly reactive chemically and bind strongly to electron donor groups containing sulfur, oxygen, or nitrogen. Biological molecules generally contain all these components in the form of thio, amino, imidazole, carboxylate, and phosphate groups. For example, the binding of silver ions to bacterial DNA may inhibit a number of important transport processes, such as phosphate and succinate uptake, and can interact with cellular oxidation processes as well as the respiratory chain. The rate of Ag(I)-induced antibacterial killing is directly proportional to the Ag(I) concentration, typically acting on multiple targets.[1] At higher silver ion concentrations, higher antimicrobial efficacy is found. The release rate of unbound, free silver ion may also be correlated to the antimicrobial activity of thus-coated devices. However, the toxic effects produced by its chronic applications limit its use in the biomedical area. Thus, a support that maintains the beneficial properties while diminishing the disadvantages is required. Water-soluble polymers (WSP) emerge as an interesting alternative.

Water-soluble polymers cover a wide range of macromolecular systems including biopolymers, such as DNA, and synthetic polymers, such as poly(acrylic acid). This class of polymers can be used in a wide range of applications due to their different structures, spatial conformation and atomic composition.

Polyelectrolytes may be distinguished from chelating polymers (polychelatogens) by their structures and atomic composition. Polyelectrolytes have charged groups or easily ionizable groups in aqueous solution, such as sulfonic or phosphoric acids, while the latter bear functional groups with the ability to form coordination bonds. The most investigated ligands present in the polychelatogens include amines, carboxylic acids, amides, alcohols, aminoacids, pyridine, thiourea, and imino.

Removal, separation, and enrichment of hazardous metal ions in aqueous solutions play an important role in the remediation of municipal and waste water. At least

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20 metals are classified as toxic and half of these are emitted into the environment in quantities that endanger human health. The heavy toxic metal cations contained in industrial effluents coming from the plants that use or produce heavy toxic metals or heavy toxic metal compounds are the main source of heavy toxic metal pollution. Specifically, the sources of metal-bearing wastewaters include: the discharge from electroless copper plating (for printed circuits, plating on plastics, *etc.*) and metal finishing industries as well as the washing effluents from metal-contaminated soil. Therefore, metal ion recovery is a key challenge from both an environmental and economic point of view. Treatment of these wastewaters depends greatly on the particular complexing agents and metal ions used as well as their concentrations. In general, they are grouped into three categories: chemical, physical, and electrochemical.[2–10] The disadvantages of two-phase separation can be avoided by using water-soluble polymeric reagents in combination with membrane filtration.[11–12] This technique called *liquid-phase polymer-based retention technique* (LPR), is based on the separation of ions bound to WSP using electrostatic or chelating groups from non complexed ions. The separation process will be successful, if the polymer reagents employed satisfy requirements such as: high affinity for the target metal ion, inactivity towards the non-target metal ion, high molecular mass, possibility of regeneration, chemical and mechanical stability, low toxicity, and low cost.

Different synthetic polymers have demonstrated good efficiency for this purpose, including poly(acrylic acid), P(AA), poly(methacrylic acid), P(MAA), poly(vinyl sulfonic acid), P(VSA), poly(styrene sulfonic acid), P(SSA), and poly(2-acrylamide-2-methyl-1-propane sulfonic acid), P(APSA).[13–18]

One of the promising applications of WSP and PMC is use in biomedical area, especially as an antimicrobial agent. A wide range of polymers have been studied as biocidal agents, where the most used polymers contain chlorine in their structures, such as poly(N-halamines)[19] or poly(hydantonines);[20] others incorporate antibiotics, such as ampicillin in its structure.[21] Polymers insoluble in water derived from pyridine and some complexes of Fe(II) and Cu(II) derived from poly(*ter*-pyridine)[22–23] generate polycations as polymers with biguanidines and oligoguanidines,[24–25] with quaternary ammonium salts and quaternary pyridinium salts, or with halogenated phosphonic or phosphonamides salts that simulate the action of detergents or surfactants.[26–27]

The aims of this work are analyze the use of synthetic anionic and cationic polymers to remove metal ions from aqueous solution, determine the functional groups involved in formation of a polymer-metal ion complex with infrared spectroscopy, and study its application as an antimicrobial agent establishing its genotoxic potential.

Materials

4-vinylpyridine (VPy), methyl iodide, [3-(methacryloylamino) propyl] trimethyl ammonium chloride (MPTA), imino diacetic acid, 2-glycidyl methacrylate 96%, 2-acrylamido glycolic acid (AAG), 2-acrylamido-2-methyl-1-propane sulfonic acid (APSA), all purchased from Aldrich Co., and alginic acid (AA) from brown algae was obtained from Sigma. Ammonium persulfate (AP) (Fluka) was used without further purification, 2,2'-azo-*bis*-isobutironitrile (AIBN). Metal ions were purchased from Merck: $Cu(NO_3)_2$ x 3 H_2O, 99%, *p.a.*; $AgNO_3$, 99.8%, *p.a.*; Sodium hydroxide (NaOH, Merck), nitric acid 70% (HNO_3, Caledon) were used to adjust pH.

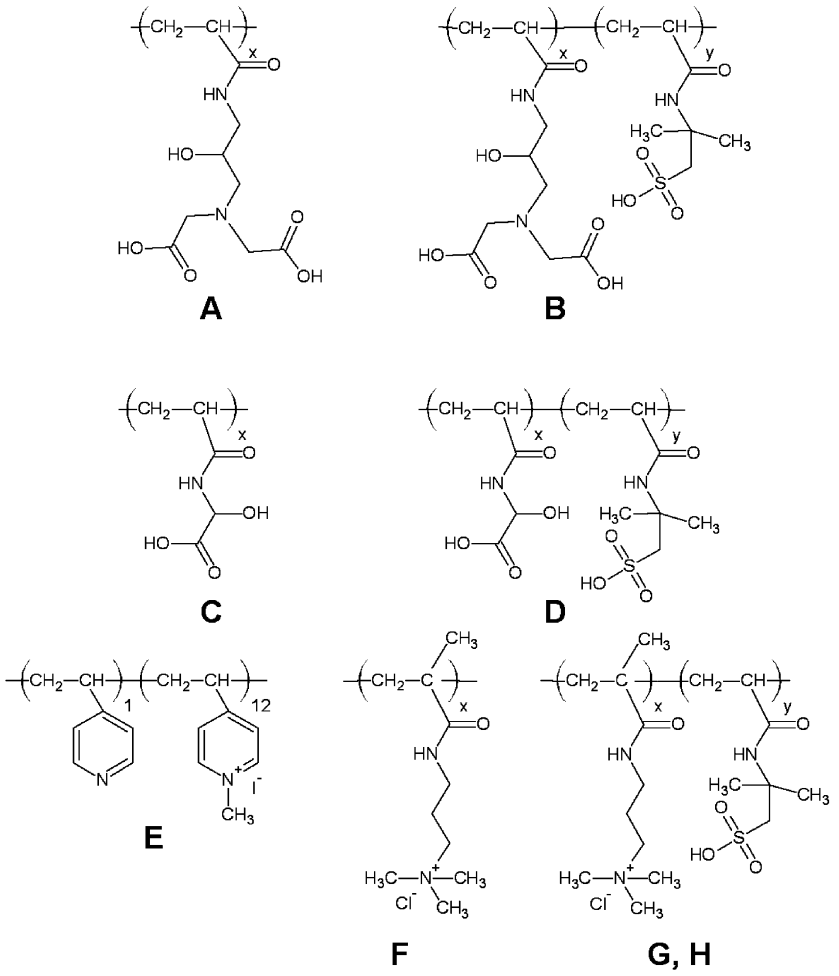
Polymer Synthesis

P(MPTA), P(MPTA-*co*-APSA), P(HMPADA), P(HMPADA-*co*-APSA), P(AGA), and P(AGA-*co*-APSA) were synthesized from their corresponding monomers by free radical polymerization using AP as initiator. All polymerization reactions were performed in a polymerization flask, AP was

added at 1 mol-%. The reaction was maintained for 24 h between 60-70 °C in N_2(g) atmosphere. P(AGA-*co*-APSA) was polymerized with a 1:1 comonomer mol ratio; P(MPTA-*co*-APSA) with a 2:1, 1:1 and 1:2 comonomer ratios. The synthesis of HMPADA is described by Chen.[28] P(VPyMe) was obtained by *N*-alkylation of P(VPy) with methyl iodide, yielding P(VPyMe)I with an 8.8% of *N*-alkylation. P(VPy) was obtained by bulk radical polymerization using AIBN (0.5 mol-%) as initiator.

Study of Metal ion Retention by LPR Technique (Washing Method)

To ensure a high level of ligand sites, the copolymer repeat unit: metal ion ratio (in mol) was 40: 1. Then, 20.0 mL of a solution containing $1.0 \cdot 10^{-2}$ mmol/L of a water-soluble homopolymer (> 100 kDa) and $2.5 \cdot 10^{-4}$ M of metal ions (5 mmol of each metal ion or 5 meq and 10 meq for mono- and di-valent metal ion respectively) are placed in the solution cell provided with a ultrafiltration membrane with a molar mass cut off, MMCO, of 10 kDa (Millipore,



Scheme 1.

Synthesized water-soluble polymers, carboxylic acid polymers, A: P(HMPADA), B: P(HMPADA-*co*-APSA), C: P(AGA), D: P(AGA-*co*-APSA); cationic polymers, E: P(VPyMe), F: P(MPTA), G: P(MPTA-*co*-APSA) 2:1, H: P(MPTA-*co*-APSA) 1:2.

Amicon). Metal ions studied were Ag(I) and Cu(II).

The pH was adjusted with dilute HNO_3 and NaOH. A washing solution was passed under constant pressure (3.5 bar of N_2) from the reservoir through the cell solution (2-4 drops by second). As the in- and out flux are rapidly equaled, the initial volume (20.0 mL) is kept constant during the experiment. Ten fractions of 20 mL were collected. Each fraction was collected in graduated tubes, and the corresponding metal ion concentration was determined in a Unicam Solaar M5 series Atomic Absorption Spectrometer (AAS).

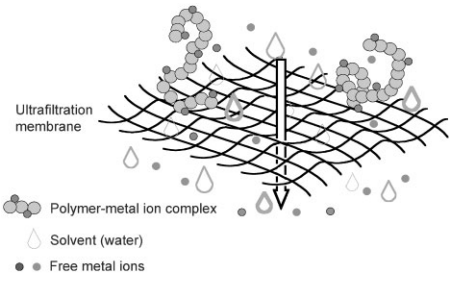
The binding and elution processes may be formulated as a chemical reaction, where a reversible reaction in combination with an irreversible transfer of metal ions across the membrane is responsible for metal ion retention (see Scheme 2). Retention, *R(Z),* is defined for any species as the fraction per unit of the species under study remaining in the cell during filtration. The metal ion (*M*) remaining in the cell during filtration consists of the sum of the metal ions bound to the polymer chain and the metal ion free in the solution. These values are a function of *F*, *i.e.* the extent of the filtration run constant during filtration; retention may be formulated as follows:

$$R(Z) = \frac{c^{free}(Z) + c^{bound}(Z)}{c^{init}}$$

where c^{free} is the concentration of M free in the solution, c^{bound} is the concentration of *M* bound to the polymer, and c^{init} is the initial metal concentration. *Z* is the valence of the metal ion considered. To obtain the retention profile, retention versus the filtration factor is plotted.

The filtration factor (*Z*) is defined as the volume ratio of the filtrate (V^f) versus volume in the cell (V^c). In this case, V^c is kept constant at 20 mL. *Z* is also a qualitative measurement of the strength of the interaction between the ligand group and the metal ion.

$$Z = \frac{V^f}{V^c}$$



Scheme 2.
Ultrafiltration principle.

Determination of Maximum Retention Capacity (MRC) by LPR Technique (Concentration Method)

To obtain polymer metal complexes, PMC, the liquid-phase polymer based retention (LPR) technique by concentration method was used. This method consists in passing a metal ion solution, at a known concentration, through a solution of water-soluble polymer (20 mL), maintaining the volume constant. To develop the enrichment (or concentration) method, only water-soluble polymers are placed in the ultrafiltration cell and the metal ion solution is placed in the reservoir. When metal ions pass through the ultrafiltration cell, the macromolecules recover the metal ions until saturation and the non-retained metal ions are collected in 5 mL and 10 mL assay tubes and quantified by AAS. Since these PMC will be used to determine antibacterial activity, an elution with 100 mL of twice-distilled water was performed after each maximum retention capacity, MRC, experiment to eliminate all the metal ions not bound to the polymer in order to only observe the polymer-metal complex's effect. The same polymer fraction, >100 kDa, and membrane, 10 kDa, were employed in this study. A blank experiment with metal ions and without polymer is required to determine the effect of ultrafiltration membrane in metal ion retention. The amount of metal ion bound to the water-soluble polymer was calculated by calculating the difference between the concentration curve slope and the blank curve.

Antibacterial Activity of Polymer, Polymer–Metal Complexes, and Free Metal Ions

To compare a toxic metal's antibacterial activity, the metal ions studied were selected for their known antibacterial activity and low toxicity level: Ag(I) and Cu(II). The antibacterial activity of polymers, the PMC, and free metal ions for *E. coli (6538P)*, a Gram-negative bacterium, and *S. aureus (ATCC)*, a Gram-positive bacterium was investigated. Antibacterial activity was evaluated using the National Committee for Clinical Laboratory Standards (NCCL) method. 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, 128, 256, 512, 1024, and 2048 μg/mL. These solutions were inoculated with the corresponding bacteria and then incubated for 24 h at 37 °C using a nutrient solution of soy tripticase. This experiment was used to determine the minimum inhibitory concentration (MIC), *i.e.*, the minimum concentration of a compound that stops the growth without necessarily kill them.

Genotoxic Activity of Metal Ion, Polymer, and Polymer–Metal Complexes

To study toxicity, the rec assay was used. This is a simple repair test that uses the *Bacillus subtillis* strains [rec(+)]. Although the rec assay is not a mutation assay, it is very useful, in addition to a mutagenic assay, for preliminary results. Using Difco nutrient broth as culture media with 0.5% glucose added (NBG) was used for overnight cultures and test media. Test media 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.

For the inhibitory 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 soft agar was distributed over the surface of a dried nutrient agar plate. Once the soft agar solidified, three 0.5 cm holes were made in each plate. Then 100 μL of polymer, polymer–metal complexes, and metal ion free solution at MIC were placed into the holes. The plates were maintained at 4 °C for 24 h, incubated at 37 °C for another 24 h, and then the inhibition halo was measured. The genotoxicity was calculated as the ratio of the inhibition diameter measured as rec(+) over rec(-).

Results and Discussion

P(MPTA), P(MPTA-*co*-APSA), P(HMPADA-*co*-APSA), P(AGA), P(AGA-*co*-APSA) are synthetic WSPs obtained by free radical polymerization, only P(MPTA-*co*-APSA) 1:1 is not water-soluble. Every polymer contains functional groups that allow metal ion interaction: P(MPTA) contains amido, quaternary amine and chloride groups, P(MPTA-*co*-APSA) additionally contains a sulfonic group; P(HMPADA) contains hydroxyl, amine (ternary), carboxylic acid and ester groups in its structure; P(AGA) contains hydroxyl, carboxylic acid and amide groups, P(HMPADA-*co*-APSA) additionally contains a sulfonic acid group; P(AGA-*co*-APSA) additionally contains a sulfonic acid group. The metal ions studied were Ag(I) y Cu(II) for the pH range 3 to 7 by the LPR technique elution method. Each functional group can be classified by the Pearson's principle: carboxylic acid, hydroxyl groups (hard bases) allow an efficient interaction with hard acids and borderline acids [Cu(II)]; a weak interaction with soft acids [Ag(I)], and borderline acids (sulfonic group) can achieve a high interaction with borderline acids.

Figure 1 shows the retention behavior at different pH for P(MPTA), P(MPTA-*co*-APSA), P(HMPADA), P(HMPADA-*co*-APSA), P(AGA), P(AGA-*co*-APSA), where it is possible demonstrate the pH dependence in metal ion retention.

A cationic polymer derived from MPTA shows a high retention only for Ag(I)

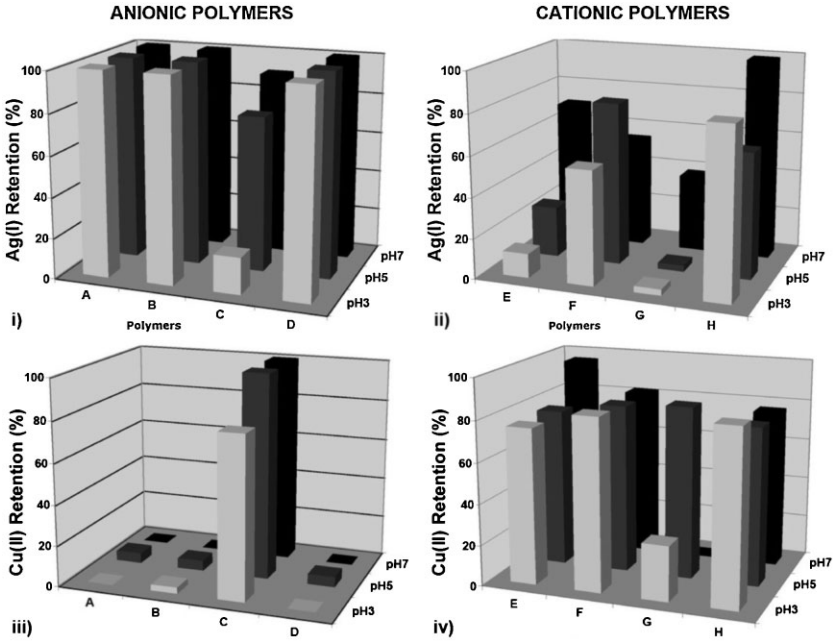
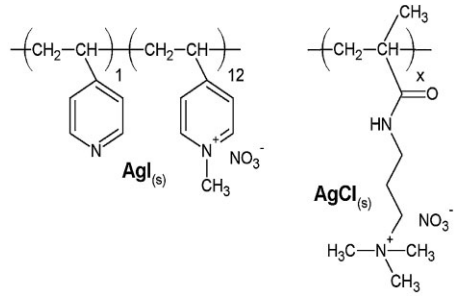


Figure 1.
Metal ion retention means LPR technique of Ag(I) [i). ii)] and Cu(II) [iii), iv)] for water-soluble polymers at different pH and a filtration factor, F, of 10. Anionic polymers, A: P(HMPADA), B: P(HMPADA-*co*-APSA), C: P(AGA), D: P(AGA-*co*-APSA); cationic polymers, E: P(VPyMe), F: P(MPTA), G: P(MPTA-*co*-APSA) 2:1, H: P(MPTA-*co*-APSA) 1:2.

(100%) due to the AgCl(s) formation, while Cu(II) ions are practically not retained (5%). Soluble copolymers derived from MPTA and APSA (mol ratios 2:1 and 1:2) show variable behavior where P(MPTA-*co*-APSA) 2:1 only retains Ag(I) (100%), and P(MPTA-*co*-APSA) 1:2 show retention similar to P(APSA).[14] At pH3, only Ag(I) was retained (18%); at pH5, Cu(II) has a retention of 78%, and Ag(I) a retention of 75%; at pH 7, both metal ions reach maximum retention, 100% for Cu(II) and a 89% for Ag(I). The copolymer with a mol ratio 1:1 not was studied because this was not water-soluble due to an internal salt formation between quaternary ammonium and sulfonate groups. The P(VPyMe) shows selective retention for Ag(I) with values of 100% at all pH, and a maximum retention of Cu(II) of 5%. This high retention for Ag(I) is due to a AgI(s) formation since the counter ion for this cationic polymer is iodide, see Scheme 3.

Anionic polymers P(AGA) show a high dependence with the pH (pKa = 4.37) for retention of metal ions achieving a maximum removal for Cu(II) at pH 5 (81%) and for Ag(I) at pH7 (40%); its copolymer shows a high interaction even at low pH. This modification is produced by AMPS incorporation since this comonomeric unit has a pKa = 1; higher retention can be obtained at lower pH. P(AGA-*co*-AMPS) achieves complete removal for Ag(I) at



Scheme 3.
Silver precipitates on cationic polymers.

different pHs, and Cu(II) maintains a constant retention at all pH. P(HMPADA) and P(AGA) present hydroxyl and carboxylic acid groups in their structures, but the former polymer incorporates a tertiary amine and a second carboxylic acid group in a small space. At pH 3, both polymers show similar retention behavior: low interactions with Ag(I) metal ion, although Cu(II) retention increases significantly. At pH 5, all retentions of P(HMPADA) are higher than found for P(AGA) and are very similar to the copolymer P(AGA-*co*-AMPS); at pH 7, all polymers show a high retention for all metal ions, reaching maximum values for Cu(II) in the case of P(HMPADA).

Since the biological activity of these polymers depends basically on the concentration, the chemical nature, especially for polymer metal-complexes where the metal ions produce the highest antibacterial agent activity, the maximum retention capacity of the metal ions by these polymers and the real metal ion content in the PMC are determined. MRC was determined by the LPR technique concentration method. The values were previously reported.[29–30]

In order to propose an interaction model, all complexes were characterized by UV-Vis spectrometry, FT-IR and Far-IR spectroscopy to establish which functional groups are involved in the complex formation. The characteristic peak for the AgNPs plasmon was not observed in the UV-Vis analysis, indicating that all silver ions are forming complexes or precipitated (AgI, AgCl) but not as Ag0, which is important due to the recognized antimicrobial activity of Ag0. Only the MRC of P(AGA)-Cu(II) could not be determined due to precipitation of PMC at a low metal ion concentration.

Two significant differences were observed between WSP and its PMC in the FT-IR of P(AGA) and P(AGA)-Cu(II),: 1) a signal attributed to HO−C=O (1760 cm^{-1}) appears as COO^- and moves to the same wave number of HN−C=O (1687 cm^{-1}) and 2) C-Ost (1300-1200 cm^{-1}) in complex show a high absorbance with respect to the homopolymer. The Far-IR spectrum shows signals at 532 and 211cm^{-1} attributed to Cu−O and Cu−N interactions; in P(HMPADA), the ester group (1724 cm^{-1}) moves to slightly lower frequencies for all complexes (1705–1716 cm^{-1}), the νO−C=O_{asym} band (1641 cm−1) decreases its frequencies (between 1593 and 1634 cm^{-1}) for PMC, the νC−N band (1403 cm^{-1}) decreases its frequencies (between 1378 and 1398 cm^{-1}) for PMC, the C−O stretching band (1177cm^{-1}) of C−OH shows a displacement to 1173 cm^{-1} for all PMC, for P(HMPADA)-Cu(II), the interaction Cu−O is placed at 381cm^{-1} and Cu-N at 202 cm^{-1}; the changes observed for P(AGA) were also observed for P(AGA-*co*-AMPS) and the Cu(II) complex, HO−C=O (1739cm^{-1}) appears as COO^- and moves to the same wave number of HN−C=O (1653cm^{-1}), C−Ost of C−OH moves slightly in complex from 1031 to 1034cm^{-1}; since a sulfonic group is a strong acid, long-range interactions are not observed.

Once the PMC is synthesized, its antibacterial properties can be studied. Different studies show the efficiency of several metal ions as antibacterial agents. Furthermore, their toxicity is known to basically take place at the cell level by diffusion towards the cytoplasm where the basic metabolic processes are altered. This toxicity can be diminished by complexing with molecules of high molecular weight since they would diminish diffusion towards the cytoplasm. The use of PMC as a possible antibacterial agent is based on the bacterial cellular wall's characteristic, which in the case of bacteria gram-negative (*e.g. E. coli*) presents an external membrane shaped by a great variety of proteins and lipopolysaccharide, while the bacteria gram-positive (*e.g. S. aureus*) do not present this external membrane although they do present a thick cap of peptidoglycan in which other acid structures are absorbed, such as teichoic acid and lipoteichoic acid.

The presence of these negatively charged structures on the bacterial cell wall should allow them to strongly interact with

Table 1.
MIC and metal ion concentration for polymers and its PMCs with Ag(I) and Cu(II).

Sample	MIC (μg/mL)		Metal ion concentration (μg/L)	
	E. coli (ATTC)	*S. aureus 6538P*	*E. coli (ATTC)*	*S. aureus 6538P*
P(VPyMe)	>128	64		
P(VPyME)-Ag(I)	>128	32	15	5
P(MPTA)	>128	32		
P(MPTA-*co*-APSA)	>128	>128		
P(HMPADA)	> 2040	> 2040		
P(HMPADA)-Ag(I)	128	128	43	43
P(HMPADA)-Cu(II)	2040	2040	35	35
P(AGA-*co*-AMPS)	> 2040	> 2040		
P(AGA-*co*-AMPS)-Ag(I)	2040	1024	0.4	0.4
P(AGA-*co*-AMPS)-Cu(II)	> 2040	1024	>0.2	0.1
Ag(I)	16	16		
Cu(II)	2040	256		

polycations by means of electrostatic forces. Additionally, the formation of bound complex or electrostatic interactions with the wall cell by means of a polycation should inhibit bacterial growth (see Table 1).

Since synthesized polymer–metal ion complexes have bactericide activity on *S. aureus* and *E. coli*, and these materials can be used as antibacterial or antiseptic agents with all the potential advantages, a genotoxicity study is necessary and important. For HMPADA homopolymer, P(VPyMe) and P(VPyMe)-Ag(I), no presence of halo inhibition was observed in either strain (see Figure 2). P(AGA-*co*-APSA) complexes were not analyzed due their high MIC. Free metal ions at studied concentrations did not show genotoxic activity (accumulative effects are not studied by this technique). As expected, PMC showed a reduction in its possible genotoxicity in comparison with its respective free metal ion. No genotoxic activity was observed for P(HMPADA) complexes where the rec(+)/rec(-) values were 1.01 for Ag(I), 0.90 for Cu(II), values far from the ratio limit (rec(+)/rec(-) = 1.20).

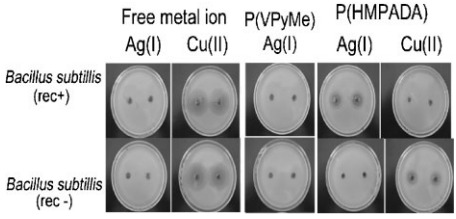


Figure 2.
Genotoxic assay for metal ions and complexes of P(VPyMe) and P(HMPADA).

Conclusion

The polymers studied for Ag(I) and Cu(II) recovery show different behavior. Anionic polymers retain close to 100% of these ions, however cationic homopolymers do not retain ions, and the high retention observed for Ag(I) was explained as due to the AgI and AgCl precipitate formed with the counter ion of these polymers. The synthesized polymers and PMC were completely soluble in aqueous media, allowing the characterization of these WSP and PMC as antibacterial agent. For both strains studied, the MICs show higher antibacterial activity for the PMC than for the WSP, with a low MIC for P(MPTA) and P(VPyMe)-Ag(I) (*S. aureus* 32μg/ml). All compounds showed low genotoxic activity.

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