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Water-Soluble Polymers and Their Polymer-Metal Ion-Complexes as Antibacterial Agents

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Summary: Natural and synthetic polymers with heteroatom, such as oxygen, nitrogen, sulfur or phosphorous, potentially can efficiently remove metal ions from aqueous solutions. The ability of synthetic polymers derived from imino diacetic acid, acrylamido glycolic copolymer, and the natural polymer alginic acid to remove metal ions from dilute solution in conjunction with ultrafiltration membranes was studied. The maximum retention capacity was determined and its polymer-metal ion-complexes were studied as possible antibacterial agents.

Keywords: alginic acid; antibacterial; metal ions; ultrafiltration membrane; water-soluble polymers

Introduction

Water-soluble polymers (WSP) cover a wide range of macromolecular systems including biopolymers such as DNA and synthetic polymers such a poly(acrylic acid). Due to its different structures, spatial conformation, and atomic composition give to this class of polymers a wide range of applications.

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

Removal, separation, and enrichment of hazardous metal ions in aqueous solutions play an important role for remediation of municipal and wastewater. At least 20 metals are classified as toxic and half of these are emitted into the environment in quantities that constitute human health risks. The toxic heavy metal cations contained in industrial effluents coming from plants using or producing heavy toxic metals and heavy toxic metal compounds are the main sources of the heavy toxic metal pollution. Specifically, metal-bearing wastewaters include discharges from electrolysis 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 methods for such wastewaters depend greatly on the particular complexing reagents and metal ions used as well as their concentrations. In general, they are grouped into three categories: chemical, physical, and electrochemical.[1–9]

From many separation techniques used industrially, membrane separation is an efficient and widely applied separation process that is comparable to other separation techniques in terms of technical and economic feasibility. Liquid-liquid extraction, sorption, precipitation, ion exchange, and others are classic preconcentration and

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separation methods for elements in geological, biological, environmental, and industrial fluids. These two-phase distributions or heterogeneous reactions with diffusion-controlled processes present several disadvantages, such as long contact times or the use of additional separations processes. These disadvantages can be avoided with the use of water-soluble polymeric reagents in combination with membrane filtration.[10,11] This technique termed *liquid-phase polymer-based retention technique* (LPR) is based on the separation of ions bound to WSP from those non-bound by mean electrostatic groups or chelating groups can be achieved by two methods. The *washing method* is a batch-like method where a liquid sample containing the polymer and the metal ions to be separated is placed in the ultrafiltration (UF) cell at a given pH and ionic strength. Subsequently, solutes, in contact with WSP and UF membrane are washed with a water solution contained in the reservoir that may reproduce the same pH and ionic strength values. The second one, the *enrichment method*, is analogous to a column method. A solution containing the metal ions to be separated is passed from the reservoir through the UF cell containing a polymer solution. Both cell and reservoir solutions may be adjusted to the same ion strength and pH. The separation process will be successful if the polymer reagent employed meet requirements such as: high affinity towards 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.

Furthermore, this method is used to synthesize polymer-metal ion complexes (PMC), which have emerged as a new generation of materials with tremendous potential in fields such as superconducting materials, ultrahigh strength material, liquid crystals, catalysts, and biocompatible polymers.[12–15] Different polymer acids such as: 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-acrylamido-2-methyl propane sulfonic acid), P(APSA)[16–21] have demostrated a good efficiency for this propose. Alginic acid is a natural polymer derived from brown algae. In acid aqueous medium, it can be found in gel form, and with the presence of Ca(II) and Mg(II) ions it forms gel in water. This polymer consists in two uronic acids, *D*-mannuronic and *L*-guloronic acids, disposed as blocks in different ratios, alternating or random form. Due to this variability, its pKa value ranges between 3.5 to 5.0.[22–24]

The aim of this work is to analyze the potential use of synthetic and natural WSPs as poly(hydroxymethyl propyl imino diacetic acid), P(HMPADA), poly(2-acrylamido glycolic acid), P(AGA), poly(2-acrylamido glycolic acid-*co*-2-acrylamido-2-methyl propane sulfonic acid), P(AGA-*co*-AMPS), and alginic acid (AA) to remove metal ions from aqueous solution, determining the functional groups involved in the formation of the polymer-metal ion complex using infrared spectroscopy and studying their application as antimicrobial agents.

Materials

Imino diacetic acid (Aldrich Co.), 2-glycidyl methacrylate 96%, (Aldrich Co.), 2-acrylamido glycolic acid, 2-acrylamido-2-methyl propane sulfonic acid, (Aldrich Co.), alginic acid from brown algae was obtained from Sigma. Ammonium persulfate (AP, Fluka) was used without further purification All metal ions were purchased from Merck: $Cu(NO_3)_2 \times 3H_2O$, 99%, p.a.; $Zn(NO_3)_2 \times 6H_2O$, extra pure; $AgNO_3$, 99.8%, p.a.; $Cd(NO_3)_2 \times 4\ H_2O$, 99%, p.a. Sodium hydroxide (NaOH, Merck), nitric acid 70% (HNO_3, Caledon) were used to adjust pH.

Polymer Synthesis

P(HMPADA), P(AGA), P(AGA-*co*-APSA) were synthesized from their corresponding monomers by radical polymerization using

AP as initiator. All polymerizations were carried out in a polymerization flask. AP was added at 1 mol-% for 24 hours between 60–70 °C under N_2(g) atmosphere.

Polymer Isolation, Purification, and Characterization

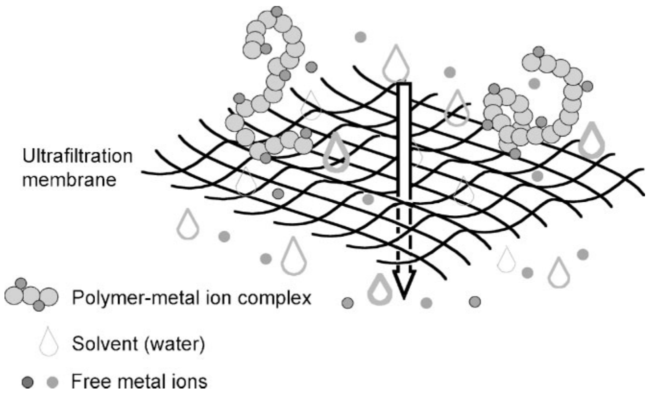
All synthesized polymers are completely water-soluble. For its isolation and purification all polymers were dissolved in twice-distilled water at 0.5 g/L, after that were fractioned using a ultrafiltration cell with ultrafiltration membranes with molar mass cut off (MMCO) of 100, 50, and 10 kDa. To obtain a purified polymer all collected fractions were lyophilized. The main polymer fraction (>100 kDa) was characterized by gel permeation chromatography, thermogravimetric analysis, and ^{1}H-NMR, FT-IR and far-FT-IR spectroscopy.

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 (0.1160 g of fraction > 100 kDa) and $2.5 \cdot 10^{-4}$ M of metal ions (5 mmol of each metal ion or 5 meq, 10 meq and 15 meq for mono- and di-valent metal ion respectively) are placed into the solution cell provided with a ultrafiltration membrane with a molar mass cut off, MMCO, of 10 kDa (Millipore, Amicon). Metal ions studied were Ag(I), Cu(II), Zn(II), and Cd(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 1). 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 ion 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



Scheme 1.
Ultrafiltration principle.

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. Retention can be plotted versus the filtration factor, and a retention profile is obtained.

The filtration factor (Z) is defined as the volume ratio of the filtrate (V^f) versus volume in the cell (V^c). 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.

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

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

To obtain PMC, the liquid-phase polymer based retention (LPR) technique by concentration or enrichment method was used. This method consists in passing a metal ion solution, known concentration, through a solution of water-soluble polymer (20 mL), maintaining constant volume. To develop this method, only water-soluble polymers are placed into the ultrafiltration cell and the metal ion solution is placed in the reservoir. When metal ions pass through the ultrafiltration cell, the macromolecules uptake the metal ions until saturation and the non-retained metal ion is collected in 5 mL and 10 mL assay tubes and quantified by AAS. Since the PMC will be used to determine its activity as antibacterial agent, an elution with 100 mL of twice-distilled water was done after each MRC experiment to eliminate all the metal ions not bound to the polymer in order to observe only the polymer-metal-ion complex‘s effect. In this study, the same polymer fraction, >100 kDa, and membrane, 10 kDa, were employed.

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, such as Ag(I), Cu(II), Zn(II), and Cd(II). The antibacterial activity of the polymers, the PMC, and free metal ions was investigated for *E. coli (6538P)*, a Gram-negative bacterium, and *S. aureus (ATCC)*, a Gram-positive bacterium. 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 hours 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 killing them.

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

To study the 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. For the test media, plates containing 25 mL of nutrient agar (2% agar) were used for the 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 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. When the soft agar was 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 hours, incubated at 37 °C for another 24 hours, 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(HMPADA), P(AGA), and P(AGA-*co*-APSA) are WSP obtained by radical polymerization; alginic acid, AA is a commercial natural polymer. All these synthetic polymers are completely soluble in water. P(HMPADA) contains hydroxyl, amine (ternary), carboxylic acid, and ester groups in its structure; P(AGA) contains hydroxyl, carboxylic acid, and amide groups; P(AGA-*co*-APSA) also contains a sulfonic group; AA presents ether, hydroxyl, and carboxylic acid groups in its structure as possible chelating or electrostatic groups. The metal ions Ag(I), Cu(II), Zn(II), and Cd(II) were studied between pH 3 and pH 7 using the LPR technique elution method. The presence of carboxylic acid and hydroxyl groups (hard base means Pearson's principle) allows an efficient interaction with hard acids and borderline acids [Cu(II), Zn(II)] and a weak interaction with soft acids [Ag(I), Cd(II)], while the sulfonic group (borderline acid) can achieve a high interaction with borderline acids.

Figure 1 shows the retention properties at different pH for P(HMPADA), P(AGA), and P(AGA-*co*-APSA). It is possible observe the pH dependence on the metal ion retention.

P(AGA) shows a high dependence on pH ($pKa = 4.37$) for metal ion retention, achieving complete removal for Zn(II) and Cd(II); its copolymer shows a higher interaction even at low pH. This modification is an effect of the incorporation of AMPS: since this comonomeric unit has a $pKa = 1$, higher retention can be obtained at lower pH with P(AGA-*co*-AMPS) completely removing Ag(I), Zn(II) and Cd(II). P(HMPADA) and P(AGA) present hydroxy and carboxylic groups in its structures, but the former polymer also incorporates a tertiary amine and a second carboxylic group in a small space. At pH 3.0, both polymers show similar behavior: a low interaction with Ag(I), Zn(II), and Cd(II) metal ions, but Cu(II) retention increases significantly; at pH 5.0, all retention values for P(HMPADA) are higher than those for P(AGA), and they are very similar to the copolymer P(AGA-*co*-AMPS); at pH 7.0, all polymers show an

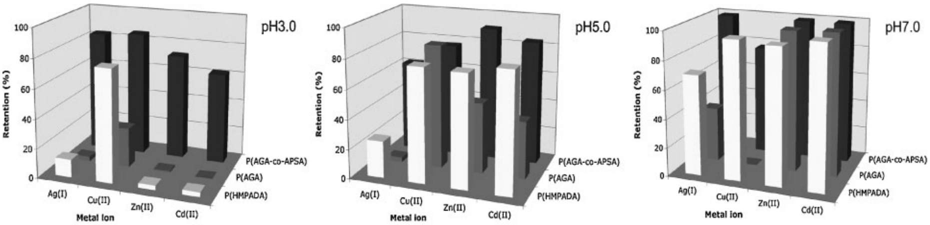


Figure 1.
Retention (%) at Z = 10 at pH 3.0, 5.0 and 7.0 for different polymers.

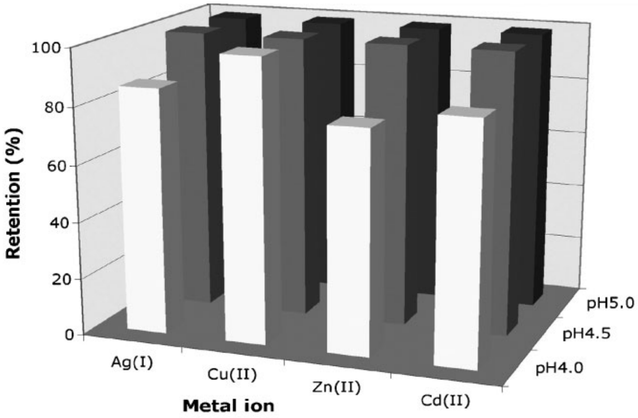


Figure 2.
Retention (%) for alginic acid at different pH and Z = 10.

excellent retention behavior for all metal ions, reaching maximum values for Cu(II), Zn(II), and Cd(II) in the case of P(HMPADA).

For AA, metal ion retention was studied at pH 4.0, pH 4.5, and pH 5.0 because the LPR technique cannot be applied because AA is found as gel below pH 4.0 and because the maximum retention is achieved at pH 5, see Figure 2. This figure shows the high retention of AA at different pHs, reaching its maximum from pH 4.0 in the case of Cu(II), and from pH 4.5 for Ag(I), Zn(II), and Cd(II) without precipitation or gel formation.

Since all metal ions are efficiently retained for all polymers, MRC was determined by the LPR technique concentration method. Ag(I), Zn(II), and Cd(II) were studied at pH 7.0 and Cu(II) at pH 5.0; in the case of AA, all metal ions were studied at pH 5.0. Table 1 and Figure 3 show the results of MRC. P(AGA) retains efficiently all metal ions, but precipitate formation prevented MRC determination for Cu(II) and all metal ions. With the copolymer containing a sulfonic acid group, the MRC values are lower than that it found for P(AGA), and these metal ion complexes can be used in other applications since precipitation is avoided. A similar situation occurs with AA where only the polymer-silver ion complex is completely water-soluble. For P(HMPADA), only the copper ion complex is not water-soluble, forming a precipitate at low metal ion concentration, while the polymer-Ag(I)-complex shows the maximum MRC.

All metal ion-complexes formed were characterized using FT-IR and Far-IR spectroscopy, establishing the functional groups involved in the complex formation, see Figure 4. Several shifts between WSP and its PMC were observed. In the FT-IR of

Table 1.
Maximum retention capacity (MRC) for Ag(I), Cu(II), Zn(II), and Cd(II) with studied polymers.

Metal ion	MRC (mg metal ion/g polymer)			
	P(HMPADA)	P(AGA)	P(AGA-*co*-AMPS)	Alginic acid
Ag(I)	342	201*	199	62
Cu(II)	17*	–*	103	3*
Zn(II)	117	226*	99	79*
Cd(II)	78	136*	40	60*

*: Maximum amount of metal ion incorporated to polymer before precipitation.

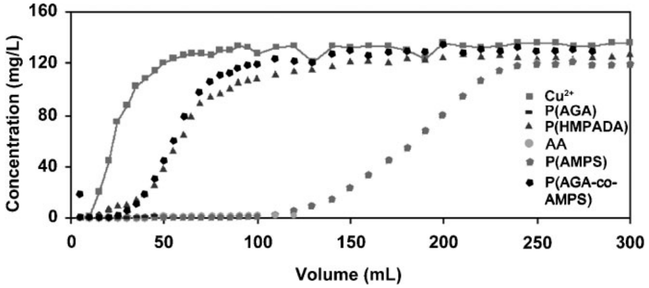


Figure 3.
Maximum retention capacity (MRC) curves for Cu^{2+} with different polymers.

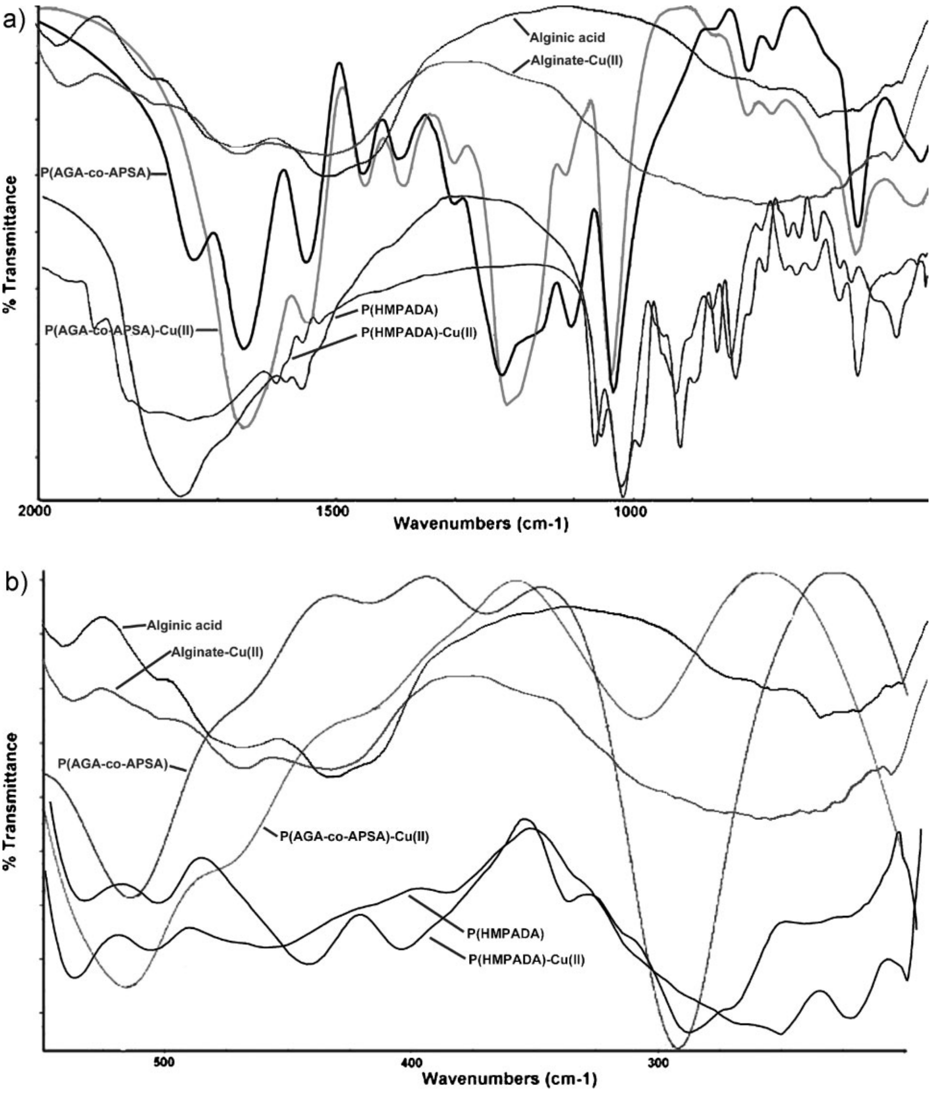
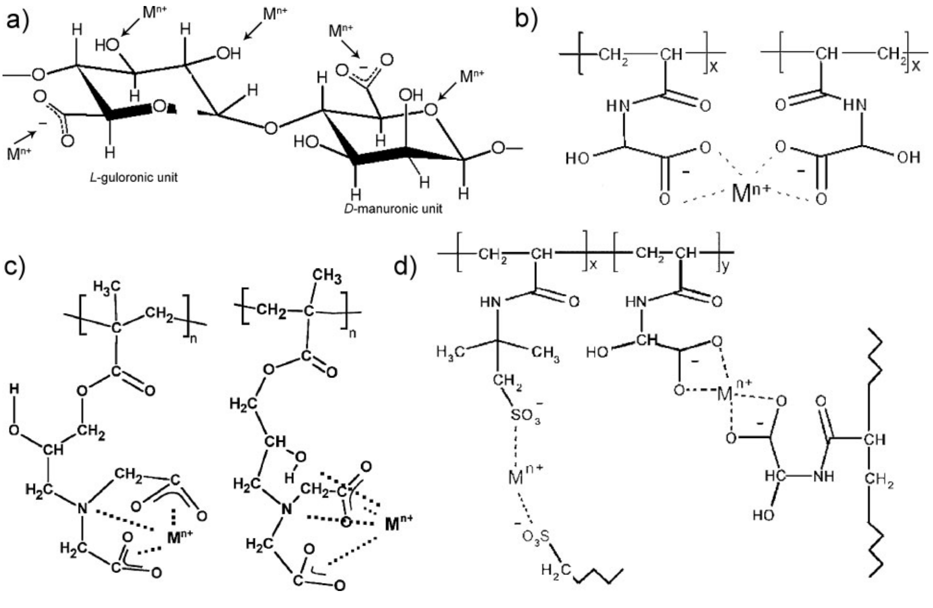


Figure 4.
a) FT-IR for polymers and polymer-Cu(II) complexes, b) Far-IR for polymers and polymer-Cu(II) complexes.

P(AGA) and P(AGA)-Cu(II), two significant differences are observed: the absorption 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 C-Ost (1300–1200 cm^{-1}) in complex shows a high absorbance with respect to the homopolymer, the Far-IR spectrum shows absorption signals at 532 and 211 cm^{-1} attributed to Cu−O and Cu−N interactions. In P(HMPADA), the ester group (1724 cm^{-1}) moves slightly to lower frequencies for all complexes (1705–1716 cm^{-1}), the νO−C=O$_{\mathrm{asym}}$ 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 (1177 cm^{-1}) of C–OH shows a shift to 1173 cm^{-1} for all PMC.

For P(HMPADA)-Cu(II), far-IR shows the Cu-O interaction at 381 cm^{-1} and Cu-N at 202 cm^{-1}. For P(AGA-*co*-AMPS) and the Cu(II) complex, the same changes that P(AGA) can be observed: HO-C=O (1739 cm^{-1}) appear as COO^{-} and moves to the same wave number of HN−C=O (1653 cm^{-1}), C-Ost of C-OH moves slightly in complex from 1031 to 1034 cm^{-1}, since the sulfonic group is a strong acid, its long-range interactions are not observed. For AA, the most significant differences involve carboxylic and hydroxy groups. A reduction in the intensity of $\nu(\mathrm{COO^{-}})_{\mathrm{sym}}$ for AA at 1730 cm^{-1} and an increase of intensity for $\nu(\mathrm{COO})_{\mathrm{sym}}$ (1620 cm^{-1}) and $\nu(\mathrm{COO})_{\mathrm{as}}$ (1460 cm^{-1}) in PMC was observed. δCOH at 1250 cm−1 are not present in PMC, νCO and δ(CCO) at 1100 cm^{-1} and 1030 cm^{-1}, respectively, reduced its intensity in PMC, the far-IR do not show significant differences. These results suggest the following patterns for polymer-metal ion interaction, see Scheme 2.

Once synthesized the polymer-metal complexes, PMC, they can be studied as antibacterial agents. Different studies show the efficiency of several metal ions as antibacterial agents. Furthermore, their toxicity is also known, taking place at the cell level basically 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



Scheme 2.
PMC interactions for a) alginic acid, b) P(AGA), c) P(HMPADA), and d) P(AGA-*co*-AMPS).

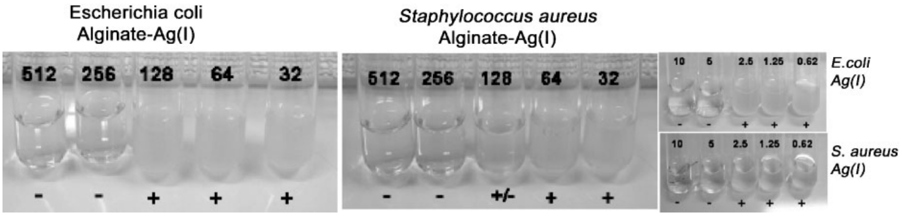


Figure 5.
Determination of minimum inhibitory concentration means dilution test for Ag(I) complex and Ag(0); (−) = without bacterial grow, (+/−) = low bacterial grow, (+) = with bacterial grow.

diminish diffusion towards the cytoplasm. The PMC, as a possible antibacterial agent, is based on the characteristic of the bacterial cellular wall, 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 (LPS), differing from the bacteria Gram-positive (*e.g. S. aureus*), which do not present this external membrane although they do present a thick cap of peptidoglycan in which other acid structures are absorbed as teichoic acid and lipoteichoic acid.

The presence of these negatively charged structures should allow them to strongly interact with polycations by means of electrostatic forces. In this respect, the formation of a bound complex or electrostatic interactions with the wall cell by means of a polycation should inhibit the bacterial growth. Figure 5 shows the result of a serial dilution test to determine the MIC of AA-Ag(I) complex against *E. coli* and *S. aureus*, and Table 2 summarizes the MIC determined for P(HMPADA), P(AGA-*co*-AMPS) and free metal ions. The P(AGA)-could not be studied using this technique because not all of the metal complexes are water-soluble.

Since synthesized polymer–metal ion complexes show a bactericide activity on *S. aureus* and *E. coli,* these materials could be used as antibacterial or antiseptic agent. With all the potential advantages, a genotoxicity study is necessary and important. For the P(HMPADA), the presence of halo inhibition in both strains was not observed (see Figure 5). Free metal ions at studied concentrations did not show genotoxic activity, including Cd(II) (accumulative effects are not studied by this technique). As expected, the PMC showed a reduction in its possible genotoxicity in comparison with its respective free metal ion. The

Table 2.
MIC and metal ion concentration for P(HMPADA), P(AGA-*co*-AMPS) and its PMCs.

Sample	MIC (μg/mL)		Metal ion concentration (μg/L)	
	E. coli (ATTC)	*S. aureus 6538P*	*E. coli (ATTC)*	*S. aureus 6538P*
P(HMPADA)	>2040	>2040		
P(HMPADA)-Ag(I)	128	128	43	43
P(HMPADA)-Cu(II)	2040	2040	35	35
P(HMPADA)-Zn(II)	> 2040	2040	239	239
P(HMPADA)-Cd(II)	>2400	4	0.3	0.3
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
P(AGA-*co*-AMPS)-Zn(II)	>2040	512	>0.2	0.05
P(AGA-*co*-AMPS)-Cd(II)	>2040	4	>0.08	0.00016
Ag(I)	16	16		
Cu(II)	2040	256		
Zn(II)	>2040	128		
Cd(II)	2040	2		

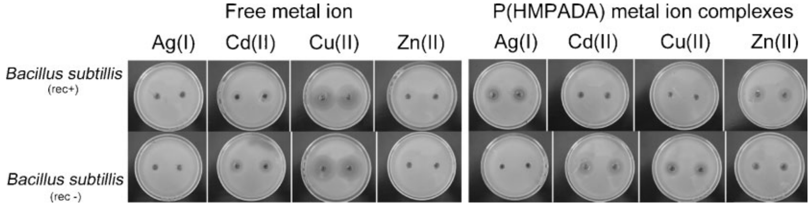


Figure 6.
rec(+)/rec(−) assay for P(HMPADA) complexes and free metal ion.

rec(+)/rec(−) values for complexes are: 1.01 for Ag(I), 0.90 for Cu(II), 1,21 fro Zn(II), and 0.95 for Cd(II). Only P(HMPADA)-Zn(II) shows an increase in the ratio up to limit (rec(+)/rec(−) = 1.20), but in this case the PMC had a higher concentration than free Zn(II) 1024 μg/mL.

Conclusions

Water-soluble polymers synthesized by radical polymerization were effective materials to remove metal ions from dilute solutions, achieving maximum retention (∼100%) for all metal ions. All these polymers can significantly accumulate metal ions in their structures, but P(AGA) and alginic acid form precipitates at very low concentration of some of these metal ions, only Ag(I) complex maintain it solubility for all polymers studied. Carboxylic acid and hydroxy groups are the main functional groups involved in complex formation. The presence of sulfonic group in P(AGA-*co*-APMPS) maintain the water solubility of PMC in comparison with the P(AGA) complexes. The bactericidal and genotoxic activities of this polyanion and its PMC were studied. The polyanions did not show antibacterial activity, and PMC showed a low bactericide effect against Gram-positive and Gram-negative bacteria, where only the P(HMPADA)-Cd(II) complex showed an important activity on *S. aureus*. All compounds, except for the Zn(II) complex, presented low genotoxic activity.

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