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ORIGINAL PAPER

Poly(L-lysine) as a polychelatogen to remove toxic metals using ultrafiltration and bactericide properties of poly(L-lysine)–Cu^{2+} complexes

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Abstract Poly(L-lysine) is a water-soluble, synthetic polypeptide containing functional amine groups that help remove di- and trivalent metal ions from aqueous solutions. This polymer's removal properties were studied under different experimental conditions: (1) competitive and non-competitive conditions; (2) different pHs; and (3) filtration factors. Under the conditions of the Liquid-Phase Polymer-Based Retention (LPR) technique, the cooper (II) ion interaction was found to be selective and efficient when compared to other divalent metal ions studied. However, interestingly, this selectivity disappeared when trivalent metal ions were present. The polymer–metal ion interactions are based on the amino groups of the side-chains as well as the polypeptide backbone chain. The removal of metal ions was strongly dependent on the pH. By structural characterization with FT-IR and EPR spectroscopy, participation of the amide and mainly amine groups was found

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to take place for the coordination. For the Cu^{2+}, coordination through four amine nitrogen donor atoms in the primary coordination sphere was detected. Antibacterial activity tests were conducted with the poly(L-lysine)–Cu^{2+} complex and showed a higher activity in comparison with the precursors Cu^{2+} and poly(L-lysine) at the same concentrations for *E. coli* (6538P), a Gram-negative bacterium, and *S. aureus* (ATCC), a Gram-positive bacterium.

Keywords Poly(L-lysine) · Water-soluble polymer · Toxic metal ions · Bactericide

Introduction

Selective separation of inorganic ions can be efficiently achieved using water-soluble polymeric reagents in combination with membrane filtration [1–3], which would occur in steps when compared with inorganic solid supports (water-insoluble) [4]. This technique, called Liquid-phase Polymer-based Retention technique (LPR), is based on the separation of non-complexed ions from those ions bound to water-soluble polymers (WSP) with chelating groups (polychelatogens) [2, 3]. The separation process is successful, if the employed polymer reagents satisfy the desired properties, such as chemical and mechanical stability, high affinity towards the target metal ion, inactivity towards the non-target metal ion, high molecular mass, regeneration, low toxicity, and low cost.

The water-soluble properties are provided by a high content of pendant hydrophilic groups (e.g., hydroxyl, amino, amide, carboxyl, and sulfonic acid groups) or hydrophilic units of the polymer backbone (e.g., ether or imino groups). A wide variety of water-soluble polymers have been utilized in the LPR process for heavy metal recovery. Three main groups of polymer reagents can be classified: *basic polymers*, such as poly(ethylenenimine), poly(vinyl amine), poly(allylamine), and other amino or imino group-containing polymers; *neutral polymers*, such as polyglycols, polyalcohols, and polyethers; and *acidic polymers*, such as poly(acrylic acid), poly(vinyl sulfonic acid), and poly(styrene sulfonic acid) [5–12].

One of the most extensively used is poly(ethyleneimine), which contains primary, secondary, and ternary amino groups [13, 14]. This polymer has been applied to remove metal ions because it possesses the basic properties, such as the physical and chemical stability, high water solubility, and a high interaction with different metal ions. However, for these polymers, the high interaction profile is accompanied by a high stability even at low pH. This property makes it difficult to cleave the complex and hence polymer regeneration.

Poly(L-lysine) is a synthetic poly(amino acid) first synthesized by Katchalski in 1947 [15], when he suggested its use as a model in protein research. Years later, Shima and Sakai discovered poly(ε-lysine) in soil bacterium *Streptomyces albulus* [16]. This homopolymer has high solubility in water and its structure contains amido (main-chain) and amino (side-chain) groups. Its complexation properties have been basically studied for Cu^{2+}, which catalyzes the oxidation of 3,4-dihydroxy-phenylalanine (DOPA) at high pH, using this reaction as a model for DOPA oxidase [17–20]. For this primary research, the knowledge of the correct

structure of the poly(L-lysine–Cu^{2+}) complex was necessary. Thus, dynamic light scattering [21], circular dichroism [22], NMR spectroscopy, and EPR [23] techniques were used to elucidate this structure. After that, copolymers, DNA-conjugated and the cell delivery with microcapsules were studied for the application in biological systems, even as an antineoplasic [24–29]. However, the possibility that surfaces treated with poly(L-lysine) allow cell adhesion [30], result then in the use of poly(L-lysine) to cover plastic and glass surfaces to increase the adhesion, growth, and differentiation of different cells types, including neuronal glyal cells, and/or transfected cells.

The aims of this work was to study the capability and/or selectivity of poly(L-lysine) to interact with 13 different metal ions of industrial, environmental or health interest, determine the functional groups involved in polymer–metal ions interaction using EPR, FT-IR, and Far FT-IR spectroscopy, and study the antibacterial activity (against gram-positive and gram-negative bacteria) of this polymer and its polymer–metal ion complexes for possible applications as biomedical material.

Experimental part

Reagents

Poly(L-lysine) was prepared according to the literature [31]. $AgNO_3$, 99.8%, p.a.; $Cu(NO_3)_2 \times 3\ H_20$, 99%, p.a.; $Ca(NO_3)_2 \times 4\ H_20$, extra pure; $Co(NO_3)_2 \times 6\ H_20$, extra pure; $Mg(NO_3)_2 \times 6\ H_20$, extra pure; $Ni(NO_3)_2 \times 6\ H_20$, extra pure; $Cd(NO_3)_2 \times 4\ H_20$, 99%, p.a.; $Pb(NO_3)_2$, 99%, p.a.; $Al(NO_3)_3 \times 9\ H_20$, extra pure; $Cr(NO_3)_3 \times 9\ H_20$, extra pure; $Fe(NO_3)_3 \times 6\ H_20$, extra pure; $UO_2(CH_3COO)_2 \times 2\ H_2O$, p.a., all from Merck; $Zn(NO_3)_2 \times 6\ H_20$, extra pure, from Aldrich. Sodium hydroxide (NaOH, Merck), nitric acid 70% (HNO_3, Caledon).

Metal ion retention by LPR technique (washing method)

To ensure a high level of ligand sites, the ratio of the copolymer repeat unit to metal ion (in mol) was 40:1. Then, 20.0 mL of a solution containing 1.0×10^{-2} mmol/L of a water-soluble homopolymer (0.0116 g of fraction > 100 kDa) and 2.5×10^{-4} M of metal ions (5 mmol of each metal ion or 5, 10, and 15 meq for mono-, di-, and trivalent metal ion, respectively) are placed into the solution cell provided with a ultrafiltration membrane with a molecular mass cut off (MMCO) of 10 kg/mol (Millipore, Amicon). Metal ions studied were separated in three classes: (a) mono and di-valent metal ions (Ag^+, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+}); (b) mono and poly-valent metal ions and (Ag^+, Cu^{2+}, Al^{3+}, Cr^{3+}, Fe^{3+}, UO_2^{2+}); and (c) biological metal ions (Cu^{2+}, Ca^{2+}, Mg^{2+}, and Fe^{3+}).

The pH was adjusted with dilute HNO_3 and NaOH. A washing solution (water at pH = 3.0, 5.0, and 7.0, depending on the metal ion) was passed through the membrane under constant pressure (3.5 bar of N_2) and then 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 (see Fig. 1). Each fraction was collected in graduated tubes, and the corresponding metal ion concentration was determined by atomic absorption spectroscopy (AAS).

The binding and elution processes may be formulated as a chemical reaction, where reversible reactions in combination with an irreversible transfer of metal ions across the membrane are responsible for metal ion retention. For any species, the retention (R_Z) is defined 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 chains 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, and retention may be formulated as follows:

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

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. The retention profiles are obtained by plotting the retention versus the filtration factor.

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.

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

Determination of maximum retention capacity (MRC) by LPR technique (concentration method)

To obtain polymer–metal complexes, the 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), keeping the volume constant. For the enrichment (or concentration) method, only the

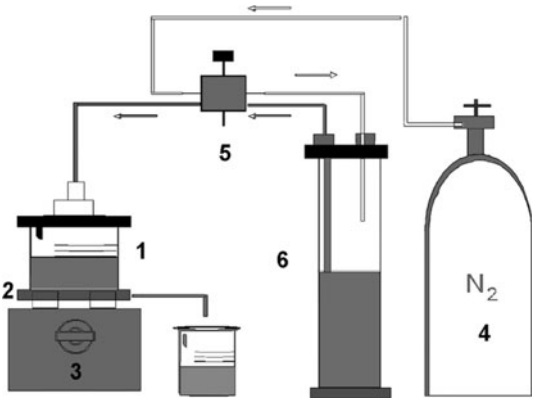


Fig. 1 LPR arrangement (*1*) filtration cell with polymeric and metal ion solution, (*2*) membrane filtrate, (*3*) magnetic stirrer, (*4*) pressure trap, (*5*) selector, (*6*) reservoir

water-soluble polymer was placed into the ultrafiltration cell while the metal ion solution was placed in the reservoir. When metal ions are passed through the ultrafiltration cell, the macromolecules uptake the metal ions until saturation and the non-retained metal ion were collected in 5 and 10 mL assay tubes and subjected to quantitative analysis by AAS. Since those polymer–metal ion complexes were used to determine their antibacterial activity, an elution with 100 mL of twice-distilled water was made after each one MRC experiments to eliminate all the metal ions not bound to the polymer and thus observe only the polymer–metal complexing effect. The same polymer fraction (>100 kg/mol) and membrane (10 kg/mol) were employed in this study. A blank experiment with metal ions and without polymer was conducted to determine the effect of the ultrafiltration membrane on metal ion retention. The amount of metal ions bound to the water-soluble polymer was calculated by the difference between the concentration curve slopes and the curves of the blank samples.

The MRC was calculated according to the relationship:

$$\mathrm{MRC} = {}^{M \cdot V}/_{\mathrm{Pm}}$$

where MRC is expressed as milligram of metal ion retained per gram of polymer (or expressed in mol), M is the metal ion concentration in mg/L, V is the filtrate volume through the membrane free of metal ion in L), and Pm is the mass of polymer in g. This value can be represented as solution enrichment, assuming a quantitative retention of different metal ions. The enrichment factor (E) was determined according to the following relationship:

$$E = {}^{P \cdot C}/_{M}$$

where P is the polymer concentration (g/L), C is the maximum capacity of the polymer (mg/g), M is the initial concentration of the metal salt (mg/L).

Antibacterial activity of the polymer, polymer–Cu^{2+} complex, and Cu^{2+}

Due to the well-known antibacterial activity of Cu^{2+} and the ability of poly(L-lysine) to migrate through lipidic bilayers [32], we studied the antibacterial activity of these compounds and the polymer–metal complex (PMC), poly(L-lysine)–Cu^{2+}. The antibacterial activity of the polymer, the polymer–metal complex, and free metal ion was investigated for *E. coli* (6538P), a Gram-negative bacterium, and *S. aureus* (ATCC), a Gram-positive bacterium. The 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 and the concentrations of these solutions were 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, and 2048 g/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 their growth without necessarily killing them.

Measurements

The FT-IR spectra were recorded on a Magna Nicolet 550 spectrophotometer. Scanning Electron Microscopy Jeol SM-6380LV. The pH was determined with a Jenco Electronics 1671 pH-meter. For the LPR technique, a membrane filtration system was employed to test the coordinating properties of the polychelatogen. Unicam Solaar M5 series Atomic Absorption Spectrometer was used for the determination of the metal ion concentrations in the filtrate. EPR measurements were made in a Bruker ESP 300 EPR instrument with a Bruker 4108 TMH/9701 cavity. The sample was transferred into a thin capillary, which was placed inside of the EPR tube. All measurements were performed at room temperature. The typical instrument settings were modulation amplitude 4.05, modulation frequency 102 Hz, time constant 2.56, receiver gain 500, scan range 100 G and scan center 3,415.2 G; one to five scans were performed depending on the signal intensity. The bacteria growth was determined by direct observation and comparison with the blank sample.

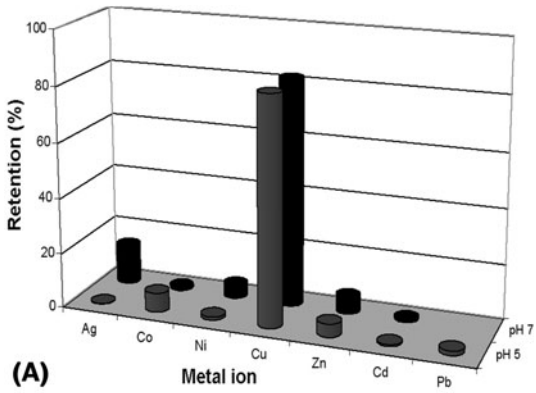
Results and discussion

Polymer–metal ion interactions

Heavy and toxic metal ion recovery from dilute solutions is a high impact research field. In this context, poly(L-lysine) with amide (main-chain) and primary amine (side-chain) groups will efficiently interact with deficient electron atoms forming stable chelates. Poly(L-lysine) has a pKa = 9 and its functional groups are classified as hard bases; according to the Pearson principle[33], they will efficiently interact with *hard acids* such as Ca^{2+}, Al^{3+}, Cr^{3+}, and Fe^{2+}, have a lower degree of interaction with *borderline acids* such as Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, and will not interact with ions classified as *soft acids* such as Ag^{+} and Cd^{2+}.

An initial study for a mixture of the ions Ag^{+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} at pH = 5.0 and 7.0 shows a specific interaction of poly(L-lysine) with Cu^{2+} with a retention value higher than 80% (see Fig. 2a) and a retention lower than 20% for all other ions. A mixture of the monovalent Ag^{+}, divalent Cu^{2+}, and trivalent ions such as Al^{3+}, Cr^{3+}, and Fe^{3+} and UO_2^{2+} were studied to corroborate selectivity for Cu^{2+}, and the results showed a high degree of interaction with poly(L-lysine), close to 100% for Al^{3+}, Fe^{3+}, and UO_2^{2+} (see Fig. 2b). The higher retention at pH 5 of Fe^{3+}, Al^{3+}, Cr^{3+}, and UO_2^{2+} over Cu^{2+} can de explained means Pearson's principle considering that poly(L-lysine) a hard base interact with hard acids and Fe^{3+}, Al^{3+}, Cr^{3+}, and UO_2^{2+} over borderline acid as Cu^{2+}.

To test the application of poly(L-lysine) for metal ion recovery from plasma, the interaction with other ions, such as Mg^{2+}, Ca^{2+}, Cu^{2+}, and Fe^{3+}, was studied at the level of blood plasma concentrations (see Fig. 3). This application could possibly be used to prevent diseases, such as Wilson's disease (chronic accumulation of Cu^{2+} in the liver) and hemochromatosis (chronic accumulation of Fe^{3+} in the liver in biological systems), although the irreversible aggregation with isolated platelets



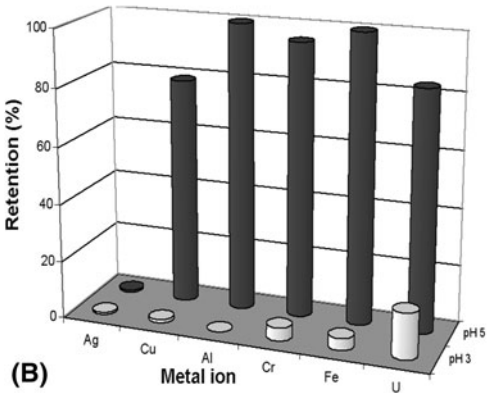


Fig. 2 Retention (%) at $Z = 10$ for poly(L-lysine) **a** at pH 5.0 and 7.0 for mono- and divalent metal ions, and **b** at pH 3.0 and 5.0 for mono- and polyvalent metal ions

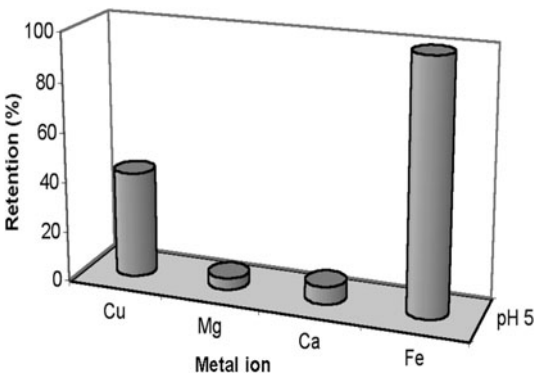


Fig. 3 Retention (%) at $Z = 10$ for poly(L-lysine) at pH 5.0 for Cu^{2+}, Mg^{2+}, Ca^{2+}, and Fe^{3+}

(platelets surface) [34] makes this potential application impossible using water-soluble poly(L-lysine).

In view of this, the Cu^{2+} complex was formed by the LPR concentration method obtaining a saturation curve for poly(L-lysine). The interaction was studied by FT-IR spectroscopy and EPR techniques. The complex formed, with a shiny blue

color, has low capacity for Cu^{2+} removal with a MRC of 48 mg/g polymer and an enrichment factor of 1.48.

Figure 4 shows the FT-IR spectra for poly(L-lysine) and its complex with Cu^{2+}. The poly(L-lysine)–Cu^{2+} complex shows significant differences with respect to the poly(L-lysine): a broad signal at 3400 cm^{-1} attributed to the amine group in the poly(L-lysine)–Cu^{2+} complex with an increased intensity for δ_{NH} at 1200 cm^{-1}, and a shift in the carbonyl signal to lower frequencies. In the far-IR spectrum, a shift of the signal was weakly observed at 452–467 cm^{-1} and was attributed to the O-metal interaction; the formation of an intense absorption band at 228 cm^{-1} corresponds to the N-metal interaction [35].

The EPR spectroscopy of a solid sample of the poly(L-lysine)–Cu^{2+} complex at the X band frequency (9.5 GHz) and room temperature showed a strong asymmetric signal centered at about $g = 2.13$. This value as well as the insufficiently resolved $g_{parallel}$ feature of the $^{63.65}Cu$ hyperfine coupling ($I = 3/2$) at the low-field side are

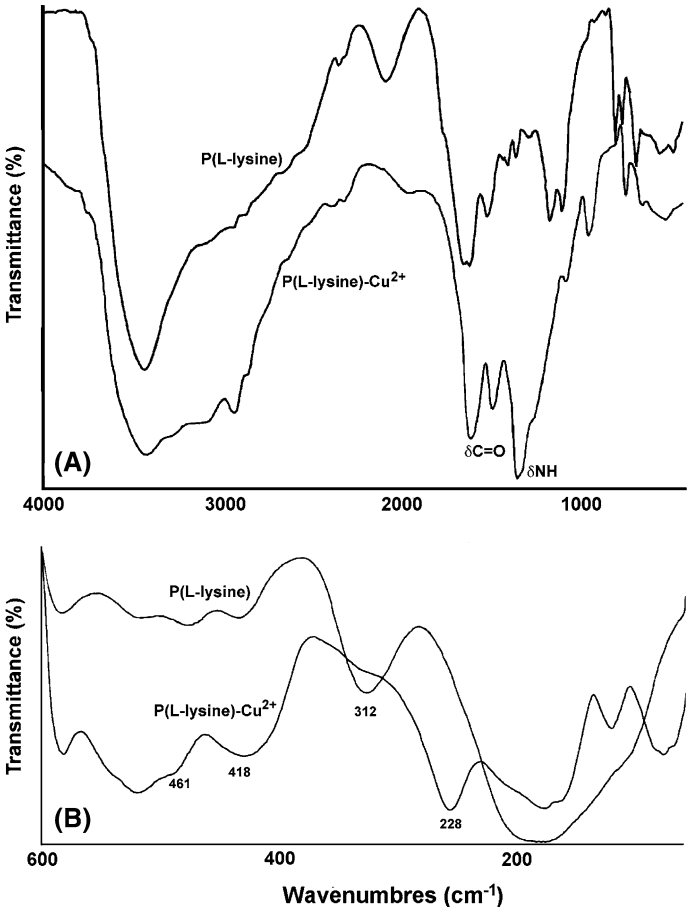
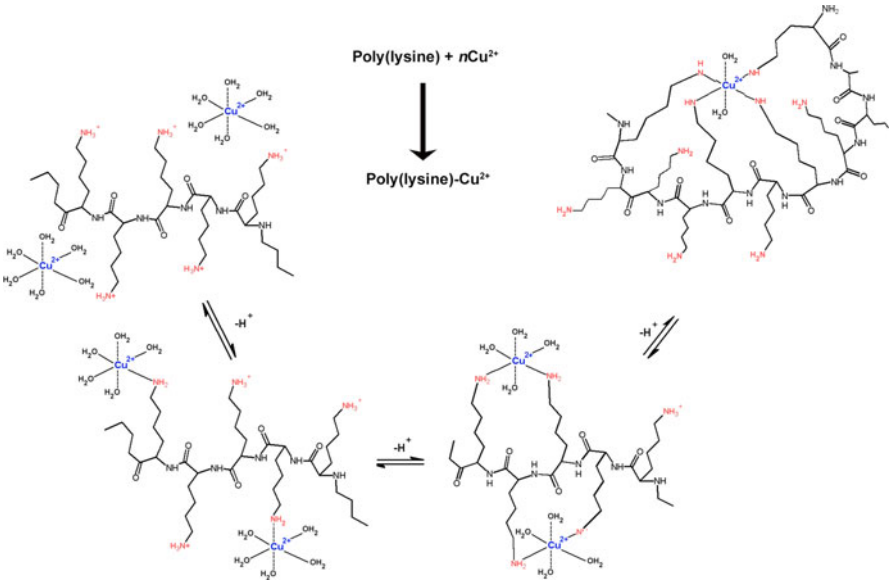


Fig. 4 **a** FT-IR spectra of P(L-lysine) and P(L-lysine)–Cu^{2+}, **b** Far FT-IR spectra of P(L-lysine) and P(L-lysine)–Cu^{2+}



Scheme 1 Interaction model of poly(L-lysine) and Cu^{2+} at different pH

compatible with high concentrations of magnetically isolated Cu^{2+} centers (d^9 configuration). The absence of deviation from the axial ($g_{perpendicular}$) splitting suggests a rather symmetrical coordination of the metal ions by four amine nitrogen donor atoms in the primary coordination sphere (see Scheme 1).

Biological applications

The use of the polymer–metal ion complex as a possible antibacterial reagent is based on the characteristics of the bacterial cell wall. The bacteria Gram-negative (e.g., *E. coli*) presents an external membrane shaped by a great variety of proteins and lipopolysaccharides (LPS), while the bacteria Gram-positive (e.g., *S. aureus*), do not feature this external membrane, although they do present a thick cap of peptidoglycans, in which other acid structures are absorbed, such 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 interaction with the wall cell by means of a polycation should inhibit bacterial growth, especially with ions with known antimicrobial activity, such as Ag and Cu. The application of poly(L-lysine) and its metal ion complexes as biocide agents is possible due to the same particular characteristics of poly(L-lysine): the ability to migrate across the bilayer membrane constituted by amino acids and the fact that the metal ions maintain their properties even when bound to the homopolymer. But the cell death mechanism belong unclear proposing four mechanisms to explain the cell death *toroidal pore*, *barrel stave*, *carpet*, and *blockade of channels* model [36].

Table 1 Minimum inhibitory concentration (MIC) of poly(L-lysine), its Cu^{2+} complex, and the free metal ion against *Staphylococcus aureus* 6538P and *Escherichia coli* ATTC

Sample	MIC (*m*g/L)		Metal ion concentration (*m*g/mL)
	E. coli (ATTC)	*S. aureus* 6538P	
Homopolymer			
Poly(L-lysine)	1024	512	–
Polymer–metal ion complex			
Poly(L-lysine)–Cu^{2+}	512	256	18
Free metal ion			
Cu^{2+}	2040	256	

The results of LPR establish that only Cu^{2+} can be studied; Ag^{+} cannot be studied due its low retention by poly(L-lysine). Antibacterial study show that the poly(L-lysine) has a higher activity than free Cu^{2+} ion, this activity is maintained and reinforced in the poly(L-lysine)–Cu^{2+} complex (see Table 1) reducing by half the concentration necessary to achieve the MIC even with a low amount of Cu^{2+}. The minor value of MIC for *S. aureus* is expected because its cellular wall has an external layer constituted by peptidoglycan, which is very flexible and contains spaces that allow the movement of the polymer or polymer–metal ion complex into the plasmatic membrane, where they are either bound to the proteins (blocking electron transport) or make this membrane permeable. Then, the possible mechanism will be the *blockade of channels* model, which involves a sequence of elementary events: (1) adsorption onto the bacterial cell surface, (2) diffusion through the cell wall, (3) adsorption onto the cytoplasmic membrane, (4) disruption of the cytoplasmic membrane, (5) leakage of the cytoplasmic contents, and (6) death of the cell. The Gram-negative bacteria have a double membrane and a thick layer of peptidoglycans, which confer a higher resistance to biocide products.

Conclusions

Poly(L-lysine) was found to interact with a high affinity (80%) and selectivity for Cu^{2+} ions in presence of others mono and di-valent metal ions. However, this selectivity is lost in presence of metal ions with higher valence: the interaction with trivalent metal ions was found to be higher for ions, such as Al^{3+} and Fe^{3+} (100%). According to the characterization of poly(L-lysine) and poly(L-lysine)–Cu^{2+} by FT-IR, Far-IR, and EPR spectroscopy in terms of the complex formation, interactions through the amine and amide groups were found to form a symmetrical coordination sphere with four amine nitrogen donor atoms.

The antibacterial activity of poly(L-lysine) was higher than found with the free Cu^{2+} ion, and this activity is maintained and reinforced in the poly(L-lysine)–Cu^{2+}-complex, especially for *S. aureus* in comparison with *E. coli*.

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