Synthesis, physicochemical and spectroscopic characterization of copper(II)-polysaccharide pullulan complexes by UV–vis, ATR-FTIR, and EPR

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Bioactive copper(II) complexes with polysaccharides, like pullulan and dextran, are important in both veterinary and human medicine for the treatment of hypochromic microcytic anemia and hypocupremia. In aqueous alkaline solutions, Cu(II) ion forms complexes with the exopolysaccharide pullulan and its reduced low-molecular derivative. The metal content and the solution composition depend on pH, temperature, and time of the reaction. The complexing process begins in a weak alkali solution (pH > 7) and involves OH groups of pullulan monomer (glucopyranose) units. Complexes of Cu(II) ion with reduced low-molecular pullulan (RLMP, $M_w \approx 6000$ g mol$^{-1}$) were synthesized in water solutions, at the boiling temperature and at different pH values ranging from 7.5 to 12. The Cu(II) complex formation with RLMP was analyzed by UV–vis spectrophotometry and other physicochemical methods. Spectroscopic characterizations (ATR-FTIR, FT-IRIS, and EPR) and spectra–structure correlation of Cu(II)–RLMP complexes were also carried out.

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1. Introduction

Biometal complexes with extracellular polysaccharides (exo-polysaccharides) like dextran, pullulan, and inulin are important in both veterinary and human medicine for the treatment of different anemia types. Pullulan is an extracellular water-soluble microbial polysaccharide produced by strains of Aureobasidium pullulans. It is now widely accepted that pullulan is a linear polysaccharide with maltotriosyl repeating units connected by $\alpha-(1\rightarrow6)$ linkages (Fig. 1a). Alternatively, the structural formula of pullulan may be presented as a regular sequence of panoses mainly bound by $\alpha-(1\rightarrow4)$ linkages. Minor structural abnormalities are reported in pullulan, such as the presence of maltotetrose residues distributed randomly along the pullulan chain (Fig. 1b). A number of potential applications have been reported for this biopolymer as a result of its good film-forming properties; pullulan can form thin films which are transparent, oil resistant, and impermeable to oxygen. Pullulan may be used as a coating and packaging material, as a sizing agent for paper, as a starch replacer in low-calorie food formulations, in cosmetic emulsions, and in other industrial and medicinal applications. According to the literature data several mono-, di- and polysaccharides like glucose, fructose, galactose, maltose, saccharose, cellulose, starch, inulin, and dextran have the capability of forming complexes with various metal ions: Fe(III), Cu(II), Co(II), Zn(II), Ni(II), Mn(II), Pt(II), and VO(II). Iron(III) complexes with different polysaccharides have special importance and they have been described in detail. Synthetic procedures for the complex formation of Cu(II) ions with polysaccharide pullulan are not available in scientific and patent literature. Due to the large number of active centers capable of bond formation between pullulan and Cu(II) ions, we have decided to investigate the preparation of Cu(II) ions complexes with reduced low-molecular pullulan. Reaction conditions of synthesis ($pH$, $T$, and $t$) and optimization of ligand/Cu(II) ratio in relation to molar mass ($M_w$) were investigated in this work. The complex formation with RLMP was analyzed by spectrophotometric UV–vis methods. The stability of complexes in the HCl solution was then investigated. We have used different spectroscopic techniques, such as Attenuated Total Reflectance–Fourier Transform Infrared microspectroscopy (ATR-FTIR), Fourier Transform–Infraed Imaging Spectroscopy (FT-IRIS), and Electron Paramagnetic Resonance spectroscopy (EPR) as the main tools to verify the conformation and the structure of this ligand type around the Cu(II) ions. Additional physicochemical characterizations were provided by pH-measurements and atomic absorption spectrometry (AAS).
2. Results and discussion

Previous experience on the synthesis of similar complexes with dextran\textsuperscript{24} pointed out to the necessity of obtaining well defined preparations of the Cu(II) complexes of potential commercial interest. In order to define the physicochemical properties of the complexes, RLMP were selected and their ligand capability was investigated.\textsuperscript{25} The results of testing and basic characteristics of synthesized Cu(II)–RLMP complexes are given in Table 1.

On the basis of the experimental results (Table 1), a reliable preparation of Cu(II)–RLMP complexes is obtained with pullulan oligomers $M_w$ 6000 g mol$^{-1}$ at boiling temperature and pH 7.5–8; within 7 min (procedure 2). At pH 7.5, the synthesis of the complexes at room temperature or in extreme conditions (autoclave, 130°C) was not completely achieved even for reaction time longer than 60 min (procedure 6). The complexes obtained under the procedures 4 and 5 (Table 1), at pH >8 present unfavorable effects of the synthesis. Comparing the obtained Cu(II)–RLMP complexes, either in solid state or in solution, various complexes colors are obtained depending on pH values (Table 1). The change of the solution color during the synthesis may be an indication that the syntheses of the complexes were successful. The obtained results showed that the color can vary from light green to dark blue in the range of pH 7.5–12. The solution of the most stable complex of Cu(II)–RLMP (procedure 2, Table 1) is green, while the alkali solution of decomposed Cu(II) ions at pH 13, where $[\text{Cu(OH)}_4]^{2-}$ ions dominate, is indigo-blue.

Water solubility of synthesized Cu(II)–RLMP complexes is different. The most soluble complex is obtained at pH 7.5 (Table 1), and this solution is stable after a period of time of six months. Complexes that are synthesized at higher pH are less soluble. The solutions of complexes obtained according to the procedure 5 (Table 1), after a longer period of time become opalescent, then start layering and precipitation are sometimes observed. Synthesis temperature significantly influences Cu(II) ion content in complexes. When the solution is warmed up to the boiling temperature, the content of bound Cu(II) in a complex is increased almost two times (13.04%) in comparison to the procedure of the synthesis at room temperature (5.31%, Table 1). However, if the synthesis conditions are more extreme (autoclave, 130°C, 1 h), the content of bound Cu(II) is decreased (3.57%). We also observed that medium pH is changed after addition of Cu(II)-salt and that Cu(II) ion content in the complex is influenced by the solution pH. In fact, when syntheses are performed at the same temperature and within the same reaction period, the highest Cu(II) ion content was obtained at pH 7.5 (Table 1). Additionally, if the solution pH values increases from 7.5 to 12, the percentage of the bound Cu(II) in the complex decreases.

Synthesized complexes differ in their stability to the hydrolytic effect of HCl. The complex of Cu(II) with RLMP obtained by the

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<th>Table 1</th>
<th>Reaction conditions related to synthesis and basic physicochemical properties of synthesized Cu(II)–RLMP complexes</th>
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<td>Procedure</td>
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Figure 1. Primary (a), and secondary (b) structure of exopolysaccharide pullulan.
procedure 2 (Table 1) shows the highest Cu(II) content (13.04%), and the highest stability on 3 M HCl hydrolytic treatment (8.5 min).

Depending on pH values, Cu(II)–RLMP complexes also behave differently considering wavelength ($\lambda$) at which they show the absorption maxima. This range of wavelengths in the visible spectra is 650–700 nm (Fig. 2).

Wide absorption bands in the visible spectra are the result of preponderance by the spin of the allowed, very close energy transfers, which contact like a unique asymmetric absorption band with 'the tail' from UV region; as the result of a charge transfer from a donor ligand atom to Cu(II) ion. 26,27 The hypsochromic effect of complexes absorption maxima, with the increase of pH solutions (Fig. 2), confirms the presence of different types of complexes. [Cu(H$_2$O)$_6$]$^{2+}$ ion absorbs at wavelength 812 nm (Fig. 2, spectrum 1), while synthesized complexes absorb within the range of 650–700 nm (Fig. 2).

**Figure 2.** Visible spectra of Cu(II) ion water solutions [Cu(H$_2$O)$_6$]$^{2+}$ (1), and synthesized Cu(II)–RLMP complexes at boiling point and pH 7.5 (2), pH 8 (3), pH 10 (4), pH 12 (5), and complex [Cu(OH)$_4$]$^{2-}$ at pH 13 (6); $c$ = 10 g L$^{-1}$.  

**Figure 3.** ATR-FTIR spectra of synthesized Cu(II)–RLMP complexes at boiling point and pH 7.5 (1), pH 8 (2), pH 10 (3), and pH 12 (4).
700 nm (Fig. 2, spectra 2–5). With the increase in the solution pH, the absorption maxima change to shorter wavelengths (blue shift) comparing with [Cu(H$_2$O)$_6$]$^{2+}$ ion. Complex that has been decomposed at pH 13 shows absorption maximum at 634 nm (Fig. 2, spectrum 6). Thereby, these spectrophotometric criteria can be adopted in order to establish the success of complex synthesis.

From the visible spectra of Cu(II)–RLMP complexes synthesized under different reaction conditions, the wavenumbers interval of the absorption maxima is found to be from 15,384 to 14,286 cm$^{-1}$. The ligand crystal field splitting energies ($\Delta$) are (Eq. 1):

\[ \Delta = \frac{\nu \times 1.2938 \times 10^{-4}}{2} \],

\[ \Delta = 1.99 \text{ to } 1.84 \text{ eV} \] (1)

According to literature data,$^{26}$ these energy values from 1.99 to 1.84 eV have been found in the area of the energy, which is characteristic of the octahedral ligand field ($\Delta_o$). d$^9$ Ion in an octahedral complex has characterized by the electron configuration t$_2^6$e$_g^3$. According to the Jahn–Teller theorem,$^{28}$ such a complex should be distorted. The Jahn–Teller effect requires that the shape of the complex changes slightly, resulting in a change in the energies of the orbitals. The resulting distortion is most often an elongation, but compression is also possible.$^{29}$ If the distortion takes the form of an elongation along the z axis (the most common distortion observed experimentally), the t$_{2g}$ and e$_g$ orbitals are affected. In octahedral complexes, where the e$_g$ orbitals are directed toward the ligands, distortion of the complex has a larger effect on these energy levels and a smaller effect when the t$_{2g}$ orbitals are involved.$^{26,29}$ This points to the possibility that in the structure of the synthesized Cu(II)–RLMP complexes, in analogy with dextran complexes,$^{24,30,31}$ Cu(II) ions are probably in the tetragonally distorted octahedral surroundings with O atoms, which can originate from a ligand or solvent.

Fig. 3 (continued)
Important results concerning the mobility and conformation of carbohydrate chains were obtained from the analysis of these compounds by ATR-FTIR and FT-IRIS spectroscopy.\textsuperscript{14,32} This is, in particular, due to the fact that steric factors and a spatial location of individual groups strongly contribute to the formation of vibrational spectra of carbohydrates.\textsuperscript{33,34} ATR-FTIR spectra may be simultaneously collected in a stepwise manner from different areas of a sample. The ATR-FTIR spectra of Cu(II)–RLMP complexes, which were synthesized from pH 7.5 to pH 12, are shown in Figure 3.

A spectroscopic FTIR study in a particular stretching and bending region of O–H (3400 and 1420 cm\textsuperscript{-1}) and C–H (2900, 1460, and 1350 cm\textsuperscript{-1}) vibrations indicates different binding between the Cu(II) ion and ligand, depending on pH and metal contents. The changes in number, frequencies, intensity, and width of the ATR-FTIR bands in a particular region of \(\nu\)(O–H) vibrations (3400 cm\textsuperscript{-1}, Fig. 3), \(\delta\)(C–H) vibrations (1500–1300 cm\textsuperscript{-1}, Fig. 3), and \(\nu\)(C–O) vibrations (1200–1000 cm\textsuperscript{-1}, Fig. 3) were related to the changes in the conformation and short-range interactions of the RLMP. The difference in frequencies, intensity, and shape of these bands in the region of \(\nu\)(O–H) and \(\delta\)(O–H), implies that in complexes that were synthesized at pH 10–12 there is a displacement of H\textsubscript{2}O molecules by O–H groups in the first coordination sphere of the Cu(II) ion. The exchange position and the intensity of complex bands can be registered in this range, where C–H and O–H bending vibrations from the CH\textsubscript{2}–OH groups take part in synthesized Cu(II)–RLMP complexes. The appearance of bands at about 1460 cm\textsuperscript{-1} and 1370 cm\textsuperscript{-1} from \(\delta\)(C–H) vibrations and the band at about 1420 cm\textsuperscript{-1} from \(\delta\)(O–H) vibrations are characteristic of one of the more possible positions of the CH\textsubscript{2}–OH group, rotating around the C5–C6 bond of the glucopyranose unit. The change of the angle between the methylene CH\textsubscript{2}–OH group and the polysaccharide chain axes consequently decreases the intensity of the appropriate IR bands (\(\nu\)(C–H) and \(\delta\)(C–H) vibration). The Cu(II) ions in a solution have a possible influence on the rotation of CH\textsubscript{2}–OH groups in the complexes. In the ATR-FTIR spectra of the synthesized Cu(II)–RLMP complexes, the absorbance bands in the carbohydrate fingerprint region 1200–800 cm\textsuperscript{-1} were sensitive to changes in a short-range order and show the influence of the solvent on the hydrogen-bonding system.\textsuperscript{7,34} The co-existence of \(\alpha\)-(1→4) and \(\alpha\)-(1→6) glycosidic linkages in the pullulan structure can be established by the appearance of a band at 935 cm\textsuperscript{-1}. The similarities of the \(\gamma\)(C–H) range indicate that there is no difference in the conformation of the glucopyranose unit in the RLMP and Cu(II)–RLMP complex molecules, and they probably exhibit \(4C_{1}\) chair conformation.\textsuperscript{30,31,35} The application of FT-IRIS system to the ligand RLMP and Cu(II)–RLMP complexes, which were synthesized at pH 7.5–12, is shown in Figure 4.

Figure 4. FTIR microscopy images (300 × 250 \(\mu m\)) of RLMP (1), and synthesized Cu(II)–RLMP complexes at boiling point and pH 7.5 (13.04% Cu) (2), pH 8 (7.26% Cu) (3), pH 10 (8.69% Cu) (4), and pH 12 (8.06% Cu) (5), were recorded in different areas of Cu(II)–RLMP complex.
FTIR microscopy images of ligand RLMP (Fig. 4.1), as well as images of the synthesized Cu(II)-RLMP complexes (Fig. 4.2–5) differ, which also indicates that the complexation process took place. The changes in color contour may show the content and distribution of copper and pullulan in Cu(II)-RLMP samples. The diameter and shape of particles for synthesized Cu(II)-RLMP complexes are different and depends on pH. ATR-FTIR spectra and FTIR microscopic images from different areas of Cu(II)-RLMP complexes are different and depends on pH. ATR-FTIR spectra and FTIR microscopic images from different areas of Cu(II)-RLMP complexes synthesized at pH 7.5–12 show high homogeneity of the sample.35,36 These IR bands in the spectra of Cu(II)–RLMP complexes synthesized at pH 7.5–12 show high homogeneity of the sample.35,36 These IR bands in the spectra of Cu(II)–RLMP complexes can be responsible for more and less ordered structures, respectively (Fig. 3).

The application of EPR spectroscopy to analysis of Cu(II)-RLMP complexes, which are synthesized at pH 8 and pH 12, is shown in Figure 5.

Cu(II) is a 3d^9 (S = 1/2; I = 3/2) ion, and this electronic configuration has been investigated in details.37–41 The ground state of the Cu(II) ion in an octahedral field has a low-lying orbital doublet 2E_g. As a result of a strong Jahn–Teller effect, EPR spectra registration at room temperature is feasible. The EPR spectra indicate the axially elongated tetragonally distorted octahedral symmetry of synthesized Cu(II) ion complexes and were typical for the Cu(II) ion with one unpaired electron in 3d subshell. Specific values of A_I and g_I for [Cu(H_2O)_6]^{2+} ion are A_I = 137 x 10^{-4} cm^{-1}, g_I = 2.41.42 EPR parameters of the spectra in Figure 5 (A_I = 187 x 10^{-4} cm^{-1}, g_I = 2.23, and g_z = 2.03), for the complexes synthesized at higher pH values, were close to the values for the frozen Cu(II)-ethylene glycol complex, thus indicating the square-planar or tetragonally distorted octahedral coordination of Cu(II) ion with four oxygen atoms in the same plane.40 The appearance and EPR parameters for the spectra of complexes obtained at pH 8 and 12 were very similar to the EPR spectra of some Cu(II) ion complexes with carboxymethyl-dextran and cellulose obtained at high pH values.39,44 Although the Cu(II) ion content of the complexes synthesized at lower pH values was much higher (up to 13.04% for the complex synthesized at pH 7.5) the EPR signal of these complexes was lacking due to strong spin–spin interactions of neighboring Cu(II) ions.42,43 and probably the formation of polynuclear Cu(II) complexes.35 took place. All EPR parameters observed from pH 7.5–12 differ, especially in A_I and g_I from the parameters of [Cu(H_2O)_6]^{2+} ion and [Cu(OH)_4]^{2-} ion (A_I = 194 x 10^{-4} cm^{-1}, g_I = 2.271).44 This indicates that O^- donors from deprotonated OH groups may participate in Cu(II) ion coordination. A decrease of g_z values with the increase of pH complex solutions also confirms the presence of different types of complexes.44,45 This conclusion is in agreement with the results obtained by UV–vis investigations.

On the basis of the results obtained by physicochemical and spectroscopic investigations (UV–vis, ATR-FTIR, and EPR) of these complexes, three different types of Cu(II) aqua and/or hydroxo complexes with deprotonated pullulan monomer unit (glucopyranose) are suggested depending on pH synthesis.35,36 These complexes originate from the displacement of H_2O molecules by O^- from deprotonated O–H groups in the first coordination sphere of the Cu(II) ion (Fig. 6).

3. Experimental

3.1. Materials and methods

Low-molecular pullulan (LMP; M_w = 6000 g mol^{-1}) with reducing groups content (RG) of 12.5% was obtained from 'Zdravlje Actavis Co.' (Leskovac, Serbia). The pullulan oligomers used in our experimental work were obtained by the rigorous purification procedures (sedimentation, fractionation, dialysis, and ion-exchange chromatography). All the other chemicals were of reagent grade purity and were used as received. Redistilled water was used for all experiments.

3.2. Reduction of low-molecular pullulan

The reduction of LMP was performed with NaBH_4 (Merck, Darmstadt). The quantity of NaBH_4 necessary for the reduction of LMP depends on the content of the reducing group RG and the total polysaccharide mass. The RG values were obtained by using Somogyi method.46 The exactly weighed amount of fresh, finely powdered NaBH_4 was added in small portions and with constant stirring into the 12.5% w/w LMP solution, heated at 40 °C on water bath. After 2 h the temperature was raised to 70 °C and the reaction mixture was vigorously agitated for 30 min, in order to remove H_2 from the solution. The reaction mixture (pH 10) was neutralized with 10% HCl solution to pH 6. The borate ions were

Figure 5. EPR spectra of Cu(II)-RLMP complex at pH 12 (8.06% Cu) (a), and pH 8 (7.26% Cu) (b).

Figure 6. Proposed structure and positions of Cu(II) ions into the polysaccharide pullulan molecule on the pH 7.5–8.
removed by passing the reaction mixture through ion exchange columns filled with Amberlite IR-120 and Amberlite IRA-410 resins. The obtained solutions were reduced to the corresponding volume by evaporation at reduced pressure. The reducing group content in the final product was determined by the Somogyi method and found to be below 0.05%.

3.3. The complex synthesis

To the solution containing 100 g of ligand (RLMP) in 0.7 L H₂O, the 0.25 L of 1.2 M NaOH solutions was added. After that, a solution containing 0.17 mol of CuCl₂·2H₂O was added dropwise under continuous stirring until the pH value of about 7 was reached. The synthesis of the complexes was performed at pH values ranging from 7–12 (adjusted by the addition of 10% NaOH solution) by boiling the solution. The change of the solution color from light blue to green indicated that the synthesis of the complexes was completed. The reaction mixture was then cooled to room temperature and neutralized with 1 M HCl. A green solution of the complexes was filtered and the complex precipitated by addition of 3 M HCl entails the decomposition of the Cu(II) complexes was performed at pH values ranging from 4000 to 400 cm⁻¹ all spectra were interactive polynomials baseline-corrected and area-normalized. A Kubelka–Munk arithmetic method was applied to enhance the resolution in this spectral region. Deconvoluted spectra were smoothed by the 40-point Fourier filter method. The spectra were analyzed using Opus software (Bruker Optics Limited).

The visible spectra of the synthesized Cu(II) complexes in water solutions were recorded on a Varian Cary 300 Systems UV–Vis Spectrophotometer, in 1 cm path length quartz cells. The region 400–900 nm was scanned at a resolution of 1 nm at room temperature. The optical blank solution was reddistilled water. The visible spectra were analyzed using WinUV software (Varian).

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References

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