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Analysis of Bioactive Olygosaccharide-Metal Complexes by Modern FTIR Spectroscopy: Copper Complexes

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1. Introduction

Spectroscopy of biomolecules has been founded at the end of last century by the researchers working in the field of optical spectroscopy applied to biosystems. Since this time, the interest of the activity has considerably grown up. Researchers have typically used traditional spectroscopic techniques, such as Raman scattering, IR absorption, UV/Vis absorption, circular dichroism, fluorescence, magnetic resonance, X-rays and neutron scattering. Recently, particular attention has been devoted to the applications of biomolecular spectroscopy in the fields of biomedical imaging, drug characterization for pharmaceutical applications, drug delivery and nanobiotechnology.

Investigations of the bioactive metal complexes are very interesting in medicine and pharmaceutical industry, with the aspects on therapy of different states of anemia or metabolism disorder. On the other hand, polysaccharides and their derivatives, as the most abundant class of biomolecules, are known to have a large variety of biological functions. Through the interaction between these polyfunctional molecules and metal ions in living organisms, the modification of the biological function of both counterparts may be expected. The polysaccharide type compounds as ligands have received considerable interest. Simple sugars and their derivatives with reduced and oxidized groups form metal ion complexes of various composition and stability. One of the known roles of the oligo- or polysaccharide complexes is the transport of metal ions through cell membranes. For example, the commercial copper preparations based on polysaccharide dextran and its derivatives are used for such purpose in both human and veterinary medicine.

In the field of biocoordination chemistry a lot of investigations are based on the synthesis and characterizations of different metal complexes of ligands they present in biological systems, or synthetic ligands, which will serve like the model-molecules for complex biomolecular structures. Bio- or synthetic ligands are mainly natural chemical compounds of macromolecular type. In this group of products of the special importance are chemical compounds of olygosaccharide pullulan, dextran and inulin with cations of the different biometals (Cu, Fe, Co and Zn). It is well known that raw microbiological exopolysaccharides dextran and pullulan, are glucose polymers with the large molar mass from a few millions g/mol, with own toxic and antigen characteristics so that they are not of pharmaceutical

importance. For commercial reasons raw polysaccharides were depolymerized to the products with adequate molar masses, with the aim of getting fractions with narrow molar mass distribution. Synthesis procedures for the complex formation of biometals with poly- or oligosaccharides are described in scientific and patent literature. However, the structure of the bioactive metal complexes with oligosaccharides has not been explained in details yet, despite a number of studies. The work represents further development in research of complex structure and pharmacobiological activity of the complexes. Some new results that are directly related to medical practice and structural physicochemistry of biomolecules based on the oligosaccharide-metal complex are presented in this publication.

Different biometals (Cu, Fe, Co, Zn) complexes with inulin, pullulan and dextran oligosaccharides, as well as reduced or oxidised derivatives, have been analyzed by IR spectroscopy. Spectra-structure correlations of the complexes have been performed by using modern spectroscopic techniques: FTIR microspectroscopy, ATR-IR, LNT-IR and D_2O-FTIR. The techniques are applying in the structure analysis of polysaccharide complexes, as well as for the confirmation of suggested types of complex structure and for the testing of homogeneities of samples. Results of IR microspectroscopic investigation shows that structural form of complexes and metal content considerably depends of constitution and ligands conformation, degree of crystallity, polymerization, polydispersity, and linearity of macromolecules. Also, stability of the synthesized complexes, as well as their pharmacological effect, depends of these parameters. FTIR investigation of the complexes by D_2O isotopic exchange proved to be a very sensitive method for determining OH group coordination and is related to the hydrogen bond strength. Results of our investigations points to the complexes are crystal hydrate molecules. Correlation of physicochemical and spectroscopic investigations of these complexes, and structure of exopolysaccharide chain, are suggesting different model structures of the synthesized complexes.

FTIR spectra and microscopy images were obtained by using an FTIR microspectroscopy system, ATR–FTIR spectrometer Bruker Tensor-27 in conjunction with a FTIR Bruker Hyperion-1000/2000 microscopy attachment equipped with the 4× viewing objective (objective magnification 4×, visible magnification 57×) and 15×IR Schwarzschild objective (objective magnification 15×, visible magnification 215×). The standard detector, a 250 μm liquid nitrogen cooled, mid-band mercury–cadmium–telluride (MCT) detector (ATR objective GMBH, Germany) with preamplifier, with the range of the IR spectrum from 7000 to 400 cm^{-1} was used. The spectra were measured with 2 cm^{-1} resolution and 200 scans co-addition. The spectrometer was linked to a PC equipped with Bruker OPUS software to allow the automated collection of IR spectra. The measurements were conducted in the reflection mode. In the region from 4000–400 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 IR spectra were imported to GRAMS/AI 7 (Thermo Galactic, USA) for peak area integration.

Thus, various tests can be performed by the Bruker Hyperion microscope, such as transmission, reflection, polarized, and ATR–IR measurements, the linear scan and mapping techniques in terms of software, and optic video technology for true video analysis. In addition, spatial resolution IR spectra and functional group imaging can also be acquired and analyzed. For measuring IR spectra by FTIR microscopy accurately, several primary parameters in the operation need to be selected and set first, which include aperture sizes, number of scans, resolution, velocity of motional mirror, and sampling background.

2. Character and spectroscopy of bioinorganic compound

Metal ions in biological systems are divided into two classes (Nakamoto, 2009). The ions of first class (K, Na, Mg and Ca) are important in maintaining the structure of proteins by neutralizing negative charges of peptide chains and in controlling the function of cell membranes that selectively pass certain molecules. In the second class, ionic forms of Fe, Co, Cu, Zn, Mn, Mo, and so on exist in small to trace quantities, and are often incorporated into proteins (metalloproteins). The latter class is divided into two categories: (A type) transport and storage proteins and (B type) enzymes. Type A includes oxygen transport proteins such as hemoglobin (Fe), myoglobin (Fe), hemerythrin (Fe), and hemocyanin (Cu), electron transfer proteins such as cytochromes (Fe), iron–sulfur proteins (Fe), blue-copper proteins (Cu), and metal storage proteins such as ferritin (Fe) and ceruloplasmin (Cu). Type B includes hydrolases such as carboxypeptidase (Zn) and aminopeptidase (Zn, Mg), oxidoreductases such as oxidase (Fe, Cu, Mo) and nitrogenase (Mo, Fe), and isomerases such as vitamin B12 coenzyme (Co).

During the last decades laboratory (with animals) and clinical researches have shown that many pathologic states of a body are accompanied by statistically reliable disturbances in the metabolism of metals at the molecular and body levels. Any chronic disease, the cause of which has not yet been established, can be due to abnormalities in metal metabolism. The determination of the amount of biometals in the body is suggested as the earliest diagnostic test of diseases (Grigorieva et al., 1983).

The metals participating in metabolism can be divided into the following groups: a) inherent in a living body and involved in the sphere of essential biofunctions (Cu, Fe, Zn, Mn, Mo, Co, Mg, Ca, K, Na); b) introduced, often toxic, whose physiological role has not been fully elucidated and their presence in the body tissue and liquids is due to their abundance in nature and wide application by people (Al, Cr, Cd, Ni, Pb, *etc.*). For the first group of metals both positive and negative balances were detected in different pathologies, and for the second group, as a rule, only the positive balance was observed. One of the reasons for the abnormal accumulation and removal of metals from a human body may be the wide application of drugs in clinics and which, by their chemical nature, are good ligand–complexing agents (up to 80% of all used drugs). Using non-steroidal antiinflammatory compounds (HL) and a copper-containing blood enzyme, ceruloplasmin (CuCPL), the ligands (drugs) were shown to take away competitively the metals from metal-containing and metal-activating enzymes: CuCPL + HL = CuL + CPL. Such an interaction results in a "discomfort" of an enzyme system in the body which is indicative of a side effect of drugs, *i.e.* complexing agents. For some diseases the shifts in metal metabolism are specific: rheumatoid arthritis (−) Fe, Zn; (+) Cu, Al, Mn, Mo, Cr; atherosclerosis (−) Cr, Mn, Zn; cancerogenesis (−) Cu, Fe, Mg; (+) Zn, Mn; diabetis (−) Cu, Mn, Cr; (+) Zn; *etc*. The correction in the concentration of these metals results in a therapeutic effect. The complex compounds of biometals with different types of drugs are the most promising tool for introducing the required metal into the body. It has been established that the application of antiinflammatory agents as complexes with some biometals decreases their toxicity and increases and prolongs their therapeutic effect (chemico-therapeutic synergism); antiulcerogenic, cytotoxic and other helpful properties, unusual to non-complexed agents, appear.

To understand the roles of these metal ions in biological systems, it is first necessary to know the coordination chemistry (structure and bonding) of metal ions in their active sites.

Such information is difficult to obtain since these active sites are buried in a large and complex protein backbone. Although X-ray crystallography would be ideal for this purpose, its application is hampered by the difficulties in growing single crystals of large protein molecules and in analyzing diffraction data with high resolution. As will be discussed later, these difficulties have been overcome in some cases, and knowledge of precise geometries has made great contribution to our understandings of their biological functions in terms of molecular structure. In other cases where X-ray structural information is not available or definitive, a variety of physicochemical techniques have been employed to gain structural and bonding information about the metal and its environment. These include electronic, infrared, resonance Raman, ESR, NMR, ORD, CD, Moossbauer spectroscopy, EXAFS, and electrochemical, thermodynamic, and kinetic measurements.

Infrared spectroscopy has been used extensively for the study of bioinorganic compound. In some cases, however, the vibrations of interest may not be enhanced with sufficient intensity. Then, one must resort to IR spectroscopy, which exhibits all vibrations allowed by IR selection rules. It should be noted, however, that IR measurements in aqueous media are generally limited to the regions where water does not absorb strongly. Furthermore, it is often necessary to use difference techniques to cancel out interfering bands due to the solvent and some solute bands. In the following, we will review typical results to demonstrate the utility of vibrational spectroscopy in deducing structural and bonding information about large and complex bioinorganic molecules. Marked progress has been made in chemistry of the bioinorganic complexes where the active site is modeled by relatively simple coordination compounds. Thus, we compare vibrational spectra of biological molecules and their model systems whenever appropriate or necessary. Since biospectroscopy is one of the most exciting areas of modern research, the volume of literature on biological compounds is increasing explosively. It is clearly not possible to cover all important topics in a limited space. Several excellent monographs (Parker, 1983; Nakamoto & Czernuszewicz, 1993) and review articles cited in each section should be consulted for further information.

Infrared spectroscopy has been used particularly for the study of polysaccharide complexes with metal ions, especially the active sites of the ions in the complexes. FTIR spectroscopy opens up new possibilities for the fine structural analysis of polysaccharides and its derivatives, the establishment of the type of bonding between the elementary links and their rotational isomerism. Weak intermolecular interactions have a significant influence on the specifically valuable properties of biological molecules and polymer compounds. We had to restrict ourselves to a few examples of wide potentialities of the method of FTIR spectroscopy in investigating the relationships between the structure and the properties of extracellular polysaccharides and its complexes with different metal ions.

3. Copper(II) ion and its significance

Copper(II) ion is a biologically active, essential ion, creating ability and positive redox potential allow participation in biological transport reactions. Cu(II) complexes possess a wide range of biological activity and are among the most potent antiviral, antitumor and antiinflammatory agents (Vosburg & Cooper, 1941). On the other hand, condensed triazoles exhibit a range of pharamacological activities such as mitotic (Jackson & Polaya,1951), hypotensive (Walker et al., 1951), CNS stimulant (Lepetil, 1975), antiflammatory (Hardtmann & Kathawala, 1977) and analgesic activites (Kathawala, 1974; Clark et al., 1997).

Copper(II) ion is an essential component of several enzymes such as ceruloplasmin, cytochrome C oxidase, lysil oxidase, superoxid dismutase and tyrosinase that are required to maintain the host homeostasis (Platonova et al., 2004; Gaiduk et al., 2009). At the same time, copper ions can be involved in the reactions producing active radicals, which affect the structure of all types of biomolecules. Therefore, it is not suprising that cells lack free copper ions, while their safe transfer is realized by a special system, metabolic copper system, some genes of which have been recently cloned.

Copper has an important role in the metabolism and transition of iron in the body. Microcytic hypochromic anemia is one of the outcomes of copper deficiency. There is a great number of hypocupremical drugs used commercially nowadays. As active substance, these drugs contain $CuSO_4$ (Expert group, 2002). In the literature is known that metal complex with polysaccharides and their derivatives are of growing importance in medicine and pharmacy. New blood substitutes with hemostimulating and antianemic function, which are complexes of dextran and pullulan with Cu(II) ion differ from the existing analogues in good bio- and hemocompatibility and more pronounced and prolonged action (Klimovich et al., 1998; Gapanovich et al., 1998). These complexes are very stable during prolonged storage and are not toxic. Copper(II) ion is used in the treatment of microcytic hypochromic anemia. It is apsorbed from the lower part gastrointestinal tract. This active pharmaceutical compound has a repetitive dose schedule (0.6-2 mg daily).

4. Bioactive copper-pullulan complex

Pullulan is a linear exopolysaccharide of α-D-glucopyranose that is often described as a α-(1→6) linked polymer of maltotriose subunits. This unique linkage pattern gives pullulan with distinctive physical properties. 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 (Deshpande et al., 1992). Pullulan is derivatized easily to control its solubility or provide reactive groups. Consequently, pullulan and its derivatives have numerous potential food, pharmaceutical, and industrial applications.

Bernier isolated water-soluble polysaccharides from the cultures of *Aureobasidium pullulans* and reported that α-D-glucopyranose is the major product of acid hydrolysis (Bernier, 1958). Based on the positive optical rotation and IR spectrum of pullulan was concluded that the polymer is a α-glucan in which α-(1→4) linkages predominate (Bender et al., 1959). Subsequent studies using IR, periodate oxidation, and methylation analysis established that pullulan is essentially a linear glucan containing α-(1→4) and α-(1→6) linkages in a ratio of 2:1 (Sowa et al., 1963). Partial acid hydrolysates of pullulan include isomaltose, maltose, panose, and isopanose (Leathers, 2003). The discovery of the enzyme pullulanase provided a critical tool for the analysis of the structure of pullulan (Wallenfels et al., 1961). Pullulanase specifically hydrolyzes the α-(1→6) linkages of pullulan and converts the polymer almost quantitatively to maltotriose (Wallenfels et al., 1965). Based on this result, pullulan is frequently described as a polymer of α-(1→6) linked maltotriose subunits (Fig. 1).

However, pullulan can also be viewed as a polymer of panose or isopanose subunits, which may reflect the biosynthetic origins of the molecule more accurately. Indeed, a number of enzymes that produce panose or isopanose from pullulan have been described since. Catley

(Catley et al., 1970) established that pullulan contains maltotetraose subunits (Fig. 2) in addition to the predominant maltotriose subunits. The frequency of maltotetraose subunits appears to vary on a strain-specific basis, from about 1% to 7% of total residues (Catley et al., 1986). The evidence suggests that maltotetraose subunits are distributed randomly throughout the molecule (Carolan et al., 1983). Unlike the maltotriose subunits in pullulan, maltotetraose residues are substrates for many α-amylases, and it has been proposed that hydrolysis of pullulan at these sites accounts for the decrease in molecular weight commonly observed in late cultures.

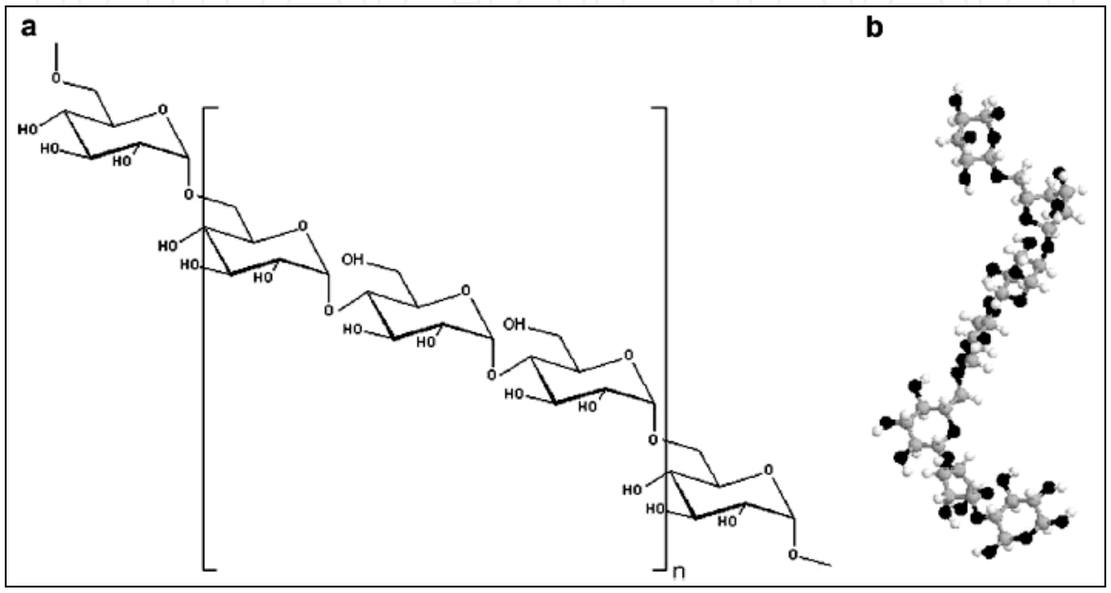


Fig. 1. Molecular structure of a representative portion of pullulan, illustrating the primary structure of repeating linkages: (a) 2D model, (b) 3D model stick and ball

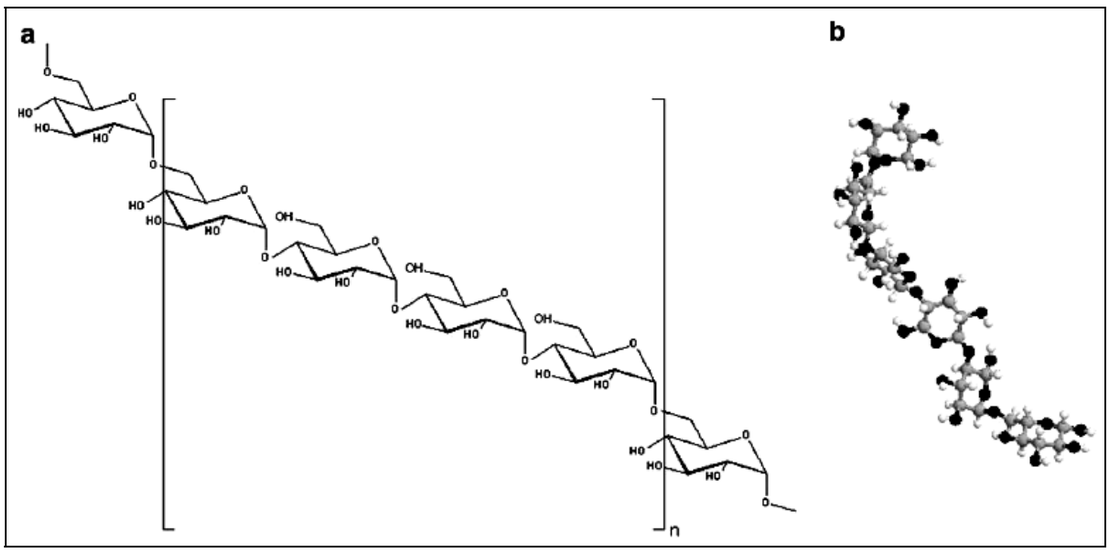


Fig. 2. Molecular structure of the secondary (minor) repeating structure of pullulan, occurring in about 1–7% of total linkage subunits: (a) 2D model, (b) 3D model stick and ball

Many types of carbohydrate derivatives (reduced or oxidized) have been synthesized for biomedical applications. In addition, polysaccharides such as chitin (Tanodekaew et al., 2004), chitosan (Pan et al., 2003; Yin et al., 2003), heparin (Ishihara et al., 2003; Kweon et al., 2003), alginate (Leonard et al., 2003; Perets et al., 2003), inulin (Nikolic & Cakic, 2007), dextran (London, 2004; Serizawa et al., 2003; Lawrence et al., 1997) and pullulan (Ilic et al., 2002; Kim et al., 2003; Nikolic at al., 2002) have been derivatized for biomedical applications. Pullulan is a polysaccharide that has been used in a drug delivery because of its solubility and biocompatibility. In addition, although the polysaccharides have many ionic groups, both anionic and cationic, pullulan is nonionic (Shingel, 2004).

Reduced low-molar pullulan (RLMP), was chosen as a new material for complexing, and the subsequent interactions with Cu(II) ions were investigated. The complexing process begins in a weak alkaline solution (pH > 7), and involves OH groups in C(2) and C(3) or C(6) pullulan monomer units (α-D-glucopyranose). Complexes of Cu(II) ion with reduced low-molar pullulan were synthesized in the water solutions, at the boiling temperature and at different pH values, ranging from 7.5–12. Cu(II) complexes were prepared from sodium salts, and investigated in the solid state. Fourier transform infrared spectroscopic data of synthesized complexes are rare in literature. FTIR spectroscopic characterization is now widely used to study the composition of the complex carbohydrate systems, the molecular interactions, a molecular orientation and conformational transitions of polysaccharides (Zhbankov, 1972; Panov et al., 1976; Panov & Zhbankov, 1988; Shingel, 2002; Zhbankov et al. 1997). The major goal of this section is to use different FTIR spectroscopic techniques (FTIR, LNT-FTIR, ATR-FTIR, and FTIR microspectroscopy) as the main tools to verify the conformation and the structure of this type of ligand around the Cu(II) ions.

Experimental.

Pullulan of average molar mass 2 x 10^5 g mol^{-1} and reduced low-molar pullulan of average molar mass 6000 g mol^{-1} was obtained from PCI ''Zdravlje Actavis Co." (Leskovac, Serbia). $CuCl_2$ x $2H_2O$ was purchased from Merck (Darmstadt, Germany). Cu(II) complex synthesis with RLMP have been described in detail by Nikolic (Nikolic et al., 2008). For FTIR sample preparation the KBr pastille method was used. Fine pulverized, water-free samples (1 mg) were mixed with potassium bromide (150 mg, Merck) stored at 80 ^{0}C for 6 h, and then pressed at 200 MPa to obtain a transparent pellet. The reference measurement was performed with pure KBr. The dryness of the pastille was controlled by the band at ca. 1640 cm^{-1}, which is associated with the deformation vibrations of the O-H bond from water molecules (Nikolic et al., 1996; Bellamy, 1954).

The FTIR spectra as an average of 40 scans were recorded at room (298 K) and liquid-nitrogen (77 K) temperature on a BOMEM MB-100 FTIR spectrometer (Hartmann & Braun, Canada) equipped with a standard DTGS/KBr detector in the range of 4000–400 cm^{-1} with a resolution of 2 cm^{-1} by the Win–Bomem Easy software. The spectrometer was purged with dry N_2. A Specac P/N 21525 variable-temperature cell was used for the LNT measurements. In the region all spectra were baseline-corrected and area-normalized. A Fourier self-deconvolution based on the Griffiths/Pariente method was applied to enhance the resolution in a spectral region of 4000–400 cm^{-1}. A gamma factor of 12 corresponding to a peak width of 24 cm^{-1} was used. Deconvoluted spectra were smoothed by the 30-point Savitzky–Golay filter method.

In addition, FTIR microspectroscopy system, ATR-FTIR spectrometer Bruker Tensor-27 in conjunction with a FTIR Bruker Hyperion-1000/2000 microscopy attachment equipped with

a 15x objective and a 250 μm liquid-nitrogen cooled, narrow-band mercury–cadmium–telluride (MCT) detector (ATR objective GMBH, Germany) with the range of the IR spectrum from 4000 to 400 cm^{-1} was used in this analysis. The spectra were measured with 4 cm^{-1} resolution and 320 scans co-addition. The measurements were conducted in the reflection mode. In the region from 4000–400 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.

Results and discussion.

The FTIR spectra of the RLMP and the synthesized Cu(II) complexes (Fig. 3) contain following characteristic bands: ν(O-H) 3400 cm^{-1}, ν(C-H) 2930 cm^{-1}, δ(HOH) 1640 cm^{-1}, δ(C-H) 1450 and 1345 cm^{-1}, δ(O-H) 1420 cm^{-1}, a complex band ν(C-O) and ν(C-C) 1200–1000 cm^{-1}, γ(C-H) 1000–700 cm^{-1}. Between FTIR spectrum of RLMP and FTIR spectra of the synthesized complex on the different pH there is a clear difference in the area of vibrations of all types of OH groups and molecules H_2O (Fig. 3). That is, in the spectrum RLMP has found the wide intensive band on the approx. 3400 cm^{-1} which is the result of valent vibrations OH groups and valent vibration of H_2O constitutional molecules. The band on the 1640 cm^{-1} is the result δ(HOH) (Nikolic et al., 1996; Bellamy, 1954; Nikolic et al., 2007; Nikolic et al., 2008).

The appearance of the spectrum in this region is different, as expected. In the spectrum of the complex which is synthesized on the pH 7.5 (Fig. 3) the centroid of this band is shifted, and decreased temperatures provoke a clear separation of two bands the frequency which is 3378 cm^{-1} and 3246 cm^{-1} (data from LNT-FTIR). By the complex which is synthesized on the pH 8 the frequencies of these bands have been 3453 cm^{-1} and 3333 cm^{-1}, 3458 cm^{-1} and 3348 cm^{-1} on the pH 10 and 3389 cm^{-1} and 3355 cm^{-1} on the pH 12. These bands are sensitive at the decreasing temperatures so those, according to this criterion, need attribute ν(OH) vibrations. These changes in ν(OH) region are results of complexing i.e. the deprotonation of the RLMP ligand OH group, most likely of different surroundings in the first coordination sphere of the Cu(II) ion. Exactly let us say we know that by the complexing Cu(II) ion with dextran (Dex) in the dependence on the pH form different types of the complex (pH 8: $Cu(II)(Dex)_2(H_2O)_2$, pH 10: $Cu(II)(Dex)_2(H_2O)(OH)$, pH 12: $Cu(II)(Dex)_2(OH)_2$) and the spectral picture in this region is very similar (Nikolic et al., 2008). If in the case of the complex with RLMP would form similar complexes, bands in this region would need the attribute valent vibrations of the OH group and coordinate molecules H_2O by the complex which is synthesized on the pH 7.5 with regard to the OH ligand group and the OH group in the first coordination sphere of the Cu(II) ion by the complex on the pH > 10.

In the spectrum presented in Fig. 4a, of the complex which synthesized on the pH 7.5, one from two previous quoted bands would originate from ν(HOH), whose correct position could not be established and this is probably a low-frequent band 3246 cm^{-1} the absence of which in the spectrum of the complex was synthesized on the pH 12. In the area of δ(HOH) vibrations unlike RLMP where spectrum has only one band on the 1645 cm^{-1}, in the spectrum of the complex which synthesized on the pH 7.5 in the area of δ(HOH) vibrations have two bands (1657 and 1642 cm^{-1}) which points to two different types of H_2O molecules (Fig. 4b). The higher frequency band 1657 cm^{-1} with the increasing pH diminishes the intensity. In other words the complex on the pH 10 and pH 12 is absent (Fig. 4b).

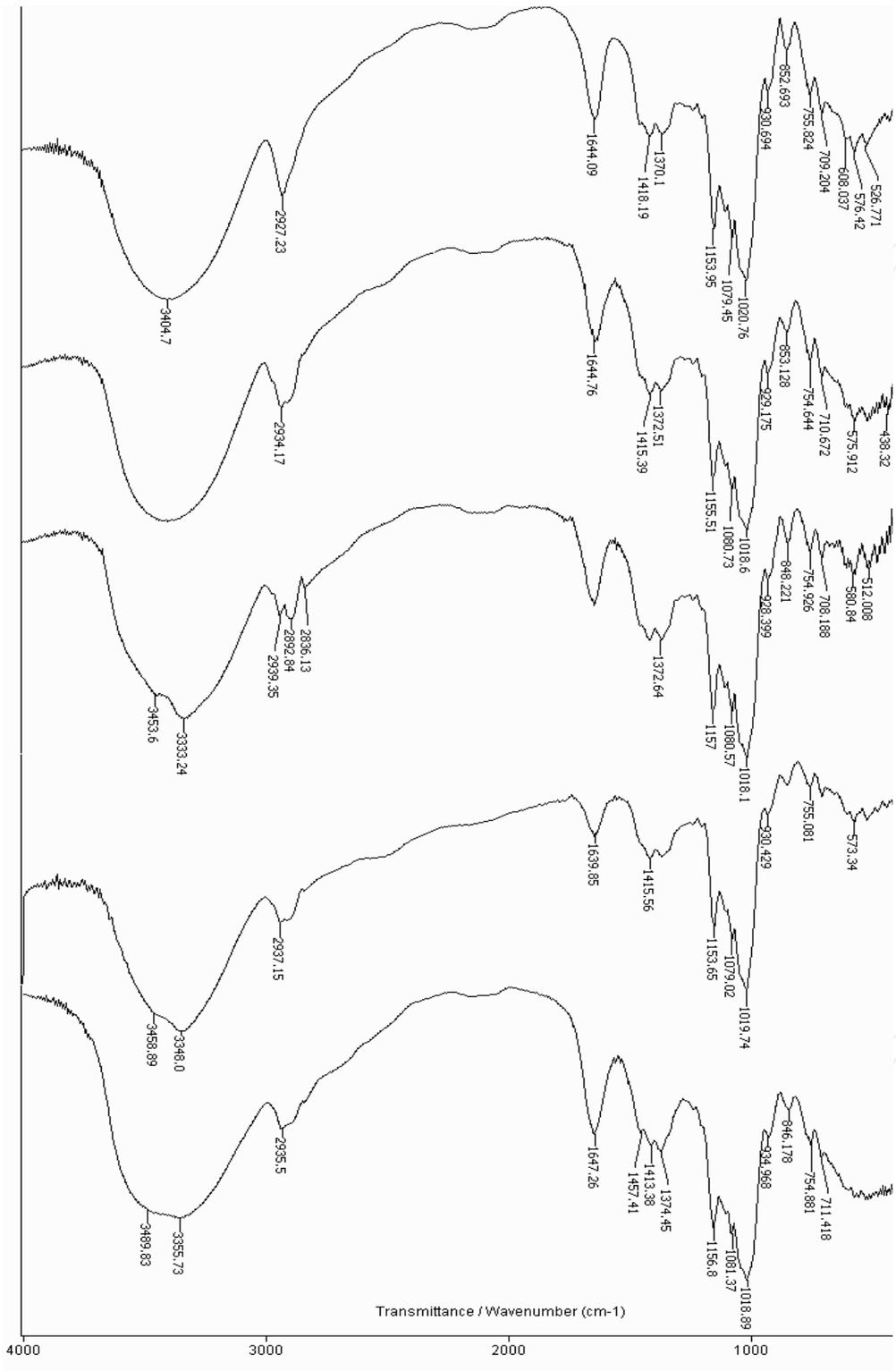


Fig. 3. FTIR spectra of RLMP, Mw = 6000 g mol^{-1} (1) and Cu(II) complexes with RLMP synthesized at boiling point and pH 7.5 (2), 8.0 (3), 10.0 (4) and 12.0 (5)

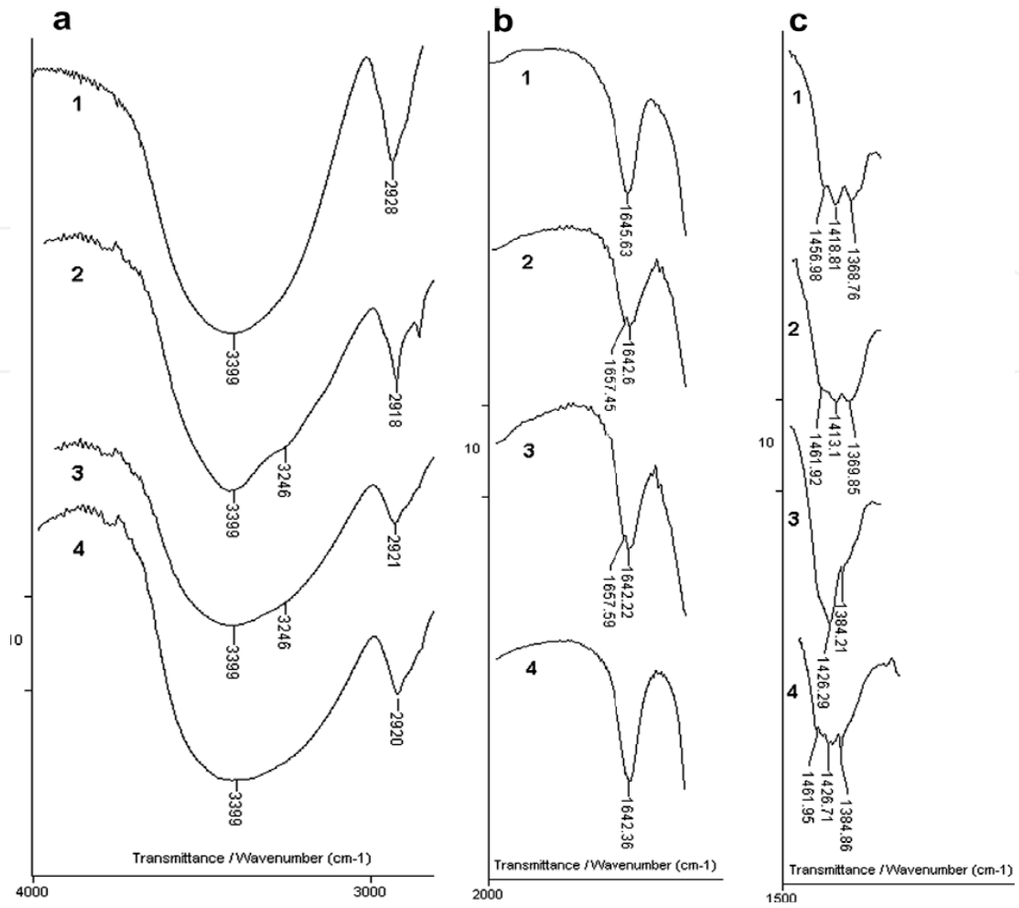


Fig. 4. Stretching (a), bending in plane (b) and bending in plane OH plane (c) region from LNT-FTIR spectra of RLMP (1) and synthesized Cu(II)–RLMP complexes at boiling point and pH 8.0 (2), 10.0 (3), 12.0 (4)

The spectral picture favors the suggested structure in Fig. 5 (type I). The complex which is synthesized on the pH 10 and pH 12 intensity bands from ν(HOH) and δ(HOH) diminishes with increasing pH. Expect the appearance band from δ(OH) from the first coordination sphere of Cu(II) ion whose intensity with increasing of pH growth; this band in the spectrum of the complex which synthesized on the pH 7.5 is absent. In addition to this, the fact is that in the area of δ(HOH) by the complex which is synthesized on the pH 10 and pH 12 only one band exists, which points to one type of H_2O molecule (like by RLMP) and band δ(OH) on the 1384 cm^{-1} (data from LNT-FTIR) (Fig. 4c).

Spectroscopic FTIR study in a particular region of O-H (3400 and 1420 cm^{-1}) and C-H (2900, 1460, and 1350 cm^{-1}) vibrations indicates different binding between the central metal ion and ligand, depending on pH and metal contents. Water protons take part in the formation of relatively weak hydrogen bonds (Nikolic et al., 1996; Bellamy, 1954; Nikolic et al., 2007; Nikolic et al., 2008). In the 1200–1000 cm^{-1} region, the spectra of RLMP and the complex comprise a number of highly fused bands. The enhancement of the resolution by using a Fourier self-deconvolution allows bands to be more accurately detected. The main bands found in the deconvoluted spectra of RLMP and the complex at ca. 1154, 1108 1079, 1042, and 1019 cm^{-1} are due to coupled valent vibrations of the C-O and C-C bonds and

deformational vibrations of the CCH, COH, and HCO bonds. The band at about 1150 cm^{-1} has been assigned to valent vibrations of the C-O-C bond and glycosidic bridge. The broad peak at 1108 cm^{-1} should most likely be ascribed to the vibration of the C-O bond at the C(4) position of the glucopyranose units (Kacurakova et al., 1996). Complex vibrations involving the stretching of the C(6)-O(6) bond with participation of the deformational vibrations of the C(4)-C(5) bond result in the appearance of a band at 1079 cm^{-1} (Sivchik et al., 1979; Nikonenko et al., 2005; Zhbankov et al. 2005; [a]Zhbankov et al., 2003; [b]Zhbankov et al., 2003; Zhbankov et al., 2000). In the spectra of Cu(II) complex with RLMP band at 1079 cm^{-1} is less pronounced than in the spectra of RLMP. In the case of pullulan complexes, part of C(6) atoms participate in the formation of the C(6)-O-Cu(II) linkages; as a result, the band intensity at 1079 cm^{-1} for the Cu(II) complex with RLMP is reduced more than in the case of RLMP. The band at 1079 cm^{-1} in the FTIR spectra of RLMP is attributed to the antisymmetric stretching vibration of C(6)-O-C(1) glycosidic bridge. These findings suggest that the 1079 cm^{-1} band for the Cu(II) complexes with RLMP can be considered as a characteristic for the type of interunit links and for the Ligand–Metal [C(6)-O-Cu(II)] linkage (Shingel, 2002; Zhbankov et al., 2000; [a]Mitic et al., 2008; [b]Mitic et al., 2008).

In the case of the Cu(II)–RLMP complexes O-H groups participate in the formation of the Cu(II)–RLMP linkages. As a result, the band frequency at 3404 cm^{-1} for ν(O-H) vibrations in RLMP is reduced to approximately 3340 cm^{-1} (Fig. 3) in Cu(II)–RLMP. These findings also suggest that the band can be considered as characteristic for the type of Metal–Ligand links. The band at about 1042 and 1019 cm^{-1} found for polysaccharide in the spectra of RLMP and the complex were shown to relate to the crystalline and amorphous phases, respectively (Smits et al., 1998). The changes in intensity of these bands are strongly associated with the alterations in the macromolecular order. These bands in the spectra of RLMP and the complex can be responsible for more and less ordered structures, respectively. The major attention was focused on the bands in the 1160–1010 cm^{-1} region because the absorbance pattern due to ring vibrations in this spectral range is known to be individual for each carbohydrate structure.

Moreover, we attempted to obtain the information about the conformations of these macromolecules in a solvent exhibiting a different influence on the system of intra- and intermolecular interactions. Special interest in the IR range for structural investigation is from 1000 to 700 cm^{-1}. In the spectra of RLMP and the complexes, bands of negligible intensity are found in the region (950, 916, 860, 760 cm^{-1}). According to the normal coordinate treatment on the RLMP model, these bands are interpreted as due to mixed CCH deformation vibrations coupled with CCO, OCO, and COC bending (Buslov et al., 1998; Zhbankov et al., 1997; Zhbankov, 1992; Zhbankov and Avsenev, 1984; Kiselev et al., 1977). Both the number and frequencies of the bands in the IR range depend on the conformation of the D-glucopyranose units. It is well known that the glucopyranose units exist in six different typical conformations (1C, C1, 1B, B1, 3B, and B3) (Panov & Zhbankov, 1976; Komar et al., 1968). The similarities of the γ(C-H) range indicate that there is no difference in the conformation of the glucopyranose unit in the RLMP and complex molecules, and they probably exhibit C1 chair conformation (916 and 850 cm^{-1}).

It appears that the intensity of the 996 cm^{-1} band in the pullulan spectra may indicate the extent of the interchain association. The band at 950 cm^{-1} belongs to the structure-sensitive region, and together with the band at 935 cm^{-1}, characterizes the type of interunit bonds and angles. The band at 935 cm^{-1} was recently used to discover the co-existence of α-(1→6) and

α-(1→4) glycosidic linkages in the pullulan structure (Shingel, 2002; Zhbankov et al., 2000). A decrease of the band at 950 cm^{-1} indirectly confirms an occurrence of the conformational transitions in polysaccharide systems owing to rotational isomerism of pyranose rings about the glycosidic bond. For pullulans the band at 900 cm^{-1} described α-(1→6) linkages. α-(1→4) linkages were observed at 925 cm^{-1} (Shingel, 2002). Ring deformations and scaffold vibrations were observed at 710, 660, 600, 570, and 525 cm^{-1}. In the experiment on the influence of the medium pH on a binding Cu(II) ion with different polysaccharides (Mitic et al., 2007; Norkus et al., 2002; Norkus et al., 2004), there is a possibility of gradual complexing, where their reforming starts at pH 8. Degradation of the Cu(II)–RLMP complex begins at pH values higher than 12. The Cu(II) ions form three different types of complexes with the deprotonated monomeric RLMP unit. Different structural models of the Cu(II)–RLMP complexes of tetragonal distorted O_h coordination in the function of pH synthesis pH 7–8 (type I), pH 8–10 (type II), and pH 10–12 (type III) are given in Fig. 5.

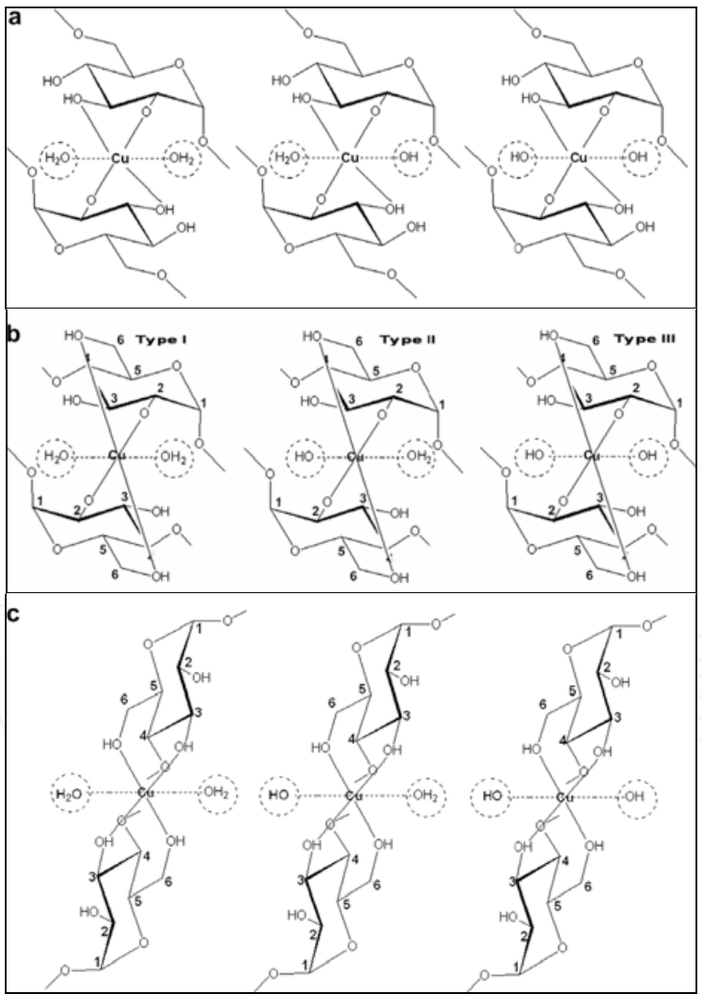


Fig. 5. Structure model of Cu(II)–RLMP complexes, with six O-donor atoms in tetragonal distorted O_h environment of Cu(II) ions, with participation of: (a) C-2 and C-3 ligand OH groups, (b) C-2 and C-6 ligand OH groups, (c) C-3 and C-6 ligand OH groups

The reactivity of the RLMP depends primarily on the reactivity of the secondary, equatorially oriented hydroxyl groups (OH-2, OH-3, OH-4 and OH-6). The contents of the primary O-H groups in RLMP are slightly increased at lower Mw (about 2%). The reactivity of the polysaccharide C-atoms was determined by 13C NMR spectroscopy for pullulan it was C(6)>C(3)>C(2)> C(4) (Mahner et al., 2001). Carbohydrates without anchoring donor groups form a very weak complex with Cu(II) ion in an aqueous solution. The availability of more than one anchoring group can, however, prevent the coordination of the alcoholic O-H groups fulfilling the coordination sphere of the metal ion. The metal interaction with the set of the non-deprotonated OAH groups increases the complex stability. Mainly, the complexes were shown to form, in different protonation states, the deprotonation processes starting from pH 7 (Norkus et al., 2002). After deprotonation of one or more alcoholic O-H groups, the Cu(II) ion complexes having anionic character are usually very stable. In some cases, the formation of various amounts of alkoxo or hydroxo bridged dimeric (or oligomeric) species can be detected. The disugars bound the metal ions less efficiently than the monomeric units, while the trimetric ligands can probably simultaneously use terminal subunits to coordinate the metal ion. In aqueous solution, the RLMP complexes are formed by the displacement of the H_2O molecules from the first coordination sphere of Cu(II) ion by the alcoholic OAH groups. In general, it seems to be true that at least three O-H groups in a favorable steric arrangement are required for the complex formation (Gyurcsik & Nagy, 2000). The general leading rule is that the sugars in pyranose form (in RLMP C1-chair conformation) contain an equatorial–equatorial–equatorial (eq–eq–eq) sequence of three adjacent hydroxyl groups. The possible coordination sites are depicted on the model given in Fig. 5.

The characterization of metal ion coordination equilibrium of polyalcohols and other sugar-type ligands, containing alcoholic and aldehyde (or ketone) oxygen donor atoms, is difficult due to the low stability of the complexes in neutral or acidic aqueous solutions (Gyurcsik & Nagy, 2000; Nagy et al., 2003). The low electron densities on these donor oxygens cause the situation, in spite of their relatively large number in one ligand molecule, that they do not readily substitute the water molecules bonded in the first coordination sphere of the metal ions. With increasing pH, however, the hydrolysis of some metal ions prevents the coordination of the organic ligands. Thus, complex formation can only be expected in strongly alkaline solutions after deprotonation of alcoholic hydroxyl groups. The fact that in solutions of carbohydrates the species are in anomeric and conformational equilibrium and the isomers interact in different ways with metal ions makes the studies even more complicated. Any shift in the above equilibrium due to the metal ion coordination, thereby resulting in the changes in the fraction of the isomers having suitable positioned sequences of alcoholic hydroxyl groups in the total concentration of the ligand, will also influence the complex stability.

The methods, such as FTIR, NMR, ESR, X-ray and UV–Vis made it possible to assign the binding hydroxyl or other groups and also to characterize the metal ion coordination of carbohydrates monitoring the ligand conformation or/and configuration changes forced by the complexation processes. FTIR spectra of the Cu(II)–RLMP complexes were recorded on room (RT) and on low nitrogen temperatures (LNT) in order to detect bands which are sensitive to the reduction temperatures respective bands which have originated from vibrations of all types OH groups and H_2O molecules. In Fig. 6 RT-IR and LNT-IR spectra of the complex synthesized on the pH 7.5 have been presented in the comparison with RLMP.

FTIR spectra show the correlation between O-H stretch frequency and the hydrogen bond strength (3400–3200 cm^{-1}) predict a red shift of the bonded O-H stretching band on cooling (from 3350 to 3246 cm^{-1}) (Fig. 6). It is expected that the non-interacting O-H group (at 3378 cm^{-1}) is much less sensitive to cooling and consequently will show smaller red shifts. The red shift of the band is an indication of the involvement of the appropriate O-H proton in a weak hydrogen bond.

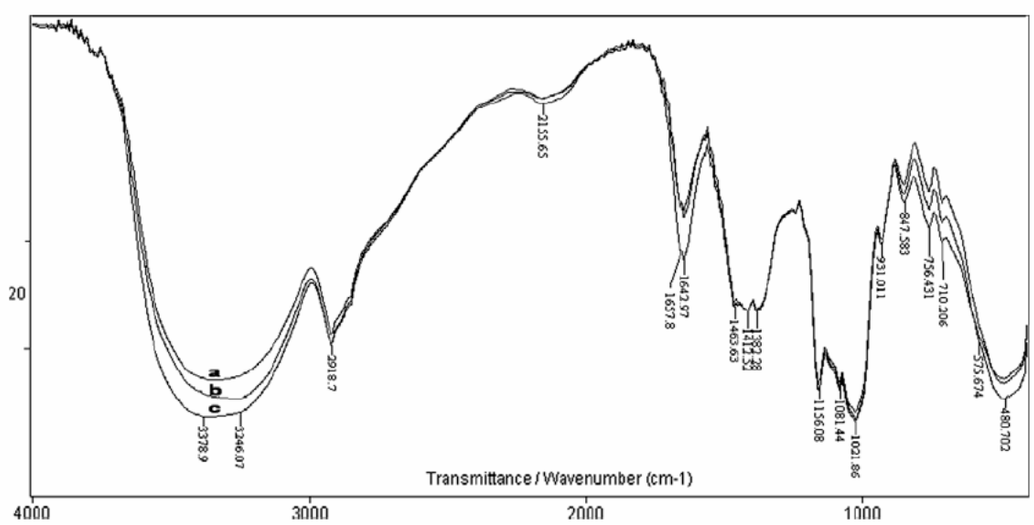


Fig. 6. Stretching and bending region from RT (a) and LNT-FTIR (b and c) spectra of Cu(II)–RLMP complex (type I, pH 7–8) (a – 298 K, b – 173 K, c – 77 K)

In the LNT-FTIR spectra of the complex was synthesized on pH 7.5, two bands (3378 and 3246 cm^{-1}) are found in the region of ν(O-H) vibrations (Fig. 6). In the low-frequency region on LNT-FTIR spectra were presented in Fig. 6, sensitive on the reduction of temperature bands in the γ(OH) bending region from librations of coordinated water molecules on frequencies 847 cm^{-1} and 756 cm^{-1}, show blue shift on cooling. The librations of the O-H group in this region of the complex synthesized on pH 12 were much less sensitive to cooling. The observation allowed one to suggest that the most probable water molecules are coordinated around Cu(II) in the complex type I (Fig. 5). The number and shape of these bands implies that in complexes type III there is the displacement of H_2O molecules by the O-H groups in the first coordination sphere of the Cu(II) ion. The LNT-FTIR results confirm the structural models of complexes presented in Fig. 5. The results obtained from the structural studies of the investigated complexes were based on other spectroscopic techniques (Nikolic et al., 2005; Mitic et al., 2004; Nikolic et al., 2004; Nikolic et al., 2006; Bartkowiak et al., 1998; Cakic et al., 2008).

The changes in number, frequencies, intensity, and width of the FTIR bands in the particular region of ν(O-H) vibrations (3400 cm^{-1}), δ(C-H) vibrations (1500–1300 cm^{-1}) and ν(C-O) vibrations (1200–1000 cm^{-1}) (Fig. 3) were related to changes in the conformation and short-range interactions of the RLMP. Very important changes can be observed in the range of 1500–1300 cm^{-1} by detailed empirical analysis. Otherwise, the FTIR range is specific of bending vibrations of CH_2-OH groups (Fig. 7). Namely, the exchange position and intensity of complex bands can be registered in this range, where C-H and O-H bending vibrations from the CH_2-OH groups take part. The change of intensity on some bands was registered

only in synthesized Cu(II)–RLMP complexes. An approximate effect exists in the stretching of the FTIR range of C-H vibrations (3000–2800 cm^{-1}, Fig. 3). The appearance of bands at about 1460 cm^{-1} and 1370 cm^{-1} from δ(C-H) vibrations and the band at about 1420 cm^{-1} from δ(O-H) vibrations are characteristic for one of more possible positions of the CH_2-OH group, rotating around the C(5)-C(6) bond of the glucopyranose unit. The change of the angle between the methylene CH_2-OH group and the polysaccharide chain axes, consequently decreases the intensity of the appropriate IR bands (ν(C-H) and δ(C-H) vibration). The Cu(II) ions in solution have a possible influence on the rotation of CH_2-OH groups in the complexes (Cakic et al., 2004; Mitic et al., 2007; Nikolic et al., 2006).

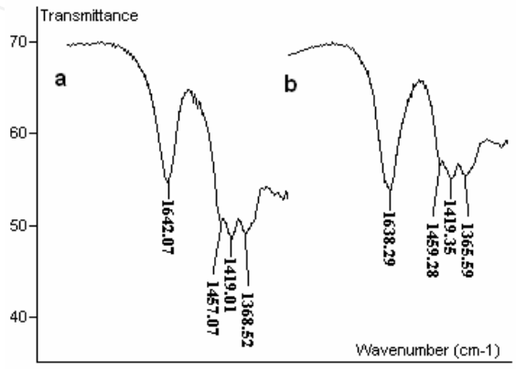


Fig. 7. Region of δ(CH) and δ(OH) CH_2OH grop vibrations in FTIR spectra of: (a) RLMP; (b) and Cu(II)–RLMP complexes

We also applied FTIR spectroscopy to determine spectral manifestation of the changes in the complex structure caused by recrystallization from D_2O and, thereby, complete the structural investigation of this modified polysaccharide. The FTIR spectra of the Cu(II) complexes with RLMP and recrystallized analogs from D_2O were analyzed in order to find the specific spectral peculiarities that allow one to obtain the information about the structure and the conformation of these macromolecules in solvents that exhibit different influences on the system of intra- and intermolecular interactions. No effect of the conformation change was observed for the recrystallized Cu(II)–RLMP complex, especially in the range of 1000–700 cm^{-1}. When the a-D-glucopyranose units with C1 chair conformations are present, the FTIR spectra exhibit one band in the region between 925–885 cm^{-1} and another one around 860–820 cm^{-1}, which are assigned to mixed CCH deformation vibrations (Shingel, 2002; Nikolic et al., 2008). The results allowed one to suggest a predominant crystalline form of the recrystallized Cu(II)–RLMP complexes.

Recently, FTIR spectroscopy was coupled with a microscope and a computer system, capable of microanalysis of minute samples by using a dedicated MCT detector. The resultant FTIR vibrational microspectroscopy can provide molecular information of samples with a high spatial resolution at microscopic level. Samples with microscopic size can be nondestructively analyzed by both vibrational microspectroscopies, particularly in the application of biomedical sciences (Kacurakova et al., 2001; Lin et al., 2007; Chiu et al., 2004; Nikolic et al., 2008). Thus, the use of vibrational microspectroscopy has extensively become a great potential over other spectroscopic techniques for noninvasive investigation of chemical components of ultrastructural samples (carbohydrates, lipids, proteins, nucleotides) (Mousia et al., 2001; Yu et al., 2005).

More recently, FTIR and/or Raman microspectroscopic imaging systems have also been developed for applying to biosciences (Gierlinger & Schwanninger, 2007; Chenery & Bowring, 2003). ATR-FTIR spectra may be simultaneously collected at a time in a stepwise manner from different areas of a sample. The absorbance ATR-FTIR spectra of Cu(II)–RLMP complex which was synthesized at pH 7.5 are shown in Fig. 8. The absorbance of a band corresponding to a specific chemical component may be plotted as a map. ATR-FTIR spectra were presented in Fig. 8(A–C) from different areas of Cu(II)–RLMP complex and show high homogeneity of the sample.

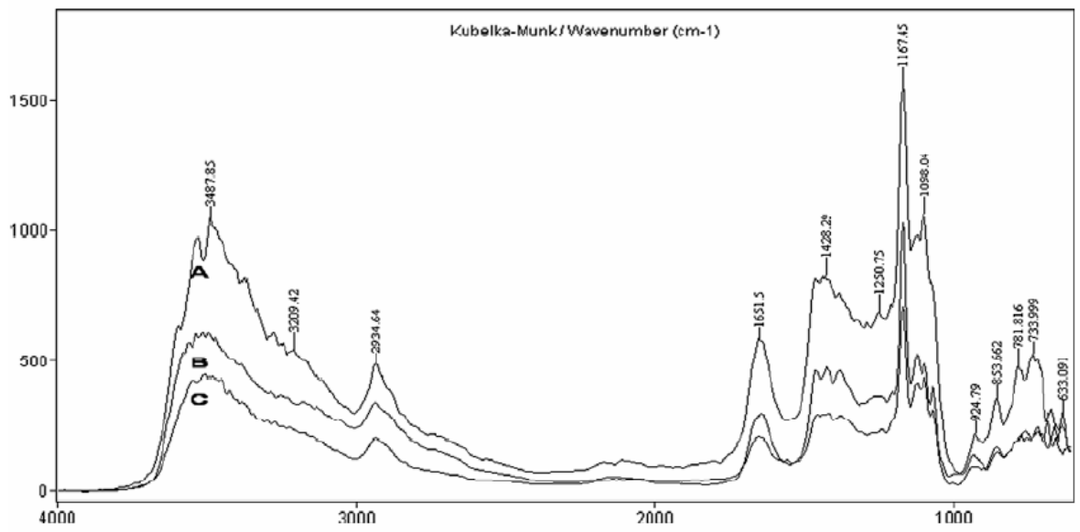


Fig. 8. ATR–FTIR spectra of Cu(II)–RLMP complex synthesized at pH 7.5 (type I) from different areas (A, B and C) of a sample

A new imaging capability has been established not only to image heterogeneous regions of the samples and simultaneously provide spectroscopic and spatial information, but also to show visually the concentrations of components and to highlight their effect from the three dimensional plot. The application of microscopic FTIR imaging system to the ligand RLMP and Cu(II)–RLMP complexes, were synthesized at pH 7.5–12, is shown in Fig. 9. FTIR microscopy images of ligand RLMP, as well as images of the synthesized Cu(II)–RLMP complexes differ which also indicates that the complexation process and the creation of coordination compounds took place. FTIR microscopy images confirmed that the changes in the intensity of the analyzed bands are strongly associated with the alterations in the macromolecular order. These bands in the spectra of the complexes can be responsible for more and less ordered structures, respectively (Fig. 9). The changes in color contour may show the content and distribution of copper, and polysaccharides in Cu(II)–RLMP samples.

Conclusions.

The complexing process begins in a weak alkali solution (pH > 7.5), and involves OH groups in C(2) and C(3) or C(6) pullulan monomer unit (α-D-glucopyranose). A part of FTIR spectra, in the range on 1000–700 cm^{-1} of Cu(II) ion complexes with RLMP, indicates no influence of complexing process on the conformation change of C1 glucopyranose units. The IR band d(HOH) at the frequency of 1640 cm^{-1} indicated the existence of water molecules in a complex structure.

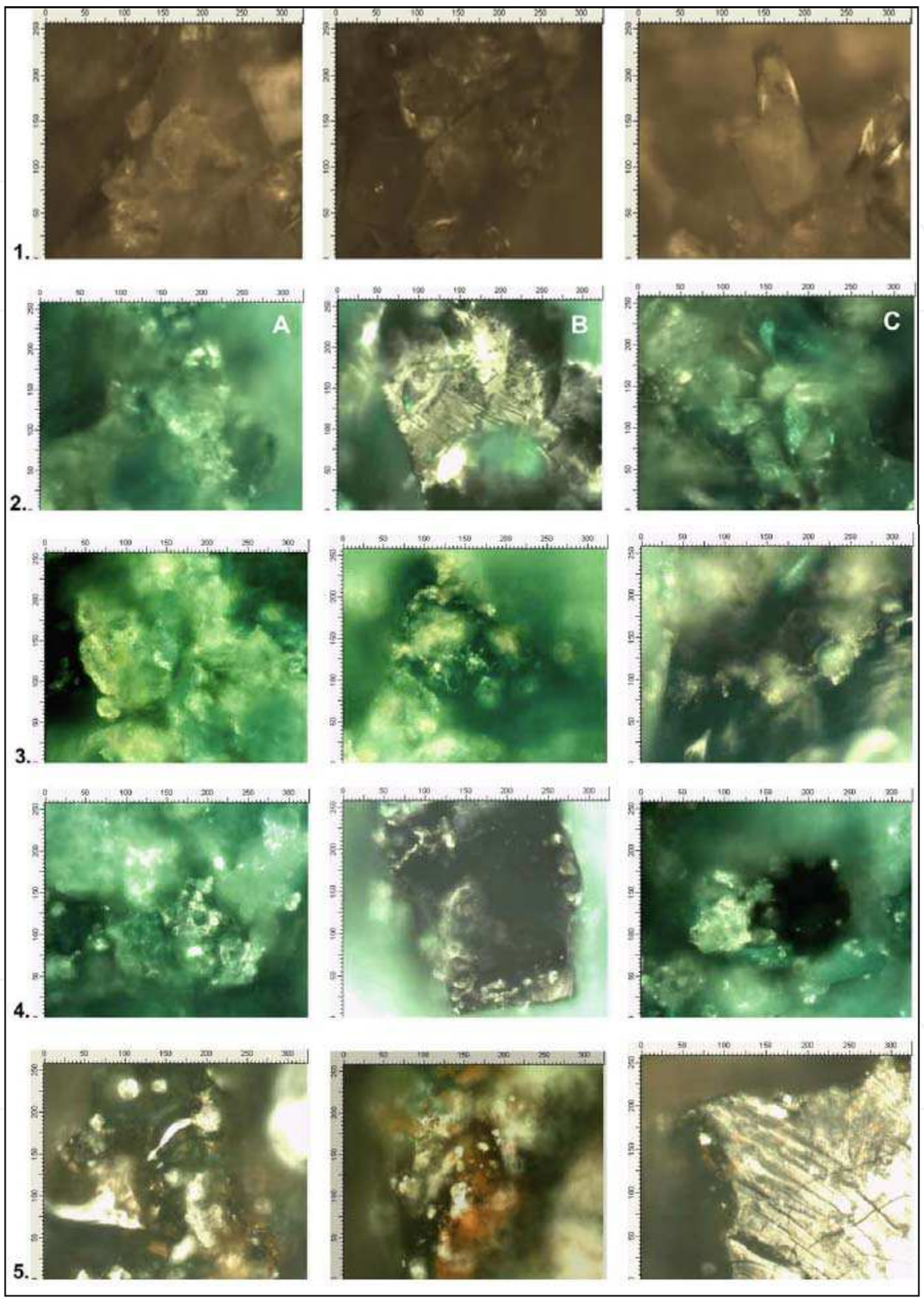


Fig. 9. FTIR microscopy images (250 μm x 300 μm) of RLMP, Mw = 6000 g mol^{-1} (1) and Cu(II) complexes with RLMP synthesized at boiling point and pH 7.5 (2), 8.0 (3), 10.0 (4), 12.0 (5)

From LNT-FTIR it follows that non-interacting OH group is insensitive to temperature variation whereas a bonded OH shows a significant red shift upon cooling. In the low-

frequency region on LNT-FTIR spectra, sensitive on the reduction of temperature bands in the γ(OH) bending region from librations of H_2O molecules on frequencies 847 cm^{-1} and 756 cm^{-1}, show blue shift on cooling.

Cu(II)–RLMP complexes are formed by the displacement of H_2O molecules from the first coordination sphere of Cu(II) ion by the OH groups. Copper(II) ion with RLMP unit (Glc) forms three different types of complex (pH 7–8: $Cu(II)(Glc)_2(H_2O)_2$, pH 8–10: $Cu(II)(Glc)_2(H_2O)(OH)$, pH 10–12: $Cu(II)(Glc)_2(OH)_2$).

The changes of the intensity on some bands were registered in RLMP complexes (in the ranges of a stretching vibration at about 2930 cm^{-1} and a bending vibration at about 1400 cm^{-1}). The bands are characteristic of one of more possible positions of the CH_2-OH group, rotating around C(5)-C(6) bond of the pullulan glucopyranose unit.

FTIR microscopy images shows more and less ordered structures of the Cu(II)–RLMP complexes. ATR-FTIR microspectroscopic data shows homogeneity of the Cu(II)–RLMP samples. The results of the FTIR spectroscopic study by different techniques allowed one to suggest a predominant crystalline form of Cu(II)–RLMP complexes.

5. Bioactive copper-dextran complex

Dextran $H(C_6H_{10}O_5)_xOH$ is a complex, branched glucan composed of chains of varying lengths (from 10 to 150 kDa). It is a polysaccharide similar to amylopectin (Belder, 1985). The straight chain consists of α-(1→6) glycosidic linkages between glucose molecules, while branches begin from α-(1→3) or α-(1→4) linkages (Fig. 10). Dextran is synthesized from sucrose by certain lactic-acid bacteria, the best-known being *Leuconostoc mesenteroides* and *Streptococcus mutans*. Dextran is an oral bacterial product that adheres to the teeth, creating a film called plaque; dental plaque is rich in dextrans. Dextran is also formed by the lactic acid bacterium *Lactobacillus brevis* to create the crystals of tibicos, or water kefir fermented beverage which supposedly has some health benefits. Dextran is freely soluble in water, methyl sulphoxide, formamide, ethylene glycol, glycerol, 4-methylmorpholine-4-oxide, and hexamethylphosphoramide. Some dextran fractions may adopt a certain degree of crystallinity and may only be brought into solution by strong heating.

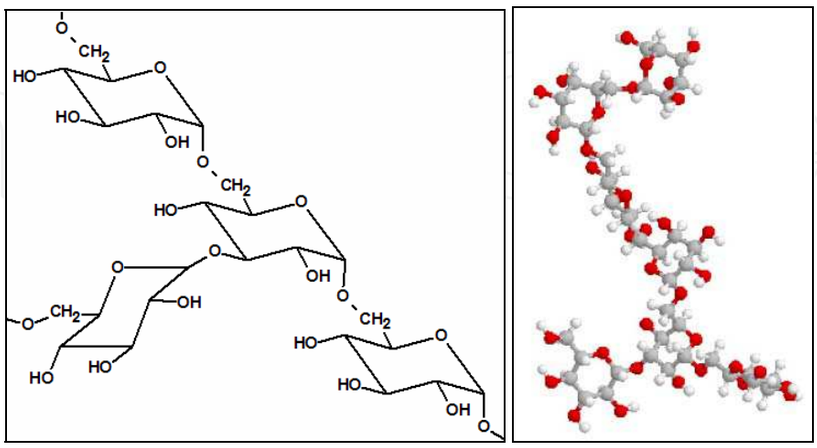


Fig. 10. Molecular structure of a dextran: (a) 2D model, (b) 3D model stick and ball

Dextran and its derivatives have been studied extensively (Zhbankov, 1972; Skornyakov & Komar, 1996). The Fourier-transform infrared spectra of a series of branched dextrans also

were examined. The IR spectra of low molar dextran have been investigated in the range between 4000 and 400 cm^{-1} (Nikolic et al., 1996). The FTIR spectrum of reduced low molar dextran is presented in Fig. 11. Information on the glucopyranosyl units conformation in the polysaccharide can be acquired in the 1000–700 cm^{-1} region in which we expect the deformational γ(CH) vibrations bands. In this particular region, as it can be seen in Fig. 11, at least two weak bands around 911 and another 850 cm^{-1} are observed, which is the proof for C-1 conformation of glucopyranoside units of dextran. In the δ(OH) range of IR spectra one band at about 1645 cm^{-1}, which is sensitive to deuteration, has appeared. The band originates from water molecules.

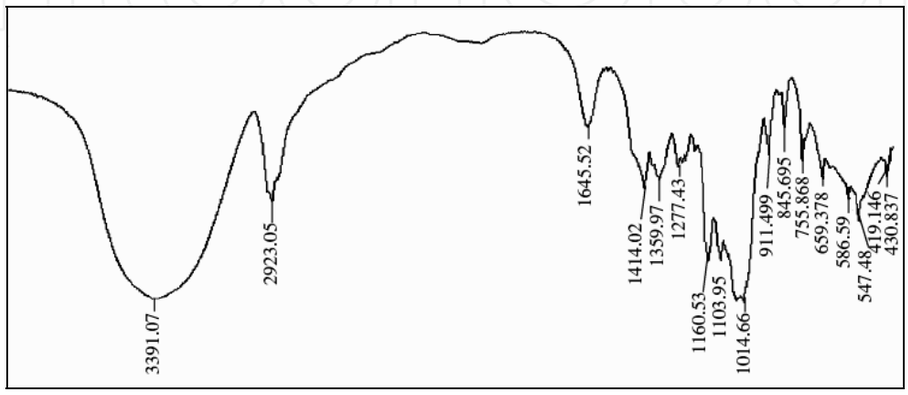


Fig. 11. FTIR spectra of reduced low molar dextran, Mw = 5000 g mol^{-1}

Dextran is used medicinally as an antithrombotic (anti-platelet), to reduce blood viscosity, and as a volume expander in anemia (Lewis et al, 2008). These agents are used commonly by microsurgeons to decrease vascular thrombosis. The antithrombotic effect of dextran is mediated through its binding of erythrocytes, platelets, and vascular endothelium, increasing their electronegativity and thus reducing erythrocyte aggregation and platelet adhesiveness. Dextran also increases blood sugar levels. Biological active polysaccharides dextran have possibility to binding different ions and metals in the solution and making of water-soluble complexes. These complexes have wide application in human medicine and veterine. These compounds have great importance in investigations today.

Copper, essential biometal for living organisms, is a hematopoetical active element of some metaloenzymes regulating the iron absorption in intestines, maintaining, at the same time, the iron in a reduced state and influencing the iron incorporation into hemoglobin (Lewis, 1995). The copper amount necessary is usually supplemented by a normal diet in both humans and animals. It is known that copper deficiency causes a number of pathological states. Complex compounds of Cu(II) ion are important for prevention and treatment of some anemia caused by iron deficiency.

The carbohydrate type compounds as ligands have been of a considerable interest. Simple sugars and their derivatives with reduced and oxidized groups form metal ion complexes of a various composition and stability. In both human and veterinary medicine commercial copper preparations based on polysaccharide dextran and its derivatives are used for such purpose (Ilic et al., 2003). According to literature data, dextran has the ability of complex formation with various biometals (Co, Zn, Ca and Mg) (Mitic et al., 2007; Cakic et al., 2006; Lugovaya et al., 1976; Gyurcsik & Nagy, 2000). Iron complexes with different polysaccharides have special importance, and they have been described in detail (Ilic et al., 2002;

Nikolic & Cakic, 2007; Pekic & Cvetkovic, 1988; Cakic & Nikolic, 2003; Nikolic et al., 2002). The interaction of Cu(II), Ni(II) and Fe(III) ions with dextran may be used for their speciation by ultrafiltration (Solpan & Sahan, 1993). Synthesis procedures for the complex formation of Cu(II) ion with polysaccharides, including dextran, are described in scientific literature (Nikolic et al., 2005; Mitic et al., 2004; Mitic et al., 2007).

In the section, we analyzed the IR spectra of Cu(II) ions complexes with reduced low molar dextran (RLMD). Fourier-transform IR spectra of dextran and its compounds with copper(II) ion, recorded at room temperature, were analyzed in order to obtain the information about the structure and the conformation of these polymer compounds. For IR sample preparation KBr pastille method was used. The IR spectra as an average of 40 scans were recorded at room (298 K) temperature on FTIR spectrometer BOMEM MB-100 (Hartman–Braun) equipped with a standard DTGS/KBr detector, in the range of 4000–400 cm^{-1} with the resolution of 2 cm^{-1}, by Win-Bomem Easy software. FTIR microspectroscopy system, ATR-FTIR spectrometer Bruker Tensor-27 in conjunction with a FTIR Bruker Hyperion-1000/2000 microscopy attachment equipped with a 15x objective and a 250 μm liquid-nitrogen cooled, narrow-band mercury–cadmium–telluride detector (ATR objective GMBH, Germany) was used also in this analysis.

Results and discussion.

The plan for the synthesis of the Cu(II) complex with reduced dextran has required a detailed analysis of the synthesis procedure; both from the aspect of the reaction conditions of the synthesis and from the aspect of obtaining the stable and commercially applicable preparation of the complex. The analysis of the synthesis of similar complexes has pointed to the necessity of defining the physicochemical properties of commercial preparations. By their correlation, the undesired effects can be eliminated and thus a considerably improved pharmacological effect of the complex.

The reactivity of the dextran primarily depends on the reactivity of the secondary, equatorially oriented hydroxyl-groups (OH-2, OH-3 and OH-4). The contents of the primary OH groups in dextran are slightly increased at lower molar masses (about 2%). As it is the case with the other glucans, the reactivity of the OH-2 group to the alkalizing reagents is higher than in the OH-3 and OH-4 groups. This is rationalized in the context of higher acidity of the OH-2 because of the proximity of anomeric centre (Gyurcsik & Nagy, 2000). When the OH-2 and OH-4 ionize, reactivity to OH-3 is reduced; however, the substitution on the OH-2 and OH-4 abolish this action and induce the successive increase in the reactivity of OH-3. On applying these generalizations, it is necessary to exercise some precaution, since the potency of the base may affect the relative and absolute reactivities of the hydroxyl. Through derivatization of the dextran to the reduced form the large number of activation centers with will be at the disposal to the copper ions for the purpose of binding the complex is expected. This creates the possibility of achieving the considerably larger stability of the synthesized complex as well as of their pharmacological effect.

For this reason, the choice and optimization of the low molar dextran in the capacity of the ligand have been made. Considering the importance of physicochemical parameters on the process and the synthesis results, the examination and optimization of ligands in relation to molar mass (*Mw*), as well as the reaction conditions of the synthesis (pH, *T* and *t*) were investigated and optimized (Mitic et al., 2007). The basic characteristics of synthesized Cu(II) complexes with RLMD are given in Table 1.

Sample	pH synth.	Complex color	Cu (%) (by AAS)	Water solubility
1	7.5	Light green	7.23	Soluble
2	7.5	Green	19.85	Very soluble
3	8.0	Green-blue	8.12	Soluble
4	10.0	Blue-green	8.20	Sparingly soluble
5	12.0	Dark blue	6.97	Slightly soluble
6	7.5	Dark green	4.08	Slightly soluble

Table 1. Characteristics of copper-dextran complexes with RLMD (M_w 5000 g/mol) as ligand, synthesized at boiling temperature

On the basis of obtained experimental results (Table 1), a favorable result of Cu(II)-RLMD complexes synthesis is obtained with dextran oligomers *M*w 5000, at boiling temperature and pH 7.5–8.0, within 7 min. Complexes obtained at pH > 8 present unfavorable effects of synthesis. Comparing the obtained complexes of Cu(II) with RLMD, either in solid state or in solution, it is obvious that, depending on pH values, various complex colors are obtained (Table 1). The change of the solutions color during the synthesis may be an indicator whether the syntheses of complexes were successful. The results obtained have shown that, in the range of pH 7.5–12, the color can vary from light green to dark blue. This is confirmed by the green solution color of the most stable complex of Cu(II) with RLMD (procedure 2, Table 1), in comparison with an indigo-blue alkali solution of decomposed Cu(II) at pH>12, where $[Cu(OH)4]^{2-}$ ions dominate.

Water solubility of synthesized complexes of Cu(II) with RLMD is different. The most water soluble complex is obtained at pH 7.5 (Table 1). The solution is permanent and stable after a longer period of time (6 months). The complexes that are synthesized at higher pH are less soluble. The solution of the complexes obtained, following the procedure 5 (Table 1), after resting for long period of time, start layering, precipitate and become opalescent. Medium pH is changed after adding Cu–salts and Cu(II) content in a complex is much influenced by it. Syntheses are performed at the same temperatures and within the same reaction period, but at different pH values (Table 1). The highest Cu(II) content was got at pH 7.5. The possibility of obtaining Cu(II)–RLMD complexes with a higher Cu(II) content has been tested with the increased concentration of Cu–salts. The expected results have not been obtained.

Solution pH probably has the influence on the way of binding of Cu(II) into a complex, i.e. on the type of a bond because, due to the change of pH value, the stability (Nikolic et al., 2006), the color and the solubility of the complex obtained are also changed (Table 1). Thus, by the increase of solution pH values from 7.5 to 12, the percentage of the bounded Cu(II) with RLMD in complex decreases. Some authors (Tolmachev et al., 1975), in the paper of influence of medium pH on binding Cu(II) with dextran, point out the possibility of gradual complexing, i.e. gradual forming of coordination bounds, where their reforming starts at pH 8. Thus, Cu(II) ions form of three different types of complexes with dextran (Norkus et al., 2002). Decomposition of Cu(II)-dextran complex begins at pH values higher than 12.

The results of ATR-FTIR spectroscopic investigations show that spectra of Cu(II)–RLMD complexes and ligand are basically similar (Fig. 12). The similarities of the bending (C–H) range indicate that there is no difference in the conformation of the glucopyranose unit in the dextran and the complex molecule (916 and 850 cm^{-1}), and they probably exhibit C-1 chair conformation (Mitic et al., 2007).

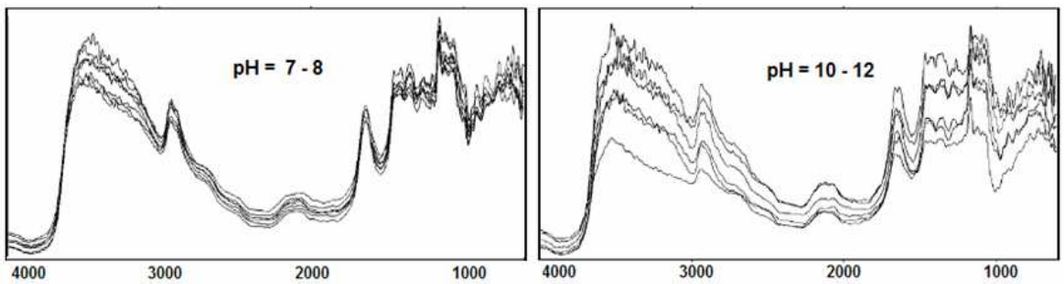
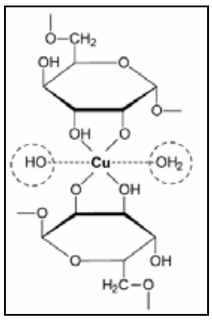
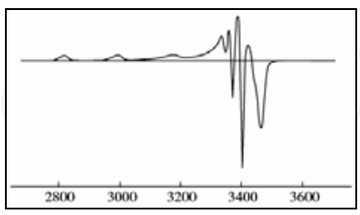


Fig. 12. ATR-FTIR spectra of Cu(II)–RLMD complexes synthesized at pH 7-8 and pH 10-12



Mid FTIR spectra of Cu(II)–RLMD complexes synthesized at different pH (pH 7–8, Fig. 13B, and pH 10–12, Fig. 13C) recorded at 298 K, show that the correlation between the O–H stretch frequency and the hydrogen bond strength. Spectroscopic IR study in particular region of OH (3400, 1420 cm^{-1}) and C–H (2900, 1460, 1350 cm^{-1}) vibrations indicates different binding between central metal ion and ligand, depending on pH and metal contents (Fig. 13). The difference in frequencies, intensity, and shape of these bands in the region 3600–3100 cm^{-1}, implies that in complexes which were synthesized at pH 10–12 there is the displacement of H_2O molecules by O–H groups in the first coordination sphere of the copper(II) ion. Dextran and complexes with Cu(II) ion have one crystallographic type of water molecule (1640 cm^{-1}). Water protons take part in the formation of relatively weak hydrogen bonds (Cakic et al., 2002; Nikolic et al., 2006; Nikolic et al., 2008).



The FTIR investigation corresponds with the results obtained by ESR spectrometry (Mitic et al., 2004), as well as with the results obtained by UV-VIS investigations (Mitic et al., 2007). ESR parameters of the spectra ($A_{||}$ = 187 × 10^{-4} cm^{-1}, $g_{||}$ = 2.23 and $g_{\perp}$ = 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 coordination of Cu(II) ion with four oxygen atoms. Although the Cu(II) ion content of complexes synthesized at lower pH values was much higher (up to 18.95% for the complex synthesized at pH 7.5) the ESR signal of these complexes was lacking due to strong spin–spin interactions of neighboring Cu(II) ions. ESR spectrum of complex containing 6.97% of copper synthesized at pH 10 is

presented to the left. The ESR spectra indicate the axial symmetry of synthesized complexes and were typical for the Cu(II) ion with one unpaired electron in 3*d* subshell. Asymmetric appearance of the hyperfine spectral lines originates from the unresolved spectral contributions of two natural isotopes, 63Cu and 65Cu. ESR spectral parameters ($A_{||}$ = 187 × 10^{-2} cm^{-1}, $g_{||}$ = 2.23 and $g_{\perp}$ = 2.03) point to the tetragonal coordination of Cu(II) with four oxygen atoms from ligands in the same plane (Mitic et al., 2004; Nikolic et al., 2004).

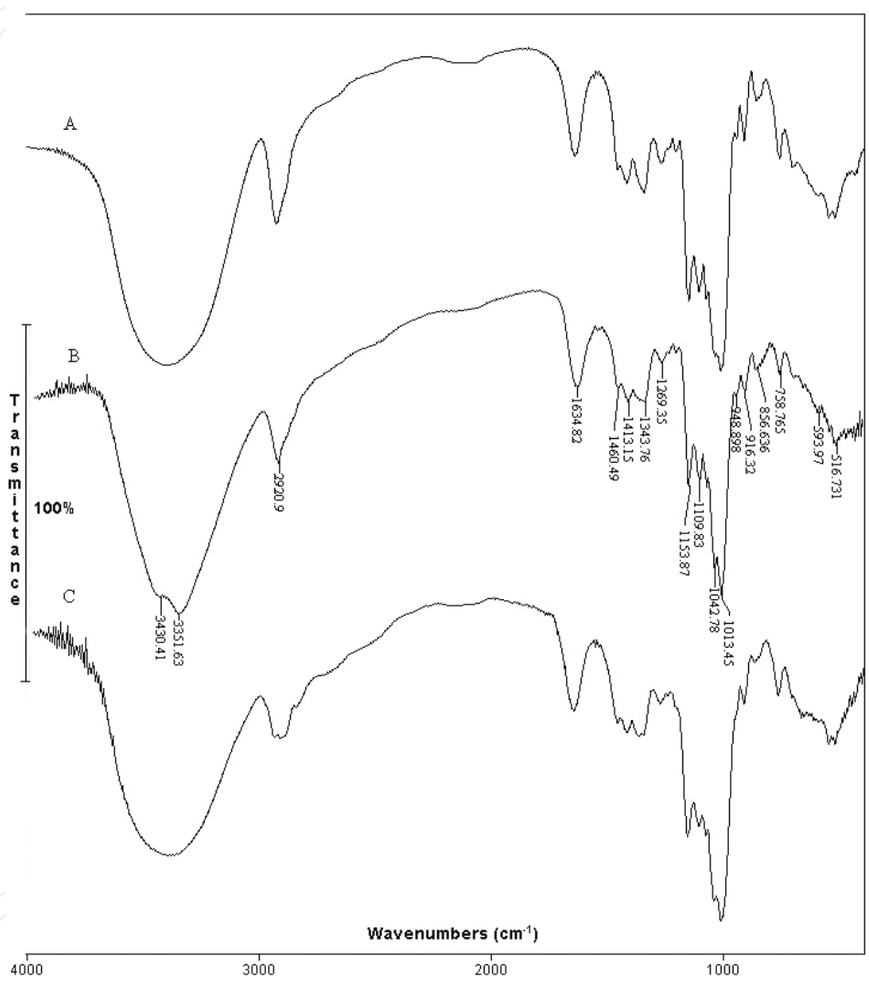


Fig. 13. FTIR spectra of: LM dextran (A); stable Cu(II)–dextran complex, with high metal content (≈ 18%), obtained at pH 7–8 (B); unstable Cu(II)–dextran complex, with low metal content (≈ 5%), obtained at pH 10–12 (C)

In addition, depending on pH values, complexes of Cu(II) with RLMD also behave differently considering wavelength at which they show absorption maximum. This range of wavelengths in the VIS spectra is 650–700 nm (Mitic et al., 2007). Hypsochromic effect of complexes absorption maximums with increase of pH solutions confirms the presence of different types of complexes. Hexaqua copper(II) ion $[Cu(H_2O)_6]^{2+}$ absorb at wavelength 812.7 nm, while synthesized complexes absorb within the ranges of 650–700 nm. With increase in solution pH the absorption maximums change to shorter wavelengths

comparing with $[Cu(H_2O)_6]^{2+}$ ion. Complex, which has been decomposed at pH values over 12, shows absorption maximum at 634 nm. Thereby, these spectrophotometric criteria can be applied for the confirmation of the success of complex synthesis. On the basis of the obtained results by spectroscopic investigations of this complexes, three different types of Cu(II) complexes structure with deprotonized dextran monomer unit (Glc–) are suggested depending on pH synthesis. At pH 7 to 8 $[Cu(Glc)_2(H_2O)_2]$ is formed; at pH 8 to 10 $[Cu(Glc)_2(OH)(H_2O)]^-$ is formed and at pH values over 10 $[Cu(Glc)_2(OH)_2]^{2-}$ is formed.

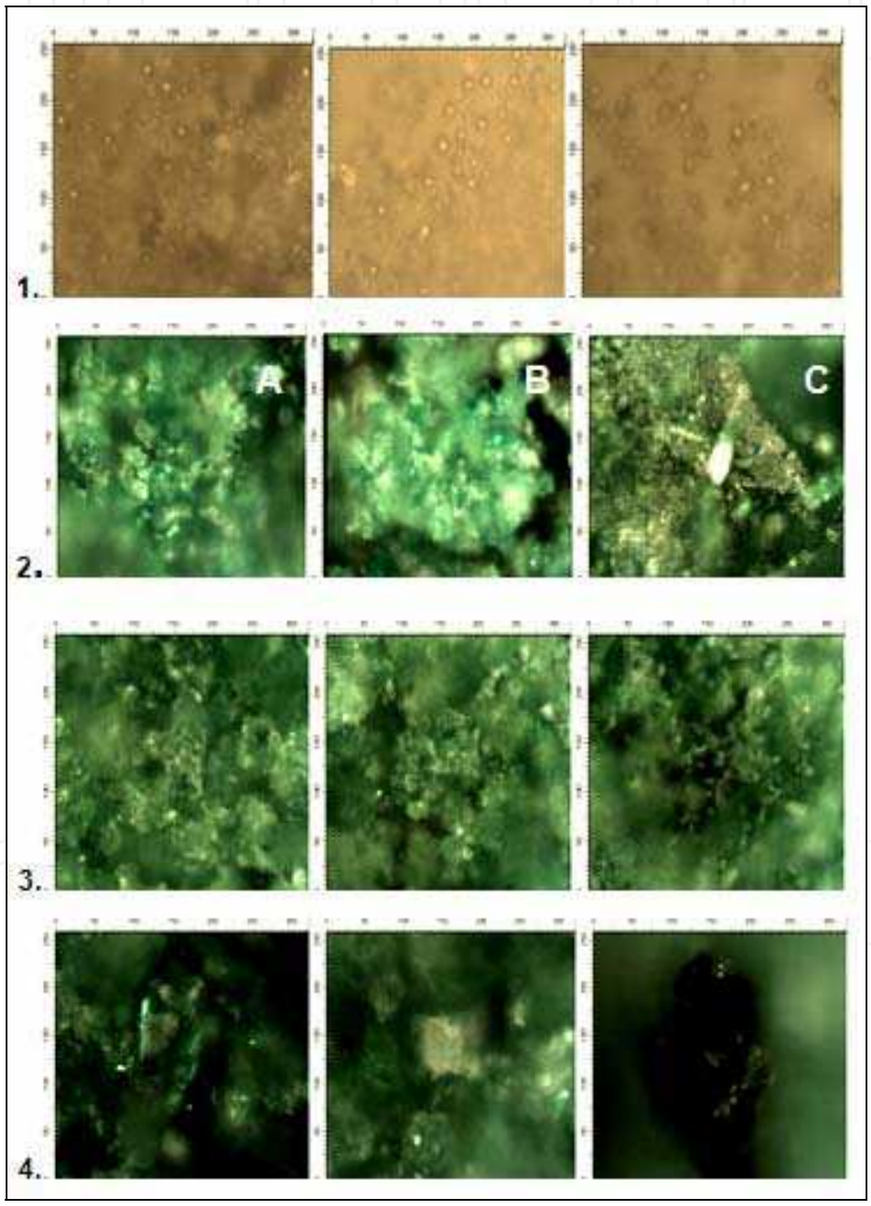


Fig. 14. FTIR microscopy images (250 μm x 300 μm) of RLMD, Mw = 5.000 g mol^{-1} (1) and Cu(II) complexes with RLMD synthesized at boiling point and pH 7-8 (2), 8-10 (3) and 10-12 (4)

The application of microscopic FTIR imaging system to the RLM dextran as ligand and Cu(II)–RLMD complexes, were synthesized at pH 7.5–12, is shown in Fig. 14. FTIR microscopy images of dextran, as well as images of the copper-dextran complexes differ which also indicates that the complexation process and the creation of coordination compounds took place (Mitic et al., 2010). FTIR microscopy images confirmed that the changes in the intensity of the analyzed bands are strongly associated with the alterations in the macromolecular order. These bands in the spectra of the complexes can be responsible for more and less ordered structures, respectively. The changes in color contour may show the content and distribution of copper, and polysaccharides in Cu(II)–RLMD samples.
After physicochemical standardization of the most stable complex obtained according to the procedure 2 (Tab. 1), the preparation for the pharmacological test was provided. The preparation was tested pharmacologically with the aim of determining systemic acute toxicity expressed as a median lethal dose (LD_{50}) and as an equivalent of Cu(II) dose per kg of a mouse body weight (Cakic et al., 2008). None of the applied complex doses at the concentration of 82–169 mg equivalent of Cu(II) per kg of a mouse's body weight was lethal in the tested mice. Therefore, in this case, a median lethal dose could not be determined. The application of higher doses has caused the mortality of one part of experimental animals. Thus, in this range, the preparation toxicity LD_{50} of 1419–1661 was determined, which corresponds to the equivalent of Cu(II) dose of 281–329 mg per kg of the body weight, in the concentration 5–20% of the complex solution. Toxicity investigations of various commercial copper salts show a wide range of values for LD_{50}. The level of the acute toxicity is higher for more soluble than for less soluble Cu(II) salts. The results of our pharmacological investigations point to the lower toxicity of Cu(II) complex with RLMD, what is much better than in the case of commercially applicable inorganic copper salts.

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