PHARMACEUTICALLY IMPORTANT COMPLEX COMPOUNDS OF SOME MICROBIAL EXOPOLYSACCHARIDES

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In the field of medicinal chemistry, a lot of investigations are based on the synthesis and characterization of different metal complexes of ligands present in biological systems, or synthetic ligands, which will serve as the model molecules for complex biomolecular structures. Bio- or synthetic ligands are mainly natural chemical compounds of macromolecular type, such as carbohydrates, proteins, and nucleic acids. In this group of products very important are chemical compounds of polysaccharide dextran, pullulan and inulin with cations of the different transition biometals (Cu(II), Co(II), Zn(II) and Fe(III)). Ligands are mainly coordinated across the donor oxygen atoms used both in veterinary and human medicine, because all systems consist of the polysaccharide as energetic source, and bioelements as significant factors in metabolic processes in the organism. For prevention of anemia, products of the biological ligands and iron are usually used and there is a lot of literature data about that. Except iron, for the successful medical treatment, the presence of other hematopoietically active biometals is necessary, such as copper, cobalt and zinc. In human and veterinary medicine, especially interesting are products with polymicroelements, which provides the complex treatment of different diseases at the same time. This study will focus on the results of investigations carried out on the metal ion complexes of carbohydrate type ligands, with special attention to potential pharmaceutical applications of the biometal complexes with polysaccharides dextran and pullulan, and their derivatives.

Key words: microbial polysaccharides, dextran, pullulan, biometals, complexes

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Introduction

The interaction of carbohydrates, as a type of polysaccharides, with transition metal ions, is a field of medicinal chemistry in constant development, due to the presence of such interactions in biological systems and multiple applications they offer in areas that are important for food, chemical, veterinary and pharmaceutical industries (1). The interaction of this type was observed at the beginning of the last century (2), but these compounds have remained mainly unexplored. One of the reasons is that the quantitative characterisation of equilibrium coordination of metal ions with polyalcohols and carbohydrates that contain only alcohol or aldehyde/ketone oxygen donor atoms is difficult because of the low stability of the compounds in neutral or acidic aqueous solutions. In spite of relatively large number of them in a single molecule ligands, small electron density of the donor atoms of oxygen causes not so easy substitution of the water molecules bound in the first coordination sphere of the central metal ion (1,3). Hydrolysis of some metal ions prevents the coordination of organic ligands, and therefore the formation of complex compounds can be expected only in alkaline solutions, after deprotonation of the hydroxyl groups.

Dextran is exopolysaccharide which represents the molecular chain of D-glucopyranose units, linked mainly by α-(1→6) O-glycosidic linkages, which may be produced by microbiological synthesis. The macromolecules of dextran, with the exception of α-(1→6) bonds, contain different amount of α-(1→2), α-(1→3) or α-(1→4) O-glycosidic bonds (4). Dextran has multiple potential for intramolecular hydrogen bonds within the crystal (5,6). Reactivity of dextran mainly depends on the reactivity and orientation of the secondary hydroxyl groups (OH-2, OH-3 and OH-4), while the reactivity of the OH groups is much higher in the equatorial (eq) than in an axial (ax) position (7). On the other hand, the reactivity of the OH-2 groups to the alkylating agents is greater than the OH-3 and OH-4. For an elementary ring of amorphous dextran, using the FTIR spectroscopic data analysis (8,9), the pattern of C-H group fragments is eq-ax-ax-ax, which corresponds to the γ1 conformation of the glucopyranose ring. As the OH groups at C-2, C-3 and C-4 atoms are spaced equatorially, complexing of M(II) ions at these locations is expected...
and probably starts at the deprotonated OH group of the C-2 glucopyranose unit of dextran (9). Polysaccharide dextran has an excellent capability of forming water-soluble complexes with the variety of biometals (Cu, Co, Zn, Mo, Ca, Mg, Fe) (10,11), as well as other metals (Tb, Al, Cd, Pb, Ni, Mn) (12, 13). Stability, solubility, absorption, and non-toxicity of the complex are important factors that must be accomplished from the point of application of these complexes with polysaccharides in veterinary and human medicine (14,15). The structure of these complexes, due to its complexity, is not fully understood, which prevents the establishment of appropriate correlation of structure and pharmacological effect.

Therefore, for the preparation of such complexes, the different oligosaccharides and polysaccharides are used. By its composition, structure and type of glycosidic bond, dextran is very similar to amorphous water-soluble linear polysaccharide pullulan. Pullulan meets all the requirements of a potential ligand for the synthesis of complexes with various biometal ions, regarding constitution (16). A linear polysaccharide pullulan can be easily depolymerized to oligomers which are easily coordinated with the metal ions. The recent literature has data on oxidized pullulan oligomers (17), but apart from these there are not enough data about the synthesis of complexes with biometals, their structure and pharmacological effect of potential pharmacological preparations based on these complexes.

It is known that the raw polysaccharides (dextran, pullulan etc.) have molecular weights in the range from $10^4$ to $10^6$g/mol, and for the complex synthesis the most suitable molecular weights are from 3000 to 10000g/mol as a narrow distribution of molar mass (18). The most suitable and the most commonly used method for depolymerization of polysaccharides is hydrolysis in aqueous solutions of hydrochloric acid. Therefore, to obtain the oligomers of corresponding polymerization degree, it is necessary to perform the optimization of the applied conditions of hydrolysis (pH, temperature and time). Bearing in mind that the m(II) metal ions are present in the system, the elimination of undesirable reduction activity of the ligand is carried out using NaBH₄ by reducing group content less than 0.05%.

According to these facts, the complex synthesis requires testing under a variety of reaction conditions (pH, temperature and time). For better clarifying the structure of these complexes, the final molar ratio of metal-ligand should be first determined, then the proportion of "bound" and "free" ligands and their molecular weight distribution. Particular attention should be paid to determine the conformation of the monosaccharide units, the degree of linearity, as well as the method of water bonding in the complex compounds.

**Carbohydrates as ligands**

In an aqueous solution, carbohydrate complex compounds are formed by replacing the molecules of water in the first coordination sphere of the metal cation by hydroxyl groups. Since the molecules of water hydrate the cations much better than the alcohol OH groups, polysaccharides do not form stable compounds with the cations in neutral aqueous solutions. They produce mainly polymeric compounds in the solid state (19). Generally, it takes at least three hydroxyl groups in a favorable steric arrangement for making compounds. The general rule is that the sugars in the pyranose form with chair conformation containing an axial-equatorial-axial (ax-eq-ax) set of three adjacent hydroxyl groups, or 1,3,5-triaxial (ax-ax-ax) hydroxyl groups or in the furanose form of three neighboring hydroxyl groups in a cis-cis arrangement, are the best coordinating ligands (1). Carbohydrates and alditols of the three-three arrangement of the hydroxyl groups form more stable complexes than those with erythro arrangement. All of the above mentioned ligands form the compounds with the metal cations in the ratio of 1:1 in a hydrophilic solvent (20,21) and provide direct evidence of coordination of the oxygen atom of non-deprotonated hydroxyl groups. The relatively low stability of a compound reflects, among the other things, a low donor ability of these O atoms.

A radius of cations is an important factor in the creation of complex compounds also. While the above mentioned ax-eq-ax and ax-ax-ax positions show similar geometry with three oxygen atoms at about 295pm, they differ in the relative directions of orbitals containing lone electron pairs and in the ability to coordinate oxygen atoms. This leads to significant differences in the coordination of metal ions with different ionic radius. The position of OH groups in ax-eq-ax position coordinates only cations having ionic radii of >80pm (22, 23). The position of the three axes found in cis-inositol preferred cations having ion radius of ~60pm, but <100pm (24). If a donor group is introduced in the sugar molecule, which, as the position of the primary coordination can support the deprotonation of the hydroxyl groups of the carbohydrate, the ability of production of the compounds is enhanced by several orders of magnitude, even in neutral or acidic solutions. These donor groups can be carboxyl, carboxymethyl, amino, thiols, phosphates or other groups. After deprotonation of one or more hydroxyl groups, the resulting transition metal compounds having an anionic character are generally very stable. The general scheme of the deprotonation of the polyhydroxycarboxylic acids' hydroxyl groups was given by Bekkum et al. (25). In some cases, the creation of different amounts of alkoxo or hydroxyl dimeric (or oligomeric) types can be detected. Ligands with a donor group are in competition with the processes of hydrolysis of the transition metal ions and keep them in solution over a wide pH range, but the hydroxyl ligand compounds are also formed among the common species. The combination of the distribution curves obtained in this way, with the spectroscopic measurements, is needed for obtaining structural information for the individual complex compounds. Spe-
ctroscopic measurements can also help in the assessment of micro- and conformational equilibrium. Determining the acidity constants of the individual anomers of the amino sugars using $^1$H-NMR spectroscopy (26), and the study of the interaction between the carbohydrate and borate using $^{11}$B- and $^{13}$C-NMR spectroscopy are good examples (27). However, the presence of more than one donor group may prevent coordination of the hydroxyl groups included in the coordination sphere of the metal ion. Also, epimerization process of the ligands is possible, which is supported by a metal ion (26,27).

Pharmacological importance of carbohydrates and their complex compounds

Carbohydrates and carbohydrate derivatives, as one of the most extensive class of biomolecules are known to have diverse biological functions. Through the interaction between these multifunctional molecules and the metal ions in living organisms, the modification of the biological functions of both sides may be expected. Although the biological significance of the metal ion compounds with carbohydrates has not been fully investigated, there are several studies which are taking place in this field. The transport of metal ions through the cell membrane is one of the known roles of oligosaccharide or polysaccharide compounds (1,3). There is the influence of carbohydrates on the distribution of Ca(II) ions in the body (28,29) and the interaction between carbohydrates and the metal ions takes place in the metalloenzymes in carbohydrate metabolism (30). Coordination of metal ions can directly affect the function of the enzyme or an appropriate orientation of the carbohydrate molecules. Study of the Zn(II) ion influence on the processes of isomerization and phosphorylation of carbohydrates also shows the creation of the metal ion compounds (31). In the study of the crystal structure of the CaZn(II) ion compound with erythromycin A, a direct coordination of the hydrocarbon part of the ligand was determined (32).

An important role of carbohydrates is to increase the solubility of potential biolgands or essential metal ions. The treatment of iron excess (33,34) or iron deficiency (15) are one of the most important clinical uses of chelating agents. It is well known that Fe(III) ion forms the compounds with oxygen based ligands, such as highly water-soluble and poorly immunogenic carbohydrates. Fe(III)-sorbitol-D-gluconic acid is the example of a widely used and tested compound (35,36). In the treatment of anemia caused by the lack of iron a polysaccharide-iron compound named "FedEx" derived from Fe(III) ions and dextran is also used (37,38). A complex compound of Zn(II)-hyaluronic acid has been patented and used as a drug for the treatment of ulcers, bedsores and especially for wounds that do not heal (39). It is also possible to synthesize chelates of the toxic metals such as Ni(II), Cd(II) and Pb(II), which assist in their elimination in the urine via the kidneys. It was found by Kohn (40) that Pb(II) ion is well coordinated by galactopyranoside uronic acid. Water-soluble polysaccharides have been tested as carriers for the magnetic resonance (MR) recording. In particular, Mn(II) and Gd(III) compounds are more efficient agents of proton relaxation than the metal chelating agents of lower mass (41). The major role of carbohydrates is to protect the cell in its environment. Surrounding itself with a layer of extracellular polysaccharides containing high water content, the microorganism ensures greater resistance against desiccation and predation by protozoans. Moreover, the anionic nature of the exopolysaccharide layer can help to capture essential minerals and nutrients. Exopolysaccharides also help to degrade certain metals due to their anionic character and their capacity to chelate metals and ions (42).

Polysaccharides and their derivatives as biopolymers

Biologically active polymers (bio-polymers) can be classified into two groups. The first group includes biopolymers whose activity is determined by their macromolecular nature and exists on the level of the polymer only. The second group is the group of synthetic, biologically active polymers, consisting of synthetic polymer-carrier hybrids with the biologically active substance, which can be both low molecular as well as high molecular weight by their nature (43). Neutral biopolymers, due to their low toxicity and high hydrophilicity, represent a good basis for creating a wide range of functional polymer-carriers and polymer hybrid systems with variable pharmacokinetics. The group of neutral biopolymers includes hydrophilic biosynthetic polysaccharides dextran and pullulan (43,44) known as plasma-expanders (8,9). In recent years, exogenous and endogenous microbial polysaccharides are of a great importance because of the wide range of applications and fundamental aspects of the biosynthesis. Potential applications of microbial polysaccharides in the pharmaceutical industry are growing rapidly (45). These polysaccharides are commercially very attractive due to their properties. They are non-toxic and biodegradable, unlike many other synthetic polymers, and they are created extracellularly during the periodic aerobic fermentation cultures of non-virulent microorganisms. Microbial polysaccharides are classified as thickening agents, suspending and stabilizing aqueous systems (xanthan gum, scleroglucan), polysaccharides which form gels (curdlan, alginuate), and the special purpose polysaccharides (dextran, pullulan, inulin, chitin, chitosan, heparin) (43,44).

The polysaccharide dextran

The structure of dextran

Dextran is homopolysaccharide which can be obtained by microbiological synthesis. It represents the molecular chain of D-glucopyranose units linked mainly by $\alpha-(1\rightarrow6)$ O-glycosidic linkages. In the macromolecules of dextran, with the exception of
α-(1→6) bonds, α-(1→2), α-(1→3) or α-(1→4) glycosidic bonds are present in a different content (46), with a degree of branching approx. 5% (Figure 1.).

The polysaccharide dextran is produced by the microorganisms *Leuconostoc mesenteroides*, *Leuconostoc dextranicum*, *Streptobacterium dextranicum* etc., in the medium comprising saccharose or other carbohydrates, which consist of D-glucopyranose units (47). Dextran obtained from various microbial strains differ from each other by the structure, physical and chemical properties, i.e. the difference in a degree of branching of macromolecules, relative content of a particular type of glycosidic bonds, solubility, molar mass, optical activity, physiological effects, etc. (4).

Biosynthesis of the raw dextran in the pharmaceutical and chemical industry is carried out in special fermenters which are filled with the substrate, which includes: saccharase, organic nitrogen compounds, promoters of bacterial growth and a certain amount of inorganic salts. After the culture of bacteria is added to the medium, at the same time cells start to proliferate, to secrete enzymes and synthesize dextran. Biosynthesis of dextran has been completed in the time period of 20 to 24 hours. The raw dextran is removed from the fermented mass by deposition of organic solvents. The molar mass of thus obtained raw dextran is in the order of several million g/mol, with a high degree of polydispersity (48). Factors that determine the nature and properties of dextran polysaccharide are: structure of molecule, the average molecular weight and molecular distribution. The raw dextran cannot be used for clinical purposes, since it possesses toxic and antigenic properties (45,47). These are the reasons why raw dextran is depolymerized and repeatedly fractioned with a hydrophilic solvent to give a fraction with a narrower distribution of the molecular weight (46,48). Dextran molecules with a large molecular weight can cause side reactions in the body because of their toxicity, whereas molecules with low molar masses are rapidly excreted from the body. Fractionation conditions need to be adjusted to obtain fractions with the narrow distribution of molecular weights (49). Dextran depolymerization can be carried out by acid hydrolysis, alcoholysis, thermal destruction of the UV-, γ- and X-radiation, and by ultrasound. In the pharmaceutical and chemical industry, acidic hydrolysis is mainly applied. As a result of acid hydrolysis there is also the decrease of a degree of macromolecule dextran branching, which is caused by the relatively high stability to acid hydrolysis of α-(1→6) bonds. Only dextran with a small degree of branching, i.e. with the content of α-(1→6) bonds over 90% are used in clinical purposes (48).
The use of dextran

Dextran, in the form of small molecular weight fractions, is commonly used in the pharmaceutical industry as an infusion solution, as a blood plasma expander and as a remedy for improving the microcirculation. This application of dextran is based on the fact that it meets certain requirements, such as high capacity of colloidal water binding, long retention time in the bloodstream, a suitable value of the viscosity, certain osmotic pressure, stability, non-toxicity and etc. The greatest clinical application involves dextran with an average molar weight of 40000 g/mol (T-40, for improving the microcirculation) and 70000 g/mol (T-70, a substitute for blood plasma) (49). The fraction of dextran T-70, with strictly controlled purity and distribution of molecular weights, is administrated intravenously as a 6% solution in saline. This standard clinical dextran is used to establish the blood pressure and circulating blood mass in the treatment of hemorrhagic shock and burns, particularly when there is not a sufficient amount of blood. The fraction of dextran T-40, like a 10% solution in 0.9% NaCl or 5% glucose, is used for the treatment of the blood rheology disturbances, in the artificial organ transplants, and for the heart and blood vessels operations. Beside clinical dextran, in pharmaceutical industry, dextran oligomers with molar masses between 3000 and 10000 g/mol are being used increasingly. These oligomers of dextran are obtained by decomposition of the larger molecular weight dextran in acidic aqueous solutions at high temperature (45,47).

The complex compounds of polysaccharide dextran

Dextran creates alkoxides with alkaline and earth alkaline metals, the so-called dextranates. Dextranates are used as intermediates in the synthesis of other dextran products. Dextran complex compounds with different metal cations have been described in the literature (1-13). The structure of most of these compounds is not exactly determined. The compounds are prepared in an interaction of dextran with the metal salts in an alkaline medium, sometimes in the presence of other compounds of low molecular weight with complexing properties. Dextran was previously reduced (NaBH4) or oxidized (NaIO4, H2O2) in some cases. It was shown in pharmaceutical practice that better pharmaceutical compositions are obtained when low molecular weight dextran with as much as possible lower the content of reducing groups, the so-called reduced low-molecular dextran (RLMD) is used for their production. Complex compounds of RLMD with Cu(II) (8,9,50,51), Co(II) (52), and Fe(III) ions (53) are known in the scientific and patent literature, and they are primarily used in veterinary medicine. The compositions with polymicroelements are of particular interest in medicine, providing at the same time more complex treatment of the diseases or they are added as dietary supplements. The application of similar multi-component compositions is particularly successful in terms when the exact dose is required to calculate the correction of the deficit of each biometal, in particular diseases, and the favored choice of the ligand in the complex which introduces biometals into the body. Products based on dextran and Fe(III) hydroxide are usually used for prevention of anemia (54), and there are a lot of literature references about them (1,15,37,38). In addition to iron, for successful treatment of specific anemia (hypochromic, microcytic, pernicious etc.), the presence of hematopoietic and other active biometals, such as copper, cobalt and zinc is necessary. The interaction of carbohydrates and their derivatives with a copper (1) is one of the most extensively studied areas of coordination chemistry. The reason for this is the presence of copper in a large number of enzymes, as well as in animal diet, that is rich in carbohydrates.

Using UV-Vis spectrophotometric assays of the interaction of Cu(II), Co(II) and Ni(II) ions with a solution of the polysaccharide dextran (55), it is ascertained that the degree of binding of the metal in complex primarily depends on the solution pH, and the content of OH groups in the first coordination sphere of metal ions. Spectrophotometric analysis of the solution of the dextran complex with the Cu(II), Ni(II), Co(II), Zn(II) and Mn(II) ions (56) revealed that in the formation of the complex participate OH groups of the C-2 and C-3 atom of dextran monomer unit (glucopyranose) and that effects that lead to a change in the conformation of macromolecules are not present. By direct polarographic and UV-Vis spectrophotometric assays of the Cu(II) ion complex with dextran in alkaline solutions (57), it was proved that the complexation of Cu(II) ions starts at a pH greater than 7. The synthesis and spectroscopic characterization of the Cu(II) ion complex compounds with a reduced low-molecular dextran is described in detail by Mitić et al. (9,58). The formation of Cu(II)-hydroxyl complexes with the deprotonated dextran monomer unit was observed in the range of pH 8-12. Synthesized Cu(II)-dextran complexes are decomposed at pH >12.

The way of binding of Cu(II) ions with a reduced dextran in the alkaline solution was analyzed using UV-Vis spectrophotometry (59). Complexes of Cu(II) ions with RLMD show different maxima of UV-Vis absorption as a function of pH (7-12). There are typical hypsochromic (blue) shifts due to the interaction of orbitals of Cu(II) ion and ligands. The results of spectrophotometric analysis indicate the possibility of forming a coordination bonds which rearrangement begins at pH >8. The models of the gradual complexation as a function of pH are proposed using the correlation of the spectra and the structure. Some articles (52,60) have described synthesis and studied the complexes of Co(II) ions with a reduced low molecular dextran, using various physicochemical and spectroscopic techniques (FTIR, ESR), in order to determine the geometry of the complexes.
The polysaccharide pullulan

The structure of pullulan

Pullulan is an extracellular water-soluble homopolysaccharide, which can be obtained by microorganisms *Aureo-basidium pullulans* (61). The chemical structure of the product depends on the type of a substrate used and the types of microorganisms (62,63). Pullulan is the neutral glucan composed of linear chains of D-glucopyranose units linked alternately by one α-(1→6) and two α-(1→4) O-glycosidic bonds according to the structure (64). Crude pullulan, which is obtained from a strain *Aureo-basidium pullulans* CH-2, depending on the production conditions (type of substrate, pH, a nitrogen source, aeration and mixing), has high molecular weight of 2×10^5 to 4×10^6 g/mol. Pullulan is often subjected to modification. Mineral acids are used in particular for depolymerization to obtain fractions of molecular weights 5000, 10000, 20000, 40000, 70000g/mol, and higher, which may be used in food, chemical, pharmaceutical and cosmetic industry. Bernier (64) isolated the water-soluble polysaccharide from the culture of *Aureobasidium pullulans*. The major product of the acid hydrolysis of that compound is α-D-glucopyranose (65). Linear homopolysaccharide of glucose, which is named pullulan, is often described as α-(1→6)-linked polymer that has maltotriose composition. There is the unique law of connectivity that gives pullulan some important physicochemical properties. On the basis of a positive optical rotation and IR spectroscopy, it was concluded that pullulan is α-glucan polymer in which α-(1→4) links are dominant (66). Later, using FTIR spectroscopy, periodate oxidation, methylation analysis, it was confirmed that the molecule of pullulan is a linear glucan consisting of α-(1→4) and α-(1→6) O-glycosidic bonds in a ratio of 2:1. Isomaltose, maltose, panose and isopanose are formed by partial acid hydrolysis of pullulan (61,66). The discovery of the enzyme pullulanase was important for founding a tool to analyze the structure of pullulan. Based on the results of structural analysis, pullulan is often described as a polymer of α-(1→6) linked maltotriose (Figure 2.), which makes it a dominant primary structure.

Pullulan may also be presented as a polymer of panose or isopanose substituents, which may reflect the biosynthetic origin of the molecule more accurately; a number of enzymes have been described as producers of panose or isopanose from pullulan. A number of studies have shown that pullulan contains tetraose substituents, which are added to two dominant substituents of pullulan. The frequency of maltotetraose substitutes depends on the specific type used, and ranges from 1 to 7% of the total residues. Maltotetraoses are randomly distributed across the pullulan molecule (61,67). In a molecule of pullulan, the remains of maltotetraose make the secondary structure, in contrast to maltotriose substituents (Figure 3.).

In a structural analysis of polysaccharides, FTIR spectroscopic techniques are generally used a lot (14,51,52,67). There is a significant influence that shows conformation of glucopyranose ring on the IR spectrum in the low frequency area from 1000 to 700cm⁻¹, because the spectral interval is sensitive to the change of the mutual arrangement of the C-H groups in the elemental ring of polysaccharides and it can be used to study conformational transitions of the D-glucopyranose rings in the polysaccharide. The band at 900cm⁻¹ (determined by the presence of α-(1→6) bond) and the band at 925cm⁻¹ (determined by the presence of α-(1→4) bond) can be observed in the IR spectra of pullulan (67). The content of the linkage type in different preparations can be determined by measuring the intensity of these bands. The reactivity of the OH group of glucopyranose units in pullulan is C-6>C-3>C-2>C-4, and it is determined by ¹³C-NMR spectroscopy, by sulphonation reaction using CISO₂H/pyridine (68,69). The average length of the repeat units in the chain can be determined, assuming that the glucopyranose rings of polysaccharides have α1 conformation, and for pullulan value is from 0.425 to 0.570nm (compared to 0.425nm with amylose and 0.570nm with dextran).
(61). On this basis it can be concluded that the pullulan chain is more elastic than that of amylose (starch polymers of glucose with α-(1→4) bond), because of longer repeating unit. The viscometric test results are consistent with the typical flexible polymer chains in the solvent. Pullulan chain flexibility is the result of the presence of α-(1→6) O-glycosidic bonds, and therefore in the aqueous solution acts as a flexible, stretchable thread (70). Kinetic studies have shown that the depolymerization reaction of pullulan is a reaction of the first order with respect to the number of broken glycoside bonds (70,71). The depolymerization rate constants of pullulan are higher than the corresponding value for dextran (70,71). The depolymerization activation energy of pullulan is substantially smaller than the corresponding values of dextran (71).

**Physicochemical properties and the use of pullulan**

Dry powder of polysaccharide pullulan is white non-hygroscopic and rapidly soluble in hot and cold water. Pullulan is non-toxic, non-mutagen, odorless and tasteless. Because of its resistance to mammals' amylase, it is low caloric and suitable for diet treatment. A solution of pullulan has a relatively low viscosity and is stable to heat and change in pH. Pullulan and its derivatives have adhesive properties. Pullulan can be used as an adhesive in dentistry, as a coating in pharmaceutical industry and as a low molecular weight filler in cosmetic products (43,61,66).

Pullulan is similar to a commercial dextran, it is a linear, neutral, soluble α-D-glucan (dextran is more branched). Pullulan fractions having a molecular weight of 30000 to 90000g/mol can be used as a substitute for blood plasma, instead of dextran. Similarly to dextran, pullulan can be easily modified to a variety of derivatives (58,72), which are generally biodegradable. Most of them have a reduced solubility in water, so reactive groups are introduced as functional groups. The esterification or etherification may decrease the aqueous stability of the pullulan progressively. Reduction provides an increase in the thermal stability of the pullulan and the reaction of carboxylation can change its solubility in cold water. Cross-linked pullulans (analogos to Sephadex) are used in the gel chromatography. Pullulan can be sulphonated, chlorinated and sulphonylated (64-66). Pullulan, which is extracellular α-polyglucan, has a uniform structure, regardless of used strains of microorganisms. Linear α-(1→4) amylose chain is interrupted by regular arrangement of α-(1→6) bonds in pullulan. This is a structural difference that gives the flexibility and increases the solubility, leading to the formation of a clear film and the characteristics of the fiber, which allows the pullulan imitation of synthetic polymers (63–66,73). Pullulan can be used for tumor cell targeting. The new anticancer polymer therapeutics were synthesized by pullulan derivatization with either doxorubicin or doxorubicin and folic acid. Pullulan being biocompatible and nontoxic is investigated for gene delivery application. Pullulan derivative which has metal chelating residues is mixed with a plasmid DNA in aqueous solution containing Zn²⁺ ions in order to obtain the conjugate of pullulan derivative and plasmid DNA with Zn²⁺ coordination. Liver targeting study focuses on the blood compatibility of the cationic pullulan, physicochemical characterization, and uptake of nano-complex by hepatocytes and in vitro transformation. Liver targeting can be achieved by using drug loaded pullulan (74,75).

**Complex compounds of polysaccharide pullulan**

The preparation of a viscous crude pullulan, which is separated from the biomass by centrifugation and purified by precipitating with an ethanol solution, is described in the literature (76). Wolf (77) described the polysaccharide pullulan as a low digested carbohydrate and its application in the food industry, especially in beverages and dietary products. Pullulan derivatives as ligands for the synthesis of platinum complexes are described in the patent literature (78). Macromolecular complex, obtained by synthesis, is soluble in the water, and it can be concluded that there is an outstanding anticancer activity and low toxicity on the basis of the pharmacological tests. The authors state that this is a new macromolecule which can be used as a remedy.

The synthesis and use of a water-soluble complex of pullulan with polyethylene glycol is described in patent literature (79). Pullulan is mentioned as one of possible ligands in addition to dextran, in the preparation of complexes with Fe(III) hydroxide in a review of the patent literature. The preparation and use of polynuclear complex of Fe(III) ions with pullulan oligomers and its reduced and oxidized derivatives in the pharmaceutical industry has been described by Ilić et al. (80). The complex was synthesized from an aqueous solution of pullulan with insoluble Fe(III) hydroxide, that is, after purifying and standardizing the preparation, subjected to pharmacological tests. Synthesized complex has potential use in prevention of iron deficiency anemia. There is a great interest in pullulan regarding its good physicochemical properties: degradability, water solubility, stability etc. In addition to cross-linked pullulan membranes (81), particular attention attracts modified pullulan by using periodate oxidation to form dialdehyde structure (82). It has a wide practical application both in chemical and in the cosmetic industry. It is commonly used for making capsules in the pharmaceutical industry. It was found that the reduced pullulan has significant advantages in the application than the native pullulan. In the pharmaceutical industry pullulan of low molecular weight is used with as much as possible lower content of reducing groups, which is the so-called reduced low mole-
cular pullulan (RLMP). There are also well-known complex compounds of Zn(II), Co(II), Fe(III) (58,72,80) and Cu(II) ions (83) with RLMP, which are used primarily in veterinary medicine, for the prevention of a condition caused by a deficit of these ions in the body.

**Conclusion**

A brief review of recent advances in complex compounds of extracellular microbial polysaccharides applications in the pharmaceutical fields has been reported. In recent years, dextran and pullulan have a great impact primarily because of the wide range of applications and because of the fundamental aspects of the biosynthesis. Despite this fact, their complex compounds with metals are not fully characterized yet and require extensive experimental work. Potential application of microbial polysaccharides and their derivatives as well as complex compounds in medicine, agriculture, food, chemical and pharmaceutical industry is growing rapidly. A study of biological systems, including metal complexes with carbohydrates, as well as the biological effects of these complexes, provides a detailed insight into the mechanism of these interactions. Further investigations with a multidisciplinary approach are imperative in order to develop novel products that can be useful as drugs.

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