

## Synthesis of some new antianemics

### I. Iron pullulan complexes of pharmaceutical interest

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First experiments were performed in order to obtain a polynuclear iron(III) complex with pullulan, which could be applied in therapy of sideropenic anemia. Complete synthesis of the complex can be realized with oligomers of depolymerized pullulan at an average molar mass 8000–10000 g · mol<sup>-1</sup>. Maximum iron contents of 51.4 mg · cm<sup>-3</sup> is effected by pullulan depolymerizates ( $M_w \approx 9000 \text{ g} \cdot \text{mol}^{-1}$ ,  $[\eta] = 0.085 \text{ dl} \cdot \text{g}^{-1}$ ) at 130 °C for 240 min, at pH = 10.5 and at an initial mass ratio Fe/ligand of 1 : 3. The effected concentrations and complex stability respond to requirements of pharmacological application of parenteral antianemic preparations.

#### 1. Introduction

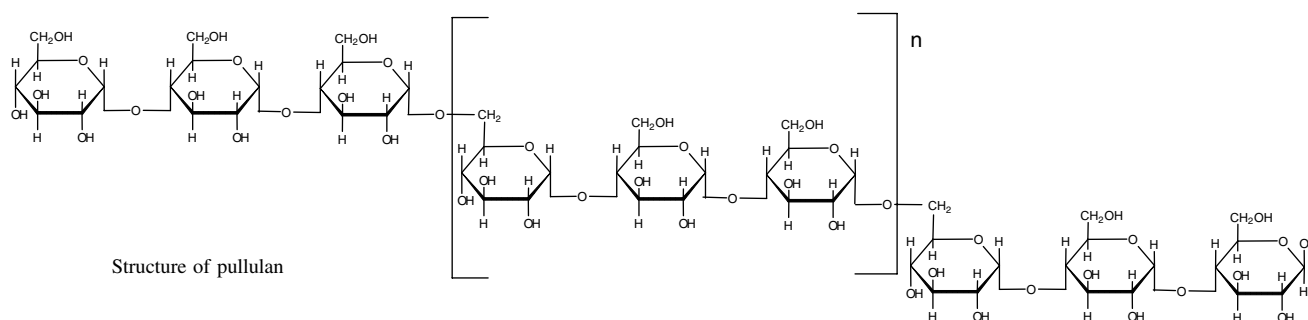
The task of creating new, therapeutically active iron(III) complexes remains a challenge, because iron deficient anemia is wide-spread in the world [1]. Numerous preparations based on organic and inorganic compounds of bi- and trivalent iron are used today for the prevention and therapy of sideropenic anemia [2]. Iron-carbohydrate complexes (i.e. iron-dextran) are preferred as the standard in modern iron therapy [3, 4]. Synthesis of these complexes with native polysaccharides is impossible. Our experience with iron dextran complex and similar preparations shows that only when oligosaccharides have limited molar mass, complexation can occur. Therefore, depolymerization yielding oligomers of well-known degree of polymerization is mandatory. Synthesis and complex stability are directly influenced by the ligands' degree of polymerization. Usable reaction conditions (pH, T, t, P) are important for iron contents in the preparation and for its toxicity [5]. The possibility of different types of carbohydrate ligands to form water-soluble complexes with Fe(OH)O in alkaline solution shows that there is no strict choice of ligands. Application of hydrogenated pullulan as a ligand is not known. Pullulan is a water-soluble polysaccharide very similar to dextran in its composition [6, 7]. Pullulan is an amorphous polymer with a linear flexible chain, the main axis of which consists of  $\alpha$ -D-glucose units. The structural unit, which is regularly repeated, is maltotriose ( $\alpha$ -1,4-triglycoside) linked by  $\alpha$ -1,6-glycosidic bonds. The interest in this polysaccharide is considerable because of its good physicochemical qualities, good degradability and wide opportunities for industrial and pharmaceutical applications [8, 9].

Pullulan has all conditions to form water-soluble complexes with iron. That's why pullulan and its hydrogenated oligomers were examined as ligands for the syn-

thesis of polynuclear antianemic complexes. Since the stability of a complex is directly affected by the characteristics of the applied ligand, the conditions of preparation and characterization of pullulan oligomers as well as the conditions of complex synthesis are optimized.

#### 2. Investigations, results and discussion

Preliminary researches of complex synthesis with different samples of native pullulan (NP-1 and NP-2) of high molar mass showed negative results. Neither with prolonged time of reaction nor under more drastic reactive conditions in an autoclave, the complex was formed. Therefore, the starting components were depolymerized to oligomers of adequate polymerization degree ( $P_w$ ) under different reactive conditions (pH, T and t). For investigating the influence of the initial molar mass of pullulan on the depolymerization rate, the progress of depolymerization of two samples was followed, the chosen molar masses 220570 and 328000 g · mol<sup>-1</sup>. The influence of pullulan concentration on its depolymerization rate in solutions with constant HCl concentration was verified for the concentrations 6.3 and 7.1%. Conditions of depolymerization, according to previous investigations [10], were chosen depending on ligand qualities and initial  $M_w$ . A temperature of 80–85 °C and a pH value of 2.0 proved to be optimal for the depolymerization. Under more drastic conditions of depolymerization (higher temperature and lower pH values), higher contents of mono- and oligosaccharides with lower  $M_w$  (below 1000 g · mol<sup>-1</sup>) appeared. These saccharides inevitably have to be removed from depolymerizates before complexation with Fe(OH)O, because a very alkaline environment under high temperature and pressure causes their decomposition to toxic products [11]. On the other hand, if the average molar mass of the applied polysac-



charide is too high, the product will be a gel, not practical for any kind of application [12].

### 2.1. Depolymerization of native pullulan

Pullulan NP-1 ( $M_w = 220570 \text{ g} \cdot \text{mol}^{-1}$ ) was depolymerized by hydrolysis in HCl/water. Depolymerization was followed by viscosimetry [13]. Conditions that allow application of this method are steadiness of pullulan in acidic water solution at  $25^\circ\text{C}$  (depolymerization would be interrupted by cooling), and the constancy of ratio between pullulan intrinsic viscosity in water and hydrochloric acid (important for calculation of average weight molar mass). The average number at molar mass, polydispersity ( $M_w/M_n$ ), and contents of reducing groups (RG) characterize some depolymerizates (Table 1).

According to previous experiences, the time of depolymerization at  $80^\circ\text{C}$  and pH 2.0 with pullulan NP-1 is limited to 90–150 min. In this interval depolymerizates with  $M_w$  between 21000 and  $13000 \text{ g} \cdot \text{mol}^{-1}$  are obtained (Table 1).

Similar  $M_w$  values with relatively small deviation were also obtained with NP-2 pullulan ( $M_w = 328000 \text{ g} \cdot \text{mol}^{-1}$ ) under the same conditions. This shows that the depolymerization velocity does not depend on initial pullulan molar mass in this interval. Therefore, in the second series, analogously to complexes when low molar dextran is used ( $M_w = 5000 \text{ g} \cdot \text{mol}^{-1}$ ), for the depolymerization of NP-2 pullulan higher temperature and longer time intervals (0–180 min) were applied at the same pH value. With this sample, in the interval of 130–180 min (Table 2), depolymerizates with  $M_w$  under  $10000 \text{ g} \cdot \text{mol}^{-1}$  could be obtained.

**Table 1: Characteristic parameters of native pullulan NP-1 and its oligomers, obtained by depolymerization with HCl (pH 2,  $80^\circ\text{C}$ , 90–150 min)**

Sample ( $c = 6.3\%$ )	t (min)	$\eta_r$	$[\eta]$ ( $\text{dl} \cdot \text{g}^{-1}$ )	$P_w$	$M_w$ ( $\text{g} \cdot \text{mol}^{-1}$ )
NP-1	—	—	—	1362	220570
Dep-1	90	1.500	0.147	125	20313
Dep-2	100	1.481	0.142	119	19291
Dep-3	110	1.462	0.138	114	18485
Dep-4	120	1.423	0.129	103	16715
Dep-5	130	1.385	0.118	90	14633
Dep-6	140	1.365	0.113	85	13718
Dep-7	150	1.356	0.111	82	13357

**Table 2: Characteristic parameters of native pullulan NP-2 and its oligomers, obtained by depolymerization with HCl (pH 2,  $85^\circ\text{C}$ , 0–180 min)**

Sample ( $c = 7.1\%$ )	t (min)	$[\eta]$ ( $\text{dl} \cdot \text{g}^{-1}$ )	$M_n$ ( $\text{g} \cdot \text{mol}^{-1}$ )	$P_w$	$M_w$ ( $\text{g} \cdot \text{mol}^{-1}$ )	$M_w/M_n$
NP-2	—	—	—	2025	328000	—
Dep-8	5	0.233	29074	249	40396	1.389
Dep-9	10	0.209	26002	212	34346	1.321
Dep-10	30	0.184	14301	175	28399	1.986
Dep-11	50	0.157	10769	138	22410	2.081
Dep-12	80	0.131	8993	106	17104	1.902
Dep-13	100	0.106	7929	77	12469	1.573
Dep-14	130	0.091	5378	61	9929	1.846
Dep-15	140	0.085	4767	55	8968	1.881
Dep-16	150	0.071	4724	42	6856	1.451
Dep-17	180	0.050	2672	25	4062	1.520

The influence of pullulan concentration on depolymerization showed slight variations within the limits of experimental error. Thus, in the observed time interval concentrations could be neglected. Depolymerization increases considerably the content of reducing saccharides. Experiments have shown that unfavorable ligand qualities for the outcome of complex preparation arise precisely from their reducing activity. In preparation with higher contents of RG, sedimentation occurs. Decreasing complex stability increases toxicity. Therefore, the reducing activity of the obtained pullulan oligomers has been decreased by  $\text{NaBH}_4$ . In accordance with kinetic laws, hydrogenation of pullulan oligomers is a pseudo first order reaction [14].

### 2.2. Synthesis of polynuclear iron (III) complexes with pullulan

On the basis of numerous data [4, 5], which refer to the synthesis of iron(III) complexes with different carbohydrates, it can be concluded that there are no fundamental differences among many quoted procedures.

The influence of molar masses of ligands on creating of complexes was investigated. Five depolymerizates with the ligands masses from  $4000$  to  $15000 \text{ g} \cdot \text{mol}^{-1}$  were used (Table 3). According to published data about dextran complexes [16], we expected a positive outcome of complex synthesis.

Data in Table 3 show that high molar pullulan does not lead to successful results. It is more convenient to use depolymerizates of  $6000$ – $10000 \text{ g} \cdot \text{mol}^{-1}$ . That confirms an earlier assumption that the average molar mass of the ligand has a limiting value which secures binding with  $\text{Fe}(\text{OH})\text{O}$  particles. In favor of that are results with depolymerizates Dep-7 and Dep-17 with masses higher or smaller than necessary. They do not clarify the reaction mixture at all (i.e. dissolve solid forms of  $\text{Fe}(\text{OH})\text{O}$ ), not even under prolonged or more drastic conditions (autoclave). In the progress of the synthesis we have observed a second expected phenomenon. Boiling the solution under atmospheric pressure does not lead to dissolution of  $\text{Fe}(\text{OH})\text{O}$  and changing of the color of the reaction system (e.g. Dep-7 and Dep-15). Under more drastic conditions (prolonged time, higher temperature and pressure), depending on pH (9.5–11.5), complexes are formed only partially (~60% of dissolved  $\text{Fe}(\text{OH})\text{O}$ ) with iron contents of  $5.9 \text{ mg} \cdot \text{cm}^{-3}$  in the soluble fraction (Dep-7). It is evident that at higher pH values the synthesis should afford higher iron contents in the complex. At the same time, with dissolution of  $\text{Fe}(\text{OH})\text{O}$  the color change from red into distinctly gray-brown shades. This might be the

**Table 3: Basic characteristics of pullulan oligomers and reaction conditions for complex synthesis**

Ligand	t <sub>DEP</sub> (min)	M <sub>w</sub> (g · mol <sup>-1</sup> )	pH	T (°C)	t <sub>SYNT</sub> (min)	+T* (°C)	+t <sub>SYNT</sub> (min)	Synth. result	Fe content (mg · cm <sup>-3</sup> )
NP-2	0	328000	10.5	80	120	140	120	—	—
NP-1	0	220570	10.5	70	120	140	90	—	—
Dep-7	150	13357	10.5	100	120	—	—	—	—
Dep-7	150	13357	9.5	70	120	120	90	—	—
Dep-7	150	13357	10.5	70	120	120	90	30%	3.2
Dep-7	150	13357	11.5	70	120	120	90	60%	5.9
Dep-15	140	8968	10.5	100	120	—	—	40%	4.8
Dep-15	140	8968	10.5	80	120	120	90	100%	31.2
Dep-16	150	6856	10.5	70	120	100	180	80%	11.3
Dep-16	150	6856	10.5	80	120	120	60	60%	5.1
Dep-16	150	6856	10.5	80	60	105	60	40%	4.5
Dep-17	180	4062	10.5	80	120	100	90	—	—

T\* and t\* relate to the synthesis of the complexes in an autoclave, at 200 kPa

result of alkaline degradation of the ligand in the progress of synthesis, which results in the presence colloidal dispersed reduced iron [5]. At complexes obtained with depolymerizates of  $M_w = 6856 \text{ g} \cdot \text{mol}^{-1}$  (Dep-16), during the first 24 hours after synthesis visible sedimentation of the solid phase occurs. That indicates insufficient stability of the complex, but with achieving a little bit higher iron contents than in the previous case. The uncompleted synthesis (~80% of dissolved Fe(OH)O), is probably due to inadequate conditions, in accordance to the ligands molar mass. Complete synthesis of complex, i.e. obtainment of a clear dark-red reaction mixture, was accomplished with depolymerizates Dep-15 and iron contents of  $31.2 \text{ mg} \cdot \text{cm}^{-3}$ .

Regarding that complex synthesis can be realized only under drastic conditions, analogously to the synthesis of dextran complex with iron [17], in further experiment the synthesis was conducted in an autoclave at 200 kPa and at 100–130 °C. Since the molar mass of the used ligand had been determined earlier, and for finding the optimum of molar mass, we used two more depolymerizates Dep-13 and Dep-20 (Table 4).

Complete reaction could be achieved with pullulan oligomers in masses of 8000–10000  $\text{g} \cdot \text{mol}^{-1}$ . Maximum iron contents of 51.4 mg per unit of preparation volume were reached by depolymerizates Dep-15. The result of synthesis with other depolymerizates is in accordance with previous facts, establishing the basis to obtain complexes with higher iron contents per volume. Possibility and result of iron pullulan complex synthesis were confirmed by FTIR spectroscopic studies [18].

### 2.3. Preliminary toxicological examinations of iron pullulan complex

For the pharmacological test, the iron complex with Dep-15 ligands (Table 4) was standardized on  $50 \text{ mg Fe/cm}^3$ , in 0.9% sodium chloride solution, at pH = 6.5 and the mass ratio Fe/ligand 1:2.84. The preparation was tested for acute toxicity (LD<sub>50</sub>) using white mice of both sexes, 18–26 g. Application was conducted intravenously (dose of 900–1900  $\text{mg} \cdot \text{kg}^{-1}$ ). None of the applied doses showed mortality within 10 days. Thus, the lethal dose could not be determined. Application of higher doses was not realizable because of the application volume, which could not be increased in consideration of standardized preparation. It can be concluded that the max. applicable dose is  $1900 \text{ mg} \cdot \text{kg}^{-1}$  at the iron concentration built-in the complex of  $50 \text{ mg} \cdot \text{cm}^{-3}$ .

The effected concentrations, complex stability, and acute toxicity respond to requirements of a pharmacological application of parenteral antianemic preparations. Our results are preliminary, but they contribute to our knowledge concerning the preparation of optimum ligands for successful synthesis and for conditions of synthesis, not only of the examined iron complex with pullulan but of similar polysaccharides. Research should be conducted to standardize partially depolymerized carbohydrates and to improve their physicochemical and pharmacobiological characteristics.

## 3. Experimental

### 3.1. Materials

Native pullulan (NP) was produced by biosynthesis with our own species (CH1, Pharmaceutical and Chemical Industry “Zdravlje”, Leskovac-Yu), using the fungus *Aureobasidium pullulans*. Two samples of pullulan were

**Table 4: Reaction conditions and characteristic parameters of complex synthesis with different pullulan oligomers of NP-1 and NP-2**

Ligand	M <sub>w</sub> (g · mol <sup>-1</sup> )	M <sub>n</sub> (g · mol <sup>-1</sup> )	[η] (dl · g <sup>-1</sup> )	RG (%)	T (°C)	t <sub>SYNT</sub> (min)	pH	Synth. result	Fe content (mg · cm <sup>-3</sup> )
Dep-7	13357	7004	0.110	5.72	100	180	11.0	—	15.9
Dep-13	12469	7929	0.106	6.34	100	180	11.0	—	16.2
Dep-14	9929	5378	0.091	7.59	120	150	10.5	+	26.3
Dep-14	9929	5378	0.091	7.59	120	180	10.5	+	28.4
Dep-14	9929	5378	0.091	7.59	130	240	10.5	+	28.6
Dep-15	8968	4767	0.085	10.74	130	210	10.5	+	38.8
Dep-15	8968	4767	0.085	10.74	130	240	10.5	+	51.4
Dep-20	8040	5020	0.079	7.01	105	120	10.5	+	28.5
Dep-16	6856	4724	0.071	12.29	100	180	10.5	—	21.3

used. Different pullulan fractions were characterized by the light-scattering method on molar mass: NP-1 ( $M_w = 220570 \text{ g} \cdot \text{mol}^{-1}$ ) and NP-2 ( $M_w = 328000 \text{ g} \cdot \text{mol}^{-1}$ ).

### 3.2. Methods

The amorphous  $\beta_2$ -form of  $\text{Fe}(\text{OH})\text{O}$ , as the only applicable form for this synthesis, was prepared by sedimentation from ferric chloride solution using sodium carbonate at room temperature [15]. The precipitate, after removing electrolytes, was directly used for the synthesis of the complex. This form of exceptional quality of  $\text{Fe}(\text{OH})\text{O}$  was used because this gel gives complexes with hydrogenated low molar dextran and dextran carboxylic acid [3].

Native pullulan was depolymerized by  $\text{HCl}$ , pH 2, 80–85 °C, 0–180 min. For obtaining fractions with as much as possible a narrow distribution of molar masses, all depolymerizates were fractionally precipitated by ethanol, in order to remove ligands of low molar masses from fractions, which were prepared for the reaction with iron. Average weight molar masses ( $M_w$ ) of the pullulan oligomers were determined by the viscosimetric method based on Khun-Mark-Houwink's equation, adapted for pullulan by Kato [19]:

$$[\eta] = 1.91 \cdot 10^{-2} \cdot M_w^{0.67} \quad (\text{in cm}^3 \cdot \text{g}^{-1}) \quad (1)$$

Average number molar masses ( $M_n$ ) and the contents of RG were determined by Somogyis method [20]. Viscosimetric measurements were performed at 25 °C using Ostwalds viscosimeter [21]. Values of specific and intrinsic viscosity  $[\eta]$  were calculated by Schultz-Blaschke's equation.

The oligomers were reduced with  $\text{NaBH}_4$  at 40 °C [22]. The quantity of  $\text{NaBH}_4$  (96% activity) necessary for hydrogenation (Q), which depends of RG contents and initial ligands mass (m), was calculated by an empiric formula:

$$Q \text{ (mg)} = 0.95 \cdot 10^{-3} \cdot \text{RG} (\%) \cdot m \text{ (g)} \quad (2)$$

The excess of unreacted  $\text{NaBH}_4$  was destroyed by  $\text{HCl}$  and by further treatment of the depolymerization solution on 70 °C.

Solutions both of depolymerized pullulan and complex were purified and deionized by acidic and alkaline ion-exchangers (Amberlit IR-120 and IRA-410). The iron content in the preparations was determined complexometrically. Chlorides in water solutions both of ligands and complexes were determined by potentiometric titration with 0.1 M  $\text{AgNO}_3$ .

Polynuclear  $\beta\text{-Fe}(\text{OH})\text{O}$  and its complexes with pullulan were synthesized according to Müller [5]. Methods of complex preparation involve neutralization of  $\text{FeCl}_3$  solution with an alkaline solution of modified pullulan. The synthesis was realized either at boiling temperature under atmospheric pressure or in an autoclave at 200 kPa until 140 °C, at a mass ratio Fe/ligand 1:3, and different reaction conditions (pH, T, t). Success of synthesis was defined as complete dissolution of the  $\text{Fe}(\text{OH})\text{O}$  gel and as changing of the light-brown color of the initially homogeneous reaction mixture into dark-red, characteristic for polynuclear iron complexes with carbohydrates.

The test of systematic acute toxicity was executed by the Litchfield-Wilcoxon method [23], on white mice of both sexes, 18–26 g. To groups of 6 mice the following doses were successively applied: 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800 and 1900  $\text{mg} \cdot \text{kg}^{-1}$  intravenously. During ten days the percentage of expiring was followed. These tests were conducted in the biomedical laboratory, Sector of applied research and development, "Zdravlje" Leskovac.

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