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Perspective

Current knowledge on biosynthesis, biological activity, and chemical modification of the exopolysaccharide, pullulan

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Abstract—The article presents an overview of the latest advances in investigations of the biosynthesis, molecular properties, and associated biological activity of pullulan. The literature survey on the pullulan biosynthesis is intended to illustrate how the great variety of environmental conditions as well as variability in strain characteristics influences the metabolic pathways of the pullulan formation and effects structural composition of the biopolymer. Molecular properties of pullulan as α-(1→4)- and α-(1→6)-glucan are discussed in terms of similarities with amylose and dextran structures, and an emphasis is made on the inherent biological activity of pullulan molecules. The author also attempts to summarize the concepts, options, and strategies in chemical modification of the biopolymer and to delineate future prospects in designing new biologically active derivatives.

Contents

1. Introduction

The first description of the water soluble, neutral polysaccharide, pullulan, should be dated back to the beginning of the 1960s when the group of Wallenfels purified and analyzed the polysaccharide from fermentation medium of *Aureobasidium pullulans*.[1] Elemental analysis revealed that the content of carbon and hydrogen atoms in this exopolymer corresponds to that known for compounds having chemical formula $C_6H_{10}O_5$. The polymer complexes Cu^{2+}, and in contrast with starch, gives no color reaction with I_2. Based on IR spectroscopic data it was concluded that both α-(1→4)- and α-(1→6)-linkages are present in the polysaccharide. The purified polysaccharide has a molecular

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weight of ca. 250 kDa and shows an optical rotatory activity of +192° in a 1 g/dL solution. This biopolymer was named pullulan.

Afterwards, several research groups reported[2,3] that the structure of pullulan could not be described as consisting solely of α-(1→4)- and α-(1→6)-linkages since a small proportion of α-(1→3)-linkages are also present in the structure. The latter finding gives rise to the opinion that different strains of *A. pullulans* produce nonidentical pullulans with respect to their structure and composition.[4,5]

Evidence of structural uniformity of pullulan produced by different strains of *A. pullulans* was provided by Taguchi et al.,[6] who showed that the structure of pullulan is not dependent on the strain used, as it was believed earlier. In the author's view, discrepancies in this question arose due to inadequate purification of pullulan from the water-insoluble acidic polysaccharides produced by *A. pullulans*. To separate pullulan from an insoluble jelly-like polysaccharide Kikuchi et al. developed the procedure of purification comprised of treatment of the culture media with acetyltrimethylammonium hydroxide.[7]

It is now widely accepted that pullulan is a linear polysaccharide with maltotriosyl repeating units joined by α-(1→6)-linkages. Alternatively, the structural formula of pullulan may be presented as a regular sequence of panoses bonded by α-(1→4)-linkages (Fig. 1). As it will be shown below, the latter representation seems to be more correct from the viewpoint of the mechanism of pullulan biosynthesis. Minor structural abnormalities are reported in pullulan, for example, a careful hydrolysis of pullulan by exo- and endoenzymes performed by Catley et al.[8] showed chain fragments resistant to the action of enzymes. Such resistance was attributed to the presence of maltotetraose residues distributed randomly along the pullulan chain. However, these structural abnormalities should not affect the overall physicochemical properties of pullulan.[8]

The producer of pullulan, *A. pullulans*, is a black yeast widely spread in all ecological niches including forest soils, fresh and sea water, plant and animal tissues, etc. Generally, the culture of *A. pullulans* is classified as nonpathogenic; however, some strains may cause health problems.[9–11] Pullulan has been commercially produced since 1976 by the Hayashibara Company Ltd (Okayama, Japan), which remains the principal supplier. Recent arrangements with Pfizer for production of consumer products such as Listerine PocketPacks® oral care strips may result in expanded markets for pullulan.

A frequently cited work reviewing the application of *A. pullulans* in biotechnology appeared in 1992,[12] but the latest thorough review of the peculiarities of the pullulan biosynthesis with this culture was published in 1981.[13] Since then, new data and developments have been reported in numerous studies, so that an overview of current knowledge on pullulan biosynthesis and applications is timely. This contribution will focus on the results of investigations carried out in the past 10 years, though important data from earlier studies will not be ignored.

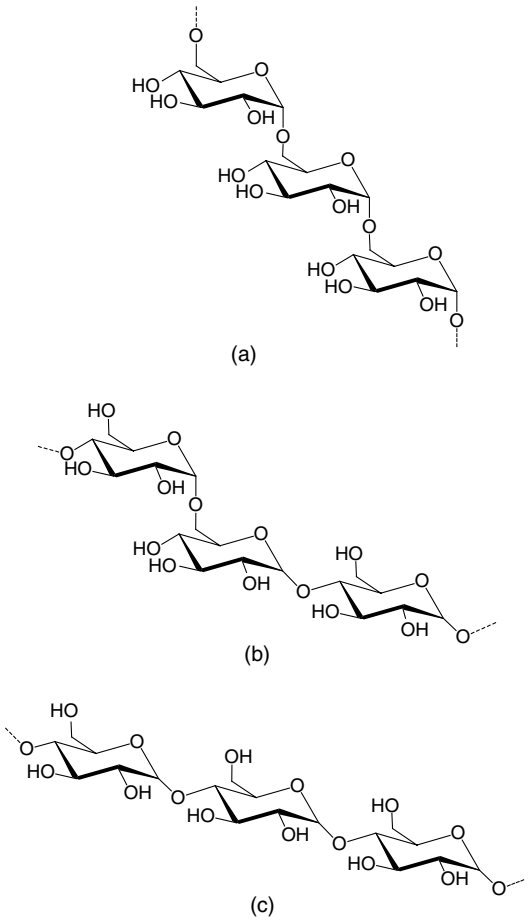

Figure 1. Structural formula of the repeating unit of pullulan (b) [α-(1→4)- and (α-(1→6)-linkages] in comparison with dextran (a) [(α-(1→6)-glucan] and amylose (c) [(α-(1→4)-glucan] structures.

The major attention in the fermentation studies of *A. pullulans* was devoted to developing optimal cultivation conditions while maintaining a high productivity of the cells. The main objectives were high yield, short fermentation time, low cost, and high purity of the final product to meet the stringent requirements for food, cosmetic, and pharmaceutical applications.

Current applications of pullulan as a low-calorie food additive with excellent film-forming properties have been well described in book chapters,[14,15] whereas its bioactive potential has attracted less attention. Accordingly, this survey focuses on the molecular properties (for the sake of clarity this is done in comparison with the well-characterized nearest analogs, amylose and dextran), associated biological activity of pullulan as well as recent advances in the biomedical

applications of this polysaccharide. By delineating the major directions in investigations of this polysaccharide, we will suggest perspectives for further work.

2. Biosynthesis of pullulan

2.1. Exopolysaccharides produced by *A. pullulans*

Even in the first works on pullulan biosynthesis researchers observed that the culture produces two different exopolysaccharides. One of these polymers corresponds to pullulan, and the second is frequently described as a water-insoluble jelly-like material. An electron microscopy study of cell walls of *A. pullulans* performed by Simon et al.[16] revealed that both pullulan and the insoluble polysaccharide are localized on an outer surface of the chlamydospores, the cells that were considered as the main polysaccharide producer on nongrowth media. The highly dense peripheral layer was ascribed to the chains of pullulan arranged in a network covering the inner layer of β-(1→3)-glucan composed of glucose and mannose[16] (Fig. 2). Elinov reported that the β-glucan produced by *A. pullulans* differs from the polysaccharides of the cell wall and has the following structure:[17]

$$\left[\begin{array}{l} \qquad\qquad [4\alpha\text{-D-Glu } 1\rightarrow 4\alpha\text{-D-Glu } 1]_n \\ \qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\quad 6 \\ \beta\text{-D-Glu } 1\rightarrow 3\beta\text{-D-Glu } 1\rightarrow 3\beta\text{-D-Glu } 1 \end{array}\right]_m$$

where $m = 4409$–$13{,}900$ and $n = 0.5$–2.

The main chain of the polymerized β-D-glucose residues is constant, whereas the branches at the C-6-position may involve up to four α-D-glucose residues, depending on the cultivation conditions.[17]

An extracellular origin of the β-(1→3)-glucan was not commonly accepted.[18] Kataoka-Shirasugi et al. have fractionated polysaccharides of the cell wall of *A. pullulans*[19] and found that some of the purified polymers were β-(1→3)-glucans with branches at the C-6 position, similar to the structure depicted above. This β-glucan may possess weak anionic properties due to the presence of malate residues linked to the polymer through ester bonds.[20]

Very little is known up to now explaining how the mechanism of biosynthesis of these jelly-like glucans is associated with the pullulan elaboration, though there were indications that the elaboration of insoluble exopolysaccharides is dependent on genetic type of *A. pullulans*.[21] In particular, it is not clear yet whether environmental conditions, for example, pH or morphological changes of the cells are responsible for its extracellular elaboration.[18,21–24] One can expect, however, that the recently resumed practical interest in this biopolymer[19,20] as a compound with antitumor activity will stimulate intense investigation and provide answers to these questions.

Besides the β-glucans, two physically distinct polysaccharides with identical chemical structures corresponding to that of pullulan were recovered from the ethanol precipitates of a cell-free broth by Madi et al.[25,26] This phenomenon was interpreted as due to the presence of pullulan fractions with different molecular weight as a result of inconsistency of the biosynthetic pathways by the fungus. However, the explanation used in those studies contradicts the well-established facts of the strictly regulated (in particular, by cell morphology) character of the polysaccharide biosynthesis, which usually produces pullulan with the narrow molecular-weight distribution.[13,17] The investigations of the rheological behavior of the pullulan broth hypothesized a complex structure of pullulan fluid where the aggregated states of the polymer in a media are sensitive to both changes in pH and mechanical impacts.[27] In view of the fact that the fractions recovered by Madi et al. were of very close molecular weight to be resolved simply by ethanol precipitation,[26] their appearance seems to be associated with the differences in the structure of the microbial pullulan suspension, as proposed by Toda et al.[27]

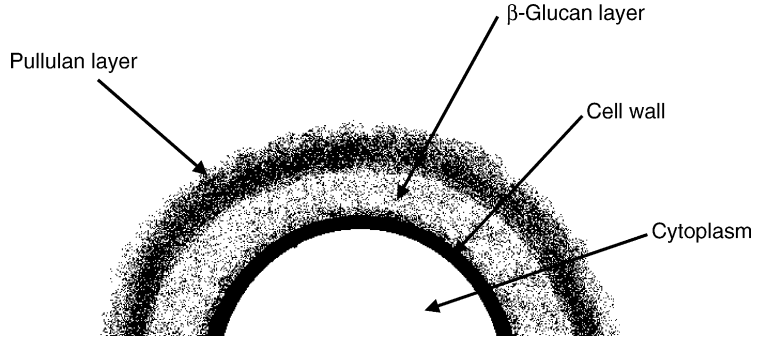

Figure 2. Schematic illustration of the localization of polysaccharides layers on the outer surface of *A. pullulans* cells.

2.2. Influence of pH and cell morphology

Relatively low initial pH (pH 2.5) suppresses synthesis of pullulan but stimulates elaboration of the insoluble glucan.[25] An optimal value of pH for the pullulan production lies in the range between 5.5 and 7.5.[28–34] There were only a few reports where highest pullulan content was achieved by cultivating the microorganism at acidic pH.[35] In the experiments with the strain, *A. pullulans* IAM 5060, Toda et al. found that even a small deviation in the pH near neutral values affects the productivity of the cell.[27]

It is of interest to note that the optimal pH established for the biomass growth is 4.5 or lower.[24,29] This difference in optimal values of pH for the pullulan synthesis and the cell mass growth indirectly correlates with the independent character of these two processes.[13] However, the relationship between morphology and the polysaccharide-producing capacity of the culture cannot be ignored since the polysaccharide elaboration is known to be associated with the specific cell morphology,[16] though the exact cellular type responsible for pullulan synthesis is still a matter of debate (see Ref. 36 and references therein). In an overwhelming number of studies, pullulan elaboration was found to occur only with the yeast-like morphology of *A. pullulans*,[36] whilst in other several papers the ability to synthesize the polysaccharide was the characteristic of the chlamydospore population.[16,37] At least there is convincing agreement among researchers that pH provokes morphological changes of cells, which in turn may additionally differentiate biosynthetic routes. The yeast-like cells at neutral pH produce pullulan of a very high molecular weight,[34] whilst combined cultivation of the mycelial and the yeast-like cellular forms can be beneficial for high pullulan concentration.[30]

2.3. Mechanism of pullulan biosynthesis

Despite intense investigations of cytological and physiological characteristics of *A. pullulans*,[12,18,23,38] the mechanism of pullulan biosynthesis is far from being fully understood. Synthesis of ATP in the cells of *A. pullulans* is realized via the pentose monophosphate route. Experiments with radiolabeled glucose added to the medium of *A. pullulans* showed that C-1 of glucose is transformed into carbon dioxide.[39] Fast increase in the concentration of CO_2, accompanied by a reduction of the dissolved oxygen concentration,[40] can be monitored by a downward turn of pH in the beginning of fermentation. It is worth mentioning that an uptake of sugars is followed by an elaboration of ethanol[41–43] that is subsequently utilized by the fungus as the concentration of dissolved oxygen increases.[43] One can expect that acidic compounds produced upon ethanol oxidation also contribute to the observed initial decrease of pH. Recovery of pH is usually observed in the late stages, owing to an increase of the mass transfer at aeration. Thus, in general, the pH profile of cultivation has a characteristic U-like pattern,[30,41,44] more or less prolonged in time, depending on the rates of substrate consumption and oxygen supply.

Pullulan can be synthesized from sucrose by cell-free enzymes of *A. pullulans* when both ATP and UDPG are added to a reaction mixture.[24] UDPG cannot be replaced by ADPG, indicating that the pullulan chains or pullulan precursors originate from UDPG. Additionally, it was proved that the processes of transglycozilation of ADP-glucose, known as the major routes of dextran and starch genesis, do not occur in a pullulan formation.[13] Certain experimental results evidenced that glucose-containing lipid intermediates play a crucial role in the pullulan biosynthesis.[45] By summarizing these data with their own results, Catley and McDowell[46] have proposed the following order of the biochemical events preceding pullulan formation (Fig. 3). The first stage is the UDPG-mediated attachment of a D-glucose residue to the lipid molecule (LPh) with a phosphoester bridge. A further transfer of the D-glucose residue from UDPG (step 2) gives lipid-linked isomaltose. In the next step, isomaltosyl participates in the reaction with lipid-linked glucose to yield an isopanosyl residue (Fig. 3). Further, isopanosyl residues are polymerized into the pullulan chain.

Besides glucose or sucrose, the culture of *A. pullulans* is able to consume mannose, galactose, fructose, and other sugars as carbon sources. The pathways of pullulan formation on these media are not clear. It is only known that, in the case of maltose-containing media, the carbohydrate metabolites needed for the polymer formation, that is, panose [α-Glc-(1→6)-α-Glc-(1→4)-α-Glc] and/or isomaltose [α-Glc-(1→6)-α-Glc], can be synthesized via a glucosyl-transfer reaction in *A. pullulans*.[47]

As reported in, Ref. 48 the pathways of pullulan production on a nongrowth medium, apart from direct conversion of glucose residues into the polysaccharide, may involve polymerization of the carbohydrate precursors stored inside the cells. It was believed that the

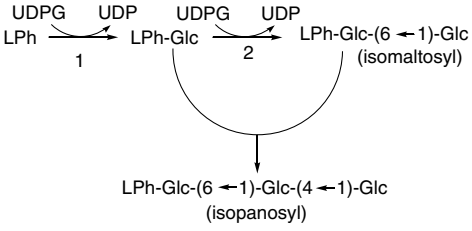

Figure 3. Scheme for the biosynthesis of the pullulan precursor (isopanose) with participation of the phospholipid intermediate (LPh) and its glucose conjugates (LPh-Glc), as proposed by Catley and McDowell.[46]

cells first accumulate sugars and use this carbohydrate reserve for pullulan production in later stages of the culture life cycle. The experimental evidence for this hypothesis was obtained by Catley and Kelly[49] and, more recently, by Simon et al.[37] who found an inverse correlation between the concentration of pullulan and the content of intracellular glycogen. Although the mechanism in accordance to which glycogen is transformed into pullulan is not well understood, the latter findings are useful to explain the independent character of the processes of pullulan production and cell mass growth.

2.4. Substrates and efficiency of pullulan fermentation

Although the literature on pullulan biosynthesis is contradictory because of differences among the numerous strains of *A. pullulans*, it was clearly demonstrated that the yield of pullulan strongly depends on the rate of substrate conversion.[13] Moreover, the concentration of the polysaccharide produced by *A. pullulans* is dependent on the carbon source. The wild strain of *A. pullulans* segregated by Bender et al.[1] consumed 22 g/dL of glucose and 20 g/dL of sucrose during 5 days for the polysaccharide production, yielding 1.3 g of the exopolymer per 1 g of dry cells. It is important to note that the exopolymer synthesis was also observed on a fructose-containing medium. On a medium containing maltose as a carbon source, the wild fungus intensively grew, but had low pullulan-producing activity.

The problem of low pullulan-producing activity was solved later by the development of several mutant strains of *A. pullulans* with improved ability to synthesize pullulan.[50–53] By using these cultures it became possible to perform large-scale fermentation processes under well-controlled conditions. Progress was particularly stimulated by the development of new fermentation reactors designed to maintain high productivity of the culture.[40] In addition, visual inspection methods[36,54] and several analytical techniques, including capillary electrophoresis and high performance liquid chromatography,[41,55,56] were applied successfully to monitor changes in the cell morphology and carbohydrate composition of the cultivation broth. In particular, chromatographic analysis revealed very rapid degradation of sucrose into glucose and fructose with subsequent utilization of resultant sugars, thus providing experimental evidence that explains the high yields of conversion of substrate into the polysaccharide.[41]

In order to reduce the cost of the fermentation product, pullulan biosynthesis from the hydrolyzates of potato starch waste was studied.[55] Fermentation of *A. pullulans* on a medium containing 20% maltose-rich hydrolyzates yields 115% higher concentration of pullulan than that obtained on glucose syrup, indicating that maltose is a better substrate than glucose for pullulan production by the studied strain of *A. pullulans*.[57] Other wastes from the agricultural and food industries, such as deproteinized whey,[58] beet molasses,[44,59] sugar cane juice, and even peat hydrolyzate[60] are also considered as economical and efficient substrates for the pullulan production. An exhaustive literature survey devoted to the use of different industrial wastes for pullulan production and the problems associated with the recovery and characterization of the final product has been recently presented.[44]

A complex monomeric composition of extracellular polysaccharides produced by *A. pullulans* may be encountered not only in the cases of waste materials, but also with the use of well-defined glucose analogs like, for example, 3-*O*-methyl-D-glucose, D-glucosamine, and *N*-acetyl-D-glucosamine.[61–63] By using these substrates, some groups have synthesized the exopolymer containing glucose and mannose, only,[62] whereas in other studies a more complicated polysaccharide molecular structure was obtained.[63]

Typical yield values of pullulan fermentation that are claimed in the literature are summarized in Table 1. The efficiency of fermentation (FE) calculated as a ratio of the amounts of pullulan obtained to the substrate loaded does not vary greatly and assumes the values between 0.40 and 0.63, indicating that the mutant strains of *A. pullulans* in current use are almost identical with respect to their polysaccharide-producing activity. One has, however, to keep in mind that pullulans produced by different strains may greatly differ in their degree of polymerization. As the pullulan yields claimed in most studies are estimated by weighting of the dry residue after removal of cells, or for instance, on the basis of the amount of maltotriose released after enzymatic hydrolysis of the polymer,[69] that is, without indication on the molecular weight, molecular-weight distribution, or degree of polymerization, the more detailed relationships between the amounts of substrate consumed and

Table 1. Characteristics of the pullulan fermentations

Initial substrate (g/dL)	Pullulan (g/dL)	FE[a]	Reference
	Glucose		
30	13.0–15.0	0.43–0.50	67
50	20.0	0.40	64
50	23.4	0.47	65
80	~50.0	0.62	66
100	23.3	0.23	56
	Sucrose		
50	30.0	0.60	68
50	30.2	0.60	41
50	30.0	0.60	31
100	34.4	0.34	56

[a] FE means fermentation efficiency calculated as a ratio of the amounts of pullulan obtained to the substrate loaded.

the polymerization degree of the final product still need to be determined. A definition of the chemical structure of the polysaccharides becomes especially important when the cultivation of *A. pullulans* is performed on the substrates with unknown chemical composition or industrial wastes since molecular structure of pullulan in this case may vary greatly.[70]

2.5. Molecular-weight distribution of native pullulan

The average molecular weight of pullulan varies in very broad ranges, from hundreds to thousands of kiloDaltons, depending on the culture strain, pH,[69,70] cultivation techniques,[71] and substrates used.[68] On a fructose-containing medium, the fungus produces high-molecular-weight pullulan, whilst the use of sucrose results in the highest yields of the polymer.[56] Nitrogen source also influences the molecular weight of the exopolymer. For certain strains of *A. pullulans* it was established that a mixture of peptone, ammonium sulfate, and urea can be the best combination with respect to the high yield and molecular weight of pullulan.[56]

In the initial stages of biosynthesis, pullulan chains produced from glucose, maltose, or sucrose are characterized by a high molecular weight, though the molecular-weight distribution (MWD) of the polymer may not be unimodal.[41,62] The molecular-weight distribution becomes narrow late in the stationary growth phase owing to increase of the relative amounts of the high- and medium-molecular-weight fractions.[41] The definition of breadth of MWD can be given in the term of polydispersity, which is usually calculated as the ratio between weight-averaged, M_w, and number-averaged, M_n, molecular weights: M_w/M_n. The values of M_w/M_n reported for pullulan lie between 2.1 and 4.1[41,56,68] and are significantly lower than the corresponding values known for other industrially important polysaccharides (amylose, dextran). A possible explanation of this may be the difference in the biosynthesis pathways or the cell morphology-regulated mechanism of pullulan formation.

2.6. Fermentation techniques

The influence of aeration on vital activity of cells producing pullulan was studied in detail.[12,18,23,38] Under anaerobic conditions, the cell population neither grows nor produces pullulan. An intense aeration during fermentation leads to a significant increase of pullulan concentration. This effect is especially pronounced on a nitrogen-rich media. An inverse effect of aeration was detected upon fermentation on the media deficient in nitrogen source, where intense aeration suppressed pullulan production.[45] Audet et al. commented that although a higher dissolved oxygen concentration favors higher productivity on a balanced nutrient medium, an intense aeration should be used with care since the molecular weight of pullulan under well-aerated conditions could be reduced.[72]

An increase of the oxygen transfer rate achievable by increasing a gas partial pressure may improve the polysaccharide-producing activity of *A. pullulans*, as it was evidently demonstrated.[73] High airflow rates and high working pressure is beneficial for the growth of cell mass and pullulan synthesis. However, when pressure exceeds the critical values of 0.5–0.75 MPa, a spontaneous aggregation of the cells with a complete cessation of pullulan production is observed. These phenomena were explained as being induced by an increased concentration of carbon dioxide at higher pressure.[73]

In order to prevent cell disintegration, cell immobilization procedures were applied to pullulan production.[74–76] The elaboration of pullulan using cells of *A. pullulans* entrapped in agarose and carrageenan was studied by West.[76] Both immobilized systems were found technically acceptable for pullulan production; however, the highest content of pullulan was obtained with the use of agarose-entrapped culture. Other researchers noticed that this method is inconvenient for pullulan production because of the several undesirable events, including the restriction of polysaccharide diffusion through microporous sorbents and the destruction of the immobilization system due to a rapid increase of the entrapped biomass.[74,75]

Another approach to stabilize growth conditions and thereby increase pullulan yield is the use of continuous fed-batch cultivation.[32] An optimization of fed-batch cultivation was performed by the investigation on the effect of feed mode and composition of the feed solution on the efficiency of pullulan fermentation.[71] The fed-batch culture gives high pullulan yields; however, the higher rates of pullulan production and substrate uptake, and the higher FE values, are characteristics of the traditional batch cultivation.[32]

In conclusion, the literature data cited here clearly indicate that the great variety of environmental conditions, as well as variability in strain characteristics, influence the metabolic pathways of the pullulan biogenesis. From the biochemical point of view, the mechanism of the pullulan biosynthesis appeared to have a very complex pattern. In this context, evident knowledge regarding the mechanism of the substrate transformations as well as the routes of pullulan genesis could help to control molecular weight, molecular-weight distribution, and architecture of pullulan directly in a course of fermentation. From the technological point of view, the clarity in this question is necessary to provide ecological safety in the biosynthesis process. As noted in Ref. 62 there is a potential possibility to modulate the structural composition of exopolysaccharides produced by *A. pullulans* by varying the chemical nature of the carbon source.

3. Molecular and hydrodynamic properties of pullulan

Pullulan structure is intermediate between amylose and dextran structures because of the co-existence of the both α-(1→4)- and α-(1→6)-linkages in a single compound (Fig. 1). The unique molecular properties of pullulan that originated from this structural feature have been studied intensively by computational methods and by various instrumental techniques.

Pullulan has been the object of numerous ^{13}C and ^{1}H NMR spectroscopy studies.[77–82] Benesi and Brant completely assigned the NMR signals for all carbon atoms in the repeating unit of pullulan,[77] and Dais et al. investigated the temperature dependence of the spin–lattice relaxation times of pullulan ring carbons.[81] It was found that α-(1→4)-maltooligomers have higher relaxation times in comparison with the corresponding isomaltooligomers, which confirms a more flexible character of α-(1→6)-linked oligosaccharides.[78,81,83] Amylose is expected to have a relatively smaller conformational space than dextran because of presence of the α-(1→4) linkage (Fig. 1). Due to the specific interunit bonding, this polysaccharide is known to adopt a double-helix conformation. In the case of dextran, the occurrence of the α-(1→6)-glycosidic bond provides for an increase of the chain mobility. Consequently, the segmental mobility of the pullulan backbone is not uniform, with the regions of increased mobility centered on the α-(1→6)-linkages.[84] On the other hand, certain computational data indicated that the pullulan macromolecule might exhibit slight helical twists,[85,86] thus mimicking the behavior of amylose. It was established[77] that the minimal fragment resembling conformational motion of the entire pullulan chain should comprise at least 15 glucose residues, whereas the corresponding lengths for both α-(1→6)- and α-(1→4)-linked polysaccharides are equal to 10 or 12 glucose residues.[77,83] Nevertheless, the computer simulation studies performed recently on the pullulan oligomers having less than 12 residues were successful in describing polymer dynamics and coincided with experimental results.[87,88]

It is important to note that both computational approaches were based on an assumption that the conformational energy of the whole pullulan chain might be presented as a sum of weight-averaged contributions of each type of glycosidic linkage.[87] In the other words, the latter definition for the entire macromolecular chain represents the principle of additivity of the chain flexibility,[89] which was first written for pullulan as[90]

$$A_p^{-1} = (2/3)A_a^{-1} + (1/3)A_d^{-1}, \quad (1)$$

where A_p, A_a, and A_d are the length of the Kuhn segment of pullulan, amylose, and dextran, respectively. As it can be seen in Table 2, the values of the Kuhn segment calculated by using Eq. 1 agree well with the experimental estimates.

Table 2. Comparison of the molecular characteristics of pullulan estimated experimentally and calculated by Eq. 1

Reference	Dextran	Amylose	Pullulan	
			Estimated	Calculated by Eq. 1
		Kuhn segment A (Å)		
89	≈13		20.7	
			16.5	22.4
91		34.2		
		Activation energy E_a (kJ/mol)		
92	32.0	52.0	39.0	36.7
93	38.6	53.2	50.6	47.4

The term of the Kuhn segment represents the minimal chain length at which directional persistence starts to dissipate, and therefore, one can expect that other measurable quantitative characteristics of motion of the entire macromolecule should obey the above relationship. Indeed, the results of the activation energy calculation (Table 2) are in fairly good agreement with the experimental values estimated by Scandola et al.[92] or Einfeldt et al.,[93] thereby confirming validity of our assumption.

Hydrodynamic and molecular characteristics of pullulan in solution were intensively studied.[94–98] Hydrodynamic properties reflect the response of the polymer to the action of solvent and hence inherently depend on the conformational flexibility of the chain. In the NMR experiments it was found that the equilibrium hydration shell of the pullulan chain in solution consists of 13 water molecules per glucose residue.[82] Based on accurate dynamic light scattering measurements, Kato et al.[95] derived the molecular weight (M)–intrinsic viscosity relationship for pullulan having M greater than 1×10^5:

$$[\eta] = 2.21 \times 10^{-2} M^{0.66} \ (\mathrm{cm^3\,g^{-1}}). \quad (2)$$

This finding is in a good accordance with both the results reported by this group earlier[94] and data of Nishinari et al.[97] The dependencies of the radius of gyration (R_g) and hydrodynamic radius (R_H) on M were found to obey the following equations:[95]

$$R_g = 1.47 \times 10^{-2} M^{0.58} \ (\mathrm{nm}), \quad (3)$$

$$R_H = 2.25 \times 10^{-2} M^{0.52} \ (\mathrm{nm}). \quad (4)$$

The exponents in Eqs. (2)–(4) confirmed that pullulan in an aqueous solution behaves as a random coil. The close value (0.595) of exponents in Eq. 3 was obtained from the size-exclusion chromatography data.[99]

In order to estimate to what extent the chain dimensions of pullulan are perturbed by the exclusion volume effect, the ratio (R_g/R_H) was examined.[95] Within the entire range of M's studied, the ratio (R_g/R_H) was

smaller than the theoretically predicted value for a linear polymeric chain at the Θ-conditions, indicating a presence of the excluded volume effect. As reported in Ref. 89 the exclusion volume effect is responsible for the nonlinear logarithmic plot of the Mark–Kuhn–Hauwink dependence ($[\eta] = KM^{\alpha}$) of pullulan within a wide range of M. Therefore, those authors proposed to describe $[\eta]$ versus M dependence by equation

$$[\eta] = 1.96 \times 10^{-2} M^{0.66\pm0.012} \ (\mathrm{cm^3\,g^{-1}}) \qquad (5)$$

for pullulan with M greater than 3×10^4, and

$$[\eta] = 6.16 \times 10^{-2} M^{0.56\pm0.042} \ (\mathrm{cm^3\,g^{-1}}) \qquad (6)$$

for pullulan with M smaller than 3×10^4.

As can be seen, Eq. 5 correlates well with the finding of Kato et al. (Eq. 2). The exponent in the Mark–Kuhn–Hauwink dependence for pullulan (0.65–0.67) is intermediate between analogous exponents described for dextran (0.50)[100] and amylose (0.68–0.70).[101]

An interesting conformational behavior of pullulan is observed in a solution of DMSO. The intrinsic viscosity of pullulan in DMSO is higher than that in water, and the exponent α in the Mark–Kuhn–Hauwink equation is equal to 0.75. However, the length of the Kuhn segment in DMSO ($A = 20$ Å) almost corresponds to that in aqueous solution.[102] It should be pointed out that the analysis of the pullulan structure by means of NMR spectroscopy revealed that the primary hydroxyl groups in the adjacent $(1\rightarrow4)$-linked glucose residues form hydrogen bonding.[103] This hydrogen-bonding pattern may exist in water, but not in DMSO, whose solvent properties are determined by the ability to break intra- and intermolecular hydrogen bonds. Therefore, dissolution of pullulan in DMSO imposes several conformational changes in the polysaccharide. Studying the flow birefringence, Pavlov and Evlampieva found that the thermodynamic swelling parameter (ε) for pullulan in a DMSO solution $\varepsilon = 0.18$, whereas in an aqueous solution $\varepsilon = 0.112$.[102] The higher value of ε for pullulan in a solution of DMSO may be attributed to a more pronounced exclusion volume effect. Similar phenomena of the chain expansion in the solution of DMSO were seen also in the cases of other polysaccharides.[104] The exponent $\alpha = 0.87$ estimated for amylose in DMSO exceeds the value obtained in aqueous solutions.[105]

As the configuration of the D-glucopyranose ring of maltotriose units is stable in DMSO,[106] an increase of the anisotropy reported for the pullulan chain[103] can be most likely interpreted as being due to changes of mutual orientation of the pyranose rings in such a polar solvent. It is worth mentioning here that a comparative analysis of the pullulan IR spectra recorded in DMSO and in water allows one to detect a pronounced increase of a band in the structure-sensitive spectral region (950 $\mathrm{cm^{-1}}$).[107] The changes in intensity of this band are interpreted as being caused by conformational transitions of the polysaccharide due to rotational isomerism of the pyranose rings about the glycosidic bond.

Important results concerning the mobility and conformation of carbohydrate chains were obtained from the analysis of these compounds by IR and Raman spectroscopy. This is, in particular, because of the fact that the steric factors and the spatial location of individual groups strongly contribute to the formation of the vibrational spectra of carbohydrates.[108] In the IR spectrum of pullulan, the absorbance bands in the carbohydrate fingerprint region 1200–800 $\mathrm{cm^{-1}}$ were sensitive to changes in short-range order and show the influence of solvent on the hydrogen-bonding system.[107] The co-existence of α-$(1\rightarrow4)$- and α-$(1\rightarrow6)$-glycosidic linkages in the pullulan structure can be established by the appearance of a band at 935 $\mathrm{cm^{-1}}$.[41]

The bands in the Raman spectra region 480–540 $\mathrm{cm^{-1}}$ are regarded as markers for the prevailing type of interunit linkages.[108,109] The most intense bands in this region correspond to the vibrations of a group of atoms involved in glycosidic linkage. In the spectra of dextran, the most intense band at 543 $\mathrm{cm^{-1}}$ is associated with vibrations of a group of atoms at the C-6 position. The line at 480 $\mathrm{cm^{-1}}$ corresponding to vibrations of atoms at the C-4 position has a low intensity and appears as a weak shoulder. Analysis of model compounds shows that the presence of a volumetric substituent at C-4 leads to the appearance of an intense band at 470–485 $\mathrm{cm^{-1}}$.[110] As it is expected, the Raman spectra of amylose contain an intense line at 480 $\mathrm{cm^{-1}}$. In the case of pullulan, both of the bands at 543 and 480 $\mathrm{cm^{-1}}$ appear in the spectra.[109] This result corresponds to the fact that both α-$(1\rightarrow4)$- and α-$(1\rightarrow6)$-glycosidic linkages are present in this polymer.

Keilich and Bittiger have applied circular dichroism (CD) to investigate the conformation of the polysaccharides that differ in the type of glycosidic linkage.[111] In the CD spectrum of dextran with benzoyl substituents at the C-2, C-3, and C-4 positions, a positive Cotton effect with an intense band at 238 nm was observed. In the spectrum of amylose substituted at the C-2, C-3, and C-6 carbons, a negative band at 235.5 nm was present. It was assumed that the configuration of the $(1\rightarrow4)$-glycosidic linkage strongly influences the optical properties of the chromophore at C-2, causing an appearance of the band at 235.5 nm.[111,112] The CD spectra of pullulan were interpreted as a superposition of the spectra of dextran and amylose, since both bands at 235 and 240 nm were registered.

4. Biological activity of pullulan

Due to a high concentration of hydroxyl groups, polysaccharides exert inherent physiological activity. The first experience of application of neutral polysaccharides

as bioactive polymers is the use of a dextran-based blood-plasma substitute, which has been in use since 1944.[15] These preparations are designed to maintain the volume of circulating blood and its osmotic pressure in cases of massive loss of blood. The therapeutic effect of these preparations is determined by molecular weight and conformational characteristics of the polymeric substance. High-molecular-weight fractions with $M =$ 200 kDa can provoke toxic reactions and preclude normalization of the blood microcirculation; therefore, the molecular weight and concentration of polymer are adjusted so as to obtain a solution with a viscosity corresponding to that of blood (2.8–4.0). Usually, this is 6 and 10 g/dL for dextran with $M = 70$ and 40 kDa, respectively.

Such preparations are widely administered all over the world and are still the subject of intense investigations.[113–120] An important therapeutic effect of the intravenously administered polysaccharides is the specific interaction with low-density lipoproteins by means of weak van der Waals forces, which results in elimination of the lipid fraction from the blood compartment.[121–124]

There have been several attempts to develop blood-plasma substitutes based on pullulan.[125–127] Japanese chemists have studied an efficiency of intravenous injections of pullulan with various M_w and M_w/M_n.[125] It was found that for pullulan samples with M_w not exceeding 15 kDa, the half-time required from injection to urinal excretion is very short, resulting only in a higher effort on the kidneys. The use of pullulan with M_w higher than 150 kDa led to the unwanted rapid increase of venous pressure. Therefore, it was concluded that pullulan suitable for intravenous injection should have a narrow MWD with $M_w/M_n = 1.2$ and M_w about 60 kDa.[125] The refined polymer should be free from low- and high-molecular-weight components.

An effect of M_w of intravenous pullulan on its pharmacokinetics has been studied in detail.[127] The preparation of pullulan with M_w of 51, 60, and 82 kDa and $M_w/M_n \approx 1.6$ were tested as the correctors for hemorrhagic shock. It was suggested that the pullulan-based preparation would exhibit a superior therapeutic efficiency in comparison with dextran due to biodegradation of the polysaccharide. These investigations have not led to the development of a blood-plasma substitute. The main results of these experiments can be summarized in the following observations: (i) in the form of 6- g/dL intravenous solutions, pullulan has a viscosity inadmissibly exceeding that of blood, and (ii) an attempt to decrease the viscosity by decreasing either the molecular weight of the polymer or its concentration in the solution leads to a rapid exclusion of pullulan from the organism, followed by the development of a secondary hemorrhagic shock.

More recently, it has been shown that pullulan exhibits a great affinity toward the liver and is effectively endocytosed by the parenchymal liver cells.[128,129] At the same time, pullulan has no mutagenic, carcinogenic, and toxicological activities.[130] Hepatic uptake of intravenously administered pullulan is markedly reduced by the co-administration of asialofetuin and arabinogalactan having a high affinity to the corresponding cell receptors. This indicates that pullulan, in contrast with dextran, binds asialoglycoprotein receptors and penetrates the hepatocyte membrane via receptor-mediated endocytosis.[131] As the binding of carbohydrates with the specific receptors is governed by the stereochemistry of pyranose rings and the mutual orientation of hydroxyl groups of carbohydrates,[132] the conformational transitions from C_{1a} to *boat* conformers available for the pyranose rings only in the case of α–$(1 \rightarrow 4)$-linked glucose residues[133] of pullulan are thought to be important factors determining the high affinity of the biopolymer toward the asialoglycoprotein receptors. The great affinity of pullulan toward liver is considered to be responsible for its short half-life period of circulation in blood.[134]

5. Chemical modification of pullulan, derivatives and their biomedical application

The differences in the type of glycosidic linkages of polysaccharides, among the physical characteristics, lead to the appearance of different chemical reactivities of the compounds due to the individual particularities in the distribution of hydroxyl groups within the pyranose rings and a specific molecular microenvironment. In the pullulan structure, approximately nine OH groups per repeating unit are available for substitution. The relative reactivities of these groups may vary greatly, depending on the polarity of the solvent and the reagents.

Bruneel and Schacht have demonstrated that the highest reactivity of a polysaccharide in the reactions of carbonation and succinoylation in DMSO solution is at the C-6 position.[135,136] Similarly, the highest reactivity of the hydroxyl group of pullulan to phenyl isocyanate in DMSO was established for the hydroxyl group at the C-6 position of the D-glucopyranose ring.[137] Carboxymethylation of pullulan in an alcohol–water mixture, however, is less pronounced at the C-6 hydroxyl group and proceeds predominantly at the C-2 position, similarly as known for dextran.[138] The product of this derivatization, carboxymethylpullulan (CMP), is a promising polymeric carrier for many drugs since an introduction of negative charges into the macromolecules of CMP results in prolonged retention of the polymer within the organism.[134]

CMP conjugates with sialyl Lewis X ligands can be advantageously applied in cases of acute inflammatory lesion to target the inflammatory sites.[139] The conjugates have been found to bind E-selectin receptors, albeit

without showing multivalent efficiency because of conformational restrictions imposed by linear pullulan chains. The interactions with the receptors are characterized by high selectivity, and, therefore, cannot be associated with physicochemical properties of the macromolecular carrier. Thus, the low binding affinity of short-chain oligosaccharide analogous of sialyl Lewis X ligands, necessitating its high-dosage administration,[140] can be sufficiently improved by the use of CMP carriers.

Several CMP–doxorubicin conjugates were synthesized and evaluated for their antitumor effects. Studied composites in the form of micelles with polysaccharide-rich cores have higher affinities toward tumor cells than doxorubicin itself, providing for a direct release of the drug at a high level.[141,142] The control of drug release from the CMP carrier can be achieved by adjusting the length of the spacer between the carboxyl group of CMP and the amino group of doxorubicin.[143] Besides having a high affinity to tumor cells, CMP is found to be selectively absorbed by the spleen and lymph nodes. The latter property of CMP toward immune tissues is thought to be a beneficial feature, allowing one to design novel carboxymethylpullulan–immunodepressant conjugates.[144]

The inherent affinity of pullulan toward liver cells has been used in preparing drug–polymer composites for hepatitis therapy.[128,129] Covalent conjugation of pullulan with an interferon–water-soluble low-molecular-weight recombinant protein that possesses both antiviral and immunoregulatory activity allows one to preserve the biological activity of the drug while enhancing its liver accumulation.[128] It has also been shown that a similar efficiency of interferon–polymer composites can be achieved through simple metal coordination of protein on the polysaccharide support. For this purpose pullulan molecules are first modified by attachment of the chelating ligand like, for example, diethylenetriaminepentaacetic acid.[129]

In order to develop a pullulan derivative that will act similarly to the well-known anticoagulant heparin and dextran sulfate, (Fig. 4) pullulan sulfation was studied.[145] The degree of substitution of pullulan in sulfation was higher than that of dextran under identical conditions, which was attributed to the presence of a higher amount of primary hydroxyl groups in the pullulan structure. In the independent study, Mähner et al.[146] have obtained sulfated derivatives of dextran and pullulan via sulfation with the SO_3–pyridine complex. Reactivity of the polysaccharide C-atoms for dextran was C-3 > C-2 > C-4, while for pullulan it was C-6 > C-3 > C-2 > C-4.[145] This finding was confirmed by Alban et al. who found that the sulfation of the OH groups occurs in the order C-6 > C-2 > C-3 > C-4 (Fig. 4), irrespective of the molecular weight of pullulan, the procedure and the degree of substitution.[147]

The final characteristics of the sulfated derivatives depend to a great extent on the temperature, duration of the reaction and the sulfation reagent used. Sulfoethylation of pullulan in aqueous alkaline solution of chloroethane sulfonate is accompanied by the degradation of the polymer,[148] whilst synthesis of pullulan sulfates with the use of SO_3–pyridine complex at 80 °C produces specimen with an average molecular weight and a radius of gyration (R_g) higher than those of the parent polysaccharide, indicating an occurrence of the intra- and intermolecular crosslinking. The products with a homogeneous distribution of the sulfate groups were obtained by continuous sulfation of pullulan at 75 °C in DMF solution, with stepwise addition of the SO_3–pyridine complex.[147] Additionally, it was shown that the highest degree of substitution was achieved by repeated (twofold) sulfation reactions.

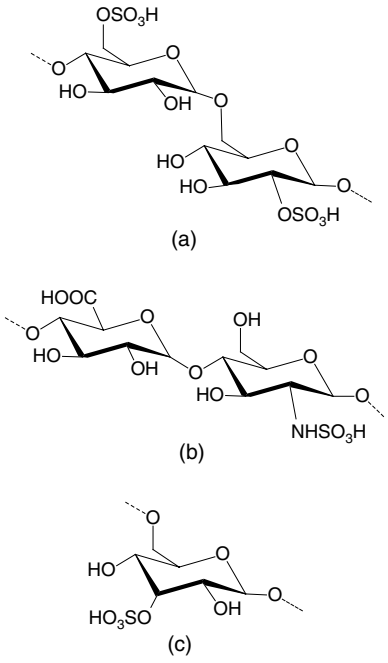

Figure 4. The structure of pullulan sulfate (a) substituted at the C-6 and C-2 positions[147] in comparison with heparin (b) and dextran sulfate (c) structural units.

The anticoagulant activity of sulfated pullulan is almost the same as that of heparin; however the action profile of these derivatives differ from that known for heparin and appears to depend on the molecular weight of polymer, degree of substitution, and distribution of the sulfated groups on the various positions of the glucose residue.[147]

An anionic pullulan derivative suitable for the production of a blood-plasma substitute was developed through the radiation-induced destruction with simultaneous modification of the polysaccharide.[149–151] A destruction of the polymer backbone in solution proceeds randomly, generating a polymeric substance with a molecular weight 55–70 kDa and the a polydispersity index of 2.0–2.5,[149] which is directly used as a base of

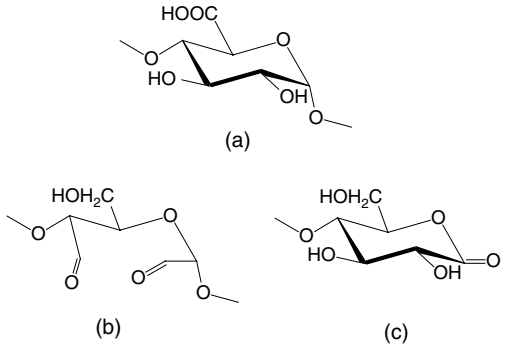

Figure 5. Modified glucopyranose elements in the structure of gamma-irradiated pullulan. Residues of uronic acid (a) in the concentration 6–17 groups per macromolecule provide for weak polyelectrolyte behavior, whereas dialdehyde structures (b) increase the flexibility of the modified pullulan chain due to ring opening.[150,151] Lactones (c) are mainly found as the end groups.

blood-plasma substitute.[152] Chemical modification of glycopyranose rings with the formation of carbonyl (dialdehyde and lactone) and carboxyl groups (Fig. 5) increases the resistance of pullulan to the action of amylase and thus helps to control the rate of its in vivo degradation in blood vessels.[152] Moreover, because of the presence of these groups, modified pullulan exhibits a pronounced effect of desegregation of platelets, as was demonstrated from in vivo and in vitro experiments.[151,153] The efficiency of a newly developed blood-plasma substitute was recently studied in dogs under the conditions of experimental shock.[153] An isovolumetric replacement of blood loss by the modified pullulan solution resulted in a rapid recovery of main the hemodynamic parameters such as index of blood circulation, rate of cardiac contractions, volumetric cardiac output, followed by normalization of the blood microcirculation.[153]

A promising route for developing novel biologically active composites is a combination of a highly hydrophilic polysaccharide chain with natural or synthetic hydrophobic compounds. The synthesis of pullulan derivatives containing chloroalkyl groups has been developed by the reaction of crosslinked pullulan microparticles with different chloroalkyl chlorides in organic basic solvents.[154] Pullulan reactivity in the chloroacetylation reaction is higher than that of dextran. This difference in reactivity correlates with the higher content of primary hydroxyl groups of pullulan in comparison with the dextran chain. This polysaccharide derivative can further be used as a macromolecular carrier of many drugs such as metronidazole, nicotinic acid, sulfathiazole.[155] Obvious therapeutic benefits can be achieved by slow release of drugs into the plasma, and thus altering their concentration profiles.

'Smart' pH-responsive polymer composites for tumor targeting have been constructed from sulfodimethoxine-conjugated pullulan acetate.[156] The particles represent micelle self-assembled structures with a size of ca. 70 nm, which is small enough to penetrate tumor sites. The outer surface of the nanoparticles is decorated by hydroxyl and carboxyl groups from pullulan acetate and sulfodimethoxine moieties.[156] The latter function influences the charge of the particles by altering ionization of surface groups in the pH range of 7.2–6.5. Ionization of the sulfodimethoxine moieties provokes a repulsion of between particles, which results in their stability above pH 7.4. An accelerated release of the drug from the sulfodimethoxine–pullulan composites is thought to be due to the deformation of the interior structure in response to the deionization of the sulfodimethoxine groups at pH below 7.2.[156]

Pullulan modified by attachment of a certain number of cholesteryl groups self-aggregates into the stable monodispersed nanoparticles of a hydrogel,[157] which forms complexes with such soluble proteins as bovine serum albumin, chymotrypsin, and insulin.[158] Nanoparticles of hydrophobically modified pullulan can find application in various fields, including drug-delivery systems and/or the thermal and colloidal stabilization of proteins.[159–161] Liposomes prepared from cholesterol-bearing polysaccharides have been tested as drug-delivery particles to target vascular cells.[162] Cholesterylpullulan bearing aminolactose or other cell-recognition elements is considered to be a good carrier of anticancer drugs.[163] In the form of a complex with oncoprotein, cholesterylpullulan was shown to be an efficient agent in cancer therapy.[164]

Hydrogel microspheres based on crosslinked pullulan, containing pendant quaternary ammonium groups with different chemical structures, have been synthesized and tested as possible bile acid sorbents.[165] Bile acids can be bound ionically to them and removed from the enterohepatic circulation by excretion. The removal of bile acids launches several biological processes having as the final result a decrease in plasma cholesterol level.[166]

Alkylperfluorinated pullulan derivatives were synthesized from pullulan and carboxymethylpullulan using a perfluoroalkyl carboxylic acid ($C_8F_{17}CH_2CH_2COOH$) and perfluoroalkylamines ($C_7F_{15}CH_2NH_2$ and C_8F_{17}-$CH_2CH_2NH_2$).[167] The intermolecular aggregation of the hydrophobized polysaccharide results in formation of compact nanoparticles. The strength of the self-association of the hydrophobically modified pullulan can be regulated by the length of the hydrophobic moiety.[167]

An introduction of a highly hydrophilic carbohydrate moiety to the hydrophobic perfluorocarbon chain leads to the formation of the fluorosurfactants. When dispersed in water and other polar solvents, these molecules aggregate into well-organized micelles.[168] Because of their high mechanical stability and chemical inertness, such systems find numerous biomedical applications. Carbohydrate-derived fluorosurfactants are used for

targeting delivery of drugs and as contrasting agents. A unique ability of fluorosurfactants to dissolve oxygen with a load exceeding 20 times that of blood plasma allows one to develop injectable forms of perfluorocarbon-based oxygen carriers[169] that can be used as the blood-plasma substitutes. These oxygen carriers are not toxic and can be removed from the organism via excretion, and in contrast with genetically engineered hemoglobin, do not show reactivity-related side effects.[170–172] In this context, investigations into the structure and properties of perfluoro-modified pullulan and other hydrophobic derivatives are of practical importance.

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