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Synthesis, characterization and solution behaviour of oxidized pullulan

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ABSTRACT

Various amounts of carboxyl groups were introduced at C-6 of the non-ionic, water soluble polysaccharide, i.e. pullulan, by applying the well-established TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl), sodium hypochlorite/sodium bromide oxidation protocol, varying the reaction time. The oxidized products, more water-soluble than pullulan, were further characterized by FT-IR, ^{1}H NMR and ^{13}C NMR techniques, in order to assess the degree of oxidation. The absolute molecular weight measurements performed using a multiangle laser light scattering molecular weight analyzer, reveals a sharp drop of the molecular weight of the samples oxidized for longer reaction times. The second virial coefficients (A_2), increased from unoxidized pullulan to the oxidized samples. Dynamic light scattering (DLS) measurements provide zeta potentials and hydrodynamic radius for all studied samples. The viscosity of the initial and oxidized pullulan dilute aqueous solutions was studied in detail. All oxidized samples except the highest oxidized pullulan sample (OxPu8) showed strong polyelectrolyte behaviour, whereas this effect is less pronounced for OxPu8 due to the high degradation of the chains. The intrinsic viscosity and the interaction parameter were determined at 25 °C as a function of solvent ionic strength according to Wolf approach. The dependence of these parameters on the salt concentration follows Boltzmann sigmoid model.

1. Introduction

Pullulan is a bacterial polysaccharide, extracted from the fermentation medium of the *Aureobasidium pullulans* (Bishwambhar, Suneetha, & Ramalingam, 2011), consisting of maltotriose units connected through α-(1 → 4) glycosidic bonds, whereas consecutive maltotriose units are linked to each other by α-(1 → 6) glycosidic bonds. Pullulan is extensively used as food ingredient and as a pharmaceutical bulking agent. The daily intake of pullulan is estimated around 10 g per day for a person based on food categories (Rekha & Sharma, 2009). The use and application of this polysaccharide is increasing rapidly as it is an important and industrially available alternative material for replacing natural gums produced from marine algae and other plants.

For some applications, there is often a need to increase the hydrophilicity of the native pullulan; this can be done by the introduction of various functional groups, such as carboxylic ones, by using chemical functionalization. The presence of ionic groups allows obtaining polymer solutions with various properties and viscosity due to polyelectrolyte swelling of macromolecules. The viscosity can be controlled by polymer concentration and solvent ionic strength. However, to the best of our knowledge, the rheological properties of oxidized pullulan have never been reported. De Nooy et al. (De Nooy, Besemer, & van Bekkum, 1994; De Nooy, Besemer, & van Bekkum, 1995) described a method for the oxidation of polysaccharides employing sodium hypochlorite, sodium bromide and catalytic amounts of TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) in homogeneous conditions, using water as solvent, at pH around 10.5. It was assumed that primary alcohol groups are selectively converted into carboxylic ones. Recently an alternative protocol for the mild and efficient oxidation of polysaccharides, particularly cellulose, mediated by N-hydroxyphthalimide (NHPI)

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and various co-catalysts has been proposed (Coseri, 2009a, 2009b; Coseri et al., 2009, 2013). Both protocols, i.e., TEMPO and NHPI, claim to be selective, namely the oxidation reaction converts only primary OH groups to carboxylic ones.

The goal of this work was to study and understand the influence of oxidation on the properties of pullulan solutions, oxidized vs. native. Pullulan was oxidized using TEMPO, sodium hypochlorite and sodium bromide. Using four different reaction times, samples of oxidized pullulan having various degrees of oxidation were prepared. The oxidation was confirmed by FT-IR, ^{1}H NMR and ^{13}C NMR analysis. Moreover, the absolute molar weight of pullulan and oxidized samples was determined by using a multiangle laser light scattering molecular weight analyzer. The properties of the pristine and oxidized pullulan dilute solutions were studied using viscometry. The presence of ionic groups on pullulan macromolecules induces new length scales; thus to obtain the intrinsic viscosity we used a model developed for polyelectrolyte solutions (Wolf, 2007). In order to screen the repulsive forces between the charged groups and decrease the osmotic pressure of free counter-ions, NaCl of various concentrations was added. The discussion will be focused mainly on the changes in the viscometric behaviour induced by introduction of —COOH groups in the pullulan structure and on the effect of salt addition to aqueous solutions.

2. Materials and methods

2.1. Materials

Pullulan sample purchased from TCI Europe was dried under vacuum at 100 °C overnight prior uses.

TEMPO (99% Aldrich), sodium hypochlorite (NaOCl, 3% chlorine, Chemical Company Romania) and sodium bromide (99% Alfa Aesar) were used as received. Distilled water of HPLC grade was used for dissolution of water soluble compounds and the viscometric analyses.

2.2. Pullulan oxidation

Oxidation of pullulan was carried out as follows: 1 g of pullulan was dissolved in distilled water (120 mL). TEMPO (0.02 g) and NaBr (0.1 g) were added, and the solution was stirred at room temperature. A 3% sodium hypochlorite solution (25 mL) was brought to pH 10 by adding 4 M aqueous HCl, and subsequently added to the pullulan solution. The pH was carefully checked with a pH metre and maintained at 10 by adding 0.5 M sodium hydroxide solution. After 10 min (sample OxPu0.1), 1 h (sample OxPu1), 4 h (sample OxPu4) and 8 h (sample OxPu8), the reaction was stopped by quenching the unreacted NaOCl with 10 mL of methanol, and then acidified to a pH around 6.8. A large volume of ethanol has been used to precipitate the reaction products. The formed precipitate was collected by centrifugation. The polymer was redissolved in water, and the solution was desalted and the oligomers were removed by diafiltration through a Millipore ultrafiltration membrane from polyethersulfone (cut-off: 10,000 g cm^{-1}) in Amicon cell equipped with a tank filled with pure water (conductivity lower than 3 μS m^{-1}). The diafiltration was stopped when the filtrate conductivity was lower than 10 μS m^{-1} and the polymer was recovered by freeze-drying.

2.3. FTIR measurements

Infrared absorption spectra of pullulan and oxidized pullulan samples were recorded using a Bruker Vertex 70 spectrometer at a scan range from 4000 cm^{-1} to 650 cm^{-1}, at a resolution of 2 cm^{-1} and 32 scans. Samples were measured as a KBr pellet.

2.4. NMR determinations

Samples were prepared by dissolving 10 mg of polymer for ^{1}H NMR and 50 mg for ^{13}C NMR in 0.7 mL of D_2O. The obtained solution was filtered through a pipette containing glass wool into a standard 5 mm NMR tube. The NMR spectra were obtained on a Bruker Advance DRX 400 MHz Spectrometer, equipped with a 5 mm QNP direct detection probe and z-gradients.

2.5. Molecular weight determinations

Absolute molecular weight measurements were done using the Brookhaven BI-MwA multiangle laser light scattering molecular weight analyzer. The instrument was equipped with the BI-MwAZP software that allows data analysis to obtain accurate molecular weight values through several methods.

In this work, the Debye plot method was used. For this purpose, the scattered light intensity was measured at 5 dilute polymer concentrations (c), within 3 mg ml^{-1} to 7 mg ml^{-1} range. All polymer solutions have been prepared using aqueous 0.1 M $NaNO_3$ solution. Prior to molecular weight determination, the polymer solution was filtered through a 0.2 μm membrane filter (Millipore) followed by a 0.025 μm membrane filter and degassed under vacuum for approximately 1 h at room temperature. This instrument allows also the determination of the second virial coefficients A_2.

2.6. Dynamic light scattering measurements

Zeta potential (ζ) was measured by using a dynamic light scattering technique (Zetasizer model Nano ZS, Malvern Instruments, UK) with red laser 633 nm (He/Ne).

Zeta potential (ζ) was calculated from the electrophoretic mobility (μ) determined at 25 °C. For $k\alpha \gg 1$ (k – Debye–Hűckel parameter and α – particle radius), the Smoluchowski relationship was used, Eq. (1):

$$\xi = \frac{\eta\mu}{\varepsilon} \quad (1)$$

where η is the viscosity, ε – dielectric constant.

The apparent hydrodynamic diameter (D_H) of pullulan and oxypullulan aqueous solutions was determined through dynamic light scattering measurements by using Zetasizer ZS apparatus during the same experiment of zeta potential evaluation. The results presented good reproducibility with the deviation of the average diameter within 5%. The temperature was maintained constant at 25 °C (±0.1 °C) with a Peltier device.

2.7. Viscometric measurements

Viscometric measurements were performed at 25 °C ± 0.01 °C using an Ubbelohde capillary viscometer (type 0a with a capillary diameter of 0.53 mm) in combination with an automatic viscosity measurement system (Lauda Instrument, Germany). Upon dilution, each polymer solution was kept about 15 min prior the measurements for equilibration. Flow times were obtained with good reproducibility; the errors were less than 1%. When aqueous NaCl solutions were used as pullulan solvent, polymer was directly dissolved in NaCl solution of a given concentration, ranging from 0 to 1.5 M.

3. Results and discussion

3.1. Characterization of oxidized pullulan

Pullulan has been oxidized by using sodium hypochlorite and sodium bromide, a protocol adapted from De Nooy et al. (De

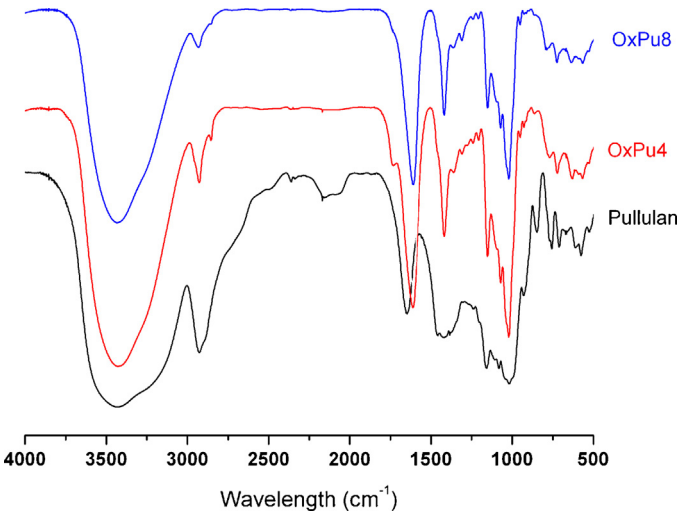

Fig. 1. FTIR spectra of unoxidized and two oxidized pullulan samples.

Nooy et al., 1994, 1995), at room temperature, and TEMPO serving as mediator for the oxidation reaction. Four samples were prepared, differing on the amount of negatively charged groups introduced after oxidation: three partially oxidized samples at C_6—OH: OxPu0.1 (reaction time 10 min), OxPu1 (reaction time 1 h), OxPu4, and one sample (OxPu8) fully oxidized at C_6—OH (reaction time 8 h, there are no unconverted primary OH groups, see NMR analyses section). The other reaction conditions are described in Section 2. Catalytic amount of TEMPO was used and the oxidant was regenerated with $^-$OCl/HOCl at pH 10. During oxidation, TEMPO is converted to the nitrosonium ion, which is the actual oxidant.

The first indication of the pullulan oxidation can be observed in FTIR, see Fig. 1. Unoxidized pullulan shows typical FTIR spectrum for this kind of polysaccharide: a broad adsorption between 3000 and 3600 cm^{-1} region due to OH stretching vibration, a band at 2926 cm^{-1} corresponding to the C—H stretching vibration, 1653 cm^{-1} adsorption band of bounded water, 1458 cm^{-1} assigned to symmetric CH_2 bending vibration and 930 cm^{-1} assigned to C—O—C stretching at α-(1 → 4)-glycosidic linkages, a marker sign of "amorphous" region (Ciolacu, Ciolacu, & Popa, 2011). After oxidation, a new adsorption band as a shoulder, around 1736 cm^{-1}, appears due to carboxylate groups. The adsorption band at 1610 cm^{-1} can be also attributed to the C=O stretching of free carboxylate groups, since the adsorption band at 1417 cm^{-1} represents the C—O symmetric stretching of dissociated carboxyl groups (Saito et al., 2009).

^{13}C NMR spectra of the initial and two oxidized pullulan samples, are shown in Fig. 2. The spectrum (a) is characteristic for the unoxidized material, showing the signal of C6 atom (6g1 → 4) at 69.26 ppm and C6 atom (4g1 → 4 and 4g1 → 6) at 63.19 ppm. Also the signals of the C1 atom around 100.68–102.99 ppm, C4 atom at 80.56 ppm and C2,3,5 atoms between 72.26 and 76.21 ppm can be observed (Arnosti & Repeta, 1995). After 4 h of oxidation (sample OxPu4), a decrease of the C6 peak belonging to the 1,4 linked α-D-glucose units are observed, concomitantly with a new peak apparition at 175.62 ppm. This is a result of the carboxylic group formation. In the OxPu8 sample, the CH_2—OH peak is absent; one can conclude that a complete conversion of OH groups to carboxylic functionality was achieved.

Fig. 3 shows the ^{1}H NMR spectra of the native pullulan, one partially C6-oxidized (OxPu4) and the fully C6-oxidized (OxPu8) samples. All spectra present a number of overlapping resonances, due to the glucose ring, between 3.4–4.1 ppm, 4.9 ppm and 5.36–5.58 ppm.

Table 1
Degree of oxidation (DO), weight average molecular weights (M_w), second virial coefficient (A_2), apparent hydrodynamic diameter (D_H), and zeta potential (ζ) for pullulan and oxidized pullulan samples.

Polymer	DO (%)	$10^{-4} \times M_w$ (g mol^{-1})	$10^4 \times A_2$ (mol mL g^{-2})	D_H (nm)	ζ (mV)
Pullulan	–	22.00	3.06 ± 0.32	41.9	2.2 ± 0.2
OxPu0.1	10	18.20	12.14 ± 0.24	48.3	−12.6 ± 0.2
OxPu1	50	11.22	23.12 ± 0.01	159	−20.4 ± 0.3
OxPu4	90	7.90	25.30 ± 0.28	208	−29.2 ± 0.4
OxPu8	100	2.80	26.14 ± 0.15	273	−34.4 ± 0.6

The integration of the peaks in the range of 3.4–4.1 ppm and 5.36–5.58 ppm, allowed to estimate the degree of oxidation as reported before by Paris and Stuart (1999). Since in the pullulan sample there are no converted CH_2—OH groups, the ratio between the 3.4–4.1 ppm and 5.36–5.58 ppm regions is the highest. Once the reaction proceeds, this ratio tends to become smaller and smaller due to the minimizing of the two protons signal to the total peaks area from 3.4 to 4.41 ppm, culminating with their total disappearance for the sample OxPu8 (see Table 1 and Supplementary content). On the other hands, with the increase of the reaction time up to 8 h, no peak of C6 (originating from 1,4 linked α-D-glucose) is observed in ^{13}C NMR spectrum of OxPu8, indicating a total conversion of primary OH groups into carboxylic ones (peak from 175.6 ppm), therefore, it can be assumed that the ratio of the peak area between 3.4–4.1 ppm and 5.36–5.58 ppm in the H NMR spectrum for this sample, of 7.60 will give a total conversion of C_6—OH to COOH groups (100% conversion). The weight average molecular weights (M_w) of the native pullulan and oxidized samples were determined and presented in Table 1. The oxidation reaction is performed under homogeneous condition; therefore, it can be assumed that the charges due to the oxidation are randomly distributed in the polymer, every third monomer being without charge. The sample OxPu0.1 shows the highest value of M_w, which is close to the value determined for pullulan. Increasing the reaction time, the value of M_w for the oxidized samples decreased, due to the degradation phenomenon, to about 79,000 g mol^{-1} for OxPu4 and 28,000 g mol^{-1} for OxPu8. It is clear that this draw in of the M_w values of oxidized pullulan is due to the longer reaction time and somehow also due to the reaction conditions (room temperature, alkaline medium). An ample study regarding the influence of the reaction conditions on the molecular weight of the sample is now in progress and the main conclusion is the occurrence of a strong degradation of the oxypullulan samples in the first part of oxidation, up to reaching a DO of about 50%.

The values of zeta-potential (ζ) for pullulan and oxidized pullulan samples were determined in deionized water at a concentration of 1 mg mL^{-1}. The results are reported in Table 1. The ζ potential has positive value in pullulan, but the introduction of the negative charges during oxidation switches the ζ potential to the negative values, ranging from −12.6 ± 0.2 mV for OxPu0.1 to −34.4 ± 0.6 mV for OxPu8 in accordance with the content of the carboxylic groups introduced into the polymeric chain.

Second virial coefficients A_2 measured using multiangle laser light scattering molecular weight analyzer shows important differences between the native pullulan and oxidized samples. These differences originates mainly on different molecular mass of the samples, and also due to the slightly different structure of the oxidized pullulan, the existence of the newly COOH moiety appeared after oxidation which induce a new behaviour of the polymer in solution.

The apparent hydrodynamic diameter (D_H) of the pullulan as determined by dynamic light scattering measurements, increased from 41.9 nm for pullulan to 48.3 nm for the sample having the lowest degree of oxidation (OxPu0.1), but reached higher values

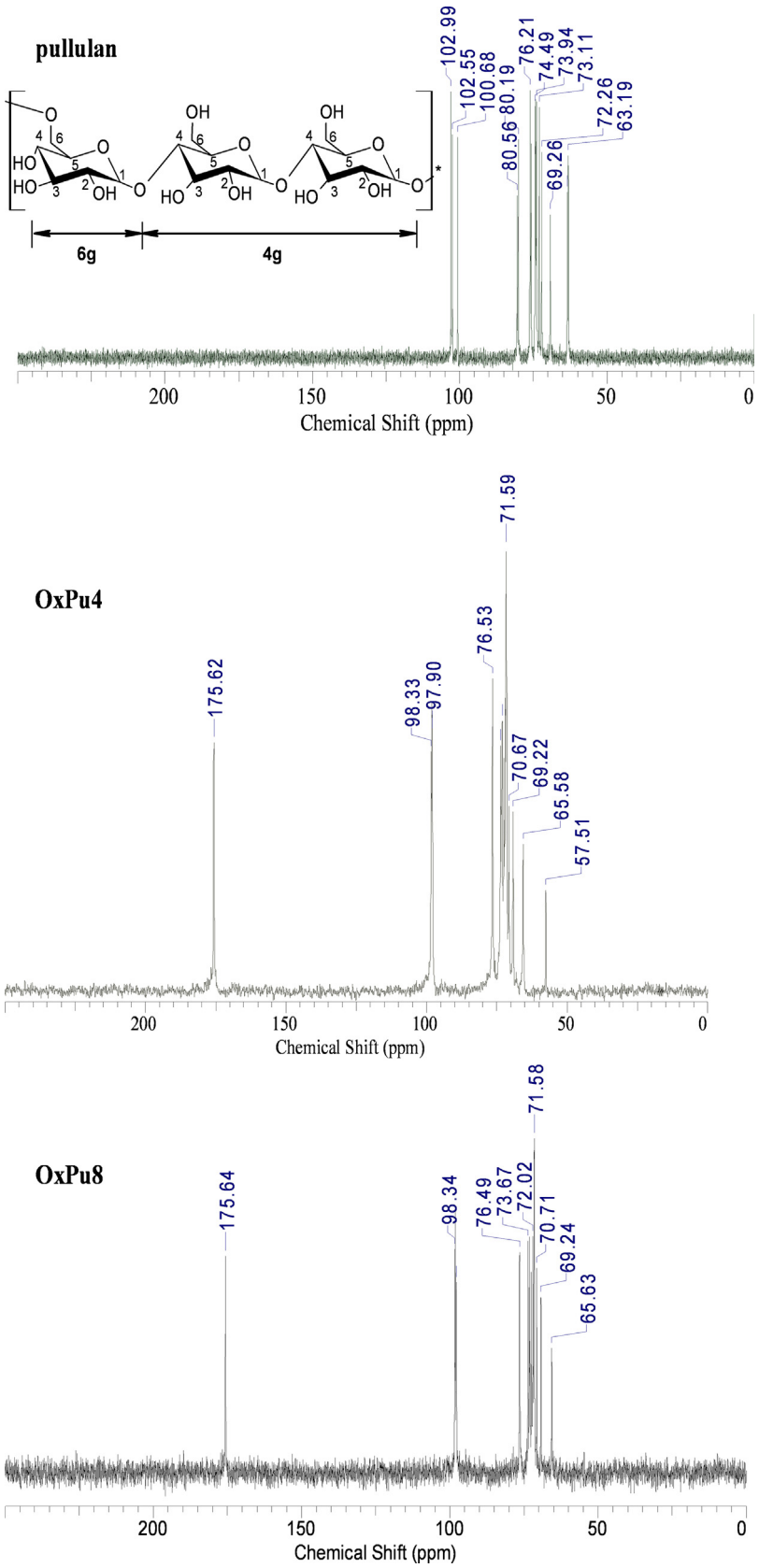

Fig. 2. ^{13}C NMR chemical shifts (ppm) (referenced to internal D_2O) of pullulan, OxPu4, and OxPu8, at 25 °C.

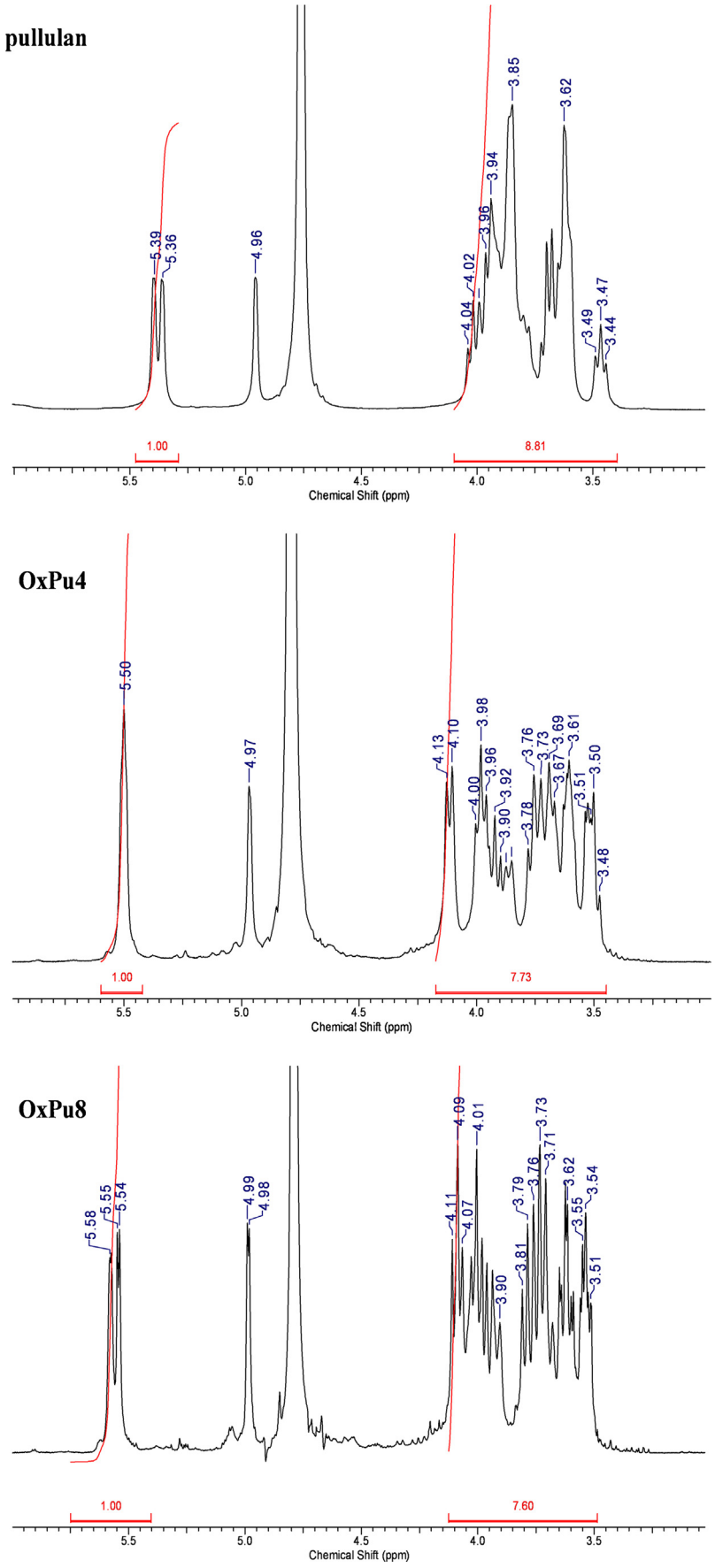

Fig. 3. ^{1}H NMR chemical shifts (ppm) (referenced to internal D_2O, 4.79 ppm) of pullulan, OxPu4 and OxPu8, at 25 °C.

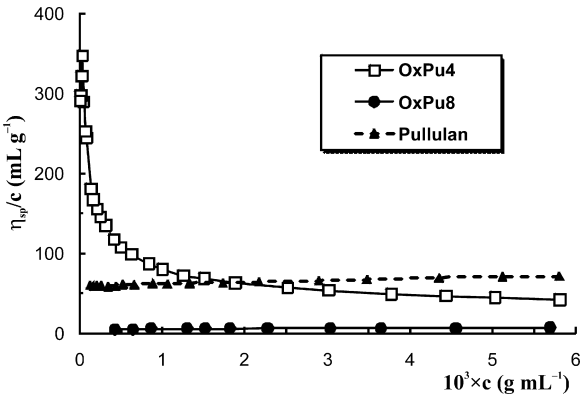

Fig. 4. Plots of the reduced viscosity as a function of concentration for the aqueous solutions of (□) OxPu4 (DO = 90%) and (•) OxPu8 (DO = 100%) at 25 °C. The lines are guides for the eye only.

for the samples oxidized longer reaction time, samples OxPu1 to OxPu8, Table 1. These larger values of the apparent hydrodynamic diameter for the samples having higher COOH content could be explained by the polyelectrolyte effect induced by pullulan oxidation and by appearance of possible supplemental hydrogen bonds between different oxypullulan chains.

3.2. Viscosity of pullulan in water and 1 M NaCl aqueous solutions

The plots of the specific viscosity (η_{sp}/c) vs. concentration (c) for pullulan solutions in water (Fig. 4) and 1 M NaCl aqueous solutions according to Huggins method are typical to neutral polymers. The dependences are linear over the investigated range of concentrations (dilute regime) and the presence of salt has no significant influence on the intrinsic viscosity ($[\eta]$) and Huggins constant (k_H). The following values were determined for pullulan solutions at 25 °C: $[\eta] = 61.5\ mL\,g^{-1}$, $k_H = 0.59$ in water and $[\eta] = 62\ mL\,g^{-1}$, $k_H = 0.61$ in 1 M NaCl.

The obtained intrinsic viscosity is in agreement with the value that can be calculated by using the following Mark Houwink dependence: $[\eta] = 2.58 \times 10^{-2}\ M^{0.66}$ ($mL\,g^{-1}$) for pullulan samples of narrow molecular weight distribution in water at 25 °C, reported by Nishinari et al. (1991) and Kato, Katsuki, and Takahashi (1984). The macromolecular chains are extremely flexible since the fluctuations between different conformations through rotations about single bonds can occur independently. In aqueous solutions, a macromolecule does not usually adopt a unique conformation, but fluctuates around it, through oscillations about the bonds to the bridging oxygen, because of collisions and thermal energy. Since pullulan contains many internal glycosidic linkages, its backbone is extremely flexible (entropic driven) and the chain adopts a random coil conformation in solution. This is reflected by the characteristic ratio, C_∞, a measure of the degree of conformational restriction or stiffness of chain, which has the value of 4.14 for pullulan in water at 25 °C (Buliga & Brant, 1987). Dais and Vlachou (2001) examined the contribution of both α-(1 → 6) and α-(1 → 4) linkages to the conformational dynamics of pullulan and compared the motional parameters, such as spin-lattice relaxation times and Nuclear Overhauser Enhancements (NOEs), with those of dextran, and amylose, considered the extremes in the conformational freedom and constraint (Dais & Vlachou, 2001). They have shown that the ring connected by α-(1 → 6) linkages exhibit a greater motional freedom than rings linked with a stiffer α-(1 → 4) bonds. Unlike α (1 → 6) linked polysaccharides, (1 → 3)-β-D-glucans due to their high rigidity in aqueous solutions forms tripe-stranded helices. These high rigid macrocyclic species are observed by TEM even after several cycles of denaturation–renaturation processes (Stokke, Elgsœter, Brant, & Kitamura, 1991). Triple helical chain conformation of β-(1 → 3)-glucan in the aqueous solution with high chain stiffness was once again proved very recently by using a combination of size exclusion chromatography (SEC), multiangle static light scattering, a differential refractometer, and a capillary viscosity detector (triple-detector size exclusion chromatography) (Li, Huang, Wang, Xu, & Zhang, 2014). The geometry of the β-(1 → 4) polysaccharides, including cellulose, galactomannans, arabinoxylans, is known to be quite similar. For these biopolymers, the chain persistence lengths are found to be ~3–5 nm, which classes them as semi-flexible or relatively stiff polymers (Picout & Ross-Murphy, 2002).

3.3. Evaluation of the intrinsic viscosity of oxidized pullulans in water. Polyelectrolyte effect

Fig. 4 shows the dependence of η_{sp}/c as a function of polymer concentration for two oxypullulan samples in water, as compared with the starting sample of pullulan. One can observe the polyelectrolyte behaviour for OxPu4 sample: viscosity increases with the decrease of polymer concentration due to an increase of the coil dimension with dilution. Oxidized pullulan behaves as a classical polyelectrolyte in dilute solutions with Coulomb repulsion between charged groups and osmotic pressure of counter-ions, both inducing the swelling of a polymer chain. It should be noted that oxidation reaction leads to pullulan degradation: the reduced viscosity of OxPu8 is very low and polyelectrolyte swelling is not detected. Polymer degradation during TEMPO oxidation is one of the major issues which researchers try to overcome. As it can be observed in Table 1, the shorter reaction time the highest the molar mass of the oxidized products is obtained. Under these circumstances, an unavoidable question naturally raises: is the degradation caused by the higher degree of oxidation or by the longer reaction time or both of them? To answer at this question, we should therefore try to modify only one parameter which can cause such a high degradation of the polymeric chains. In this context, a study is now in progress in order to evidence the chain degradation during the oxidation reaction, when a same sample (with a given degree of oxidation) is exposed on the reaction medium for the different reaction times. This approach can contribute to a better understanding the competition between oxidation and degradation phenomena during the TEMPO mediated oxidation of pullulan at high reaction times and values of DO, respectively.

The Huggins plot (Fig. 4) does not allow the direct determination of the intrinsic viscosity for polyelectrolyte solutions, due to the upward curvature at low concentrations. In order to overcome this difficulty, the obtained viscometric data were analyzed using the approach developed by Wolf (2007) which quantitatively describes the behaviour of uncharged macromolecules (Brunchi, Bercea, & Morariu, 2010; Bercea, Morariu, & Rusu, 2013) and polyelectrolytes solutions of different ionic strength (Eich & Wolf, 2011; Ghimici, Nichifor, Eich, & Wolf, 2012; Morariu, Brunchi, & Bercea, 2012). The intrinsic viscosity was thus evaluated from the initial slope of the dependence of natural logarithm of the relative viscosity ($\ln \eta_{rel}$) as a function of the polymer concentration (Fig. 5) according to the following equation, Eq. (2) (Eckelt, Eckelt, & Wolf, 2012):

$$\ln \eta_{rel} = \frac{c[\eta]}{1 + b_w c[\eta]} \quad (2)$$

where b_w is the viscometric interaction parameter which has the same physical meaning as k_H.

For charged macromolecules, b_w is given by the contributions of Coulombic interactions and those of the Van der Waals forces (Bercea, Nita, Eckelt, & Wolf, 2012; Nita, Chiriac, Bercea, & Wolf, 2013). Large Coulombic interactions give lower values of the viscosity as compared with the exponential increase of the viscosity

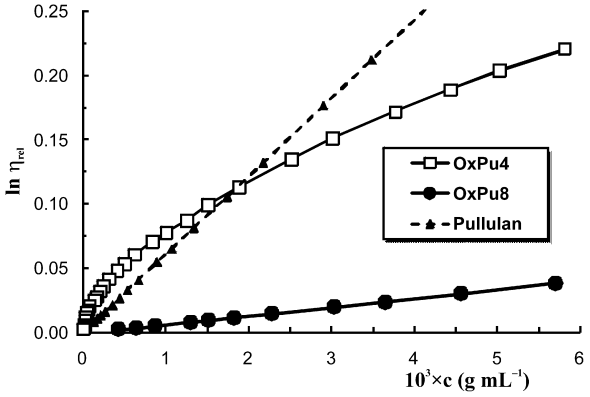

Fig. 5. Evaluation of the viscometric data according to Eq. (3) for the aqueous solutions of (□) OxPu4, (●) OxPu8 and (▲) pullulan at 25 °C. The lines are calculated by means of Eq. (2) using the parameters of Table 2.

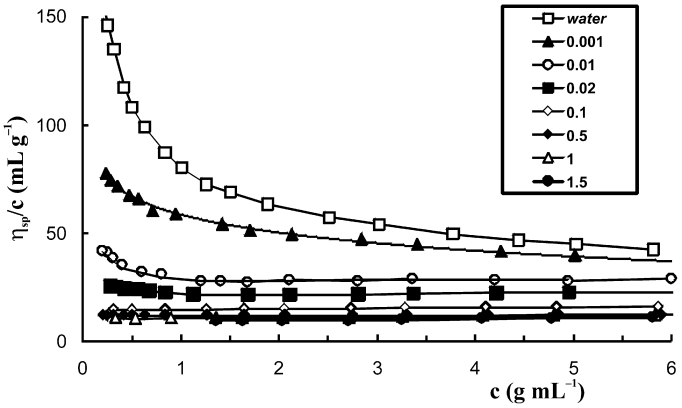

Fig. 6. Reduced viscosity vs. polymer concentration for solutions of OxPu4 in aqueous NaCl solutions (salt concentration expressed as mol L^{-1}). Solid lines correspond to reduced viscosity dependence on polymer concentration calculated according to Wolf approach.

with the rise of polymer concentration and this behaviour are due to the screening of the electrostatic repulsion.

Fig. 5 shows the evaluation of the viscometric data according to Eq. (2) for OxPu4, OxPu8 as well as for the initial pullulan sample. The $[\eta]$ and b_w values were determined by the best fit of Eq. (2) with the experimental data of the $\ln \eta_{rel}$ vs. c dependence and the obtained results are given in Table 2. For the initial pullulan sample, the intrinsic viscosity in water determined with Huggins and with Wolf approaches is nearly the same. The intrinsic viscosity of OxPu4 in water is higher than the corresponding value of non-modified pullulan, reflecting a strong swelling of the macromolecule due to the presence of carboxylic groups. $[\eta]$ value of OxPu8 in water is almost four times lower than that of OxPu4 and two times lower than of the non-oxidized pullulan sample, demonstrating that long oxidation reaction time leads to polymer degradation. The oxidation reaction during 4 h also degrades the polymer backbone (relative viscosities of OxPu4 are lower than the ones of non-modified pullulan at the same concentration), but polyelectrolyte swelling in water is "dominating" the decrease of the chain length.

3.4. Viscometric behaviour of oxidized pullulan in aqueous NaCl solutions

Fig. 6 presents the viscometric data obtained for OxPu4 in aqueous NaCl solutions of various salt concentrations. Solid lines correspond to reduced viscosity calculated according to Wolf approach. The values of $[\eta]$ and b_w are collected in Table 2.

The increase of salt concentration leads to a gradual decrease in the reduced viscosity and in the polyelectrolyte swelling of the macromolecule. At salt concentration equal and higher than 1 M, OxPu4 behaves as a neutral polymer. As a consequence, the intrinsic viscosity of OxPu4 decreases significantly with salt concentration up to 1 M NaCl (Table 2). Similar results have been reported for cationic polyelectrolytes (Ghimici, Nichifor, & Wolf, 2009; Ghimici et al., 2012) and sodium pectinate samples (Eich & Wolf, 2011).

OxPu8 in NaCl aqueous solutions was also investigated and $[\eta]$ and b_w values are given in Table 2. In 1 M NaCl the polyelectrolyte effect is suppressed, and the intrinsic viscosity is reflecting only coil dimensions. Despite the fact that DO is higher for this sample as compared with OxPu4, the decrease of the intrinsic viscosity in 1 M NaCl solution as compared with aqueous solution is smaller than for OxPu4. This is due to the degradation phenomenon during oxidation, the molecular weight reduces dramatically and a strong decrease of the intrinsic viscosity in 1 M NaCl with reaction duration occurs: from 62.1 mL g^{-1} for non-modified pullulan to 11 mL g^{-1} for OxPu4 and 6 mL g^{-1} for OxPu8.

Table 2 also shows a dimensionless parameter, $c_{max}[\eta]$, which is the "reduced concentration" calculated from maximum experimental concentration used in viscosity measurements (c_{max}) multiplied by the intrinsic viscosity. The starting concentration for each experiment was around 6×10^{-3} g mL^{-1}, and for OxPu4 we can observe a decrease of this parameter with the increase of salt concentration. At a given salt concentration $c_{max}[\eta]$ becomes constant when there is a complete screening of all charge–charge interactions and the lower asymptote limit is reached (Figs. 6 and 7).

The intrinsic viscosity of OxPu4 solutions as a function of NaCl concentration is shown in Fig. 7 in double logarithmic coordinates. The intrinsic viscosity in pure water ($c_{salt} \to 0$) is indicated by the upper asymptote. The lower asymptote is reached at 1 M NaCl ($c_{salt} \to \infty$). The experimental data can be fitted with Boltzmann sigmoid, Eq. (3) (Eich & Wolf, 2011; Ghimici et al., 2012), see the solid line in Fig. 7:

$$\log[\eta] = \log[\eta]_{c_{salt}\to\infty} + \frac{\log[\eta]_{c_{salt}=0} - \log[\eta]_{c_{salt}\to\infty}}{1 + \exp\left\{\log\left(H\frac{c_{salt}}{c_{salt}^{ip}}\right)\right\}} \quad (3)$$

where c_{salt}^{ip} represents the inflexion point of the dependence shown in Fig. 7 and H is an adjustable parameter. The value of H was found equal to 1 for oxypullulan solutions, in agreement with previous observations made in the study of charged dextran derivatives

Table 2
Parameters of Eq. (2) obtained from the best fit to viscosity experimental data by viscometry and the maximum reduced concentration, $c_{max}[\eta]$, covered by each experimental investigation.

Sample	DO (%)	Mol NaCl	$[\eta]$ (mL g^{-1})	b_w	$c_{max}[\eta]$
Pullulan	0	0.0	61.5	0.101	0.370
		1.0	62.0	0.101	0.367
OxPu01	10	0.0	88.8	3.998	0.553
OxPu1	50	0.0	101.5	2.944	0.696
OxPu4	90	0.0	106.4	4.180	0.618
		0.001	66.6	3.577	0.406
		0.01	28.3	2.084	0.170
		0.02	21.8	1.884	0.131
		0.1	14.6	1.064	0.086
		0.2	12.0	0.550	0.076
		0.5	11.6	0.256	0.075
		1.0	11.0	0.194	0.064
		1.5	10.9	0.173	0.063
OxPu8	100	0.0	28.6	7.337	0.175
		0.1	8.5	1.514	0.051
		1.0	6.0	0.612	0.034

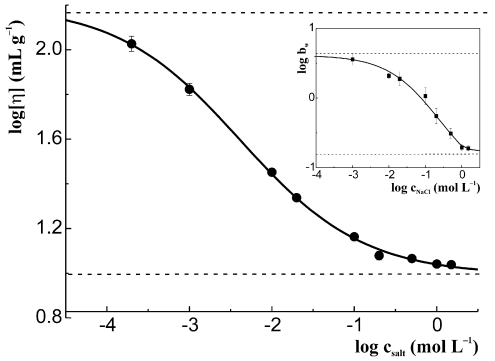

Fig. 7. Intrinsic viscosity of the sample OxPu4 as a function of the salt concentration, c_{NaCl}, at 25 °C. Solid line is the best fit of Boltzmann sigmoid. Inset: the same but for the interaction parameter b_w.

(Ghimici et al., 2012). The point of inflexion of the Boltzmann sigmoid can be considered as the salt concentration for which the intrinsic viscosity of a charged macromolecule is the most sensitive to the changes in salt concentration (Eich & Wolf, 2011; Ghimici et al., 2009, 2012).

The double logarithmic dependence of the interaction parameter, b_w, on NaCl concentration, c_{NaCl}, is depicted in the inset of Fig. 7. The behaviour is similar to that obtained for intrinsic viscosity.

By analysing the dependence between the intrinsic viscosity on the ionic strength for anionic polysaccharides, Smidsrød and Haug (1971) developed a method for evaluating the chain stiffness from viscometric data at different concentrations of added monovalent salt. This approach was applied to oxypullulan samples.

The polymer coil density increases with increasing the ionic strength (I) of the solution due to the increase of the intramolecular interactions. The intercept of the dependence of $[\eta]$ as a function of $I^{-1/2}$ gives the intrinsic viscosity extrapolated to infinite ionic strength, $[\eta]_\infty$ according to the following relationship (Smidsrød & Haug, 1971):

$$[\eta] = [\eta]_\infty + S \cdot I^{-1/2} \quad (4)$$

A plot of $[\eta]$ as a function of $I^{-1/2}$ for a sample of oxypullulan with high DO is shown in Fig. 8.

Although the electrostatic contribution to the excluded volume is not completely inhibited at infinite ionic strength (Skolnick & Fixman, 1977; Bercea, Nichifor, Eckelt, & Wolf, 2011), the $[\eta]_\infty$ value determined as being 6.475 mL g^{-1} (Fig. 8) could be interpreted as the viscosity under theta conditions ($[\eta]_\theta$) because the electrostatic repulsions between protonated amino groups are largely suppressed at infinite ionic strength. The slope S is related to the stiffness of the molecule, B, as:

$$B = \frac{S}{([\eta]_{0.1})^{1.2}} \quad (5)$$

where B is the stiffness parameter and $[\eta]_{0.1}$ represents the intrinsic viscosity at $I = 0.1$ M.

The estimated stiffness parameter was 0.0921 for OxPu4, suggesting that oxypullulan chains with high DO preserve the high flexibility of pullulan macromolecules. This B value is higher than those reported for chitosan: $B = 0.063$ (Morariu et al., 2012), carboxymethylcellulose: $B = 0.065$ (Smidsrød & Haug, 1971), and much higher as compared with the value of 0.005 reported for xanthan (Tinland & Rinaudo, 1989) which adopts a stiff conformation, 0.0055 obtained for mechanical inflexible DNA chains (Smidsrød & Haug, 1971) or 0.018 reported for flaxseed polysaccharide (Goh, Pinder, Hall, & Hemar, 2006) considered a semi-flexible polymer.

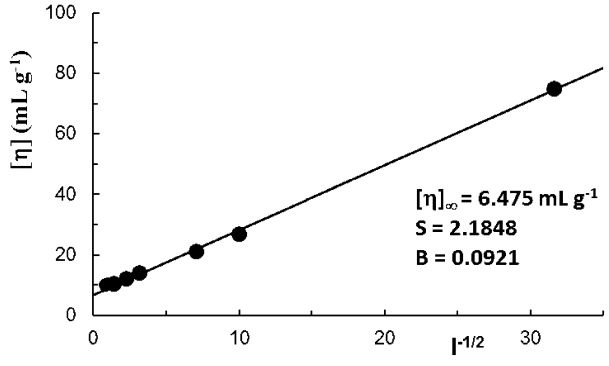

Fig. 8. Dependence of the intrinsic viscosity on the ionic strength for OxPu4. The line represents the fit of the experimental data according to Eq. (4).

4. Conclusions

Four oxidized pullulan samples, having different degrees of oxidation, which depend on the reaction time, were synthesized and characterized by FTIR and ^{13}C NMR techniques. The oxidation reaction was carried out for 10 min, 1, 4 and 8 h respectively, at room temperature, pH = 10, using TEMPO as mediator, sodium bromide, and sodium hypochlorite (10 mmol/g pullulan) as actual oxidant.

The viscometric behaviour of native and oxidized pullulans was investigated at 25 °C in water and NaCl solutions. Longer reaction times (8 h) lead to complete conversion of primary OH groups to carboxylic ones, but also to highest polymer degradation. Shorter reaction times (10 min, one and 4 h) resulted in charged pullulan demonstrating strong polyelectrolyte swelling in water.

The intrinsic viscosity of oxidized pullulans was determined using Wolf approach (Wolf, 2007). Despite degradation during oxidation, the intrinsic viscosity of oxidized pullulan (OxPu4 with DO of 90%) in water is higher than that of initial pullulan sample: polyelectrolyte swelling "dominates" the effect of degradation. Upon the addition of NaCl, the intrinsic viscosity gradually decreases to reach a constant value by addition of 1–1.5 M NaCl that are one order of magnitude lower as compared to the one in pure water. At these high salt concentrations, oxypullulans behave as neutral polymers. The dependence of oxidized pullulan intrinsic viscosity as a function of NaCl concentration was described by Boltzmann sigmoid.

The stiffness parameter was estimated as being 0.0921, suggesting that oxypullulan chains preserve the high flexibility of pullulan macromolecules.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.04.060.

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