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Synthesis of amphiphilic 6-carboxypullulan ethers

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ABSTRACT

Hydrophobically modified polysaccharides that contain carboxyl groups possess exceptional features for drug delivery and other applications. Carboxyl groups were introduced at C-6 in the pullulan backbone by applying the well-established oxidation with TEMPO and NaOCl/NaBr. The oxidized product, 6-carboxypullulan, is even more water-soluble than pullulan. Consequently, further chemical modifications have been mainly restricted to reactions that can be performed in water or under heterogeneous conditions. We find that the TBA salt of 6-carboxypullulan is soluble in a range of organic solvents and can be reacted homogeneously with various alkyl halides in DMSO and sodium hydroxide at 40 °C to yield 6-carboxypullulan ethers. Complete substitution (DS 7 per trisaccharide repeat unit) was achieved upon reaction with iodoethane, while products from reaction with longer chain alkyl halides (propyl and butyl derivatives) achieved DS up to about 3. The amphiphilic products have impressive surfactant properties.

1. Introduction

Pullulan is a non-ionic water-soluble polysaccharide which is produced from starch by the yeast-like fungus *Aureobasidium pullulans* (Bauer, 1938; Bernier, 1958). It consists predominantly of maltotriose units, i.e. units of three 1,4-linked α-D-glucose molecules, which are polymerized in a linear fashion via 1,6-linkages, as shown in Fig. 1 (Bender & Wallenfels, 1959). Pullulan has low toxicity and has been used for more than 20 years as an additive in the food industry (Leathers, 2003). It biodegrades in the body and does not evoke an immune response. It has also been shown to be non-toxic when administered intravenously (Yamaoka, Yasuhiko, & Ikada, 1993). In view of the attractive characteristics of pullulan and the possibility of chemical modification to suit the desired application, there have been many reports of the synthesis of new pullulan derivatives with application in drug delivery (Rekha, 2007; Shingel, 2004). Pullulan is concentrated disproportionately in the liver after intravenous administration, and so has been studied as a promising polymeric carrier for liver-related diseases (Hosseinkhani, Aoyama, Ogawa, & Tabata, 2002; Xi et al., 1996).

Most pullulan modifications are intended to reduce its water solubility or to introduce charged or reactive groups for functionality (Akiyoshi, Yamaguchi, & Sunamoto, 1991; Hirakura, Nomura, Aoyama, & Akiyoshi, 2004; Jung, Jeong, & Kim, 2003). For drug delivery applications, the ability of the drug carrier to swell or disperse in water is often more desirable than water solubility (Kost & Langer, 2001). Highly water soluble polymer carriers tend to release drugs quickly, while polymers that only swell or disperse in water have the ability to provide slow drug release (Edgar, 2007).

Polysaccharides that have been hydrophobically modified and contain carboxyl groups are commonly used in drug delivery systems because of their ability to provide pH-controlled drug release (Dulong, Le Cerf, Picton, & Muller, 2006; George & Abraham, 2006; Lu et al., 2009; Posey-Dowty et al., 2007). For example, hydrophobic drugs often are released from carboxyl-containing polysaccharide matrices only at the neutral pH of the small intestine and colon, when the carboxyl group becomes ionized and the polymer swells, thus limiting exposure of the stomach to the drug. Moreover, polymer–drug interactions also play an important role in drug delivery systems. An important example is the strong interaction of carboxyl groups with amines (many drugs contain amine functional groups) by hydrogen bonding. The presence of hydrophobic groups is also important; hydrophobicity will enhance miscibility with hydrophobic drugs, and slow their release. Due in part to these valuable features, polysaccharide derivatives, especially

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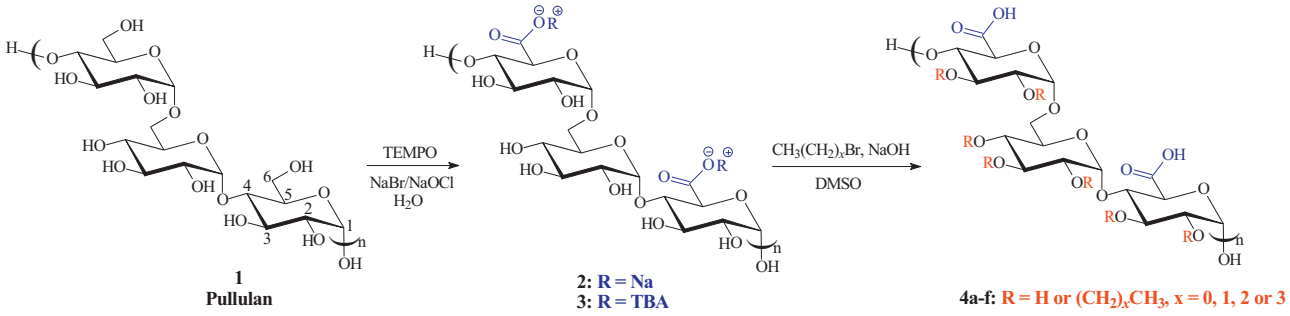

Fig. 1. Pullulan oxidation and synthesis of 6-carboxypullulan ethers.

cellulose derivatives containing pendant carboxyl groups, have been recently explored with success for amorphous solid dispersion (ASD) of drugs. These drug–polysaccharide matrix dispersions have shown the ability to significantly enhance solution concentrations and stability of many otherwise poorly soluble drugs by forming a matrix of drug, in a metastable amorphous form, entrapped within the polymer (Konno, Handa, Alonzo, & Taylor, 2008). Hydroxypropylmethylcellulose acetate succinate (HPMCAS) (Friesen et al., 2008) and cellulose acetate adipate propionate (CAAdP) (Ilevbare, Liu, Edgar, & Taylor, 2012; Kar, Liu, & Edgar, 2011) are examples of carboxylated cellulose derivatives that combine several of those attractive drug delivery functions and are promising polymers for drug delivery formulations.

Introduction of carboxyl groups to the non-ionic pullulan backbone should give an anionic derivative with interesting properties for drug delivery applications. The most widely investigated pullulan derivative containing a carboxylic acid group is carboxymethylpullulan (CMP) (Dulong et al., 2006). CMP is a promising polymeric carrier for many drugs since its high proportion of negative charges results in prolonged retention of the polymer within the organism (Yamaoka et al., 1993). CMP has been hydrophobically modified by esterification of the carboxyl groups with long alkyl chains (Henni-Silhadi et al., 2007). These derivatives self-assemble in aqueous media and efficiently solubilize hydrophobic drugs.

In the early 1990, Denooy, Besemer, and van Bekkum (1995) described the TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy free radical)-mediated selective oxidation of various water-soluble polysaccharides with hypochlorite/bromide as the regenerating oxidant. The reaction is performed under homogeneous conditions in water and primary alcohol groups are selectively oxidized to yield carboxylates. The regioselectivity of the reaction is essentially complete, and chemoselective, favoring oxidation of the primary OH to a carboxylic acid; partial oxidation to aldehyde, or oxidation at the secondary OH groups to ketones, is minimal. Such high chemo- and regioselectivity is useful for potential use of oxidized pullulan derivatives in formulations that might reach the circulation, since full characterization and structural control on such polymers is important for regulatory approval. Oxidation of pullulan by this methodology has been reported, but modified 6-carboxypullulan compounds have not been studied much for their biomedical applications, although they may have great potential for use in drug delivery systems (Paris & Stuart, 1999; Yang, Du, Huang, Wan, & Wen, 2005). One possible reason for this is the fact that 6-carboxypullulan is even more water soluble than pullulan, therefore further chemical modification is mainly restricted to reactions which can be performed in water or under heterogeneous conditions. Additionally, highly water-soluble polysaccharide derivatives may not be highly miscible with hydrophobic drugs, and may give faster than desired release profiles (and/or with inadequate pH responsiveness) for particular drugs.

In this work we introduced carboxyl groups to the pullulan backbone by applying the selective TEMPO oxidation. We then explored methods for conversion of the oxidized pullulan product, 6-carboxypullulan, to its tetrabutylammonium (TBA) salt, seeking enhanced organic solubility that would permit more facile reactions of the remaining pullulan OH groups with electrophiles. We pursued this strategy by attempting homogeneous reaction of 6-carboxypullulan salts with various alkyl halides in DMSO, employing sodium hydroxide as base, as a route to the potentially useful 6-carboxypullulan ethers.

2. Experimental

2.1. Materials and methods

Pullulan (Mw = 450 kDa, Mn = 200 kDa) was from the Hayashibara Company (Okayama, Japan) and was dried under vacuum at 120 °C overnight prior to use. Water was deionized. TEMPO (99%, Aldrich), sodium hypochlorite (NaOCl, 14.5% chlorine, Alfa Aesar), NaBr (99%, Alfa Aesar), ethyl acetate (HPLC grade, Fisher), tetrabutylammonium fluoride trihydrate (99%, TBAF), pyridine (Py), tetrabutylammonium hydroxide (TBAOH, 1.0 M in water, Fluka Analytical), ethylene glycol (EG, laboratory grade, Fisher), and lithium chloride (99%, LiCl) were used as supplied. Dimethylsulfoxide (DMSO, HPLC grade, Acros) was dried using 4 Å molecular sieves. Dimethylacetamide (DMAc, HPLC grade, Fisher) and dimethylformamide (DMF, Fisher) were used as supplied. Bromoethane (98%, Alfa Aesar), bromopropane (98%, Aldrich), bromobutane (99%, Aldrich), iodomethane (99%, Aldrich), iodoethane (98%, stabilized with silver, Acros Organics) and iodobutane (98%, stabilized, Acros Organics) were used as supplied. Proton exchange resin was DOWEX 50WX8 100–200 (H) from Alfa Aesar. Deuterium oxide (99.9 atom % D; D_2O) containing 0.75% 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt and d_6-DMSO for NMR were acquired from Sigma–Aldrich. Trifluoroacetic acid (99%) used for ^{1}H NMR was from Acros and KBr used for FTIR analysis was obtained from International Crystal Laboratories.

For NMR analysis, samples were prepared by dissolving 8–10 mg (for ^{1}H) or 50–80 mg (for ^{13}C) of polymer in 0.7 mL of D_2O or d_6-DMSO. The solution was filtered through a pipette containing glass wool into a standard 5 mm NMR tube. ^{1}H and ^{13}C NMR spectra were acquired on Varian INOVA or Varian UNITY 400 MHz spectrometers with 32–128 scans for ^{1}H and minimum of 10,000 scans for ^{13}C. Chemical shifts are reported relative to the solvents, except for ^{13}C spectra acquired in D_2O, when TMS is used as the reference.

Degree of substitution (DS) values of the 6-carboxypullulan ethers are described as per trisaccharide repeat unit, with a

maximum DS of 7 with respect to etherification. DS values were calculated by ^{1}H NMR using the following formula:

$$DS = \frac{17A}{3B - 2A}$$

This formula was derived from the following relation:

$$\frac{3DS}{17} = \frac{A}{B - (2/3)A}$$

Each OH substitution in the pullulan backbone with an ethyl, propyl or butyl group brings to the pullulan backbone 3 methyl protons (3DS) and there are 17 protons (C–H) resulting from each pullulan trisaccharide repeat unit, regardless of its DS. A is the integration of the methyl peak of the alkyl group, and was observed at 0.87 ppm for butyl, 0.93 ppm for propyl and 1.11 ppm for ethyl in the ^{1}H NMR spectra of the respective 6-carboxypullulan ether. 6-Carboxypullulan ether backbone protons were quantified by integration of the peaks observed in the 2.6–6.1 ppm region (B) minus the integration of the protons from the O—CH_2— of the respective alkyl group, that overlap with the backbone protons (2/3A). For each ether substituent, there will be one methyl (3H) per overlapping methylene group (2H).

Ether DS was calculated by ^{1}H NMR, with the exception of methyl pullulan-6-carboxylate, whose DS was calculated by quantitative ^{13}C NMR (data not shown) because the methyl resonance overlapped with anhydroglucose ring protons in the ^{1}H NMR spectrum.

FTIR spectra were acquired using a Thermo Electron Nicolet 8700 instrument in transmission mode. Samples were prepared using the KBr pellet method. 6-Carboxypullulan ether samples (1 mg) were mixed with 99 mg of KBr using a mortar and pestle. The mixture was compressed in the sample holder between two screws to form a KBr disk. Sixty-four scans were obtained for each spectrum.

Dynamic light scattering (DLS) data were obtained using a Malvern Instruments Zetasizer Nano-ZS. Polymer solutions with different concentrations were prepared by dissolving the polymer in water.

Dialysis was performed against water in a 4 L beaker using dialysis tubing (MWCO 3.500 Da) for 3 days, replacing the water twice each 24 h.

Freeze-drying of pullulan derivatives was performed using Labconco Freezone 4.5 lyophilizer.

Solubility testing on 6-carboxypullulan ethers samples was performed by adding 5 mg of sample to a glass vial, then adding 1 mL of solvent. The mixture was subjected to vortex mixing for 5–10 min at room temperature, and then solubility was judged by visual examination.

Hydrolysis of 6-carboxypullulan ethers to determine the degree of esterification (ester DS): the 6-carboxypullulan ether sample (30 mg) was stirred in NaOH (0.1 M, 5 mL) at room temperature for 20 h. After the hydrolysis reaction, the resulting solution was dialyzed against water for 24 h and freeze-dried.

2.2. Synthesis

2.2.1. Oxidation of pullulan with TEMPO and NaOCl/NaBr

Pullulan (2.0 g, 7% water, 11.5 mmol anhydroglucose units) was dissolved in 250 mL of demineralized water in a 3 neck round bottom flask equipped with 2 addition funnels. TEMPO (0.040 g, 0.26 mmol) and NaBr (0.20 g, 1.9 mmol) were added, and the solution was cooled in an ice bath to approximately 2 °C. A 15% NaOCl solution (12 mL, 25 mmol) was brought to pH 9.4 by adding 4 M aqueous HCl and also cooled in an ice bath. This solution was added to one of the addition funnels. A 0.5 M aqueous NaOH solution was added to the other addition funnel. The solution of NaOCl was slowly added to the reaction mixture and the pH was maintained at 9.4 during the oxidation by concomitantly adding the aqueous NaOH. After approximately 1 h, 15.0 mL of 0.5 M NaOH had been added and the reaction was quenched by adding methanol (5 mL) and neutralized by adding 4 M HCl. Then, $NaBH_4$ (0.5 g, 13 mmol) was added and the solution was stirred overnight at room temperature. The reaction mixture was brought to pH 6 by adding 4 M HCl, and the oxidized polymer was desalted by dialysis, after which the solution was freeze-dried and the polyelectrolyte isolated in a yield of 95% (based on the ideal molar mass of completely oxidized pullulan).

Pullulan. ^{1}H NMR (400 MHz, D_2O): δ 5.61–5.47 (m, 2 H-1), 4.98–4.9 (m, H-1), 4.11–3.4 (m, H2–H6). ^{13}C NMR (400 MHz, D_2O): δ 100.12 (C-1), 99.66 (C-1), 97.83 (C-1), 77.65, 77.27, 77.35, 73.26, 72.96, 71.62, 71.53, 71.42, 71.24, 71.01, 70.24, 69.38, 66.38, 60.62 (C-6), 60.31 (C-6).

6-CO_2NaPull. ^{1}H NMR (400 MHz, D_2O): δ 5.67–5.42 (m, 2 H-1), 5.05–4.9 (m, H-1), 4.18–3.2 (m, H2–H6). ^{13}C NMR (400 MHz, D_2O): δ 176 (C=O), 175.83 (C=O), 99.35 (C-1), 98.14 (C-1), 97.82 (C-1), 76.58 (C-4), 76.39 (C-4), 73.78 (C-3), 73.32 (C-3), 73.18 (C-3), 72.72 (C-5), 72.16 (C-5), 71.77 (C-2), 71.73 (C-2), 71.64 (C-2), 70.73 (C-4), 69.29 (C-5), 65.62 (C-6). Carbon peaks were assigned based on literature values (Denooy et al., 1995).

2.2.2. Etherification of 6-carboxypullulan TBA salt

6-Carboxypullulan Na salt (CO_2NaPull) was dissolved in water and passed through an ion exchange column. A solution with pH = 3–4 containing the protonated form of 6-carboxypullulan (CO_2HPull) was obtained. To convert CO_2HPull to CO_2TBAPull, aqueous TBAOH was added dropwise with continuous stirring to this solution until the pH was approximately 8. The resulting mixture was dialyzed against water for 48 h and freeze-dried. The final product, CO_2TBAPull, was a white solid.

CO_2TBAPull. ^{1}H NMR (400 MHz, d_6-DMSO): δ 5.18–2.98 (m, CO_2TBAPull backbone), 3.18−3.14 (m, $N(\underline{CH_2}CH_2CH_2CH_3)_4$ of TBA), 1.6−1.52 (m, $N(CH_2\underline{CH_2}CH_2CH_3)_4$ of TBA), 1.3 (tq, $N(CH_2CH_2\underline{CH_2}CH_3)_4$ of TBA), 0.92 (t, $N(CH_2CH_2CH_2\underline{CH_3})_4$ of TBA). ^{13}C NMR (400 MHz, d_6-DMSO): 171.98 (C=O), 171.49 (C=O), 98–97.51 (m, 3 C-1), 78.98 (C-4), 78.18 (C-4), 73.85–69.3 (m, C2–C5), 65.97 (C-6).

2.2.3. General procedure for the etherification of 6-carboxypullulan TBA salt

2.2.3.1. Synthesis of 6-carboxypullulan ethers by reaction with alkyl bromides and iodobutane. 6-Carboxypullulan TBA salt (0.250 g, 0.25 mmol) and DMSO (40 mL) were added to a 100 mL 3-neck round bottom flask under nitrogen containing a magnetic stirrer. The reactions performed with a low boiling point alkylating reagent were equipped with a reflux condenser. Pulverized NaOH (0.370 g, 9.25 mmol) was added to this clear solution and the suspension was stirred for 1 h at 40 °C. The reaction solution became more viscous, with a jelly-like appearance. The alkylating reagent (9.25 mmol) was added dropwise within 10 min; the mixture became fluid and was stirred for 2.5 h at 40 °C. The reaction mixture was poured into 120 mL of ethyl acetate and the resulting precipitate was filtered and washed with extra ethyl acetate. The product was then dissolved in water, passed through a column containing a proton exchange resin and the resulting solution was dialyzed against water for 3 days and freeze-dried to yield the 6-carboxypullulan ethers in protonated form.

2.2.4. Synthesis of 6-carboxypullulan ethers by reaction with iodomethane and iodoethane

Etherification of 6-carboxypullulan TBA salt with iodomethane and iodoethane furnished high DS products that could not be precipitated in any organic solvent upon reaction work up. Thus, the

experimental procedure for the isolation of these products was slightly different from the one described above. Upon reaction completion, instead of pouring the reaction mixture into ethyl acetate, the reaction mixture was dialyzed against water for 3 days. The resulting solution was passed through a column containing a proton exchange resin, then freeze-dried to yield 6-carboxypyllulan ethers in protonated form.

Methyl-6-CO_2HPull: ^{1}H NMR (400 MHz, d_6-DMSO): δ 5.96–5.95, 5.84–5.83, 5.57–5.55, 5.05–5.03, 4.8–4.78, 4.19–4.15, 3.94–3.01, 2.83–2.78 (m, backbone and CH_3 of methyl). Quantitative ^{13}C NMR (400 MHz, d_6-DMSO): 169.16, 162.16 (C=O), 108.26 (C-1), 102.81 (C-1), 97.07–95.1 (m, C-1), 85.25–65.85 (m, C2–C6 backbone), 59.76–54.37 (CH_3 of methyl).

Ethyl-6-CO_2HPull: ^{1}H NMR (400 MHz, d_6-DMSO): δ 6.1–2.6 (m, backbone and CH_2 of ethyl), 1.08 (s, CH_3 of ethyl). ^{13}C NMR (400 MHz, d_6-DMSO): 170.3 (C=O), 99.0 (C-1), 96.93 (C-1), 96.54 (C-1), 81.77–65.07 (m, C2–C6 backbone and CH_2 of ethyl), 15.4 (CH_3 of ethyl).

Propyl-6-CO_2HPull: ^{1}H NMR (400 MHz, D_2O): δ 6.1–4.9 (m, backbone) 4.67–2.82 (m, backbone and $\underline{CH_2}CH_2CH_3$ of propyl), 1.62 (s, $CH_2\underline{CH_2}CH_3$ of propyl), 0.93 (s, CH_3 of propyl).

Butyl-6-CO_2HPull: ^{1}H NMR (400 MHz, d_6-DMSO): δ 6.1–4.9 (m, backbone) 4.67–2.82 (m, backbone and $\underline{CH_2}CH_2CH_2CH_3$ of butyl), 1.59 (s, $CH_2\underline{CH_2}CH_2CH_3$ of butyl), 1.37 (s, $CH_2CH_2\underline{CH_2}CH_3$ of butyl), 0.92 (s, CH_3 of butyl). ^{13}C NMR (400 MHz, d_6-DMSO): 170.38 (C=O), 99.2–96.69 (3C-1), 80.89–65.08 (C2–C6 backbone and $\underline{CH_2}CH_2CH_2CH_3$ of butyl), 32.10 ($CH_2\underline{CH_2}CH_2CH_3$ of butyl), 18.97 ($CH_2CH_2\underline{CH_2}CH_3$ of butyl), 13.93 (CH_3 of butyl).

3. Results and discussion

3.1. Pullulan oxidation

Our approach to hydrophobically modified anionic pullulan derivatives began with oxidation with TEMPO and NaOCl/NaBr to obtain 6-carboxypullulan (2) (Fig. 1) (Denooy et al., 1995). The potential for complete oxidation of pullulan to its carboxylate, and the high regioselectivity of this reaction for C-6 were the motivation to employ this methodology to obtain pullulan derivatives containing carboxyl groups.

Pullulan was completely oxidized at C-6 as indicated by the ^{13}C NMR spectrum (Fig. S1 in Supplementary Data). Peaks at 60.6 and 60.3 ppm arising from the primary hydroxyl carbons (C-6, Fig. 1) in pullulan were no longer present in the product spectrum, while two carbonyl peaks from the new, chemically distinct carboxylic acids appeared at 175.8 and 176 ppm in the oxidized pullulan.

3.2. Synthesis and characterization of 6-carboxypullulan ethers

The oxidized pullulan product was recovered from the reaction mixture mainly as the sodium salt of the carboxylic acid, which was not soluble in DMSO. The etherification of 6-CO_2NaPull was first attempted by reacting it as a suspension in DMSO with NaOH (added in a small amount of water) and 1-bromobutane for 20 h at room temperature (Table 1). No reaction occurred under heterogeneous conditions, and only starting material was recovered. When the reaction began homogeneously with the CO_2NaPull dissolved in water (40 mL) and the bromobutane was added in DMSO (40 mL), CO_2NaPull immediately precipitated from the reaction mixture and the reaction could not be continued (results not shown).

6-CO_2NaPull was then treated with a proton exchange resin, converting it to its protonated form (6-CO_2HPull). Neutralization of the CO_2HPull with TBAOH yielded 6-carboxypullulan-TBA salt (6-CO_2TBAPull). The solubility of 6-carboxy pullulan vs. counterion (H, Na, or TBA) was then investigated (Table 2). In previous studies

Table 1
Optimization of reaction conditions for etherification of 6-carboxypullulan with bromobutane.

6-Carboxypullulan	Temp. (°C)	Time (h)	DS
6-CO_2NaPull	rt	20	No reaction
6-CO_2TBAPull	rt	20	0.41
6-CO_2TBAPull	40[a]	20	3.4
6-CO_2TBAPull	40	3.5	3.6
6-CO_2TBAPull	60[a]	20	2.91

[a] Only 1 h at this temperature.

from our laboratory, Pawar and Edgar (2011) found that alginic acid, another poly(uronic acid), could be converted to its TBA salt to enhance its organic solubility. Natural alginic acid, a copolymer of β-D-mannuronic acid and its C-5 epimer, α-L-guluronic acid, is insoluble in all common organic solvents, but could be dissolved as its TBA salt in a variety of polar aprotic solvents containing TBAF. The results from our solubility tests indicated that conversion of 6-carboxypullulan to its TBA salt provides similar solubility benefits (Table 2). Indeed, 6-CO_2TBAPull is soluble in DMSO without the need to add TBAF. This difference in solubility is believed to be because of the disruption of H-bonding interactions between the carboxylic acid and the hydroxyl groups. Furthermore, the remarkable solubility of 6-CO_2TBA-pullulan in organic media is believed to be due to the increased hydrophobicity conveyed by the *n*-butyl chains of the TBA anion and to the disruption of H-bonding by this bulky group.

The ^{1}H NMR spectrum of 6-CO_2TBA-pullulan in d_6-DMSO is shown in Fig. 2. The polymer backbone peaks appear in the region 2.8–5.5 ppm and overlap with 2 hydrogens from the butyl chain of the TBA [$N(\underline{CH_2}CH_2CH_2CH_3)_4$]. The other protons from the butyl chains of TBA appear at 0.9, 1.3, and 1.6 ppm. Integration of the TBA peaks against the polymer backbone demonstrates that all carboxyl groups are present as TBA salts.

It is well known that etherification is most facile in homogeneous solution (Durand & Dellacherie, 2006). Therefore, we performed etherification with the 6-CO_2TBAPull (Fig. 1), which was initially dissolved in DMSO (Table 2). After addition of NaOH, equilibrium is established between the TBA and Na forms of oxidized pullulan and the solution becomes viscous (due to partial formation of insoluble 6-CO_2NaPull), but no precipitate forms in the reaction mixture; the presence of the TBA ion keeps the carboxypullulan in solution. Upon addition of the alkyl halide, the reaction mixture turns fluid (the etherified product is soluble in DMSO). This procedure allows for the reaction to take place homogeneously and etherification proceeds successfully.

Process experiments were performed in order to optimize the reaction conditions (Table 1). When the etherification was

Table 2
Solubility of 6-carboxypullulan-protonated form and as Na and TBA salts.

	6-CO_2NaPull	6-CO_2HPull	6-CO_2TBAPull
Water	O	O	O
DMSO	X	Ø	O
DMF	X	Ø	O
DMSO/TBAF	X	X	O
DMF/TBAF	X	X	O
MeOH	X	X	O
EtOH	X	X	Ø
Ethylene Glycol	X	O	Ø
DMAc	X	X	Ø
ACN	X	X	Ø
Pyridine	X	X	X
THF	X	X	X

O = soluble, Ø = partially soluble, X = insoluble; DMSO: dimethylsulfoxide; DMF: dimethylformamide; TBAF: tetrabutylammonium fluoride; MeOH: methanol; EtOH: ethanol; DMAc: dimethylacetamide; ACN: acetonitrile; THF: tetrahydrofuran.

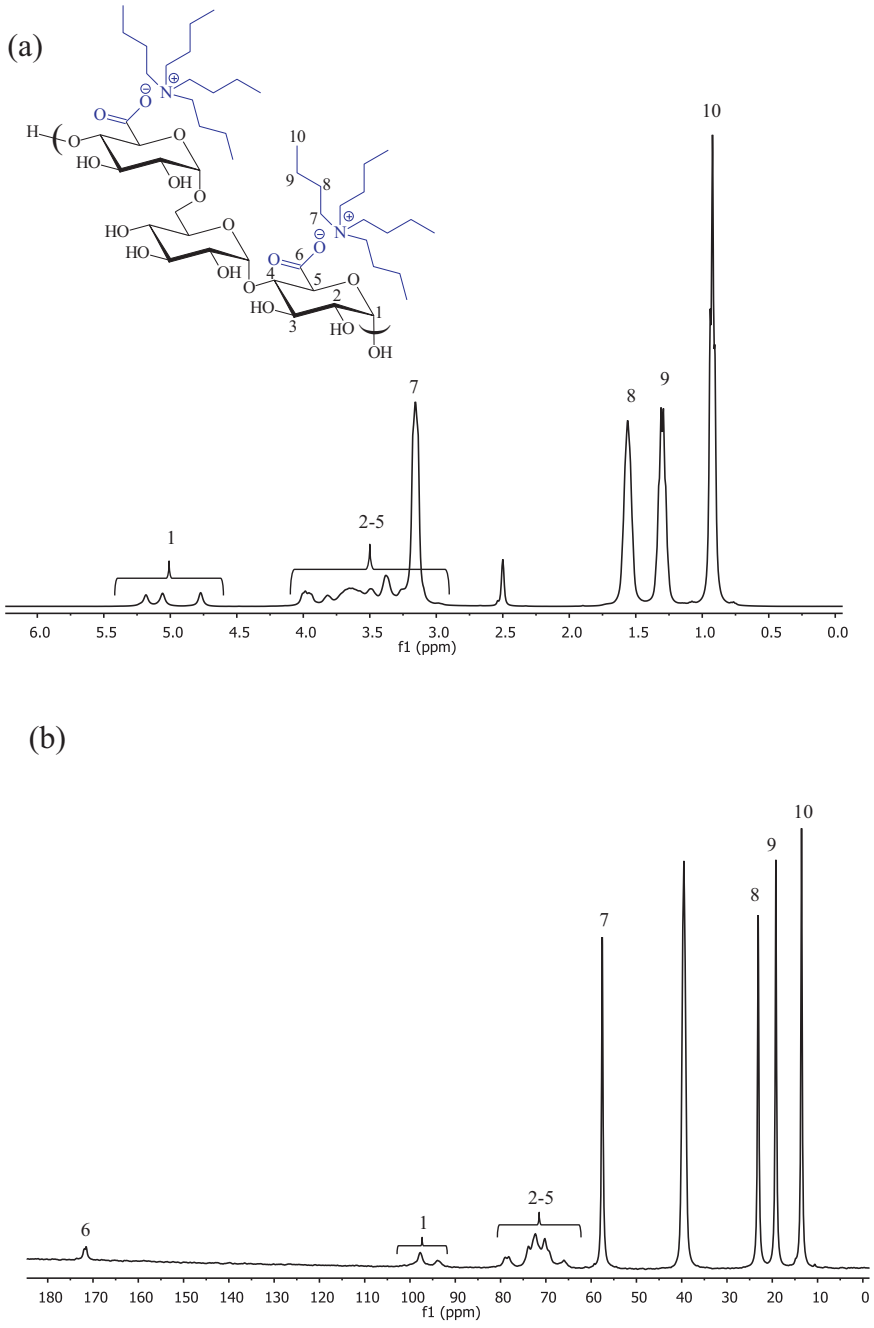

Fig. 2. (a) ^{1}H and (b) ^{13}C NMR spectra of 6-CO_2TBA pullulan in d_6-DMSO.

conducted with 6-CO_2TBAPull in DMSO at room temperature for 20 h, a DS of 0.41 was obtained. When the reaction was allowed to proceed at 40 °C for 1 h after the addition of NaOH, followed by reaction with bromobutane for 20 h at room temperature, the DS increased to 3.4. To optimize the reaction time, another experiment was conducted where, after reaction with NaOH for 1 h at 40 °C, bromobutane was added and the etherification was continued for 2.5 h, still at 40 °C. The DS obtained under these conditions (DS = 3.6) was similar to when the etherification was carried out for a much longer time at room temperature, indicating that high DS products can be obtained at shorter reaction times. In another experiment, the temperature was also increased to 60 °C during the reaction with NaOH, but no increase in DS resulted (DS = 2.91). If water was present during the etherification of 6-CO_2TBAPull, a sticky precipitate was formed that prevented further reaction. This is probably due to ion exchange forming 6-CO_2NaPull, which is insoluble in DMSO but soluble in water. Therefore, all experiments were carried out in dry DMSO and 6-CO_2TBAPull and NaOH were dried before use.

We explored the scope of the reaction by homogeneous etherification of 6-carboxypullulan TBA salt with different alkyl halides in DMSO with NaOH at 40 °C for 3.5 h to furnish a variety of 6-carboxypullulan ethers. Measured DS and solubility properties of the products are summarized in Table 3.

For the reactions performed with longer chain alkyl halides, the products, propyl pullulan-6-carboxylate (propyl-6-CO_2HPull, 4b) and butyl pullulan-6-carboxylate (butyl-6-CO_2HPull, 4c, 4f), had a similar DS of around 3, and were partially soluble in water. Solubility in DMSO and DMF depended on the chain length and DS of the ether substituent. Butyl-6-CO_2HPull was soluble in DMSO and

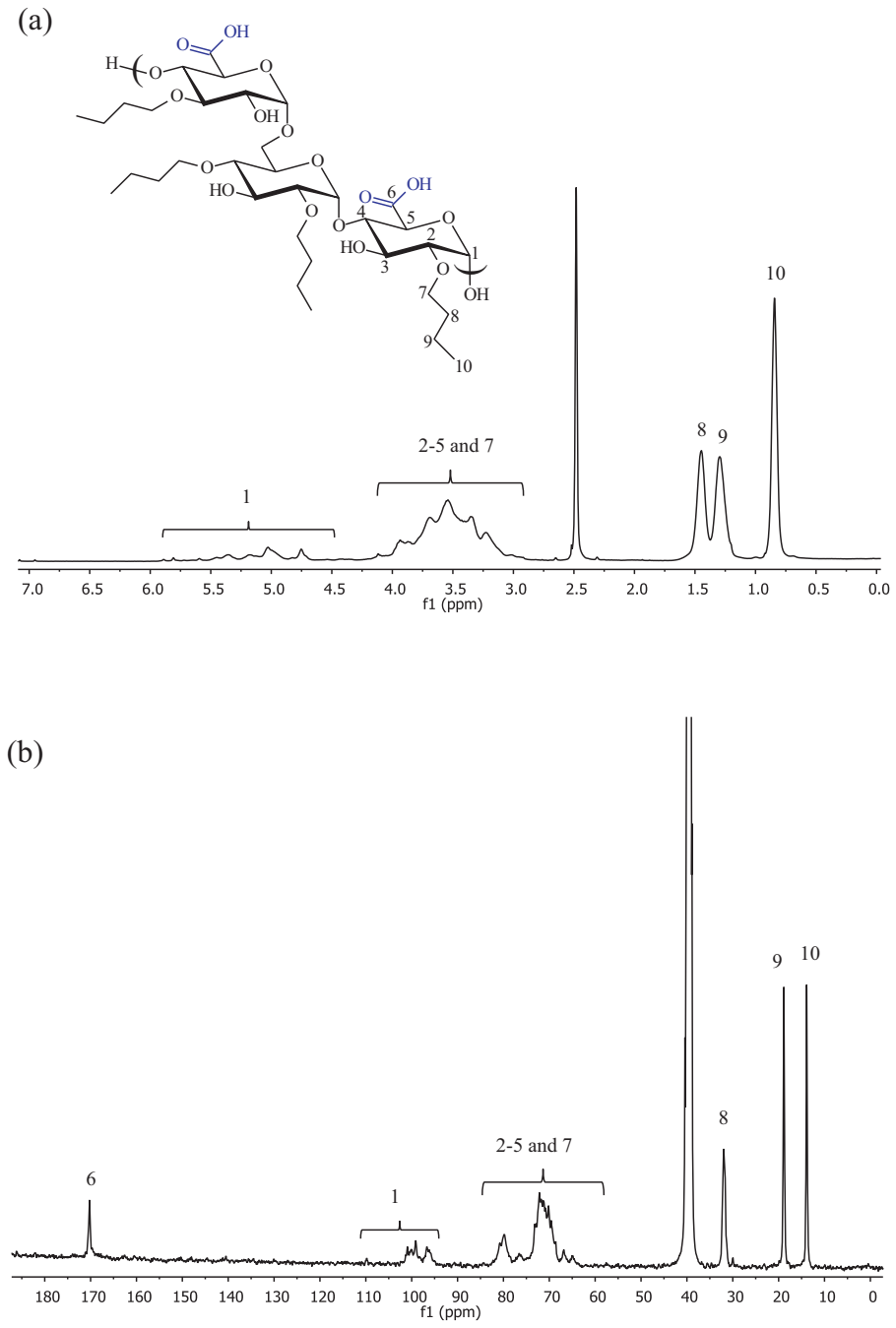

Fig. 3. (a) ^{1}H and (b) ^{13}C NMR spectra of butyl pullulan-6-carboxylate in d_6-DMSO.

DMF, but not the pullulan ether with the less hydrophobic propyl groups (which had similar DS). Reaction with shorter chain alkyl halides (methyl and ethyl), afforded products with higher DS, indicating that the smaller reagent more readily accesses the pullulan hydroxyl groups. The DS was around 5 for the reaction with bromoethane and full substitution (DS = 7) was achieved upon reaction with methyl iodide. These products (methyl-6-CO_2HPull, 4d and ethyl-6-CO_2HPull, 4e) still have partial water solubility and in this case, the much higher DS led to products that also had good organic solubility, despite the lower hydrophobicity of the methyl and ethyl groups.

Chemical structure of all carboxypullulan ether products was confirmed by ^{1}H and ^{13}C NMR, except for propyl-6-CO_2HPull. Due to the poor solubility of propyl-6-CO_2HPull, it was not possible to acquire a ^{13}C NMR spectrum, which requires a higher sample concentration, thus, its chemical structure characterization was restricted to ^{1}H NMR. Fig. 3a shows the ^{1}H NMR spectrum of butyl-6-CO_2HPull. The region between 2.8 and 6.1 ppm corresponds to the backbone protons in the butyl-6-CO_2HPull which overlap with 2 protons of one CH_2 from the butyl group ($\underline{CH_2}CH_2CH_2CH_3$). The other butyl group peaks are at 0.8, 1.3 and 1.4 ppm. The ^{13}C NMR spectrum of butyl-6-CO_2HPull (Fig. 3b) shows anomeric carbon peaks between 94 and 104 ppm. The other backbone carbon peaks are between 60 and 85 ppm and overlap with the CH_2 carbon of the butyl group ($\underline{CH_2}CH_2CH_2CH_3$). The other peaks from the butyl group are at 13.89, 18.87 and 33.72 ppm. The carboxylic acid carbonyls appear as a slightly broadened single peak at 170.9 ppm.

Table 3
Properties of 6-carboxypullulan ethers.

Product		Alkylating reagent	DS[a]	Solubility		Yield[c] (%)
				Water	DMSO or DMF	
4a	Ethyl-6-CO_2HPull	Bromoethane	5.12	Ø	O	53
4b	Propyl-6-CO_2HPull	Bromopropane	3.37	Ø	X	48
4c	Butyl-6-CO_2HPull	Bromobutane	3.6	Ø	O	46
4d	Methyl-6-CO_2HPull	Iodomethane	7[b]	Ø	O	45
4e	Ethyl-6-CO_2HPull	Iodoethane	7.36	Ø	O	45
4f	Butyl-6-CO_2HPull	Iodobutane	3.22	Ø	O	49

[a] Calculated from ^{1}H NMR spectra (see Section 2), with exception for product 4d.
[b] Based on quantitative ^{13}C NMR.
[c] Based on the molar mass of product with DS shown in this table.

We must consider the possibility that, under these reaction conditions, there may also be formation of ester groups by reaction of the alkyl halide with the carboxylate group of 6-carboxypullulan. Etherification should be the major reaction occurring since, in the presence of NaOH, deprotonation of the hydroxyl groups should occur and once the alkoxide is formed, this is a stronger and more reactive nucleophile than the carboxylate toward the alkylating reagent. The ^{1}H and ^{13}C NMR spectra showed single peaks for the carbons of the alkyl and the carbonyl groups, which is a strong indication that mainly etherification is taking place. Furthermore, the chemical shifts for those groups, although they should occur within a similar range, also indicate that the proton and carbon peaks observed in the ^{1}H and ^{13}C NMR spectra correspond to the carboxylic acid carbonyl and alkyl groups from the ethers. The carbonyl peaks for the 6-carboxypullulan ether products appear consistently at 171 ppm. The ^{13}C NMR chemical shifts for acid and ester carbonyls are expected at 170–180 ppm and 165–170 ppm respectively (Silverstein, Bassler, & Morrill, 1991).

The IR spectrum also showed strong evidence that the carboxyl groups are mostly not esterified. The carbonyl stretching absorption is one of the strongest IR absorptions, and is very useful in determining functional group identity. The carbonyl peak for esters should appear between 1750 and 1735 cm^{-1} and for acids 1700–1750 cm^{-1} (Silverstein et al., 1991). There is a small overlap in the frequency range for the carboxylic acid and ester carbonyls, thus, in order to be more precise in the carbonyl identification, IR was performed with the sodium salt of the 6-carboxypullulan ethers. The carbonyl absorbance so observed (around 1600 cm^{-1}) is readily identified as carboxylate rather than ester (Fig. 4). The carbonyl peak for the protonated carboxylic acid appears at 1727 cm^{-1} for CO_2HPull and at 1728 cm^{-1} for the alkylated CO_2H pullulan (Fig. S4a and b in Supplementary Data). Furthermore, there is no absorbance at around 1200 cm^{-1}, which is characteristic for C–O stretch in esters.

In order to quantify any possible carboxylate esterification reaction, alkaline hydrolysis of selected 6-carboxypullulan ethers was performed. The 6-carboxypullulan ether was stirred for 20 h in NaOH (0.1 M) in order to hydrolyze any esters that might be present. After hydrolysis, the resulting solution was dialyzed against water for 24 h and freeze-dried. The product was analyzed by ^{1}H NMR, and the DS was calculated and compared to the DS observed prior to hydrolysis. This experiment was performed with butyl-CO_2HPull (4c), ethyl-6-CO_2HPull (4a) and ethyl-6-CO_2HPull (4e), which were representative samples of DS around 3, 5 and 7 respectively. The results showed that the DS of the samples before and after the hydrolysis changed by only a small amount (up to approximately 0.3) for the 3 samples (Table S1 in Supplementary Data). This indicates that carboxyl esterification is at most a minor side reaction, in agreement with the observations from the NMR and IR characterization. If desired, any ester groups can be saponified by a short alkaline post-treatment as described above.

3.2.1. *Determination of critical aggregation concentration of 6-carboxypullulan ethers*

Amphiphilic polymers have the ability to form micellar aggregates in aqueous environments (Gref et al., 1994; Jung, Jeong, Kim, & Kim, 2004). Hydrophobized polysaccharides (Akiyoshi, Deguchi, Moriguchi, Yamaguchi, & Sunamoto, 1993; Nishikawa, Akiyoshi,

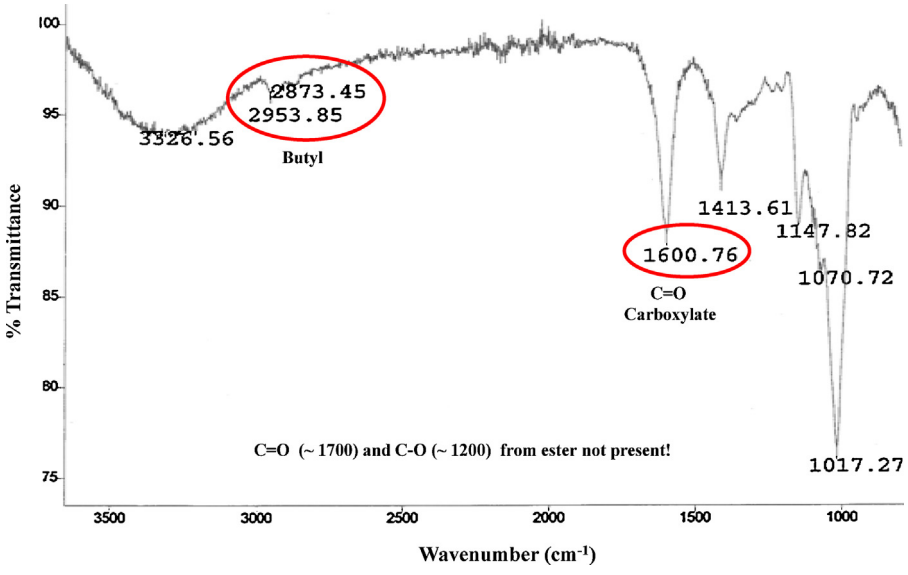

Fig. 4. IR spectrum of butyl pullulan-6-carboxylate.

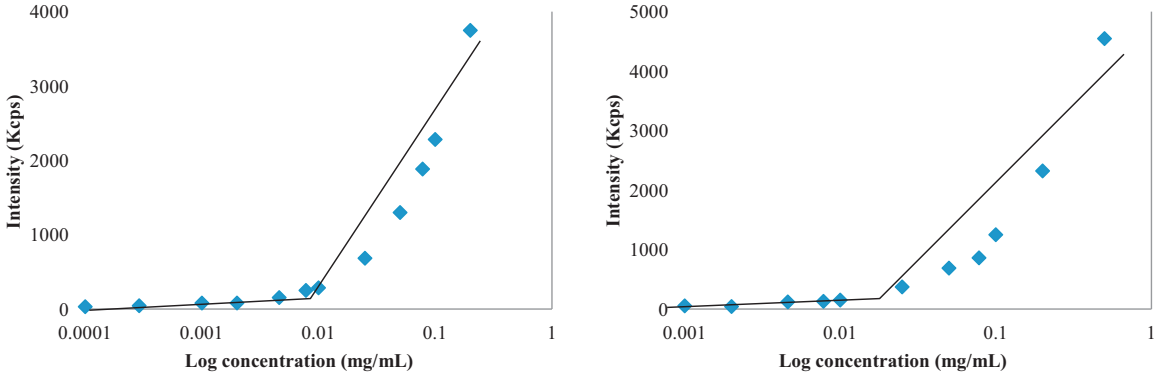

Fig. 5. Determination of CMC for (a) propyl-6-CO_2HPull and (b) butyl-6-CO_2HPull.

& Sunamoto, 1994) have received considerable attention due to their self-assembling characteristics with potential in drug delivery. We therefore investigated the self-assembly behavior of two 6-carboxypullulan ethers in water by dynamic light scattering (DLS).

A plot of the intensity of scattered light as a function of polymer concentration is shown in Fig. 5a for propyl-6-CO_2HPull and Fig. 5b for butyl-6-CO_2HPull. These results indicate that 6-carboxypullulan ethers are forming micelles at very low concentrations and the slope change in the crossover region could be related to the critical micelle concentration (CMC) value. For propyl-6-CO_2Pull, the CMC obtained was 7.8 μg/mL and for butyl-6-CO_2Pull, 25 μg/mL. The mean hydrodynamic diameter obtained using DLS for both samples in water was between 170 nm and 300 nm. The CMC values encountered for our 6-carboxypullulan ethers were within the same range obtained for other hydrophobically modified pullulans that showed interesting physicochemical properties. For example, amphiphilic cholesterol-modified pullulan had a CMC of 10 μg/mL (Akiyoshi et al., 1993). Poly(DL-lactide-co-glycolide)-grafted pullulan derivatives had CMC values ranging from 5.4 to 17 μg/mL and were successfully used in the incorporation and release of adriamycin (Jeong et al., 2006).

4. Conclusion

Carboxyl groups were introduced to the pullulan backbone by oxidation with TEMPO and NaOCl/NaBr. Oxidation was complete and selective for C-6, as expected. The oxidized product, 6-carboxypullulan, is even more water-soluble than pullulan, but we found that the TBA salt of 6-carboxypullulan is soluble in many organic solvents. Therefore, a range of 6-carboxypullulan ethers was synthesized by reaction of 6-carboxypullulan TBA salt homogeneously with various bromo- and iodoalkane reagents in DMSO and sodium hydroxide at 40 °C. Complete substitution (7 per trisaccharide repeat unit) was achieved upon reaction with iodoethane, vs. DS 5 upon reaction with bromoethane. Reaction with longer chain alkyl halides led to products with maximum DS ca. 3. Polymer structures were confirmed by ^{1}H and ^{13}C NMR as well as IR spectroscopy. Solubility properties strongly depend on the size of the alkyl group and also on the DS. We investigated the possibility of ester formation by reaction of the alkyl halide with the carboxyl group in the 6-carboxypullulan by analysis of the NMR and IR spectra, and by alkaline hydrolysis, which showed that at most only a small amount of carboxylate esterification occurs under these conditions. The amphiphilic character of these polymers led to formation of micellar aggregates, and very low critical micelle concentrations were found for selected 6-carboxypullulan ethers. We have not yet successfully measured the molecular weight of the 6-carboxypullulan ethers because of their strong tendency to self-aggregate in all solvents tested. 6-Carboxypullulan ethers with interesting properties can be obtained by a simple methodology, so the next step will be evaluation of these polymers for drug delivery and other applications.

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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.2012.12.029.

References

Akiyoshi, K., Deguchi, S., Moriguchi, N., Yamaguchi, S., & Sunamoto, J. (1993). Self-aggregates of hydrophobized polysaccharides in water – formation and characteristics of nanoparticles. *Macromolecules*, *26*, 3062–3068.
Akiyoshi, K., Yamaguchi, S., & Sunamoto, J. (1991). Self-aggregates of hydrophobic polysaccharide derivatives. *Chemistry Letters*, *20*, 1263–1266.
Bauer, R. (1938). Physiology of Dematium pullulans de Bary. *Zentralbl. Bacteriol. Parasitenkd. Infektionskr. Hyg. Abt.*, *2*, 133–167.
Bender, H. L., & Wallenfels, J. K. (1959). Pullulan, an extracellular glucan from *Pullularia pullulans*. *Biochimica et Biophysica Acta*, *36*, 309–316.
Bernier, B. (1958). The production of polysaccharides by fungi active in the decomposition of wood and forest litter. *Canadian Journal of Microbiology*, *4*, 195–204.
Denooy, A. E. J., Besemer, A. C., & van Bekkum, H. (1995). Highly selective nitroxyl radical-mediated oxidation of primary alcohol groups in water-soluble Glucans. *Carbohydrate Research*, *269*, 89–98.
Dulong, V., Le Cerf, D., Picton, L., & Muller, G. (2006). Carboxymethylpullulan hydrogels with a ionic and/or amphiphilic behavior: Swelling properties and entrapment of cationic and/or hydrophobic molecules. *Colloids Surfaces A*, *274*, 163–169.
Durand, A., & Dellacherie, E. (2006). Neutral amphiphilic polysaccharides: Chemical structure and emulsifying properties. *Colloid and Polymer Science*, *284*(5), 536–545.
Edgar, K. J. (2007). Cellulose esters in drug delivery. *Cellulose*, *14*, 49–64.
Friesen, D. T., Shanker, R., Crew, M., Smithey, D. T., Curatolo, W. J., & Nightingale, J. A. S. (2008). Hydroxypropyl methylcellulose acetate succinate-based spray-dried dispersions: An overview. *Molecular Pharmaceutics*, *5*, 1003–1019.
George, M., & Abraham, T. E. (2006). Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan – a review. *Journal of Controlled Release*, *114*, 1–14.

Gref, R., Minamitake, Y., Peracchia, M. T., Trubetskoy, V., Torchilin, V., & Langer, R. (1994). Biodegradable long-circulating polymeric nanospheres. *Science*, *263*, 1600–1603.

Henni-Silhadi, W., Deyme, M., Boissonnade, M. M., Appel, M., Le Cerf, D., Picton, L., & Rosilio, V. (2007). Enhancement of the solubility and efficacy of poorly water-soluble drugs by hydrophobically-modified polysaccharide derivatives. *Pharmaceutical Research*, *24*, 2317–2326.

Hirakura, T., Nomura, Y., Aoyama, Y., & Akiyoshi, K. (2004). Photoresponsive nanogels formed by the self-assembly of spiropyrane-bearing pullulan that act as artificial molecular chaperones. *Biomacromolecules*, *5*, 1804–1809.

Hosseinkhani, H., Aoyama, T., Ogawa, O., & Tabata, Y. (2002). Liver targeting of plasmid DNA by pullulan conjugation based on metal coordination. *Journal of Controlled Release*, *83*, 287–302.

Ilevbare, G. A., Liu, H. Y., Edgar, K. J., & Taylor, L. S. (2012). Understanding polymer properties important for crystal growth inhibition-impact of chemically diverse polymers on solution crystal growth of ritonavir. *Crystal Growth and Design*, *12*, 3133–3143.

Jeong, Y.-I., Na, H.-S., Oh, J.-S., Choi, K.-C., Song, C.-E., & Lee, H.-C. (2006). Adriamycin release from self-assembling nanospheres of poly(DL-lactide-co-glycolide)-grafted pullulan. *Journal of Pharmaceutical Sciences*, *322*, 154–160.

Jung, S. W., Jeong, Y. I., & Kim, S. H. (2003). Characterization of hydrophobized pullulan with various hydrophobicities. *International Journal of Pharmaceutics*, *254*, 109–121.

Jung, S. W., Jeong, Y. I., Kim, Y. H., & Kim, S. H. (2004). Self-assembled polymeric nanoparticles of poly(ethylene glycol) grafted pullulan acetate as a novel drug carrier. *Archives of Pharmacal Research*, *27*, 562–569.

Kar, N., Liu, H. Y., & Edgar, K. J. (2011). Synthesis of cellulose adipate derivatives. *Biomacromolecules*, *12*, 1106–1115.

Konno, H., Handa, T., Alonzo, D. E., & Taylor, L. S. (2008). Effect of polymer type on the dissolution profile of amorphous solid dispersions containing felodipine. *European Journal of Pharmaceutics and Biopharmaceutics*, *70*, 493–499.

Kost, J., & Langer, R. (2001). Responsive polymeric delivery systems. *Advanced Drug Delivery Reviews*, *46*, 125–148.

Leathers, T. D. (2003). Biotechnological production and applications of pullulan. *Applied Microbiology and Biotechnology*, *62*, 468–473.

Lu, D. X., Wen, X. T., Liang, J., Gu, Z. W., Zhang, X. D., & Fan, Y. J. (2009). A pH-sensitive nano drug delivery system derived from pullulan/doxorubicin conjugate. *Journal of Biomedical Materials Research Part B*, *89B*, 177–183.

Nishikawa, T., Akiyoshi, K., & Sunamoto, J. (1994). Supramolecular assembly between nanoparticles of hydrophobized polysaccharide and soluble-protein complexation between the self-aggregate of cholesterol-bearing pullulan and alpha-chymotrypsin. *Macromolecules*, *27*, 7654–7659.

Paris, E., & Stuart, M. A. C. (1999). Adsorption of hydrophobically modified 6-carboxypullulan on a hydrophobic surface. *Macromolecules*, *32*, 462–470.

Pawar, S. N., & Edgar, K. J. (2011). Chemical modification of alginates in organic solvent systems. *Biomacromolecules*, *12*, 4095–4103.

Posey-Dowty, J. D., Watterson, T. L., Wilson, A. K., Edgar, K. J., Shelton, M. C., & Lingerfelt, L. R. (2007). Zero-order release formulations using a novel cellulose ester. *Cellulose*, *14*, 73–83.

Rekha, M. R., & Sharma, C. P. (2007). Pullulan as a promising biomaterial for biomedical applications: A perspective. *Trends in Biomaterials and Artificial Organs*, *20*, 116–121.

Silverstein, R. M., Bassler, G. C., & Morrill, T. C. (1991). *Spectrometric identification of organic compounds*. New York: John Wiley & Sons.

Shingel, K. I. (2004). Current knowledge on biosynthesis, biological activity, and chemical modification of the exopolysaccharide, pullulan. *Carbohydrate Research*, *339*, 447–460.

Xi, K. L., Tabata, Y., Uno, K., Yoshimoto, M., Kishida, T., Sokawa, Y., & Ikada, Y. (1996). Liver targeting of interferon through pullulan conjugation. *Pharmaceutical Research*, *13*, 1846–1850.

Yamaoka, T., Yasuhiko, T., & Ikada, Y. (1993). Body distribution profile of polysaccharides after intravenous administration. *Drug Delivery*, *1*, 75–82.

Yang, J. H., Du, Y. M., Huang, R. H., Wan, Y. Y., & Wen, Y. (2005). The structure-anticoagulant activity relationships of sulfated lacquer polysaccharide – effect of carboxyl group and position of sulfation. *International Journal of Biological Macromolecules*, *36*, 9–15.