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Studies on physico-chemical and antibacterial properties of grafted pullulans solutions

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ABSTRACT

The physico-chemical properties of three grafted pullulans (P) having linked poly(3-acrylamidopropyl)trimethylammonium chloride (pAPTAC) as side chains (P-g-pAPTAC1, P-g-pAPTAC2 and P-g-pAPTAC3 with 22.53, 29.05, and 34.51 (wt.%) of pAPTAC content in polymer, respectively) and possessing polyelectrolyte character were determined by light scattering analysis. All grafted pullulan aqueous solutions were tested in the presence of 0.5 M NaCl, KCl, $NaNO_3$ or KNO_3. The biggest associations were recorded in 0.5 M NaCl aqueous solutions for P-g-pAPTAC1, P-g-pAPTAC2 and P-g-pAPTAC3 according to the maximum values for R_g extracted from MALLS (multiangle laser light scattering) measurements. Also, the dominant conformation in salted solution of these polyelectrolytes was random coil as Debye plot analysis revealed. Antibacterial activity was tested by Kirby–Bauer diffusion method and all grafted pullulans dissolved in aqueous solutions of 0.5 M NaCl have developed inhibition zone against *Staphylococcus aureus* (ATCC 25923).

1. Introduction

According to literature, the low molecular weight antimicrobial agents have some disadvantages, such as toxicity to the environment and short-term antimicrobial capacity. To overcome these problems, antimicrobial functional groups can be introduced into polymer molecule [1].

Pullulan is a neutral polysaccharide, synthesized by *Aureobasidium pullulans* fungus, with no antibacterial activity [2]. This natural polymer is biodegradable, commercially available at relative low price and possesses nine biodegradable hydroxyl groups on structural unit susceptible for chemical modification [3].

The chemical modification of natural polymers is a promising route for production of new biomaterials with specific properties (solubility, biodegradability, chemical or thermal stability, and mechanical behavior). Graft-copolymerization of pullulan with poly(ethylene glycol) PEG, poly(L-lactide) PLLA and poly(*N*-isopropylacrylamide) PNIPAAm have been reported [4–6]. In these cases, the authors have proposed to analyze the hydrophilic–hydrophobic balance, solubility in different solvents or flocculation capacity.

The graft-polymerization of vinylic monomers (possessing strong quaternary ammonium groups) on pullulan is a relative facile, inexpensive and "friendly" method for introducing a great density of charged functions and in the same time, a certain hydrophobicity induced by the alkyl chain of the monomer unit. By this method, it can be inserted a huge number of pendant ammonium groups on a single OH groups of pullulan. Generally, in design of antimicrobial systems, researchers try to achieve a balance between hydrophilicity and hydrophobicity, useful characteristics which favor the adsorbtion of biocidal agents on the surface of bacteria. The adhesion of antimicrobial agents to bacterial cells is caused by electrostatic interaction, hydrophobic interaction and other forces of interaction such as hydrogen bonding and van der Walls forces.

The pullulan and modified pullulan solutions could be characterized by laser light scattering and chromatographic methods like static light scattering (SLS), dynamic light scattering (DLS) and size exclusion chromatography coupled with multiangle laser light scattering (SEC–MALLS), either they are dissolved or not in salted solutions [5,7]. Based on these methods, important characteristics of polymers in solution were determined (Z-averaged root-mean-square radius of gyration R_g, hydrodynamic radius R_h, weight-average molecular weight M_w, polydispersity index M_w/M_n, structure sensitive ratio $\rho = R_g/R_h$, power law exponent a from conformational plot). All mentioned solution properties are useful to design new polymeric systems based on natural substances such as polysaccharides, for drug carriers, desalinization and purification of wasted waters or extensively fertilized soils.

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The polysaccharides are well known to possess a high tendency to associate, caused by the abundance in hydroxyl or amino groups present in the macromolecules, that easily undergo hydrogen bonding. The external monovalent and divalent counterions play an important role in solution properties of polysaccharides dissolved in salted aqueous solution as a result of the change in the effective charge density of the polysaccharide chains. Knowing the effect of Na^+ and K^+ cations on conformational properties of polysaccharides, other authors have studied solution properties of chitosan, k-carrageenan, flaxseed gum, sodium alginate and galactomannans [8–12].

Many literature results support the essential importance of polycationic structure in antimicrobial activity. A higher positive charge density leads to strong electrostatic interaction. Therein, the positive charge is associated with degree of substitution (DS) of pullulan, which affect the positive charge density. Polymeric quaternary ammonium salts represent a special class of antimicrobial substances continuously developing. They are used as biostatic or biocide agents against Gram negative or Gram positive bacteria and other microorganisms [13–18]. For the P-g-pAPTAC copolymers here analyzed, we expect that a more hydrophilic backbone would improve the biocompatibility of antimicrobial polymers, while side chain modification could be exploited to tune antimicrobial activity.

In the present study, the results obtained by SLS for three pullulan derivatives bearing poly(3-acrylamidopropyl)trimethylammonium chloride (pAPTAC) side chains, in terms of salt influence on solution properties were presented. The characterization of these polyelectrolytes needs to screen at least long range electrostatic repulsion and to work in the presence of external salt (low molecular electrolyte). Therefore, two alkali chloride salts (NaCl; KCl) and two alkali nitrate salts ($NaNO_3$; KNO_3) were tested. Also, antibacterial activity of these polycationic compounds against *Staphylococcus aureus* (ATCC 25923) was reported for the first time.

2. Experimental section

2.1. Material and solution preparations

Pharmaceutical grade purity pullulan (P) with weight-average molecular weight M_w = 200,000 g mol^{-1} was purchased from Hayashibara Lab., Ltd. (Okoyama, Japan) and used as received. Its derivatives, namely P-g-pAPTAC1, P-g-pAPTAC2 and P-g-pAPTAC3 were obtained by grafting (3-acrylamidopropyl)trimethylammonium chloride APTAC (75 wt.% aqueous solution) from Aldrich and using potassium peroxydisulfate KPS (Fluka) as initiator; the graft-polymerization conditions were presented in other works [19,20]. Fig. 1 shows the chemical structure of grafted pullulans.

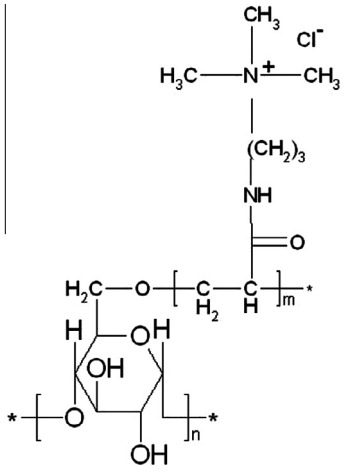

Fig. 1. Chemical structure of grafted pullulans.

These three derivatives correspond to three combinations of molar ratios between reactants (P:APTAC:KPS about 1:0.7:0.05, 1:1.4:0.05, and 1:0.7:0.13 respectively; P refers to the structural unit of pullulan). The estimated grafted content of pAPTAC in P-g-pAPTAC1, P-g-pAPTAC2 and P-g-pAPTAC3 was 22.53, 29.05, and 34.51 (wt.%) respectively. It was calculated from ^{1}H NMR spectrum in deuterated water (on Bruker Avance DRX-400 NMR spectrometer) [19] by using the equation:

$$g\%\ \text{grafted pAPTAC} = \frac{(A_{1.9}/2) \times 206.72}{(A_{5.6} + A_{5.2}) \times 368.72} \times 100 \quad (1)$$

where $A_{1.9}$ is area of the integral value corresponding to the methylene protons of grafted pAPTAC units, $A_{5.6}$ and $A_{5.2}$ are the areas of the integral values of H_1 and H_6 protons of pullulan moieties. The monomer molar mass is 206.72 and the molar mass of grafted structural unit of pullulan is 369.72. The weight content of pAPTAC in the grafted copolymers was calculated using nitrogen analysis and equation proposed by Kjeldahl method:

$$\%\ \text{grafted pAPTAC} = \frac{\%N}{14.01} \times 206.72 \quad (2)$$

The light scattering methods allow the exploration of different associations of biomacromolecules, but require suitable solvents for the molecular dissolution of components. In case of polymers with polyelectrolyte behavior, the addition of salts causes screening of charges on polymer chains and thus provides extended conformation of macromolecules desired for the above-mentioned measurements. The initial tests were performed on 0.1 M NaCl aqueous solutions and the negative A_2 values suggested attraction between the macromolecular chains and aggregation of them. This result showed that isn't good solvent from thermodynamically point of view for tested polymers. Therefore, all solutions were prepared at room temperature in aqueous solutions of 0.5 M salt using NaCl, KCl, $NaNO_3$, and KNO_3 respectively (from Fluka), and purified water (Millipore Simplicity-UV device) and then stirred about 24 h for homogenization. In all cases, the solvent and the stock solutions were filtered by 0.02 μm, and 0.45 μm Whatman filters, respectively. The dilute solutions were gravimetrically prepared for refractometric and laser light scattering measurements in range of 1×10^{-3}–2×10^{-4} g mL^{-1}. The quartz scintillation vials used for static light scattering measurements were provided by Hellma GmbH & Co., KG, Germany.

For antimicrobial susceptibility tests of the grafted pullulans, a reference strain of *S. aureus* (ATCC 25923) was used. The Mueller–Hinton agar medium was prepared in the laboratory according to the Clinical and Laboratory Standards Institute (CLSI) [21]. The bacterial inoculum was obtained from cultures aged for 18 h and pre-incubated at 37 °C. Three milliliters of suspension with about 10^5 CFU mL^{-1} were spread onto Petri plates containing Mueller–Hinton agar medium. After 10 min, the excess was removed by aspirating with a pipette, and then plates were left for 30 min to allow the microorganism to adhere to the medium. Next, two sterile steel cylinders (internal diameter of 0.5 cm and height of 1 cm) were placed on each Petri plates. 0.2 mL of the test solution was then pipetted into each cylinder. For each grafted pullulan, the test solutions included about 50 mg mL^{-1} and 25 mg mL^{-1}, respectively. For antibacterial test, the polymers were dissolved in aqueous solutions of 0.5 M NaCl (itself a component of nutritive culture medium). To evaluate bacterial growth, the plates were incubated for 24 h at 37 °C. The antibacterial activity of the polymers was then determined by measuring the diameter of the inhibition area (in mm) around each cylinder.