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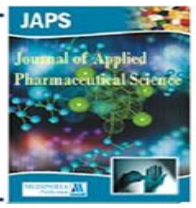

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The role of microbial pullulan, a biopolymer in pharmaceutical approaches: A review

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ABSTRACT

The article presents an overview of the latest advances in investigations and application of the pullulan from microbes (*Auriobasidium sps.*) and its derivatives in the biomedical and pharmaceutical field and how this polysaccharide can be of use as a promising biomaterial in the coming future. So many papers were published during the last four decades indicated that pullulan the unique biomaterial which has so many applications in food, cosmetics, biomedical and pharmaceutical industries . The unique linkage α (1→4) and α (1→6) pattern of pullulan endows the polymer with distinctive physical traits, including adhesive properties and the capacity to form fibers. Due to its non-toxic, non-immunogenic, non-mutagenic and non-carcinogenic nature recently there is an attempt to explore this polysaccharide for various medical applications including targeted drug and gene delivery, tissue engineering, wound healing and also even in diagnostic applications like perfusion, receptor, and lymph node target specific imaging and vascular compartment imaging using it as quantum dots.

Key words: Pullulan, Biopolymer, Tissue engineering, Quantum dots, Biomaterial.

INTRODUCTION

Biopolymer as biomaterial is a non-viable material used in medical devices intended to interact with biological system. In present day uses of biopolymers as biomaterials is increasing its demand. Biopolymers produced by a wide variety of microorganisms, are generally water soluble gums which have novel and unique physical properties. Because of their wide diversity in structure and physical characteristics these polysaccharides have found a wide range of applications in the food, pharmaceutical and other industries. Pullulan is one of the biopolymer purified from the fermentation medium of the *Aureobasidium pullulans* (bishwambhar et al, 2011). This polymer is consisting of maltotriose units connected by an α (1→4) glycosidic bond, whereas consecutive maltotriose units are connected to each other by an α (1→6) glycosidic bond. The use and application of pullulan is rapidly emerging as a new and industrially important source of polymeric materials which are gradually becoming economically competitive with natural gums produced from marine algae and other plants(Suneetha et al,2010). A film based oral care product 'Listerine' produced from pullulan is commercialized in many countries now a day (Tsujisaka et al, 1993). More over capsules that is made from pullulan are used instead of gelatin for addressing a variety of cultural and dietary requirements, including those of vegetarians, diabetics and patients with restricted diets.

There is a twenty year history of safe use in Japan as a food ingredient and as a pharmaceutical bulking agent. The daily intake of pullulan would be up to 10 g per day for a person based on food categories and usage which was estimated by FDA (Rekha et al, 2007).

Pullulan is highly water soluble hence it is used as a carrier for drug and helps in controlled release in plasma. Mostly hydrophobized pullulan is used as drug delivery carriers. Pullulan can be chemically modified to produce derivatives with low solubility or a modified polymer that is completely insoluble in water (Tsujisaka et al, 1993). Pullulan derivatives are developed and their applications towards the above mentioned aspects were also studied by various groups (Akiyoshi et al, 1998). Pullulan derivatives were also obtained by polysaccharide derivatisation with targeting agents, which were used to produce self-organized drug loaded nanogels for receptor mediated cancer cells targeting (Park et al, 2006; Kim et al, 2001). Grafting of synthetic polymers on natural polysaccharides has been widely used as one of the most convenient ways to combine the advantages of natural and synthetic macromolecules. A number of papers have been published on the grafting polymerization on to pullulan (Masci et al, 2002; Ohya et al, 1998; Jiao et al, 2004; Wu et al, 2009). In particular, pullulan has been investigated as a macromolecular platform to construct colloidal drug delivery formulations such as pH-sensitive nanoparticles, assemblies and bioconjugates for anticancer drug delivery (Suginoshita et al, 2002; Na et al, 2007). Pullulan is known for its specificity for liver and this property is exploited for liver targeting (Kaneo et al, 2001; Tanak et al, 2004). Cationic pullulan based gene delivery vectors are reported previously by other groups (Jo et al, 2006; Juan et al, 2007). Spermine pullulan was developed and its *in vivo* transfection efficiency was established. Commercial production of pullulan began in 1976 by the Hayashibara Company, in Okayama, Japan (Tsujisaka and Mitsuhashi, 1993).

BIOMEDICAL AND PHARMACEUTICAL APPLICATION OF PULLULAN

The use of pullulan in biomedical field is increasing contemporarily due to its non-toxic, non-immunogenic, biocompatible and inert nature. In comparison to dextran, the degradation rate of pullulan in serum is faster than that of dextran. The degradation index is 0.7 after 48 hour incubation while for dextran it is 0.05 (Bruneel et al, 1993).

Tissue engineering and grafting

The bulk and surface properties of bio materials used for medical implants have been shown directly influences and some cases control the dynamic interaction that takes place at tissue-implant interface. These characteristics and changes in characteristics that may takes place over time *in-vivo* should be known for designing biomaterial for specific applications and the same thing can be easily done for the pullulan. Carboxymethylated pullulan conjugated with heparin was developed by various groups and its properties towards tissue engineering applications was investigated. 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 (Na et al, 2006). Surface modification is a major tool for the tissue engineering purposes. Nine hydroxyl groups are available for substitution reactions on the repeating unit. They are distinguished by their position on the glucosidic moiety OH-2, OH-3, OH-4 and OH-6 with the ratios 3, 3, 1 and 2 respectively and their substitution is listed in table-1. Heparin-conjugated pullulan inhibited the proliferation of smooth muscle cells *in vitro* and thus can be used for the proliferation of vascular endothelial cells and to inhibit the proliferation of smooth muscle cells.

Consideration of grafting parameters

The increase in weight of the grafted pullulan over that of the pullulan indicated the grafting of concerned groups on to pullulan. The graft yields were characterized by the following parameters (Gao et al, 1998).

$$\text{Grafting ratio \% G} = \frac{\text{Weight of grafted chains}}{\text{Weight of pullulan}} \times 100$$

$$\text{Grafting efficiency \% E} = \frac{\text{Weight of grafted chains}}{\text{Weight of polymer formed}} \times 100$$

$$\text{Grafting ratio \% G} = \frac{\text{Weight of polymer formed}}{\text{Weight of monomer formed}} \times 100$$

The scanning electron micrographs of pullulan and copolymerized pullulan with different grafting ratio of 29% and 41% is given in Fig.1 in which the grafting is observed nicely.

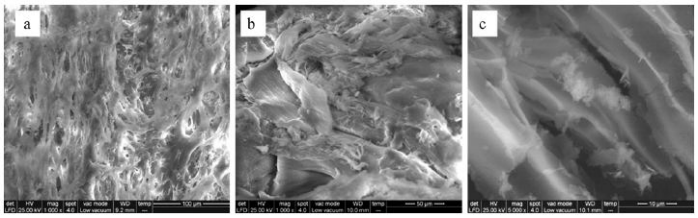

Fig.1: Scanning electron micrographs of pullulan (a), and copolymerized pullulan with different grafting ratio: 29% (b) and 41% (c). (Source: Marieta et al, 2011)

Pullulan as a carrier for drug delivery

To achieve drug delivery, stimuli-sensitive polymer systems have been intensively exploited as candidate materials (Kim et al, 2001; Kurkuri et al, 2004). Most part of pH and temperature sensitive microspheres used for the controlled delivery of drugs are not biodegradable. As studied by Gheorghe et al, 2008 in order to confer their temperature sensitivity, poly(N-isopropylacrylamide-co-acrylamide) was grafted onto pullulan microspheres. Then, the pH-sensitive units (–COOH) were introduced by reaction between the remaining –OH groups of the pullulan with succinic anhydride. The grafted pullulan microspheres are more hydrophilic than pullulan microspheres, their swelling degree as well as water regain increase significantly. Thus a pH and temperature sensitive pullulan microspheres for controlled release of drugs can be prepared (Gheorghe et al, 2008) whose scanning electron micrograph is given in Fig.2. The use of

pullulan, a member of the extracellular polysaccharide family often used in the last decade in pharmaceutics confers to microspheres stability, biocompatibility, and biodegradability (Fundueanu et al, 2003; Mocanu et al, 2004).

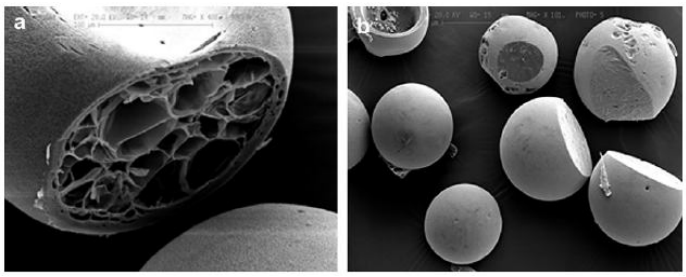

Fig.2: Scanning electron micrographs of pH and temperature sensitive pullulan microspheres prepared before (a) and after (b) grafting. (Source: Gheorghe et al, 2008).

Liver targeting of drug loaded pullulan

Liver binding affinity of the modified pullulan was tested *in vitro* on hepatocytes and *in vivo* on mice. The complex was found to be stable in the presence of plasma as observed from gel retardation assay(Xi et al,1996). This study focuses on the blood compatibility of the cationic pullulan, physico-chemical characterization and uptake of the nanocomplex by hepatocytes and *in vitro* transfection (Rekha et al, 2009).

Pullulan based anti-cancer drug

Two new anti-cancer polymer therapeutics were designed for tumour cell targeting. The bioconjugates were synthesized by pullulan derivatisation with either doxorubicin or doxorubicin and folic acid. Pullulan was activated by periodate oxidation and functionalized by reductive conjugation with cysteamine and 1.9 kDa PEG $(NH_2)_2$ (Scomparina et al, 2011). These findings suggest that the novel doxorubicin–pullulan bioconjugates possess suitable properties for passive tumour targeting. On the other hand, folic acid conjugation has been found to have limited effect on selective cell up-take.

Hydrophobised pullulan conjugates for drug delivery ; A Recent Development

Numerous papers deal with pullulan hydrogels as drug delivery systems, particularly in the form of microgels and nanogels. Obvious therapeutic benefits can be achieved by slow release of drugs into the plasma, and thus altering the concentration profiles of the drugs(Wooram et al,2010). Hydrogel nanoparticles of cross linked pullulan with glutaraldehyde have been prepared in order to develop a DNA carrier system, improving gene loading efficiency, controlled-release properties, biocompatibility and enhanced stability (Gupta et al, 2004). In particular, amphiphilic pullulans obtained from cholesteryl, acetyl or chloroacetyl graftings onto the hydroxyl groups form nanogels that are able to trap hydrophobic molecules, proteins or peptides, and nucleic acids. Moreover, hydrophobised pullulan-based nanogels interact also with various molecular assemblies such as liposomes and oil-water emulsions (Taniguchi et al, 1999). As a consequence, hydrophobised pullulan conjugates were used as drug targeting carriers for bioactive substances, such as metronidazole, nicotinic acid, sulfathiazole, mitoxantrone or epirubicin through their binding to various hydrophobic substances and soluble proteins as well as in biotechnology as artificial molecular chaperones in the presence of β-cyclodextrin or for the formation of hybrid nanogels. They can be used as polymeric nanocarriers in cancer chemotherapy protein stabilisation and artificial vaccines. Stimuli responsive nanogels such as pH-responsive thermo responsive and photo responsive nanogels were also designed using a similar self-assembly method (Mocanu et al, 2002; Sungwon et al, 2008; Wang et al, 1999)

The most of the cited paper in the field of hydrophobised pullulan reports about the self-assembly of cholesteryl-bearing pullulan as stable hydrogel nanoparticles in which pullulan chains were non covalently cross linked by associating cholesteryl moieties as given in Fig.3.

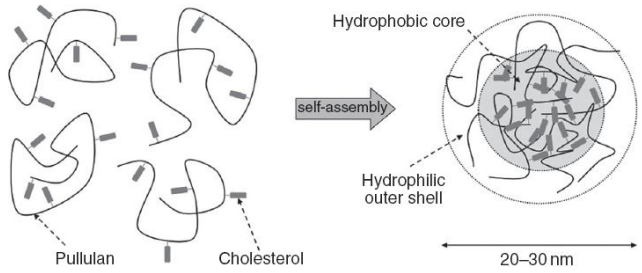

Fig.3: Formation of cholesterol-pullulan conjugate-based nanoparticles by self-aggregation in aqueous solution (Source: Lee et al, 2004).

There are about 100 glucose units between cholesterol moieties on the pullulan backbone. Eventually, recent studies have shown that cholesterol-bearing pullulan would be of great interest in the fight against Alzheimer's disease (Ikeda et al, 2009). These cholesterol-pullulan conjugate-based nanoparticles also have applications in the prevention of bone resorption when complexed to a tumour necrosis factor-α and receptor activator of nuclear factor-κβ ligand antagonist that prevents the reduction in bone mineral density (Alles et al, 2009). More recently, cholesterol-pullulan conjugated nanogels were used as a new vehicle for oro-digestive vaccine therapy, which is a developing area of interest in needle-free vaccine. Intranasal injection of antigen alone does not induce a high level of antigen, and adjuvant is always needed. In the same way, a chemically modified pullulan (palmitoyl derivative (*O*-palmitoylpullulan)-entrapped bovine serum albumin (BSA) as an antigen model was used for liposome coating. The polysaccharide could produce better IgG and IgA titre levels as compared to plain alum-adsorbed BSA. When administered by oral route, the plain liposomes containing BSA produced higher IgG and IgA levels as compared to control (Cui et al, 2002).

Application of Pullulan in gene delivery

Gene therapy is another area where the application of pullulan is being explored. Gene delivery is usually mediated by endocytic pathway. Efforts for gene therapy using virus have been performed but the major drawback that viruses are known to be

immunogenic, disease causing and can be hazardous. So attempts to develop non-viral vectors are taken and cationic derivatives of natural polymers are investigated. Pullulan being biocompatible and non-toxic is investigated for gene delivery application. Pullulan derivative which has metal chelating residues and mixed with a plasmid DNA in aqueous solution containing Zn^{2+} ions to obtain the conjugate of pullulan derivative and plasmid DNA with Zn^{2+} coordination (Hosseinkhani et al, 2002). More over pullulan is known for its specificity for liver and this property is exploited for liver targeting (Kaneo et al, 2001; Tanaka et al, 2004) .Recently the cytotoxicity and blood compatibility of cationic pullulan derivatives were assessed and compared to polyethyleneimine. Based on the cytotoxicity and blood compatibility test results it was taken for further studies.

Table-1: Chemical structures of the most common pullulan derivatives for grafting and surface modification.

Types of Reactions	Substituted Pullulan (P-OH)	References
Etherification	P-O-CH_3 (permethylation)	(Na et al,2003)
	P-O-CH_2-COOH (carboxymethylation	(Glinel et al, 2000)
	P-O-$(CH_2)_{1-4}$-Cl chloroalkylation)	(Mocanu et al, 1999)
Esterification	P-O-CO-$(CH_2)_{2-1-4}$-CH_3 (alkoylation)	(Na et al,2003)
	P-O-CO-CH_2-CH_2-COOH (succinoylation)	(Henni-Silhadi et al,2008)
	P-O-CO-CH_2-CH_2-CO-cholesterol	(Bruneel et al, 1995)
Urethane derivatives	P-O-CO-NH-CH_2-CH(OH)-CH_3	(Keilich et al,1993)
	P-O-CO-NH-R (R = phenyl or hexyl)	(Shibata et al,2001)
	P-O-CO-NH-phenyl	(Muroga et al,2006)
Oxidation	P-COOH (C^6 oxidation)	(Denooy et al,1995)
	Glycosidic ring opening (periodate oxidation)	(Keilich et al,1993)
Copolymerisation	P-O-CH_2-O-CO-C$(CH_3)_2$-G Where G is any groups like poly(methacrylate), poly(methylmethacrylate),	(Bontempo et al,2006;
Chlorination	P-CH_2-Cl (C^6 substitution)	(Morimoto et al,1990)

Core-shell Pullulan-based Nanoparticles for Bio-imaging

Recently nanotechnology is being investigated for successful and earlier detection of cancerous growths in the body. Quantum dots are nano-size semiconductor particles which have currently attracted lots of attention in the biological field. To the target cells with quantum dots or as a coating of iron oxide particles dedicated to medical imaging or hyperthermia treatments is the subject of the more recent works. In 2003, Song and co-workers used pH-responsive nanoparticles of pullulan acetate derivatives to target colon cancer cells in mice. They obtained labeling efficiencies above 90%. Endocytosis of these Quantum dots into the cell is usually low and for bio-imaging purposes there should be detectable amount. Hasegawa et al, 2005 developed cholesterol pullulan and amino-group-modified cholesterol pullulan nanogel as a novel carrier to deliver into Quantum dots cells in comparison to conventional cationic liposome which has the disadvantage of forming aggregates once it is internalized in the cell. They reported that the intensity of fluorescence per cell of CHPNH2– Quantum dots nanoparticle was comparable to that of liposome– Quantum dots complex and particles with higher number of amino groups showed fluorescence up to 3.4 times than that of the control.

More recently, the cellular uptake and the cytotoxicity of acetylated pullulan-coated super paramagnetic iron oxide nanoparticles have been examined (Gao et al, 2011) and their hyperthermic effect on tumour cells (KB) has been evaluated. Hyperthermia using magnetic nanoparticles is a very promising treatment for cancer based on the hypothesis that cancerous cells are more sensitive to an increase of temperature than normal cells.

CONCLUSION

Research studies in the field of polysaccharides have revealed that pullulan is a unique polysaccharide with a variety of potential industrial and medical applications. Despite of a large number of valuable applications, the major constraint prevailing on the use of pullulan is its cost, which is three times higher than the price of other polysaccharides such as dextran and xanthan. Thermal stability and elastic property of pullulan allows them to be utilized in many different ways. Engineering innovations and improved production by hyper producing strains could be beneficial to improve the economics of the production, thereby opening new way for pullulan utilization.

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