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Formulation and Characterisation of Pullulan Acetate Nanoparticles Loaded with 5-Fluorouracil

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Abstract: *This study was geared to analyze the preparation methods of pullulan acetate-based nanoparticles loaded with 5-fluorouracil, as well as the potential of pullulan as a biopolymer matrix for obtaining nanoparticles applied in the delivery of anticancer drugs (5-FU). Various methods were used to produce pullulan acetate-based nanoparticles loaded with 5-FU, including nanoprecipitation, modified nanoprecipitation, and double emulsion. Pullulan was previously chemically modified with acetic anhydride, dimethylformamide and pyridine, and yielded pullulan acetate. Pullulan was made using the Aureobasidium pullulans strain through a fermentation procedure. UV-Vis Spectrophotometric and dynamic light scattering (DLS) methods were used to assess entrapment effectiveness, size, and polydispersity index (PDI) of pullulan acetate-based nanoparticles loaded with 5-FU. Based on the properties of the nanoparticles obtained, the optimum preparation method was chosen. The maximum entrapment efficiency was found in pullulan acetate nanoparticles loaded with 5-FU generated by a double emulsion method. The mean hydrodynamic size and PDI of all nanoparticles were adequate. The best formulation showed faster 5-FU release profile in acid phosphate-buffered saline (pH 5) than in phosphate-buffered saline pH 7.4. According to the findings, pullulan derivatives have a great potential for producing nanoparticles that might be used to deliver anticancer medicines.*

Keywords*: polymeric nanoparticles, pullulan, antitumor drugs, cancer*

1. Introduction

Cancer represents one of the major and challenging pathology worldwide. The conventional treatment of cancer involves surgery, radiation therapy and chemotherapy. The most-common anticancer substances are 5-fluorouracil (5-FU), gemcitabine, doxorubicin, cisplatin, oxaliplatin, methotrexate, etc [1-4]. 5-FU is an anticancer substance which is utilized for the medical care of various forms of cancer (for eg. gastrointestinal cancer, breast carcinoma, ovarian cancer, skin cancer, etc.). 5-FU is an antineoplastic cytostatic, pyrimidine analog. Its anticancer activity is due to the conversion of fluorouracil to active metabolites (including 5-fluorodeoxyuridine and 5-fluorouridine) in human tissues. 5-Fluorodeoxyuridine inhibits thymidylate synthetase and blocks the reaction of the conversion of deoxyuridine acid to thymidyl acid, which creates a lack of thymidine required for nucleic acid synthesis. 5-Fluorouridine replaces uridine in the RNA chain, thus disrupting protein synthesis. Also, 5-FU has variable oral bioavailability (in the range of 0% to 80%), easily penetrates histohematic barriers, including the blood-brain barrier, and is distributed in tissues (tumors, bone marrow, liver, etc.), but has non-selective distribution, short pharmacological half-life and various toxicity. These properties limit 5-FU therapeutic applicability.

To overcome these limitations, innovative drug delivery systems offer a number of design opportunities for engineering the delivery of a specific active substance with enhanced therapeutic effects [5-7]. Nowadays, many studies in cancer therapy are focused on polymeric and/or biopolymeric nanoparticles, due to their features, like: capacity to protect their cargo from degradation, improve bioavaila-

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bility, absorption, and penetration, and deliver regulated and targeted distribution [8-11].

Pullulan is a nontoxic, nonmutagenic, noncarcinogenic, nonimmunogenic, biocompatible, and biodegradable biopolymer generated by *Aureobasidium pullulans* strains. Because of its great solubility in water, pullulan cannot self-associate to form nanoparticles in aqueous solutions. As a result, a number of studies [12-15] have concentrated on pullulan derivatization in order to expand its biomedical potential. The biomedical community has studied pullulan derivatives such as pullulan acetate, cholesterol-bearing, perfluoroalkylated pullulan, and others [15-17].

The primary objective of the present paper was to evaluate the potential of pullulan as a biopolymer matrix for the synthesis of nanoparticles that could be applied to the delivery of anticancer agents, and preparation methods of pullulan acetate-based nanoparticles loaded with 5-FU. 5-FU-loaded pullulan acetate-based nanoparticles are produced by a variety of methods, including nanoprecipitation, modified nanoprecipitation, and double emulsion [18]. The entrapment effectiveness, mean hydrodynamic size, and polydispersity index (PDI) were assessed using spectrophotometric and dynamic light scattering (DLS) methods. The best preparation method was selected based on the properties of the nanoparticles. In addition, the effects of the two solvents on the formation of nanoparticles and the effects of the addition of the stabilizer Pluronic F127 were evaluated. Also, it was investigated for the best formulation the release behaviour in phosphate-buffered saline.

2. Materials and methods

2.1. Materials

5-FU, Pluronic F127(non-ionic surfactant), pyridine, acetic anhydride, N,N-dimethylformamide (DMF) and acetone were acquired from Sigma-Aldrich (Merck Group, Darmstadt, Germany). The *Aureobasidium pullulans* strain was used to manufacture pullulan through a fermentation procedure [19]. Pullulan was chemically modified with acetic anhydride, dimethylformamide and pyridine, and yielded pullulan acetate.

2.2. Preparation of Pullulan Acetate Nanoparticles Loaded with 5-FU via nanoprecipitation method

5-FU was dissolved in pullulan/DMF solution (10:1 w/w polymer:drug). Organic phase was poured into water or 3% Pluronic F127 aqueous solution and agitated at 700 rpm at 25°C until the organic solvent was completely evaporated. To separate the free 5-FU from the nanoparticles, the suspension was centrifuged (10,000 rpm) for 30 min at a temperature of 4°C. The precipitate-containing nanoparticles (coded NP1@5FU_met1 and NP2@5FU_met1 in Table 1) were redispersed in distilled water after the supernatant was removed.

2.3. Preparation of Pullulan Acetate Nanoparticles Loaded with 5-FU via modified nano-precipitation method

5-FU was precisely weighed and dissolved in distilled water (10:1 w/w polymer:drug). Dropwise additions of 5-FU aqueous solution to the polymeric solution were made; afterwards the obtained suspension was stirred 15 min at 700 rpm at 25°C for homogenization. Organic phase was poured into water or 3% Pluronic F127 aqueous solution and agitated at 700 rpm at 25°C until the organic solvent was completely evaporated. To separate the free 5-FU from the nanoparticles, the suspension was centrifuged (10,000 rpm, 30 min, 4°C). The precipitate-containing nanoparticles (coded NP1@5FU_ met2 and NP2@5FU_met2; Table 1) were separated from the supernatant and redispersed in distilled water.

2.4. Preparation of Pullulan Acetate Nanoparticles Loaded with 5-FU via double-emulsion method

Pullulan acetate (50 mg) was dissolved in acetone or DMF (5 mL). An aqueous solution of 5-FU (polymer:drug = 10:1 (w/w)) was poured dropwise into polymeric solution; the combination was stirred for 15 min at 700 rpm at room temperature (25°C). The primary emulsion was created by dropping the organic phase into water or 3% Pluronic F127 aqueous solution. After that, the primary emulsion was

homogenized for 15 min at room temperature (25°C) while stirring at 700 rpm. The primary emulsion was sonicated for 30 min in an ultrasonic device filled with ice to obtain the secondary emulsion at a controlled power of 50% of amplitude. The secondary emulsion was agitated at 700 rpm until the organic solvent was completely evaporated. To separate the free 5-FU from the nanoparticles, the suspension was centrifuged (10,000 rpm, 30 min, 4°C). The precipitate-containing nanoparticles (coded NP1@5FU_ met3, NP2@5FU_met3, NP3@5FU_met3, and NP4@5FU_met3; Table 1) were redispersed in distilled water after the supernatant was removed.

Table 1. Preparation parameters of pullulan acetate nanoparticles loaded with 5-FU

Sample Code	Preparation method	Solvent	Stabilizer	$m_{polimer}$:$m_{5\text{-}FU}$ (g/g)
NP1@5FU_met1	nanoprecipitation	DMF	-	10:1
NP2@5FU_met1	nanoprecipitation	DMF	Pluronic F127	10:1
NP1@5FU_met2	modified nanoprecipitation	DMF	-	10:1
NP2@5FU_met2	modified nanoprecipitation	DMF	Pluronic F127	10:1
NP1@5FU_met3	double-emulsion method	DMF	-	10:1
NP2@5FU_met3	double-emulsion method	DMF	Pluronic F127	10:1
NP3@5FU_met3	double-emulsion method	Acetone	-	10:1
NP4@5FU_met3	double-emulsion method	Acetone	Pluronic F127	10:1

2.5. Characterization of pullulan acetate nanoparticles loaded with 5-FU

An indirect method was used to assess the entrapment efficiency. The quantity of 5-FU loaded in the pullulan acetate nanoparticles was calculated as the difference between the initial quantity of 5-FU and the quantity of 5-FU in the supernatant. An UV-Vis spectrophotometer was used to measure the amount of 5-FU in the supernatant at 266 nm. The given values are the average of three replicate samples with standard deviation. The DLS approach was used to measure mean hydrodynamic size and PDI (Particle Size Analyser Beckman-CoulterN4PCS-Submicron, Paris, France).

2.6. *In Vitro* 5-FU release study from pullulan acetate nanoparticles

The *in vitro* release of 5-FU study from pullulan acetate-based nanoparticles was performed by using the dialysis membrane method with maintained sink conditions. 0.5 mL pullulan acetate-based nanoparticles was put in dialysis bags (molecular weight cut-off 14,000) and immersed in 100 mL 0.1 M phosphate-buffered saline with various *p*H (5 and 7.4). The experiments were performed at a temperature of 37°C under continuous stirring (100 rpm/min) with medium replenishment. The released drug was determined by spectrophotometry.

2.7. Statistical analysis

Experiments were made in triplicate; results were displayed as mean value ± SD and were considered significant at p lower than 0.05.

3. Results and discussions

The main objective of this paper was to evaluate the possibilities of pullulan as a biopolymer matrix for obtaining nanoparticles with anticancer drug delivery applications, as well as to evaluate the preparation procedures for 5-FU-loaded pullulan acetate-based nanoparticles. To achieve this, polymeric nanoparticles containing 5-FU were produced using three methods: nanoprecipitation, modified nanoprecipitation, and double-emulsion. To evaluate the preparation processes, entrapment effectiveness, mean hydrodynamic size, and PDI were assessed. The values of entrapment efficiency, mean hydrodynamic size, and PDI of pullulan acetate nanoparticles loaded with 5-FU are shown in Table 2.

Table 2. Mean hydrodynamic size and PDI of pullulan acetate nanoparticles loaded with 5-FU

Sample code	EE (%)	Size (nm)	PDI
NP1@5FU_met1	26.01 ± 0.15	222.50 ± 0.11	0.32 ± 0.04
NP2@5FU_met1	26.11 ± 1.01	225.11 ± 0.26	0.31 ± 0.02
NP1@5FU_met2	30.42 ± 2.18	254.30 ± 0.21	0.28 ± 0.02
NP2@5FU_met2	31.25 ± 0.15	258.41 ± 0.23	0.27 ± 0.05
NP1@5FU_met3	44.01 ± 2.21	298.51 ± 0.06	0.27 ± 0.07
NP2@5FU_met3	45.05 ± 1.15	301.92 ± 0.08	0.28 ± 0.04
NP3@5FU_met3	49.01 ± 0.25	302.51 ± 0.51	0.26 ± 0.05
NP4@5FU_met3	50.22 ± 0.05	303.92 ± 1.02	0.24 ± 0.03

3.1. Entrapment efficieny

The entrapment efficiency of pullulan acetate nanoparticles loaded with 5-FU obtained by the double-emulsion technique had the highest value (EE = 50.22 ± 0.05 %). The entrapment effctiveness is determined by the polymer, surfactant, active substance properties. The different solvent affinities of the 5-FU and pullulan explain the moderate entrapment efficiency values of formulations prepared by nanoprecipitation and modified nanoprecipitation. Samy et al. [20] obtained comparable results for the entrapment efficiency of 5-FU-chitosan nanoparticles via the ionic gelation method (EE = 21.82%). The effect of two different organic phase solvents (acetone and DMF) on the pullulan acetate nanoparticles loaded with 5-FU prepared by the double-emulsion technique was investigated. Higher entrapment efficiency values showed the formulations prepared with acetone as the organic phase solvent. The effect of adding a stabilizer, Pluronic F127, during nanoparticle preparation on formulation characteristics was also investigated. No considerable differences in entrapment efficiency values between the formulations prepared with and without Pluronic F127 were observed.

3.2. Mean hydrodynamic size, and PDI

Since the size of nanoparticles impacts the half-life circulation and cellular uptake is essential in assessing the performance of a nanosystem. The homogeneity of the system is also an important criterion for assessing its performance. The PDI is a size-based indicator of sample heterogeneity; PDI < 0.1 suggests monodisperse samples, while PDI > 0.7 indicate polydisperse samples. One can observe in Table 2 that pullulan acetat nanoparticles had mean hydrodynamic sizes below 310 nm. All pullulan acetate nanoparticles loaded with 5-FU had PDI<0.35, with minimal variation between samples, suggesting that the systems were moderately homogeneous.

3.3. Stability

The pullulan acetate nanoparticles loaded with 5-FU were kept for a period of three months at a temperature of 4°C to analyze their stability, and the entrapment efficiency values were determinated after 3 months; data is shown in Figure 1. Pullulan acetate nanoparticles loaded with 5-FU produced using Pluronic F127 showed better stablility in comparision with samples prepared without Pluronic F127 having almost the same quantity of medication loaded after that time. In nanoformulations the rate of sedimentation induced by gravitation is lower than the diffusion rate and Brownian motion, hence no agglomeration in the visual appereance of pullulan acetate nanoparticles was noticed during storage.

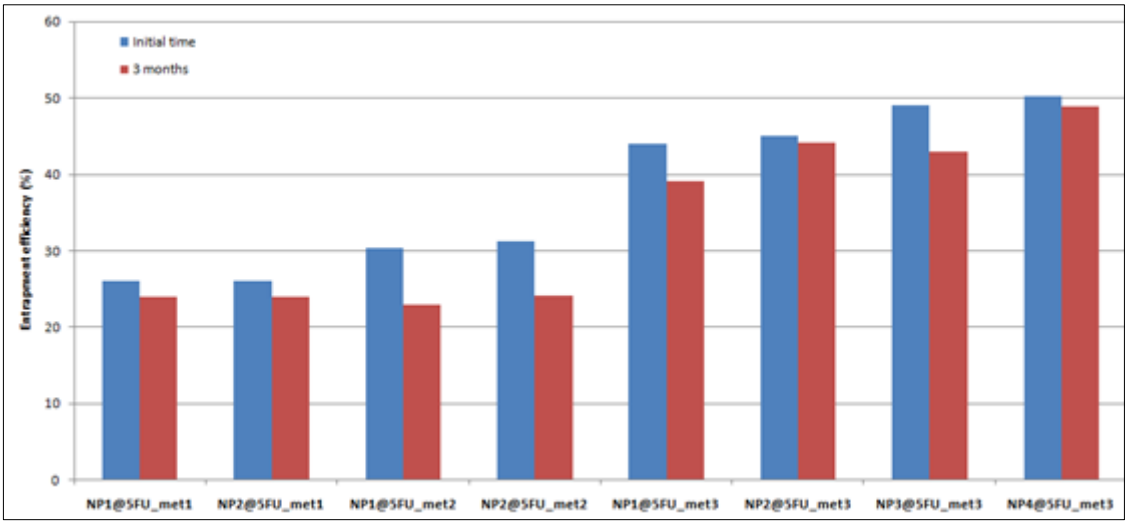

Figure 1. Comparision between the entrapment efficiencies values of the nanoformulations at 3 months vs initial time

3.4. Drug release from pullulan acetate nanoparticles

In vitro 5-FU release from pullulan acetate nanoparticles was determined in phosphate-buffered saline (*p*H 5; *p*H 7.4) at 37°C, and the results were displayed in Figure 2. The *p*H 5 was selected to simulate the environment of cancer cells, which present slightly acidic *p*H, while *p*H 7.4 simulate the normal cells environment. The release study was performed on the best formulation according to entrapment efficiency and stability (NP4@5FU_met3). As shown in Figure 2, pullulan acetate nanoparticles displayed an initial rapid release of drug and reached in six hours at a cumulative drug release value of 24.7% for buffer *p*H 7.4 and respectively 40.5% for phosphate-buffered saline *p*H 5. The initial rapid release is probably explained by release of the 5-FU crystals displayed on the surface of pullulan acetate nanoparticles. Pullulan acetate nanoparticles showed fastest 5-FU release rate in acidic than in neutral medium. To investigate the drug release mechanism from pullulan acetate nanoparticles the experimental data obtained was analyzed using various kinetic models (Hixson-Crowell, first-order, zero-order, Higuchi, Korsmeyer-Peppap). Table 3 presents the correlation coefficients of mathematical models. High correlation coefficients were obtained for Korsmeyer-Peppas and Higuchi models ($R^2 > 0.9$). For these models the coefficients were also listed; k_H, is the parameter of the Higuchi model; k_{KP} is the Korsmeyer-Peppas constant and it depends on the nanoparticle features, and *n* is a parameter that presents the nature of the release process. The parameters n of the Korsmeyer-Peppas model were lower than 0.45 in both media, suggesting the release is described by the Fickian diffusion. The k_H value of the Higuchi model is lower for the 5-FU release in neutral than in acidic medium, indicating a less intense burst effect.

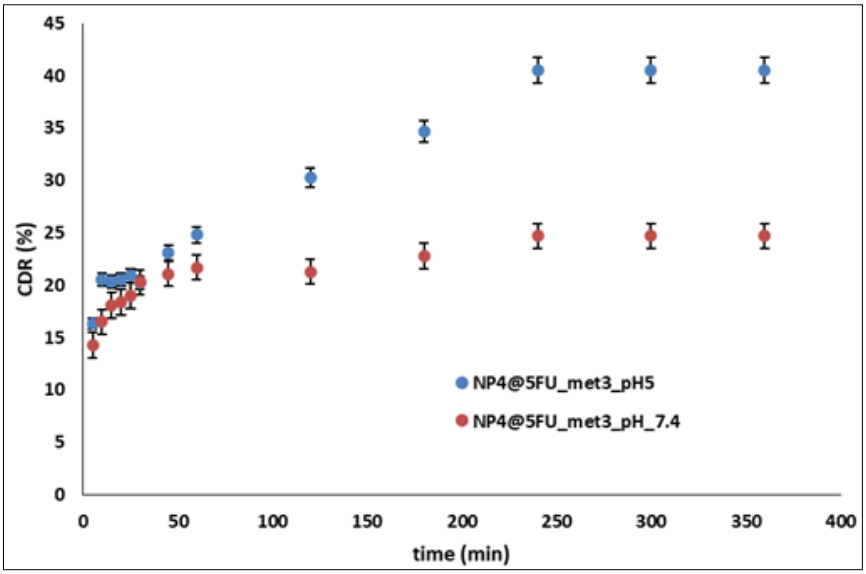

Figure 2. Release of 5-FU from pullulan acetate nanoparticles

Table 3. Analysis of drug release mechanism

Model	pH 7.4	pH 5
	R^2	R^2
Hixson-Crowell	0.7016	0.7828
zero-order	0.691	0.7279
first-order	0.7069	0.8079
Korsmeyer-Peppas	0.9174 (n = 0.12; k_{KP} =0.12)	0.9120 (n = 0.21; k_{KP} = 0.15)
Higuchi	0.9030 (k_H = 4.93)	0.9705 (k_H = 12.923)

4. Conclusions

Three approaches were used to successfully generate pullulan acetate nanoparticles loaded with 5-FU: nanoprecipitation, modified nanoprecipitation, and double-emulsion. Entrapment efficiency, mean hydrodynamic size, PDI and stability of nanoparticles were all assessed. Based on the properties of the nanoparticles obtained, the optimum preparation method was chosen. The mean hydrodynamic size and PDI of all nanoformulations were adequate. The maximum entrapment efficiency was found in pullulan acetate nanoparticles loaded with 5-FU generated by a double emulsion. The best formulation showed faster 5-FU release in acid medium than in phosphate-buffered saline *p*H 7.4, following a diffusion driven mechanism for both media. According to the findings, pullulan derivatives have a great potential for producing nanoparticles that might be used to deliver anticancer medicines.

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