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Article

Pullulan as a stabilizer agent of polymeric nanocapsules for drug delivery

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Polymeric stabilizers have received attention in the preparation of nanostructured systems due to their ability to enhance formulation stability. Considering this, the objective of this work was to prepare poly(ε-caprolactone) nanocapsules using the pullulan as a polymeric stabilizer. The nanocapsules were prepared using the interfacial deposition method of preformed polymers and they were characterized in terms of pH, average diameter, polydispersity index, zeta potential, beclomethasone dipropionate content, encapsulation efficiency, photostability and drug release profiles. The formulations showed physicochemical characteristics consistent with nanocarriers for drug delivery such as: average diameter lower than 270 nm, polydispersity indexes lower than 0.2, negative zeta potential (-22.7 to -26.3 mV) and encapsulation efficiencies close to 100%. In addition, the nanocapsules were able to delay the beclomethasone dipropionate photodegradation under UVC radiation and by the dialysis bag diffusion technique, the nanocapsules were able to prolong the drug release. Thus, pullulan could be considered an interesting excipient to formulate polymeric nanocapsules.

Uniterms: Pullulan. Nanocapsules/diffusion technique. Pullulan/polymeric stabilizer. Biocompatible polymers. Beclomethasone dipropionate/nanocapsules.

INTRODUCTION

Nanocapsules are colloidal drug delivery systems characterized by a liquid core surrounded by a polymeric shell (Mora-Huertas, Fessi, Elaissari, 2010). The core is usually composed of liquid lipids but may also present a hydrophilic nature. In such nanostructures the drug is confined in the core and/or absorbed in the polymeric wall (Schaffazick *et al.*, 2003; Kaur *et al.*, 2014).

The nanocapsules preparation requires some raw materials including polymer, oil and a combination of surfactant/cosurfactant. Biodegradable polyesters such as poly(ε-caprolactone), poly(lactide) and poly(lactide-co-glycolide) are commonly used (Mora-Huertas, Fessi, Elaissari, 2010). Regarding the core composition, a variety of synthetic and vegetable oils are reported; however, medium chain triglycerides are the most employed ones due to their low toxicity and high capacity to solubilize a wide range of lipophilic drugs (Preetz *et al.*, 2008). Oil as well as W/O cosurfactants (common sorbitan esters and phospholipids) are also core constituents. The main surfactant, in its turn, acts on the particle surface and plays a major role as a nanocapsule stabilizer agent, being poloxamer 188 and polysorbate 80 more commonly employed (Mora-Huertas, Fessi, Elaissari, 2010). However, such surfactants are known to present some incompatibilities with preservatives and other raw materials (Allen, 2004). As an alternative, polymeric emulsifiers have gained much attention in the preparation of dispersed systems due to their capacity to enhance formulation stability (Bobin, Michel, Martini, 1999).

Pullulan (figure 1) is an extracellular polysaccharide elaborated by the *Aureobasidium pullulans* fungus. This water-soluble biopolymer presents a linear structure composed of repeating maltotriose units connected by α-1,6-glucosidic bonds (Singh, Saini, Kennedy, 2008). Because of its non-toxic, non-carcinogenic and non-immunogenic properties, the pullulan is being studied for pharmaceutical and biomedical applications (Choudhry *et al.*, 2012). The pullulan efficacy as an emulsifier was

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demonstrated in the emulsification of turmeric oleoresin (Kshiragar *et al.*, 2007); however, no report on the nanoparticles preparation using pullulan as a stabilizer agent has been found. On the other hand, hydrophobically modified pullulan derivatives were employed to prepare nanostructured systems (Hassani, Hendra, Bouchemal, 2012; Park *et al.*, 2012).

In this way, this work aimed to prepare polymeric nanocapsules using pullulan as a stabilizer replacing polysorbate 80, a traditional surfactant. Besides this change, beclomethasone dipropionate was selected to be incorporated in nanocapsules as a drug model due to its therapeutic potential, and because it is a corticosteroid drug widely used as anti-inflammatory agent in the topical treatment of rhinitis and asthma (Brown, George, 1972). The developed formulation was characterized in terms of pH, particle size and polydispersity index, zeta potential, drug content, encapsulation efficiency, photostability and *in vitro* drug release.

MATERIAL AND METHODS

Material

Beclomethasone dipropionate (BD) was obtained from Henrifarma (São Paulo, Brazil). Poly(ε-caprolactone) (PCL) was supplied by Sigma Aldrich (São Paulo, Brazil), sorbitan monooleate (Span 80®) was purchased from Delaware (Porto Alegre, Brazil) and pullulan was generously gifted by Corn products (São Paulo, Brazil). Medium chain triglycerides (MCT) were obtained from Brasquim (Porto Alegre, Brazil). HPLC-grade methanol was supplied by Tedia (Rio de Janeiro, Brazil). All other solvents and reagents were of analytical grade and used as received.

Analytical procedures

The experiments were performed on a LC-10A HPLC system (Shimadzu, Japan) equipped with a LC-20AT pump, a UV-VIS SPD-M20A detector, a CBM-20A system controller and a Rheodyne valve sample manual injector with 50μL loop. The separation was performed at room temperature using a RP C_{18} Phenomenex column (250 mm x 4.60 mm, 5 μm; 110 Å) coupled to a C_{18} guard column. The isocratic mobile phase consisted of methanol and water (85:15 v/v) at a flow rate of 1.0 mL/min. BD was detected at 254 nm with a retention time of 5.9 min. The method was linear (r=0.9993) in the concentration range of 5.0-25.0 μg/mL and precise (RSD<0.96% for repeatability and RSD<1.97% for intermediate precision).

Nanocapsule suspensions preparation

The nanocapsules were prepared in triplicate by the interfacial deposition of preformed polymer method (Fessi, Puisieux, Devissaguet, 1988). Two phases were separately prepared: the organic one consisted of acetone, MCT, Span 80®, PCL and BD, the other was an aqueous dispersion of pullulan. Both phases were kept under magnetic stirring for 60 min. The organic phase was maintained at 40 °C while the aqueous phase remained at room temperature. In sequence, the organic phase was poured in the aqueous one and the resulting mixture was

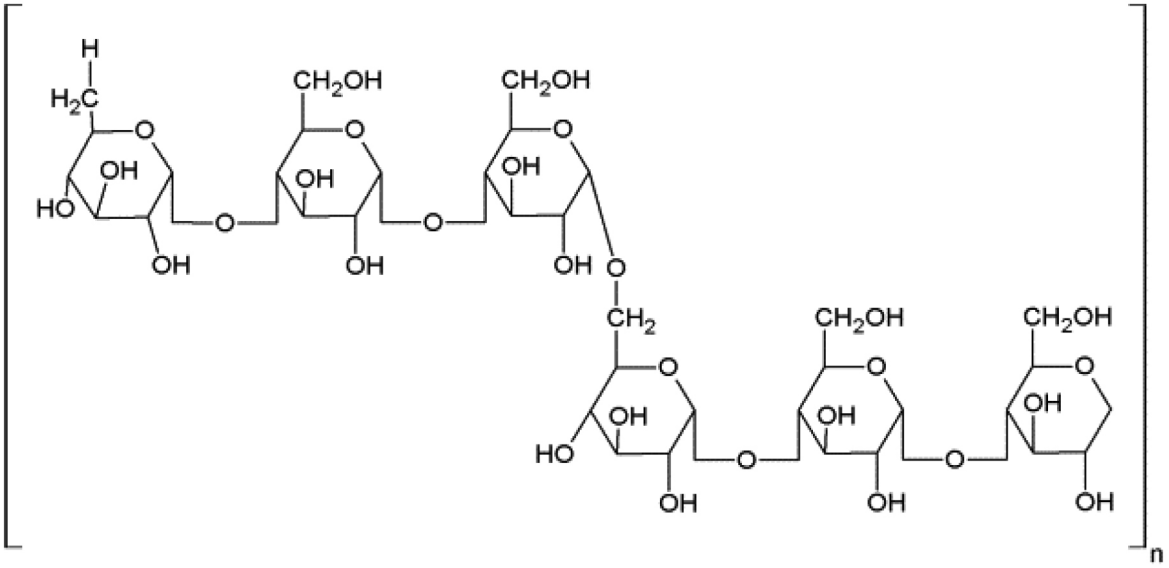

FIGURE 1 - Pullulan chemical structure.

kept under moderate magnetic stirring for 10 min. Then, the acetone and part of the water were eliminated under reduced pressure to achieve 10 mL of final volume. For comparison purposes, the formulations were prepared without the drug (blank nanocapsules, NC-B). The quali- and quantitative composition of each formulation is displayed in Table I, and still considering comparative purposes, the nanocapsules were also prepared using polysorbate 80 (NC-0.5-P80 and NC-B-P80).

Nanocapsule suspensions physicochemical characterization

After preparation, these formulation characteristics were evaluated: pH, particle size, polydispersity index, zeta potential, drug content and encapsulation efficiency. The pH was directly determined in the samples through a calibrated potentiometer (pH 21 model, Hanna Instruments). Particle sizes and polydispersity indexes were measured by photon correlation spectroscopy (25 °C) (Zetasizer Nanoseries, Malvern Instruments). Prior analysis, the samples were diluted in ultrapure water (1:500). The Zeta potential was evaluated by electrophoretic mobility (Zetasizer Nanoseries, Malvern Instruments) after the samples dilution (1:500) in 10 mM NaCl solution. For the BD content determination, an aliquot of nanocapsule suspension was diluted in methanol (15 µg/mL) and the sample was kept 10 min in an ultrasound, followed by 20 min of centrifugation at 3,500 rpm. Lastly, the sample was filtered through a regenerated cellulose membrane (0.45 µm) and injected into the HPLC system. The encapsulation efficiency was evaluated by the ultrafiltration/centrifugation technique using Amicon® 10,000 MW devices (Millipore). The amount of 200 µL of nanocapsule suspension was placed in the device and the centrifugation was carried out at 2,200 xg for 10 min. The free drug was determined in the ultrafiltrate using the HPLC method described above and the encapsulation efficiency (EE%) was calculated from the difference between the total and the free drug concentration (Equation 1).

$$EE\% = \frac{\text{Total drug content} - \text{Free drug content}}{\text{Total drug content}} \times 100 \quad (1)$$

Photostability study

The BD photodegradation incorporated into nanocapsules (NC-0.5) was studied using an UV artificial lamp (Phillips TUV lamp-UVC long life, 30 W). For comparison purposes, a BD methanolic solution (BD-MS) and a BD methanolic solution covered with aluminum foil (dark control) were evaluated as well. The samples were placed in plastic cuvettes perfectly stoppered and then exposed to UV radiation in a mirrored chamber (1 m x 25 cm x 25 cm), at a fixed distance. After 5 h of exposure, 300 µL of the samples were withdrawn, diluted in methanol (final BD concentration: 15 µg/mL), and injected into the HPLC system according to the previously described method. The experiment was performed in triplicate.

In vitro drug release study

The BD *in vitro* release from nanocapsules was evaluated by the dialysis bag diffusion technique. The formulation NC-0.5 and a BD ethanolic solution at 0.5 mg/mL (BD-ES) were placed in a dialysis bag (MWCO 10,000, Spectra Por 7) and the system was immersed in 150 mL of water with 30% ethanol (37 °C, 50 rpm magnetic stirring) to maintain the sink conditions. At predetermined intervals, 1 mL of the dissolution medium

TABLE I - Quali- and quantitative composition of nanocapsule suspensions

	NC-0.5	NC-1.0	NC-B	NC-0.5-P80	NC-B-P80
Organic phase					
Acetone (mL)	27	27	27	27	27
PCL (g)	0.1	0.1	0.1	0.1	0.1
MCT (µL)	330	330	330	330	330
BD (g)	0.005	0.010	-	0.005	-
Span 80 (g)	0.077	0.077	0.077	0.077	0.077
Aqueous phase					
Water (mL)	53	53	53	53	53
Pullulan (g)	0.077	0.077	0.077	-	-
Polysorbate 80 (g)	-	-	-	0.077	0.077

was withdrawn and replaced by the same volume of fresh medium. The drug released percentage was determined by the previous mentioned HPLC method. The mean calculated values were obtained from 3 replicates.

Drug release profiles were analyzed by model-dependent approaches: first order equation (Equation 2) and Power law model (Equation 3).

$$\frac{M_t}{M_\infty} = 1 - \left[e^{-kt} \right] \quad (2)$$

$$ft = \frac{M_t}{M_\infty} = a.t^n \quad (3)$$

where M_t is the amount of drug released at time t, M_∞ is the initial concentration of the drug, k is the kinetic rate constant, f_t is the ratio of absolute cumulative amount of drug released at time t and at infinite time, a is a constant incorporating the carrier's structural and geometric characteristics, and n is the release exponent, indicative of the drug release mechanism. The fit of the experimental data to the models was performed using the Scientist 2.0 software (Micromath, USA).

Statistical analysis

The results were expressed as mean ± standard deviation. The software used was GraphPad Prism Program, version 5. For the variance analysis (ANOVA) and post-hoc multiple comparisons. The release profiles were analyzed by the two-way ANOVA followed by the post hoc Bonferroni test, while the results of the photostability study were analyzed by the one-way ANOVA, followed by the post hoc Tukey test. In all cases, $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Polymeric nanocapsules using pullulan as a stabilizer agent were prepared by the PCL interfacial deposition, a traditional method. Several attempts have been made to optimize the pullulan amount; however, formulations prepared with concentrations lower than 0.6% precipitated when the evaporation process happened while concentrations upper to 1% resulted in the formation of precipitates immediately after preparation. In this way, the formulations were prepared with 0.77% of pullulan, the same concentration generally employed to prepare PCL nanocapsules using polysorbate 80.

After preparation, regardless of the drug presence, the nanocapsule suspensions showed a milky appearance with an opalescent bluish reflection (Tyndall effect). These characteristics are in accordance to other nanocapsule formulations reported in literature (Mora-Huertas, Fessi, Elaissari, 2010; Schaffazick *et al.*, 2003). In pilot studies, it was observed that there was no formation of nanocapsules without pullulan. Such fact shows the importance of this polysaccharide for the nanocapsule preparation. The Pullulan was essential for interfacial tension reduction allowing the formation of the nanocapsules shell/core structure without precipitation.

Table II displays the physicochemical characteristics of the prepared formulations in comparison to those of nanocapsule suspensions prepared employing polysorbate 80. The pH determination revealed that the samples possess values close to the neutrality, which is in accordance to other nanocapsule suspensions prepared with PCL (Ourique *et al.*, 2008; Almeida *et al.*, 2009). As for the particle sizes evaluation, nanocapsules showed an average diameter smaller than 270 nm. Regarding the literature, polymeric nanocapsules usually have dimensions between 100-500 nm (Quintanar-Guerrero *et al.*, 1998). Our findings evidenced that the addition of a polymeric emulsifier did not alter the particle sizes when compared to nanocapsules prepared with polysorbate 80 as an emulsifier, and with some reports presented in the literature (Ourique *et al*., 2008; Fontana *et al.,* 2009). Furthermore, polydispersity indexes were lower than 0.2 indicating a narrow size distribution. Concerning the zeta potential, nanocapsules showed high negative values, that is, higher than formulations prepared with polysorbate 80 (zeta potential < -12 mV). As higher the value of the zeta potential was (in modulus), stabler was the nanocapsule suspension due to the repulsive interactions among the particles (Hans, Lowman, 2002). In relation to the drug content, the formulations NC-0.5 and NC-1.0 were close to the theoretical value, with encapsulation efficiencies greater than 99%. It is relevant to mention that in previous trials, PCL nanocapsules prepared with polysorbate 80 were able to incorporate only up to 0.5 mg/mL of drug, evidencing that pullulan enhanced the PCL nanocapsules loading capacity. However, the visual monitoring of the formulations prepared with pullulan revealed that NC-0.5 is stabler than the NC-1.0 because this formulation presented precipitates along the storage time. For this reason, studies on the photostability and drug release profiles were performed only for the NC-0.5 formulation.

In order to evaluate the PCL ability in nanocapsules prepared with pullulan to protect BD against photodegradation, the NC-0.5 samples were exposed to UVC radiation. After 5h of exposure, approximately

TABLE II - Nanocapsules physicochemical characteristics

Sample	Actual drug content (mg/mL)	pH	Particle size (nm)	Polydispersity index	Zeta potential (mV)
NC-B	-	6.6 ± 0.2	242 ± 11	0.18 ± 0.01	-26.3 ± 4.6
NC-0.5	0.49 ± 0.01	6.2 ± 0.2	270 ± 2	0.19 ± 0.02	-22.7 ± 1.4
NC-1.0	0.99 ± 0.01	6.7 ± 0.4	269 ± 2	0.20 ± 0.01	-23.1 ± 0.7
NC-0.5 P80	0.50 ± 0.02	6.9 ± 0.3	218 ± 18	0.14 ± 0.07	-11.1 ± 3.4
NC-B-P80	-	6.5 ± 0.1	291 ± 65	0.22 ± 0.03	-12.3 ± 8.6

19% of the initial BD concentration associated to nanocapsules remained intact (Figure 2). On the other hand, about 5% of the drug in methanolic solution was maintained under the same experimental conditions. For the dark control, the BD concentration was close to 100%, which discards the influence of chamber temperature on drug degradation.

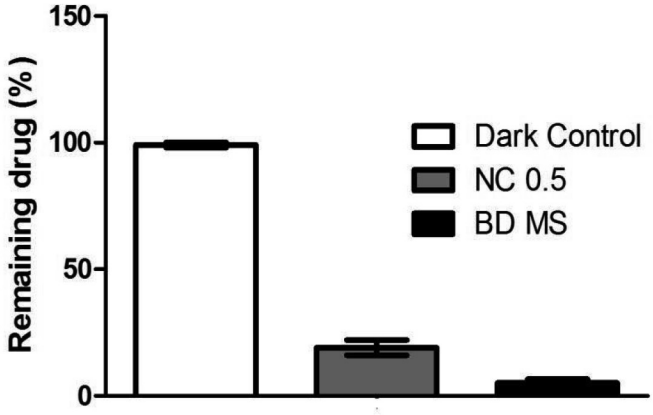

FIGURE 2 - Nanocapsules beclomethasone dipropionate content (NC-0.5), methanolic solution (BD-MS) and dark control under UVC radiation.

Concerning *in vitro* drug release experiments, Figure 3 shows that NC-0.5 was able to control BD release in comparison to the ethanolic drug solution. In a 24-hour period, 99.59% of the free drug was released while 40.48% of BD was released from nanocapsules in the same period, showing that the nanocarrier prolongs the release.

Mathematical modeling of drug release profiles indicated that both samples followed a first order kinetics (r>0.99), which means that BD release occurred in a single step without a rapid initial release. In relation to the drug release mechanism, the release exponent n obtained from Power law indicates that the BD release from nanocapsules is driven by anomalous transport (n=0.67). According to the literature, systems presenting spherical geometry, n=0.43 indicates Fickian diffusion, while n≥0.85 is related

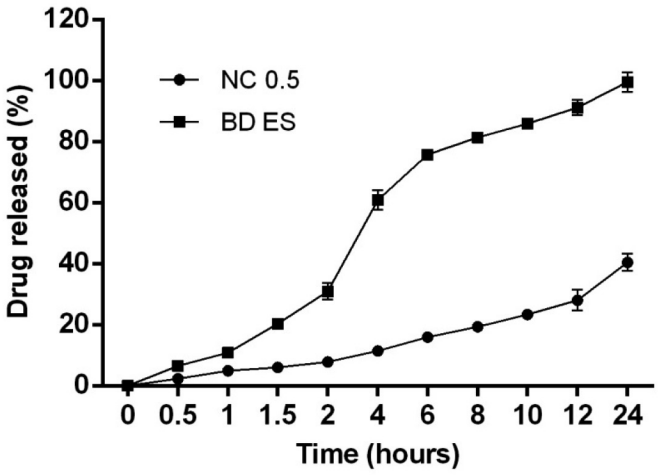

FIGURE 3 - *In vitro* beclomethasone dipropionate release profiles from nanocapsules (NC-0.5) and ethanolic solution (BD-ES) through the dialysis bag method (n=3).

to case II transport (Peppas, 1985). Values between these limits suggest anomalous transport, i.e., the drug release initially depends on the polymer relaxation followed by Fickian diffusion (Dey *et al.*, 2009).

CONCLUSION

The use of pullulan as a stabilizer agent for polymeric nanocapsules is reported for the first time. The prepared formulations showed compatible features with other nanostructured systems. The results of zeta potential and drug content indicated that the pullulan is an advantageous stabilizer in comparison to polysorbate 80. The photostability as well as the *in vitro* drug release profiles suggest that nanocapsules prepared with pullulan can be considered promising drug carriers.

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