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FORMULATION OF LIPOSOMES FROM LEVAN

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Abstract: Levan is a fructose polysaccharide that has several biotechnological applications. The elasticity of levan properties allows its use in various industries, such as pharmaceutical, food and cosmetic products. In the pharmaceutical industry, the use of liposomes allows the controlled and targeted release of drugs in their targets. This study proposes the formulation of levan-containing liposomes and characterization of these nanomolecules as a possible instrument for cancer therapy. Levan loaded liposomes (Lev-LIPO) were prepared according to the thin film formation method. The physical-chemical characterization was performed immediately after its preparation.
Keywords: Liposome, Levan, controlled release, fructan.

INTRODUCTION

Levan is formed by transfructosylation reactions and consists mainly of fructose units connected in bonds.: β→(2,6) (YOO et al., 2004). Levan can be isolated from the secondary metabolism of some microorganisms, such as:*Acetobacter xylinum* (AHMED et al., 2014), *Bacillus subtilis var. natto* (WU; CHOU; SHIH, 2013), *Zymomonas mobilis* (VIGANTS et al., 2013), *Microbacterium laevaniformans* (PARK et al., 2003), *Bacillus amyloliquefaciens* (TIAN et al., 2011), *Bacillus methylotrophicus* (ZHANG et al., 2003) and *Halomonas sp*. (POLI et al., 2009).

This fructose polysaccharide generated interest from researchers due to its wide applicability. In the pharmaceutical industry, the antitumor activity of levan produced by *Zymomonas mobilis* has been reported in vitro against A549 cells (human lung adenocarcinoma), HepG2/C3A (human liver hepatocellular carcinoma), AGS (human gastric adenocarcinoma) and MCF-7 cells (human breast adenocarcinoma) without the toxicity demonstrated by commonly used anticancer agents. used (CALAZANS et. al., 1997, 2000; YOO et.al., 2004).

Liposomes are microscopic vesicles composed of one or more concentric lipid bilayers separated by an aqueous medium. They can encapsulate hydrophilic and/or lipophilic substances, the former being inserted into or absorbed into the aqueous compartment while the lipophilic substances into the membrane (Allen & Cullis, 2013). As they are biodegradable, biocompatible and non-immunogenic, they are highly versatile for applications in therapeutic and analytical research (Balocco et al. 2018). Therefore, they are prominently considered as carriers for the localized and controlled release of drugs (Batalha et al. 2015; De Freitas et al. 2019).

The great advantage of liposomes is, in fact, that they allow the retransmission of the active ingredient to the site to be treated and promote the controlled release of the drug (Mufamadi et al. 2011; PABST et al. 2014), allowing the administration of larger doses of the drug. product avoiding the side effects seen in conventional therapy (Batista et al. 2007).

One of the best-known observations in the field of research on liposomes is that they are injected intravenously and rapidly absorbed by macrophages in the reticuloendothelial system, such as the liver and spleen (SERCOMBE et al. 2015). Since this feature is advantageous for targeting antigens or immunomodulatory agents to macrophages, many investigators have used liposomes as adjuvants, depots for the slow release of antigens, and targeting agents for the delivery of antigens to macrophages, which are one of the cell-presenting cells of antigens (Batalha et al. 2015; De Freitas et al. 2019).

This study proposes to carry out the formulation of a liposome containing levan, offering a new delivery system and controlled release of pharmacological compounds.

MATERIAL AND METHODS

LEVAN PRODUCTION

Levan was produced by the *Zymomonas mobilis* strain ZAG-12 (UFPEDA 241) by batch fermentation (CALAZANS, 2000) and a 100 g/L solution of levan in MilliQ® water was prepared. This material was fractionated by precipitation by the gradual addition of ethanol. Levan precipitated with 70% ethanol (w/w) was the fraction used in this work, as it showed a better recovery rate compared to the other fractions, and provided sufficient quantity for the subsequent steps. Soybean phosphatidylcholine (PC) (Epikuron 200, 98% PC) was obtained from Lucas Meyer (Hamburg, Germany). Chloroform, methanol, potassium phosphate and sodium hydroxide were purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade and commercially available.

LEV-LIPO FORMULATION

The manufacturing process of levan-loaded liposomes (Lev-LIPO) was based on lipid film formation followed by sonication (ANSELEM et al., 1993). The lipid constituents of soy phosphatidylcholine, cholesterol and stearylamine (7:2:1 lipid 42 μmol/10 μl phosphate buffer) were dissolved in a mixture of chloroform/methanol (3:1 v/v) under moderate magnetic stirring for 16 minutes. After solubilization, the organic solution was subjected to evaporation (Rotaevaporator Buchi) under reduced pressure at 37 °C with stirring at 120 rpm for sufficient time for solvent evaporation and lipid film formation.

The dried lipid film was hydrated by adding an aqueous phase (10 mL) consisting of 0.2M phosphate buffer solution (pH 7.4) containing levan, under gentle agitation until complete dissolution, leading to the formation of Multilamellar Vesicles (MLV). Different concentrations of levan contained in the buffer and liposome control solution (without levan) were tested to optimize the development of Lev-LIPO.

Small Unilamellar Vesicles (SUV) were obtained by VLM ultrasound with an ultrasonic probe (Vibra Cell Model CV17 Bioblock Scientific) for 300 seconds, pulse modal at 50 Hz. Finally, the liposome suspension was stored at 4±1 °C in appropriate vials.

PHYSICOCHEMICAL CHARACTERIZATION AND EVALUATION OF THE STABILITY OF THE LEV-LIPO LIPOSOME

Physicochemical analysis of the liposomes was performed at regular intervals immediately after preparation. Several characteristics, such as macroscopic and microscopic appearance, morphological examination, changes in pH and encapsulation rate were analyzed.

Liposomal formulations were subjected to accelerated and long-term stability tests in order to verify the durability of the formulation. The purpose of accelerated stability tests is to subject the preparations to stress-related conditions to simulate processes such as sterilization, transport and storage. 2mL aliquots of different LEV-Lipo were subjected to centrifugation at 820 xg for 1 h (Sorvall Super T21), mechanical stress at 160 revolutions per minute for 48 h at 37±1 °C (Polytest® 20 Bioblock Scientific) and freezing cycles and thawing (16 h at 4±1 °C and 8 h at 25±1 °C) (Batista et al. 2007; Sercombe et al. 2015).

Long-term stability in liposomal suspensions was examined for sample storage at 4±1°C. The physicochemical properties of the preparations were evaluated at 1, 7, 15, 30, 60, 90, 120 and 180 days after the formulation of the preparations or until the appearance of signs of instability.

The particle size and surface charge potential of the liposomes were determined

with the aid of a Nanozetasizer® (Nano-ZS90, Malvern, UK). Liposomal samples were diluted in water to ensure an adequate particle count for the technique that was performed. The distribution and mean diameter of the particles, as well as their standard deviation and Polydispersity Index (PDI) were calculated.

The surface charge of levan-charged liposomes was determined by measuring the zeta potential (δ) using a combination of two techniques: electrophoresis and laser dopplervelocimetry. The results show an average of at least three measurements from different samples for an equivalent lot of liposomes.

The evaluation of levan release in liposomes was performed by dissolving the liposomes with methanol P.A (1:1), extraction and direct quantification of levan by High Performance Liquid Chromatography (HPLC) using the Detector Refractive Index (RDI). The in vitro release profile of Lev-LIPO was evaluated using the dialysis method.

The encapsulation rate of levan in liposomes was determined after ultrafiltration/ ultracentrifugation using units: Ultrafree® ((Millipore, USA) at 13,128 x g for 60 minutes at 4°C. The resulting filtrate was analyzed by colorimetric and chromatographic methods and the levan encapsulation ratio calculated based on the total levan content in the liposome formulation.

STATISTICAL ANALYSIS

Data were presented as mean ± SD. Statistical significance was assessed using a t test, standard unpaired Student test. All procedures were performed in triplicate to assess the reproducibility of results.

RESULTS AND DISCUSSION

FORMULATION, PHYSICO-CHEMICAL CHARACTERIZATION AND STABILITY OF LEV-LIPO

The concentration of Lev-LIPO with 20 mg/mL of levan has not maintained its stability after the transport simulation test to confer mechanical stress with noticeable physicochemical changes after centrifugation or the effects of freeze-thaw cycles, the formulations initially showed a macroscopic appearance similar to a fluid colloidal suspension, from opaque to transparent, with a bluish appearance for 45 days (Table 1). This may indicate lipid degradation inducing fatty acid synthesis. The main route of degradation of the lipid medium leads to the synthesis of fatty acid formation, which impairs the electrical conductivity and gradually reduces the pH of the preparations (PONTES et al., 1999). The results suggest that high concentrations of levan cause instability in liposomal formulations.

Lev-LIPO had an average particle size of 313.03 ± 151.28 nm (Table 1). The size of Lev-LIPO (in the range of 200 to 400 nm). Furthermore, the in vitro levan release kinetics of Lev-LIPO showed an initial burst of 8.96 ± 2.96% (Fig. 1) in the first hour followed by a slow and controlled release of the drug during the second phase (8-72 h) with a precise speed of 0.34 mg/min (Fig. 2). The amount of levan loaded diffused (94.1%) in 72 h. This suggests that Lev-LIPO could be used as a potential drug delivery system.

The encapsulation ratio of levan in liposomes was 87,6 ± 1,2% (Table 1).

CONCLUSION

A liposome formulation was carried out containing the fructose polysaccharide levan, with a controlled and constant rate release. The liposome presented

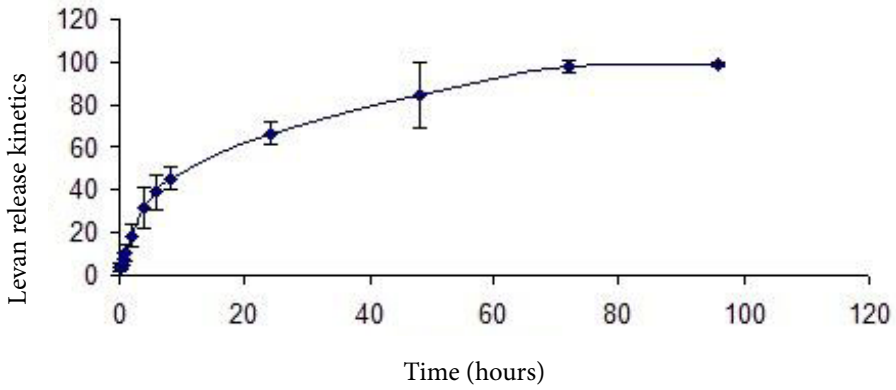

Figure 1 - Levan release kinetics

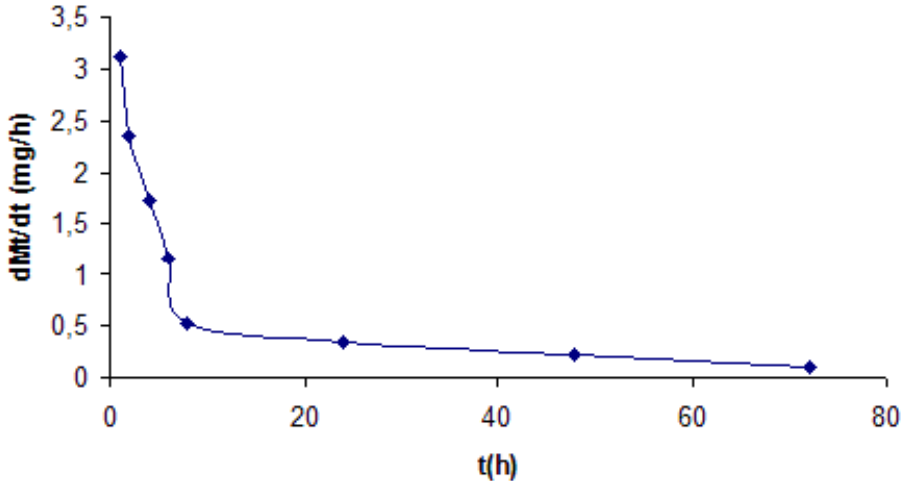

Figure 2 – Speed of levan release in liposomes.

Concentration of Levan (mg/mL)	Long term stability	Acceleration stability	Particle size (nm)[1]	Zeta potential (mV)[1]	Encapsulation rate (%)[1]
20	Estable for 45 days	Precipitated	313.03 ± 151.28	+ 2.51 ± 0.10	87.6 ± 1.2

1 – X (Average) ± SD (Standard deviation).

Table 1 – Physicochemical characterization and stability of Lev-LIPO

promising biotechnological and pharmaceutical possibilities, mainly in its use of nanotechnology, for the formulation of liposomes with targeting and moderating the release of levan in the human body.

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