Research article

Development of lipid nanoparticles with nystatin for an antifungal action

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Abstract

Fungal diseases currently affect about a quarter of the population worldwide. Fungi infections caused by *Candida albicans* have been described as a significant concern to public health. The spectrum of clinical diseases caused by this fungi species range between vulvovaginal candidiasis, oral candidiasis, candidemia and mucositis. The emergence of resistance mechanisms towards antifungal therapy greatly hampers the successful management of illness and patient outcomes. Nystatin, an antifungal drug, is categorized as a class IV of the Biopharmaceutical Classification System, presenting low aqueous solubility and low intestinal permeability. Nowadays, the emerging platform of nanotechnology and lipid nanoparticles, notably solid lipid nanoparticles (SLN), has been subject to growing attention, owing to the promising properties of vectorizing a substantial variety of pharmaceutical drugs and improving therapeutic index. Due to its hydrophobic properties, nystatin was encapsulated in SLN. The aim of this work was to formulate SLN formulations loaded with nystatin by different methods (high-speed homogenization and ultrasonication) with optimization of several parameters and formulation of 2 gels (one of them containing nystatin-loaded nanoparticles).

Initially, 3 lipids were used: Compritol® 888 ATO, cetyl palmitate and Precirol® ATO 5 and, after the study of several parameters (size, encapsulation efficiency (EE) and polymorphic behaviour of the lipids), Precirol® ATO 5 was chosen as the lipid with the most satisfactory results. The results of the present work showed that the high-performance liquid chromatography (HPLC) method to assay nystatin was linear, specific, and presented repeatability. The average diameter of empty nanoparticles (NPs) and with drug (Precirol-NYS NPs) was, respectively, 306 nm and 260 nm and an EE of 67.8%. Regarding stability, SLN with drug proved to be more stable than SLN without drug over a period of 1 month. The polymer used for the formulation of gels was the polymer commonly known by the trade name Carbopol® 940. The final formulation yield of “0.5% carbopol gel” and “0.5% carbopol gel + 10% NYS NPs” were 87.2% and 91.39%, respectively.

Keywords: *Candida*; nystatin; SLN; stability; Carbopol® 940.

Introduction

Recent studies have estimated that there are approximately 8.7 million eukaryotic organisms on the planet, of which 7% are fungi, amounting to approximately 611,000 species. Of these species, 600 are pathogenic and responsible for infections in humans. Fungal infections are a global health problem and affect millions of patients each year [1]. Currently, antifungal agents on the market can be divided into four classes depending on their therapeutic target: azoles, echinocandins, polyenes, and allylamines. However, these four antifungal drug classes have some drawbacks, such as their limited spectrum of action, evolving resistance mechanisms, or drug-drug interactions. Additionally, physicochemical properties and the similarity of fungal cells to animal cells can cause selectivity problems, which can affect clinical efficacy and efficiency [2].

Nystatin (NYS) (chemical structure: $C_{47}H_{75}NO_{17}$), the first antifungal polyene, was discovered in 1950 by Brown and Hazen, a chemist and a bacteriologist, respectively [3]. It is a macrolide polyene derived from polyketides, isolated from the microorganism *Streptomyces noursei* [4]. NYS has an amphipathic nature due to its seven free hydroxyl groups and a side chain consisting of a mycosamine (lactone) residue that has a free amine group. Its main antifungal action is against *Candida* species, but it is also effective against *Aspergillus*, *Coccidioides*, *Cryptococcus*, *Histoplasma* and *Blastomyces* [5]. NYS appears as a hygroscopic yellow powder with a suggestive smell of cereals and an unpleasant taste. [6]. It belongs to class IV of the Biopharmaceutical Classification System and has low aqueous solubility and low intestinal permeability [7]. NYS is poorly soluble in polar and non-polar solvents due to the presence of polar hydroxyl, carboxyl groups, and an amino and lactone group in the side chain, in contrast to the hydrophobic group (polyene group) [8]. It is practically insoluble in water, slightly soluble in n-propyl alcohol, methyl alcohol and n-butyl alcohol and insoluble in chloroform, ether and benzene. Its physicochemical properties are affected by exposure to sunlight, heat, and air. Therefore, it should always be stored in airtight conditions and protected from light [9]. NYS can be administered orally but its absorption in the gastrointestinal tract is very low, so its action will only be local. Excretion occurs with the drug in an unchanged form. NYS has not yet been used systemically due to its worrying infusion-related reactions

and dose-limiting toxicities, particularly to the erythrocytes [10, 11]. The antifungal mechanism of action is directly related to its amphiphilic structure, which facilitates binding to ergosterol in the membrane, disrupting both its integrity and fluidity, and eventually leading to fungal cell death [10].

Nanomedicine offers a solution to the limitations of many drugs in their free form, such as short bloodstream lifespan, low solubility in water, or poor bioavailability, which can result in adverse effects [12, 13]. Solid lipid nanoparticles (SLN) are a type of lipid nanoparticle (NP) that is composed of solid lipids at room temperature, forming a crystalline matrix [13]. These nanocarriers typically range in size from 100-300 nm [14]. Despite having advantages over other nanosystems, such as inferior toxicity, enhanced stability of degradation-prone drugs, and greater ease of controlling drug release, SLNs have some drawbacks, such as low drug loading capacity and high water percentage, but the most concerning disadvantage is the possibility of drug expulsion during storage [15]. Several factors, such as the physical-chemical structure of the matrix or the crystalline state of the lipid, can influence the amount of encapsulated active substance [16].

There is an increasing number of studies and progress being made in developing new drug delivery systems, including those for antifungal drugs. While several commercial formulations are available on the market for treating diseases caused by the *Candida* fungus, they all present associated toxicity issues and low bioavailability due to the characteristics of NYS. One way to overcome this problem is by vectorizing NYS into SLNs, which can increase the effectiveness and efficiency of therapy and make systemic treatment possible. Several studies with other lipid nanoparticles have shown significant reductions in toxicity while improving antifungal activity through NYS vectorization [5] [17] [18]. There are also ways to mask the unpleasant taste of NYS, and it can even be prescribed in the form of buccal tablets for treating oral candidiasis [19].

The most common forms of NYS presentation in the pharmaceutical industry are semi-solid formulations such as gels or creams, as previously mentioned. According to the *Portuguese Pharmacopoeia*, a gel is a colloidal system that can be either semi-solid or solid and composed of one or more drugs, excipients, and adjuvants. Gelling agents are typically proteins or carbohydrates that provide firmness to the formulation, which, when dispersed in a liquid vehicle, can form a three-dimensional network inside the liquid. Topical preparations commonly use preservatives such as methylparaben, propylparaben, sorbic acid, benzoic acid, quaternary ammonium salts and phenols [20]. *Samein* developed a gel formulation called SLN-NYS gel, which contains encapsulated nystatin and is intended for topical administration against *Candida albicans*. The encapsulation efficiency (EE) of SLN-NYS was more than 89%, and its mean diameter was 210.17 nm. The hydrogel was formulated using Carbopol® 940 polymer and had a drug content in the gel greater than 98%. The rheologic behavior of the gel showed a shear-thinning flow. *In vivo* tests conducted on rabbits demonstrated that the skin irritation score was 0.00 [21].

The rise in antifungal resistance and the unique physicochemical characteristics of NYS make it crucial to develop a pharmaceutical formulation that can overcome these challenges and enhance the therapeutic effect.

The goal of this work was to develop hydrogels containing nystatin-loaded SLNs intended for later application in cutaneous candidiasis. Though this is neither the first work with this aim nor the first applying the experimental method described [21-24], our SLN formulations contained a mixture of ingredients whose collective usage has not yet been employed in nystatin-loaded SLN hydrogels to date, namely the combination of commercial solid lipid Precirol® ATO 5 with the surfactant Tween 80.

Materials and Methods

Preparation of SLN-NYS

For the preparation of nanoparticles (NPs), the following compounds were used: NYS, which was kindly provided by Fagron Ibérica SAL, Spain. Compritol® 888 ATO, Precirol® ATO 5, and Cetyl Palmitate, which were provided by Gattefossé, France. Polysorbate 80 (Tween 80), which was supplied by Acofarma, Portugal. Ultrapure water was obtained using Direct-Q® Ultrapure Water Systems.

The SLN formulations were prepared using the hot homogenization method followed by ultrasonication. For high-speed homogenization, it was necessary to heat the oil phase (solid lipid + surfactant + active substance) in a water bath to a temperature of 5 to 10 ºC above the melting point of the solid lipid (76ºC, 65ºC and 60ºC, respectively for Compritol® 888 ATO, Precirol® ATO 5 and cetyl palmitate).

A study by *Yanzhi Wang et al.* concluded that adding the surfactant in the lipid phase (like we did) translates into SLNs of smaller diameter, slightly higher ZP, in absolute value, and a greater EE in relation to the opposite technique. These results can be explained by the fact that the lipid and the drug come into contact right away during the preparation, which causes better homogenization, division, and EE of the SLN [25].

In our experimental procedure, the aqueous phase (ultrapure water) was also heated to the same temperature of the oil phase and later added to the oil phase. This step is followed by agitation of the formed emulsion with the help of a high-speed homogenizer, a T25 Ultra-Turrax® Dispersing Instrument (IKA Labortechnik). Then, ultrasonication was performed using the ultrasonic processor Model Vibra-Cell™ VCX 130 with a 6 mm probe from Sonics & Materials, for 15 minutes at 70% amplitude. The time and speed (in revolutions per minute, rpm) were different for each lipid, with the selected conditions shown in **Table I**. The emulsions were finally cooled in ice to allow the NPs to solidify quickly and were subsequently stored in the cold at 4°C, without direct contact with light.

Table I. Time and speed (in revolutions per minute, rpm) for the three lipids.

Lipid	Lipid phase		Lipid phase + aqueous phase	
	Speed (rpm)	Time (min)	Speed (rpm)	Time (min)
Compritol® 888 ATO	11600	7	14000	8
Cetyl Palmitate	12000	7	15000	7
Precirol® ATO 5	9000	7	13000	5

The appearance of SLN was an off-white color. The texture was smooth and

homogeneous, visually clear and free of any visible particles.

Nystatin assay method

Several HPLC methods to quantify nystatin can be found in the literature [26-28]. We developed a simpler, quicker and more reliable in-house method to quantify nystatin in the formulations. Some operating conditions were adopted from the literature, such as the C18 column type, detection wavelength of 305 nm, and a flow rate of 1.0 mL/min [29]. However, for the remaining conditions, different tests were carried out to optimize the method, such as the choice and percentage of eluents and the injection volume. Initially, the mobile phase consisted of 70% acetonitrile (ACN), obtained from Honeywell (Seelze, Germany), and 30% ultra-pure water acidified to pH 3 with glacial acetic acid, procured from VWR Chemicals (Rosny-sous-Bois, France). However, it was found that the chromatographic behavior was not ideal in terms of retention time and peak areas [30]. Therefore, the organic solvent was changed to methanol (MeOH), obtained from Honeywell (Seelze, Germany), based on retention time and consistent results with scientific literature. According to *Llabot et al.*, NYS is a highly insoluble molecule, and using MeOH as the organic solvent in a higher percentage results in symmetrical peaks at the intended retention time. A flow rate of 0.6 mL/min caused long retention times, while increasing the flow rate to 1.2 mL/min resulted in poor drug resolution [31].

To determine the suitable percentage of MeOH, six different percentages (65%, 70%, 75%, 80%, 85%, and 90%) were tested, and the decision was based on these key features: lowest total run time, asymmetries lower than 1.5, plates count superior to 2000 and a capacity factor (k') value near the k' for the solvent peak, which must be at least greater than 2 [32]. The percentage of 75% MeOH was chosen based mostly on the reduction in total run time and eluent cost. The injection volume adopted was 10 µL, a choice based on the reduced coefficient of variation it presented in all parameters compared to the other injection volume of 1 µL.

To sum up, a reverse-phase column Acclaim™ 120 C18 LC Column (5 µm ultrapure silica particles, 120 Å, 100 mm x 4.6, Thermo Fisher Scientific; Waltham, MA, USA) with a flow rate of 1.0 mL/min was employed. The mobile phase consisted of a mixture of 75:25 methanol and of Milli-Q® water acidified to pH 3.0 with glacial acetic acid. Injections were made in triplicate, with a volume of 10 µL, a total run time of 5

minutes and a detection wavelength of 305 nm. The results were analyzed by Chromeleon™ 7.2 SR4 Chromatography Data System (CDS) Software (Thermo Fisher Scientific, Waltham, MA, USA). NYS solutions preparation and analysis were performed on the same day and under light protection.

Quantification of encapsulation efficiency (EE)

Another in-house method was developed to successfully quantify the drug's encapsulation efficiency in the SLN formulations, by indirect method. This involved diluting 2 mL of NYS-loaded SLNs in 10 mL of ultrapure water, vortexing for 30 seconds, removing 0.5 mL of the mixture, and diluting it with 8 mL of a solvent mixture (60:10:30 MeOH: dimethylsulfoxide: ultrapure water). The resulting mixture was placed in an Amicon® tube Ultra-0.5 and centrifuged at 4000 G for 15 minutes. The supernatant was then collected, filtered, and injected in the HPLC system for further analysis. The determination of encapsulation efficiency by indirect method followed this equation:

$$EE\ (\%) = \frac{[Drug]_{total} - [Drug]_{free}}{[Drug]_{total}}$$

Preparation of SLN-NYS gels

For the preparation of SLN-NYS gels, the following compounds were used: Carbopol® 940, kindly offered by Vaz Pereira, Portugal); methyl parahydroxybenzoate, propyl parahydroxybenzoate (Acofarma, Portugal) and 10% sodium hydroxide solution (NaOH), prepared in the Pharmaceutical Technology Laboratory, FFUP, Portugal.

Two gels were formulated. One formulation "without NYS nanoparticles" (GEL) and another formulation with the encapsulated drug (GEL-0.5-SLN-NYS), with the aim of comparing the rheological and organoleptic characteristics of the two. For each gel, Carbopol® 940 was first grounded in a porcelain mortar with water. The paraben concentrate was mixed with purified water and added to the previous Carbopol® preparation with stirring. The preparation was left to stand for 24 hours, and then the aqueous 10% NaOH solution was slowly added. For GEL-0.5-SLN-NYS, the base mass and the SLN-NYS formulation mass were mixed in the Gako Unguator Mixing Device for 2 minutes at a speed of 600 rpm.

The yield (percentage ratio between the actual amount of hydrogel obtained and the theoretical amount of hydrogel expected) of the GEL and GEL-0.5-SLN-NYS preparations was 87.2% and 91.39%, respectively.

Results

Validation of the nystatin assay method

One of the objectives of this work was to validate the analytical method to assay nystatin. The developed method demonstrated sensitiveness, specificity and selectiveness. Linearity was ensured by preparing a calibration curve, which certified the method's linearity over a good range of concentrations (10.3-35.5 µg/mL). This parameter is useful as it guarantees proportionality between sample concentration and area, the analyzed parameter [30].

Six stock solutions with escalating concentrations were prepared by dilution of the appropriate amount of nystatin in 10 mL of dimethylsulfoxide (DMSO), following by 30 minutes magnetic stirring and final storage in the cold, with all the procedures being carried out under light protection. Six standard solutions (10.3; 16.3; 20.3; 25.1; 31.1; 35.5 µg/mL) were afterwards prepared through a 1:100 dilution with a solvent mixture of 60:10:30 (MeOH:DMSO:Ultrapure water) of the previously-prepared stock solutions

The choice of this solvent mixture for dilution, instead of just employing DMSO, was made because DMSO is frequently used in HPLC at relatively low concentrations or in combination with other compatible solvents to achieve the desired solubility of the analytes without compromising the chromatographic performance. NYS is insoluble in water and methanol, but highly soluble in DMSO. DMSO is a colorless organosulfur solvent that is soluble in both polar and nonpolar compounds, miscible in most organic solvents, and also miscible in water. However, DMSO has a higher viscosity than many other common HPLC solvents such as water, methanol, and acetonitrile. When used in high concentrations, the increased viscosity can significantly raise the back pressure in the HPLC system, leading to poorer instrument performance and even causing apparatus damage [13].

Finally, each standard solution was filtered using a 0.45 µm PTFE filter, OlimPeak®, and injected into the HPLC system in triplicate.

Figure 1. (a) presents the calibration curve obtained, with an equation of y = 0.557x-2.159, a determination coefficient (R^2) of 0.990, a correlation coefficient of 0.995, and a sum of squared deviations (SQD) of 1.383. In Through **Figure 1. (b)**, there is possible to observe that the residuals are

reduced and randomly distributed, as expected. The limit of detection and limit of quantification obtained were 5.87 and 17.78 µg/mL, respectively.

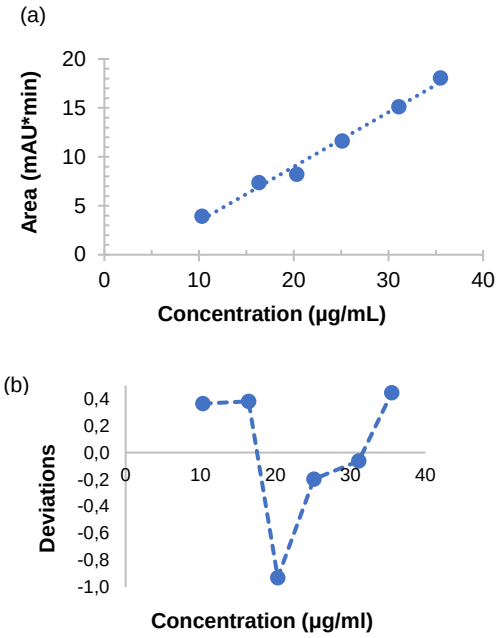

Figure 1. (a) Calibration curve for NYS. Line equation: y= 0.5572x - 2.1899; R^2=0.99, R=0.995 and SQD=1.383; (b) Graphic representation of deviations from linearity.

Selection of lipid

In order to select the lipid, it was necessary to evaluate the polymorphic behavior of the lipids through Differential Scanning Calorimetry (DSC). The size of nanoparticles was evaluated, using Dynamic Light Scattering (DLS). DLS was also used to evaluate the polydispersion index (PI). The zeta potential (ZP) was evaluated using Electrophoretic Light Scattering (ELS).

Firstly, DSC is a thermal analysis technique used to study thermal transitions in materials, including lipids. It measures the heat difference between a sample and a reference as they are subjected to a temperature-controlled scan. To observe the melting point of lipids, the graph of specific heat as a function of temperature is analyzed. The melting point will appear as a characteristic endothermic (heat absorption) peak, representing the transition from solid to liquid. The polymorphic behavior of the three lipids was analyzed (**Figure 2**), and it was observed that there was no alteration in the crystalline structure for Compritol® 888 ATO in cycle 1, 2 and 3, as the peaks and areas were similar. For Precirol® ATO 5 and Cetyl Palmitate we observed different results in the cycles.

In **Figure 2** the thermograms of all the lipids analyzed individually can be seen, whilst in **Figure 3** the thermogram of all the lipids + drug is depicted. It can be observed that the onset values increased slightly (temperature where the melting point begins), and the enthalpy values, which corresponds to the peak area, decreased, which indicates a small interaction of NYS in the behavior of lipids.

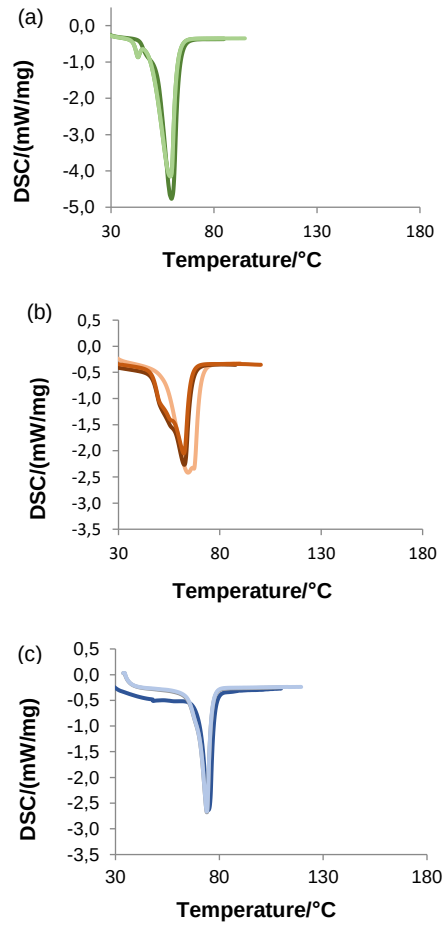

Figure 2. (a) Cetyl Palmitate Thermogram; (b) Precirol® ATO 5 Thermogram; (c) Compritol® 888 ATO Thermogram.

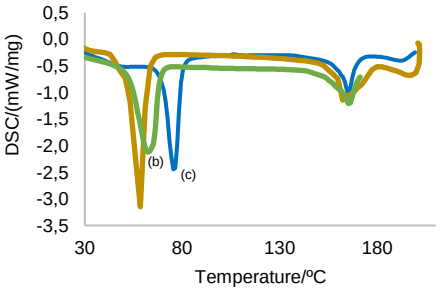

Figure 3. Thermogram of each lipid studied with NYS. (a) Cetyl Palmitate + NYS; (b) Precirol® ATO 5 + NYS; (c) Compritol® 888 ATO + NYS.

The analysis of **Figure 4** leads to the conclusion that Precirol® ATO 5 lipid permits mean diameter values closer to the target values (between 100-300 nm), while cetyl palmitate lipid obtained larger particle sizes than the target values [14]. It was found that all the PI values were within the intended range (<0.3) and the PZ values were high and negative (-24 mV for Compritol® 888 ATO, -29.5 mV for Cetyl Palmitate and -37.7 mV for Precirol® ATO 5) indicating high stability of the SLNs.

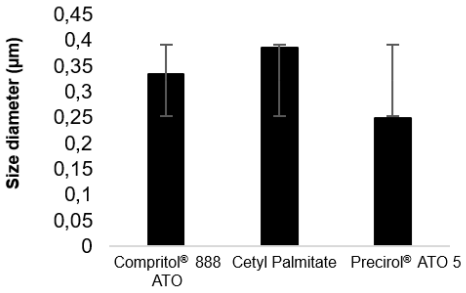

Figure 4. Mean diameter (in µm) of drug-free SLN using each lipid, determined by DLS. The mean of 3 replicates is depicted, along with standard deviation bars.

With that being said, cetyl palmitate was discarded due to the previously demonstrated values, which led to the conclusion that the other two obtained better results since one of the objectives of this study was the selection of only one type of lipid through elimination steps. The EE, determined using the indirect method, of new SLN formulations containing Compritol® 888 ATO or Precirol® ATO 5 with NYS was determined, obtaining values of 47.19% and 50.66%, respectively. The mean diameter values, ZP and PI were also determined and are shown in **Table** II.

Based on these results, Precirol® ATO 5 was chosen as it permitted more satisfactory results: lower mean diameter values and higher ZP absolute values.

Table II. Mean diameter, in µm, of the SLN obtained with Compritol® 888 ATO (SLN COMP-NYS) and with Precirol® ATO 5 (SLN PREC-NYS) and also the values of ZP and PI.

	Mean diameter (nm)	ZP	PI
SLN COMP-NYS	386	-24.36	0.209
SLN PREC-NYS	295	-26.47	0.293

Stability study

SLNs with (NP PREC-NYS) and without the drug (NPs-PREC) were prepared to study physical and chemical stability and also analyze any changes that may occur in the drug after preparation and during storage at 4ºC for 1 month at intervals of 0, 15, and 30 days. The same analytical techniques were used to characterize the physicochemical properties of the particles - mean diameter and polydispersity index values – **Figure 5** and **Figure 6**.

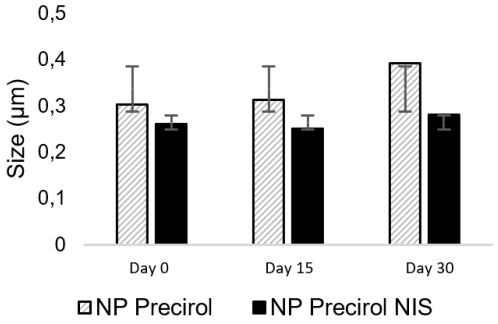

Figure 5. Mean SLN diameter (DLS) of the PREC-NYS SLNs and NP PREC formulations, at the 0, 15th and 30th day. Average values +/- standard deviations are represented.

In **Figure 6**, the PI of both SLN formulations determined by DLS is shown and it can be observed that the values remain constant over the storage time (for drug-loaded SLN formulations) and the range of values is within the expected range. The same conclusion can be drawn from the non-meaningful particle size variations over 1 month time in **Figure 5**.

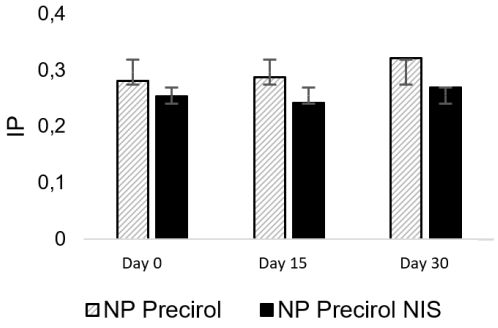

Figure 6. PI values from PREC-NYS SLNs formulations and SLN made up of Precirol® ATO 5, at the 0, 15th and 30th day. Average values +/- standard deviation are represented.

The analysis of thermal events during heating revealed several endothermic peaks, shown in **Figure 7**,

demonstrating enthalpy values and peak area similar to those shown in **Figure 2** - b) and **Figure 3**, (polymorphic behavior of the Precirol ATO 5). In **Figure 3**, the drug was in its free and non-encapsulated form, making it possible to observe the endothermic peak around 170ºC, whereas in **Figure 7** the drug is successfully encapsulated, as evidenced by the disappearance of that characteristic peak and the overlapping thermograms of blank and nystatin-loaded nanoemulsions. The values of onset, enthalpy and peak area also remain constant throughout the month of storage, concluding that the NYS-loaded SLN formulations maintain stability and do not release the drug. The enthalpy and peak values also remained constant over time, further supporting the conclusion of SLN stability.

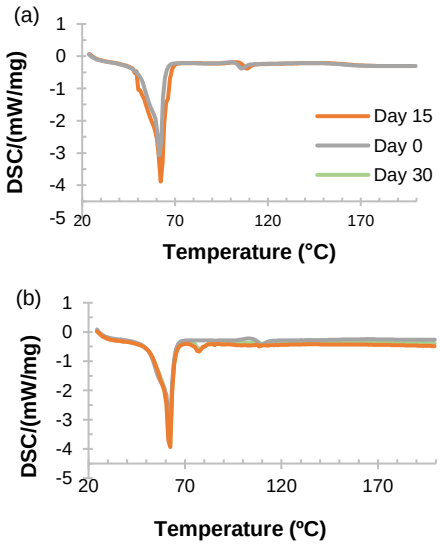

Figure 7. (a) Thermogram of the SLN PREC on days 0, 15 and 30; (b) Thermogram of the PREC-NYS SLNs on days 0, 15 and 30.

Regarding the EE, on days 0, 15 and 30, the encapsulation obtained was 67.8%, 43.7% and 43.6%, respectively. The authors believe that one of the reasons for this encapsulation decrease lies in sample loss during handling. It is plausible that reusing the Amicon® Ultra Centrifugal Filters has caused degradation of the filter membrane, affecting their ability to concentrate, purify, and filter samples, potentially leading to carryover, and impacting the purity and accuracy of subsequent filtrations. In addition, one of the most worrying about nystatin is its tendency to self-aggregate due to its low water solubility, thus causing reduced encapsulation and possible toxicity [3]. Strategies to decrease nystatin's likelihood to aggregate may be the inclusion of certain surfactants in the formulations, such as Cremophor® RH 40 [33], a poloxamer [34] or a polysiloxane-tromethanol surfactant [35].

Rheological study of gel

The Malvern Kinexus® Lab+ Rheometer is a robust and reliable tool for rheological analysis. Both gels at 0, 7 and 15 days did not show thixotropy and were non-Newtonian fluids with shear

thinning behaviour (viscosity decreasing as the shear rate increased).

The final appearance of both gels is shown in **Figure 8**, in day 0, 7 and 15, where GEL-0.5-SLN-NYS reflects the encapsulation of the drug-loaded SLN formulation in the gel, whilst GEL means a blank SLN formulation (without drug) incorporated within the gel.

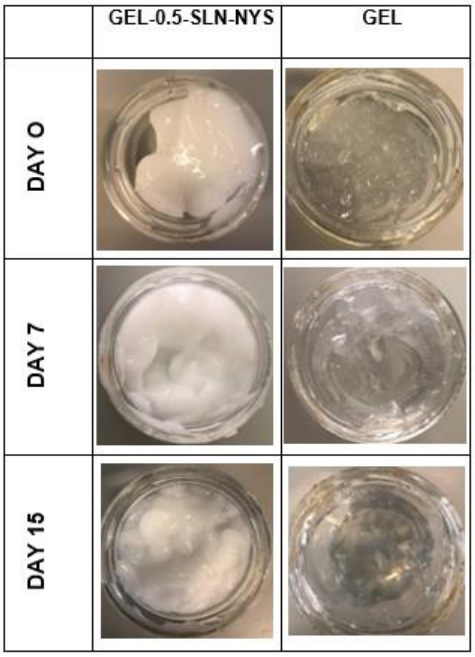

Figure 8. Preparation and characterization of Hydrogels. On the left is the nystatin-loaded SLN Gel (GEL-0.5-SLN-NYS) and on the right is the SLN gel without nystatin.

Organoleptic analysis of the formulations was conducted at production time (day 0) and 7 and 15 days later. Visual analysis was used to evaluate color, appearance, texture, and odor. The GEL had a translucent color, odorless and homogeneous appearance, while the GEL-0.5-SLN-NYS presented a white appearance.

In **Figure 9**, it can be seen that there is maintenance of the viscosity values for the four indicated shear rates and the viscosity values for the GEL-0.5-SLN-NYS at 0.1, 1, 10 and 100 s^{-1} shear rates, were slightly lower compared to the other gel, demonstrating that the presence of SLN influenced the viscosity value.

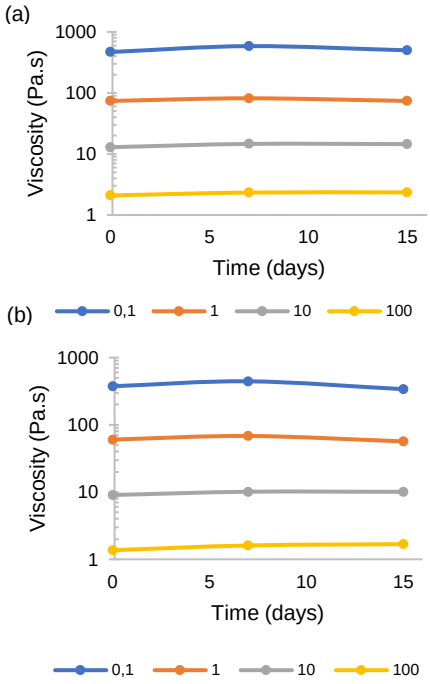

Figure 9. Viscosity as a function of time for different cutting rates; (a) GEL; (b) GEL-0.5-SLN-NYS.

Additionally, **Figure 10**, shows the viscosity in the 3 analysis times as a function of temperature. It can be seen that in the gel without NPs, **Figure 10. (a)**, the initial viscosity is lower at day 0 but on the following days the viscosity increases and, compared to GEL-0.5-SLN-NYS, the viscosity values are

higher at days 7 and 15. This may be due to the fact that during the resting period the network of polymeric chains interconnect through cross-links, making the gel more consistent and causing an increase in the viscosity value from day 7. For gel with loaded SLN-NYS, the viscosity values do not vary much depending on the temperature or over the days.

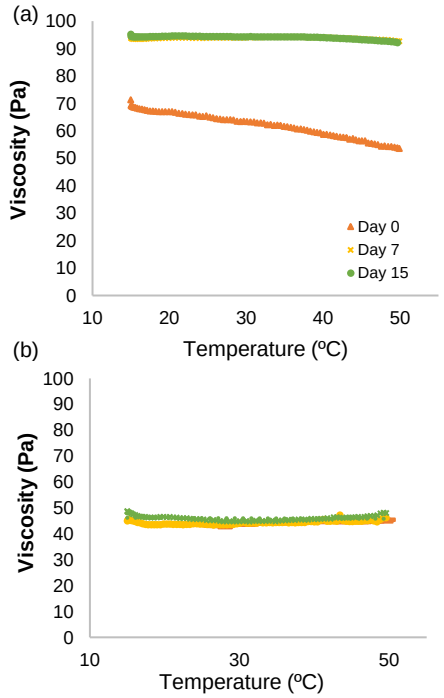

Figure 10. Determination of the effect of temperature (a) GEL; (b) GEL-0.5-SLN-NYS. The days are highlighted in different colours and geometric figures. Day 1 (▲) orange, day 7 (●) yellow, day 15 (▄) green.

The viscosity values are constant and lower than the other gel, thus concluding that the presence of NPs influences the viscosity values.

This goes to show that the encapsulation of nystatin in pharmaceutical forms, such as a hydrogel, provides greater stability and protection to the drug, preventing its degradation and ensuring a longer shelf life of the medication.

This can also predict a controlled release of the active ingredient in the body, reducing potential side effects and enhancing the treatment's effectiveness, as nystatin in its free form exhibits low bioavailability [3].

There are a few published studies on nystatin-loaded hydrogels [21-23]. All concluded that the hydrogels containing nystatin presented pseudoplastic rheologic behaviour and remained stable over time without a significant increase in particle size or entrapment efficiency. Additionally, these studies showed sustained drug release and antifungal effectiveness against *Candida albicans*, through either *in vivo* [22, 23] or *ex vivo* testing [21].

Conclusion

Based on the analysis of the results, it can be concluded that the NYS-loaded SLN formulation is stable over a storage period of one month at 4ºC, as

confirmed by the sligh variations in mean diameter and PI values and the absence of drug expulsion as indicated by the DSC analysis. However, a decrease in the EE was observed over time, resulting in possible agglomeration of SLN over 30 days, which suggests the need for further optimization of the formulation to maintain drug encapsulation efficiency over a longer period, through the employment of a higher surfactant concentration, surfactant's replacement, or even addition of a co-surfactant.

In terms of the gel formulation, the addition of SLN-NYS did not significantly affect the rheological behavior of the gel, which remained a shear thinning fluid without thixotropy. However, the presence of SLN influenced the viscosity values of the gel, which were lower compared to the gel without SLN. The gelling agents are generally proteins or carbohydrates and will give firmness to the pharmaceutical form which, when dispersed in a liquid vehicle, will have the ability to form a three-dimensional network inside the liquid itself. In terms of viscosity, it can be seen that in GEL the initial viscosity is lower on day 0, but on the following days the viscosity increases and, in comparison with GEL-0.5-SLN-NYS, the viscosity values are higher on days 7 and 15. This could be due to the fact that, during the rest period, the network of polymeric chains interconnect through cross-links, making the gel more consistent and causing an increase in the viscosity value from day 7.

Overall, the results suggests that the SLN-NYS formulation can be a potential candidate for topical delivery of NYS, and further optimization of the formulation and gel properties can lead to a more effective and stable product. *In vitro* antifungal activity studies are also needed to confirm these promising results.

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Conflict of Interest

The authors declare no conflict of interest.

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