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Solid lipid nanoparticles made of trehalose monooleate for cyclosporin-A topic release

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ABSTRACT

The aim of this work was the preparation of solid lipid nanoparticles, based on trehalose monooleate, loaded with cyclosporin-A for potential treatment of psoriasis. Trehalose was esterified with oleic acid in order to obtain a more lipophilic compound suitable as a lipid matrix for the formulation of a new type of solid lipid nanoparticles. The ester formation was confirmed by FT-IR and ^{1}HNMR. The solid lipid nanoparticles, based on trehalose monooleate, were successfully prepared with and without cyclosporin-A, using the microemulsion technique. Further characterizations were performed by differential scanning calorimetry (DSC). The drug release from the SLNs was then evaluated through the Franz diffusion cells, using both dialysis membranes and rabbit ear skin. With the aim to verify the localization of SLNs in the skin layers, additional investigations using confocal microscope and stripping tape test were carried out. Finally, a dermatological formulation, based of SLNs loaded with cyclosporin-A, was prepared and tested through Franz diffusion cells. The obtained results indicate the possibility of using these nanoparticles as vehicle of cyclosporin-A for topical treatment of psoriasis, reducing the side effects due to systemic absorption of cyclosporin-A and, at the same time, increasing its concentration at skin injury level.

1. Introduction

The skin, a protective barrier involved in many important physiological functions such as fluid homeostasis, thermoregulation, immune surveillance, provides a unique delivery pathway for therapeutic and active substances [1,2]. These functions are related to the skin's multiple layers, whit each layer associated with highly specialized cells and structures. The permeation barrier properties of human skin are mostly attributed to the top layer of the epidermis, the stratum corneum (SC) [3]. In fact, the specific composition and structure of the SC lipids selectively and effectively inhibit the penetration of chemicals [4,5]. However, these properties also limit the drug delivery across the SC so that the treatment of several skin diseases, such as psoriasis, are still limited, waiting for the unmeet need of treatments based on safer and more efficient transdermal formulations. Psoriasis is a skin disease and is one of the most common inflammatory disorders, that regards between 1 and 3% of the population. It is characterized by epidermal hyperproliferation and dermal inflammation. Its etiology is unknown but seems that genetic factors play a role. Psoriasis can affect only a small area of the skin (< 2% of body surface area) but often, the disease can be quite severe, affecting a large portion of the skin. Furthermore, psoriasis is, unfortunately, associated with systemic metabolic disorders including an increased prevalence of the metabolic syndrome, obesity, diabetes, dyslipidaemia, and cardiovascular disease [6,7,8,9,10]. One of the most effective drugs available for psoriasis treatment is cyclosporin-A (CsA) [11,12] a potent immunosuppressive agent with a selective and reversible inhibitory effect on T-cell functions, especially interleukin-2 production. CsA, for many years, is used in treatment of severe psoriasis and in a large array of autoimmune and inflammatory skin diseases. In general, it has proven effective but its long-term systemic administration gives harmful side effects like nephrotoxicity, hypertension, hyperlipidaemia, hyperkalaemia, paraesthesia, granulomatous, hepatitis, etc [13,14].

For these reasons topical approaches targeting the drug to the basal layer of epidermis, where psoriasis originates, would be desirable. However, beside the limited permeability of the SC, the CsA topical penetration is not readily achieved also due to its rigid cyclic structure and high molecular weight [15,16].

Extensive research has been performed to overcome the SC barrier and deliver drugs across the skin using physical (iontophoresis,

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electrophoresis and sonophoresis) and chemical techniques (penetration enhancers) or a combination of both [17]. However, chemical enhancers induce irritation and physical methods cause damage and disruption of the skin barrier [18,19]. In this context novel nanocarrier systems could have an important impact on clinical practice. In particular, solid lipid nanoparticles (SLNs) are generating a great interest in the pharmaceutical field [20,21,22,23–25]. The use of SLNs as topical drug carrier systems are due to several advantages. Indeed, these system increase the drugs permeation of the while limiting the damage to the skin barrier function [26,27,28,29].

The aim of the present work was to design and prepare nanoparticles based on a new trehalose monooleate ester to facilitate the topical administration of CsA. Trehalose has been chosen for the composition of nanoparticles in view of its properties: it is a disaccharide widely used in pharmaceutical field for its biocompatibility, bioprotective properties and for its stabilizing effect on bioactive substances [30,20,31].

Also oleic acid (OA) was used in combination with threalose [32] for SLNs matrix obtainment. OA is an unsaturated free fatty acid that has shown to play a beneficial role in protecting substances from free radicals and possesses the ability to modulate inflammation and enhance reparative respons*e in vivo* [33,34]. In addition, OA has been used as an emulsifying or solubilizing agent and a skin penetration enhancer in pharmaceutical products [35,36].

Furthermore, a dermatological formulation based of SLNs loading cyclosporin-A, was prepared and tested through Franz diffusion cells. The obtained results indicate the possibility of using these nanoparticles for cyclosporin-A administration and treatment of psoriasis, with the advantage of increasing the drug concentration at the injury skin site while reducing its systemic absorption.

2. Experimental section

2.1. Materials

All solvents of analytical grade were purchased from Carlo Erba Reagents (Milan, Italy) and Fluka Chemika-Biochemika (Buchs, Switzerland): dichloromethane (DCM), chloroform, n-hexane, concentrated sulfuric acid (96% w/w), 1-butanol and methanol. Trehalose (PM 378.33 g/mol); oleic acid (PM 282.46 g/mol); dicyclohexylcarbodiimide, DCC, (PM 206.33 g/mol); dimethylaminopyridine, DMAP (PM 122.17 g/mol); Polyoxyethylene sorbitan monolaurate (Tween 20, PM 1227.54 g/mol, 1.1 g/mol); biliary acid biliary acid (PM 521.69 g/mol), coumarin-6 were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA). Cyclosporin-A (PM 1202.61 g/mol) was purchased from Farmalabor srl (Milan, Italy). Sepigel™305 (polyacrylamide, isoparaffin C13-14, 7-laurate),methyl paraben, propylparaben. The cellulose acetate membrane 6-27/32" (MWCO: 12000–15000Da) was purchased by Medicell International LTD.

2.2. Instruments

The infrared spectra on potassium bromide (KBr) pellets were performed using a FT-IR Jasco 4200 spectrophotometer in the range 4000-400 cm^{-1}. UV–Vis spectra were carried out using UV/Vis JASCO V-530 spectrophotometer using quartz cells with a thickness of 1 cm. The ^{1}H NMR analysis were performed using a Bruker VM 30 spectrometer; the chemical shifts are expressed in δ and related to the solvent. Dimensional analysis was performed using a Brookhaven 90 Plus Particle Size Analyzer. The differential scanning calorimetry (DSC) was performed with DSC 200 PC NETZSCH at heating rate of 10 °C/min. The samples were freeze-dried by Micro Freeze-drying Modulyo, Edwards. Confocal analysis was carried out using Leica TC-SP2 Confocal System; Leica Microsystems Srl, Milan, Italy.

Table 1
Amount of reagents used for SLNs preparation.

Trehalose monooleate (g)	tween 20 (g)	1-butanol (g)	biliary salt(g)	water(g)	Cyclosporin-A (g)
0.36	0.208	5.02×10^{-2}	0.0884	0.611	0.0936

2.3. Esterification of oleic acid with trehalose

In a three-necked flask fitted with a reflux condenser, dropping funnel and magnetic stirrer, accurately flamed and maintained under inert atmosphere, trehalose (1 g, 2642 10^{-3} mol), DCC (1.091 g, 5.286 10^{-3} mol), and DMAP (0.0323 g, 2.64310^{-4} mol, 1:20 ratio with DCC moles), were solubilized in DCM dry (240 ml). The solution was kept under stirring for 30 min at 0 °C and then at room temperature until complete dissolution of DMAP and DCC. Subsequently oleic acid (1.493 g, 1.32 10^{-3} mol) was added drop by drop. The reaction was left at room temperature under stirring for 3 h and monitored by thin layer chromatography (TLC/silica gel, eluent mixture n-hexane-chloroform 1:9). The solution was then filtered to remove dicyclohexylurea. The reaction solvent was removed by evaporation under reduced pressure. The obtained trehalose monooleate, solid and yellow in color, was characterized by FT-IR, ^{1}H NMR and ^{13}C NMR spectroscopies.

2.4. Preparation of solid lipid nanoparticles (SLNs)

SLNs were prepared by a microemulsion technique at moderate temperature according to Gasco [37] using the reagents as shown in Table 1. Briefly, the trehalose monooleate, in the presence or not of cyclosporin-A, was melted at 70–75 °C. A warm water solution of sodium taurocholate, butanol and Tween 20 was then added to obtain an optically transparent system. The warm microemulsion was immediately dispersed in cold water (~2 °C) under high-speed homogenation at 8000 rpm for 15min. The volume ratio of warm microemulsion to cold water was 1:20. The empty and loaded SLNs dispersions were washed two times using an Amicon TCF2A ultrafiltration system. The same method was employed for the preparation of coumarin-6 loaded SLNs.

2.5. SLNs characterization

2.5.1. Particle size

Particles size was determined by dynamic light scattering at 25 °C by measuring the autocorrelation function at 90° scattering angle. Cells were filled with 100 µl of sample solution and diluted to 4 ml with filtered (0.22 µm) water. The polydispersity index (PI) indicating the measure of the nanoparticle population distribution was also determined. Six separate measurements were made to derive the average. Data were fitted by the method of inverse "Laplace transformation" and Contin [38].

2.5.2. Entrapment efficiency determination

The entrapment efficiency of SLNs was evaluated through a spectrophotometer UV–Vis. Briefly, the amount of not encapsulated drug in the SLNs was removed by centrifugation (at 8000 rpm for 30 min) and filtration. Subsequently the obtained samples were diluted in methanol (1:9) and analyzed. The absorbance of the samples was measured using quartz cells with a thickness of 1 cm and operating at specific wavelengths of cyclosporin-A (λ = 210 nm). The entrapment efficiency (EE) (%) is the percentage of active substance encapsulated in SLNs expressed referring to the initial drug amount used. This is calculated using the following equation:

$$EE\% = \frac{gf}{gi} \cdot 100 \quad (1)$$

where **gi** indicates the grams of Cyclosporin-A initially used and **gf** the final amount effectively entrapped into nanoparticles.

2.5.3. Differential scanning calorimetry (DSC) analysis

DSC of drug loaded SLNs, blank SLNs and oleate of trehalose was carried out. Measurements were performed at heating rate of 5 °C/min from 0 to 200 °C. Scans of each component were carried out under a flux of nitrogen. Each sample was analyzed at least in triplicate.

2.6. Skin permeation experiments

2.6.1. In vitro skin permeation studies

The skin permeation studies were performed ($n = 6$) using full thickness rabbit ears skin and cellulose acetate synthetic membranes for 24 h. Vertical Franz diffusion cells having a diffusional surface area of about 0,85 cm^2 were mounted on the assembly with temperature maintained between 33.5 and 36.9 °C to mimic physiological conditions. Receptor medium (6.0 ml) was filled with NaCl solution 0.9% containing 20% ethanol to increase the solubility of cyclosporin, under magnetic stirrer in order to maintain sink condition. After equilibration of the membranes (rabbit ears skin or dialysis membrane) SLNs suspension containing 0.005 g of drug was placed in the donor compartment and covered with a laboratory film (parafilm, Chicago) which prevented evaporation during the study. A solution of cyclosporin-A (1%, w/w) in olive oil was used as the control formulation as reported in many previous studies [39,40,41]. At specific time intervals, samples (1 ml) were withdrawn from receptor compartment and replaced with fresh receptor medium. Samples were analyzed by UV–Vis spectrophotometry for cumulative amount of drug permeated.

2.6.2. Quantification of drug in skin using tape stripping

At the end of the permeation experiment, the excess formulation was removed from skin surface. The skin was washed 3 times with phosphate buffer (pH 7.4) and dried. Then the stratum cornuem (SC) was separated from dermal tissues (epidermis and dermis; E + D) using serial tape-stripping involving the removal of 15 strips using adhesive tape (Scotch 845 Book Tape, 3M). Previous studies have demonstrated that 15 strips are sufficient to separate the SC from the epidermis and dermis [42,39]. The cyclosporin-A in the adhesive tapes was extracted by vortexing adhesive tapes with acetonitrile for 2 min and the acetonitrile solution was then filtered with 0.45 μm membrane and analyzed by UV–Vis spectrophotometry. The remaining epidermis and dermis were cut in small pieces, vortex-mixed for 5 min in 1 ml acetonitrile, and sonicated for 40 min. The solution was filtrated using 0.45 μm membranes, and analyzed by UV–Vis spectrophotometry [43].

2.6.3. Localization of nanoparticles in skin (CLSM study)

Confocal Laser Scanning Microscopy (CLSM) was carried out to see the depth of penetration of the SLNs [39]. To achieve this aim coumarin-6 dye loaded nanoparticles were prepared. Coumarin-6 (0.05% w/v) solution in propylene glycol was used as control. Propylene glycol was used to solubilize coumarin-6. The dye loaded SLNs were applied on the skin and kept for 24 h as in the permeation experiment. At the end of the experiment, the excess formulation was removed from the skin surface. The skin was washed 3 times with phosphate buffer (pH 7.4) and dried. Specimens were embedded in optimal cutting Temperature compound (Tissue-Tek, Sakura Finetek Europe, Alphen aan den Rijin, The Netherlands) and store at −80 °C. Cryostat-cut skin sections (16 μm thick) were mounted on slides and nuclei counterstained with Vectashield solution containing 1.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, CA, USA). Images were acquired using a confocal microscope (Leica TC-SP2 Confocal System, Leica Microsystem Srl, Milan, Italy).

2.7. Preparation of a dermatological formulation enriched with trealose-SLNs containing Cyclosporin-A

The cream consistis of purified water suspension of trehalose-SLNs containing cyclosporin-A, sweet almond oil, Sepigel™305 (polyacrylamide, isoparaffin C13-14, 7-laurate), purified water Ph.Eur, methyl paraben, propylparaben. The lipiodic phase was added to the aqueous ones to which previously the SLNs dispersion was put in. Finally, after addition of Sepigel™305 was added and the obtained biphasic system was mixed with Citounguetor (Triad Scientific, Inc. 6 Stockton Lake Boulevard Manasquan, NJ) for 2 min.

2.8. Statistical analysis

Data are expressed as mean (standard error of the mean of N replicates per experiment). Statistical analysis was carried out by Student's *t*-test using the GraphPad Prism 4 software program. $P < 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. Esterification of trehalose with oleic acid

The esterification reaction between trehalose and oleic acid was conducted in order to obtain a compound lipophilic enough to be used as a lipid matrix in the formulation of our SLNs carrying a potent immunosuppressant: Cyclosporin-A. This synthesis, conducted in dry dichloromethane at room temperature [44], has allowed the derivatization of the alcoholic function of trehalose to obtain a compound with a yield of 86% containing in its structure oleic acid (Scheme 1).

The product formation was confirmed by FT-IR, ^{1}H NMR and ^{13}C NMR spectroscopy. FT-IR (KBr) ν (cm-1): 3499, 3365, 3204, 2909, 2840, 1738, 1147.^{1}HNMR (CD_2Cl_2) δ (ppm): 0.87 (3H), 1.20–1.45 (20H), 1.5–2.0 (6H), 2.26 (2H), 3.17–3.2 (4H), 3.45–3.85 (6H), 4.45–4.50 (4H), 5.37 (2H). ^{13}C NMR (CD_2Cl_2) δ (ppm):14, 22.6, 25.3, 29, 29, 29.2, 29.8, 32, 34, 62, 63, 71.2, 74, 76, 76.5, 77, 78, 82, 102, 102.4, 130.5, 172.9.

In particular, the FT-IR spectrum of the ester (c) was compared with that of trehalose (b) and oleic acid (a) (Fig. 1).

This spectrum shows the presence of a new band at 1738 cm^{-1} attributable to the stretching vibration of the C=O of the ester (Fig. 1 curve c) and the presence at 3200 cm^{-1} and 3500 cm^{-1} of a vibration attributable to the stretching of the O—H of the trehalose glucosidic rings.

3.2. Preparation and characterization of SLNs

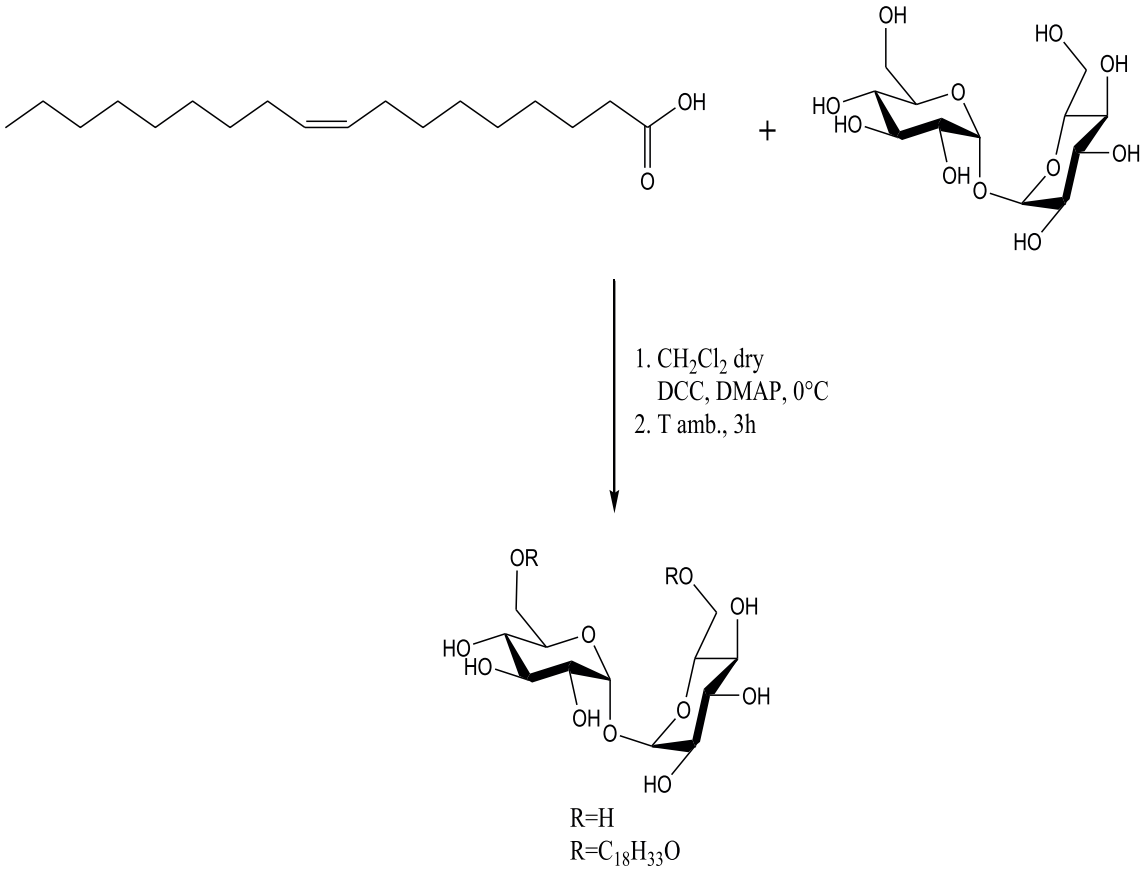
3.2.1. Entrapment efficiency and size analysis

The SLNs based on trehalose monoleate and loaded with Cyclosporin-A, were prepared successfully through the microemulsion technique with high encapsulation efficiency that was found to be 92%. This is a satisfying result possibly due to the lipophilic features of Cyclosporin-A, that well suit the lipophilic nature of our nanoparticles.

Light Scattering analysis allowed to determine the average diameter of the nanoparticles and their polydispersity index (PI) as shown in Table 2. These PI values are indicative of a good homogeneity in the distribution of particle size.

3.2.2. Differential scanning calorimetry (DSC) analysis

Fig. 2 shows the DSC thermograms of trehalose monooleate (curve a), empty (curve b) and drug loaded nanoparticles (curve c). Cyclosporin-A in the pure form exhibits a melting peak at 139 °C indicating the crystalline nature of the drug. Cyclosporin-A loaded into trehalose monooleate nanoparticles shows a slight transition peak confirming its presence also on the surface of the SLNs. However, as underlined in the next section, this amount is negligible ($1 \cdot 10^{-7}$ g vs. 0.043 g of



Scheme 1. Schematic representation of monooleate of trehalose synthetic route.

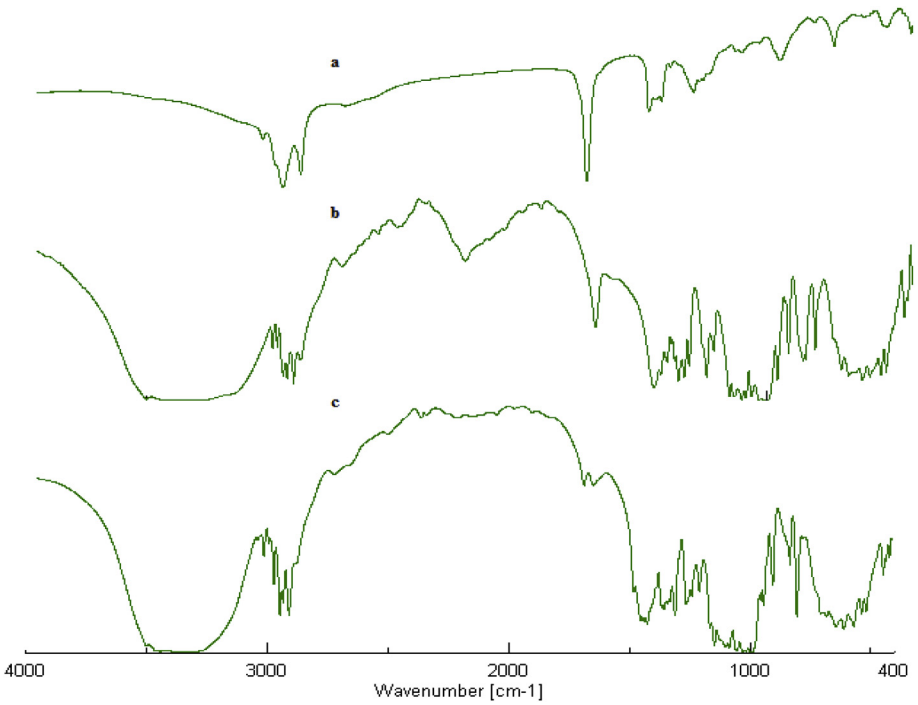


Fig. 1. FT-IR spectra of (a) oleic acid, (b) trehalose and (c) trehalose monooleate.

Table 2
Light scattering analysis.

Formulation	Mean particle size (nm)	Polydispersity Index (PI)
Empty SLNs	341.1 ± 3.2	0.291 ± 0.005
Loaded SLNs	378.4 ± 8.4	0.291 ± 0.014

Table 3
Amount of Cyclosporin-A permeated from the control and SLNs in the SC, Epidermis + Dermis and Receptor.

Formulation	SC (μg/cm^2)	E + D (μg/cm^2)	Receptor (μg/cm^2)
Control	30 ± 3.45	2.5 ± 1.7	0.9 ± 0.04
Cys A SLNs	150.89 ± 4.0	13 ± 2.6	0.1 ± 0.02

cyclosporin actually loaded into the nanoparticles).

3.3. Skin permeation experiments

3.3.1. In vitro permeation studies

The drug release from nanoparticles has been evaluated by means of the Franz Diffusion Cells, dialysis membranes or rabbit ear skin. Cyclosporin-A is a substance characterized by an absorbance range (195–215 nm) and this could cause interference by other skin components [45]. Indeed, in the skin there are lipids, proteins and other constituents that in some cases absorb at similar wavelength. The presence of these components in the Franz cell receptor compartment is due to the ethanol present in the solution (0.9% NaCl/ethanol 8:2) used to solubilize SLNs, which causes greater mobility of phospholipids [46]. The use of ethanol is dictated by the cyclosporin-A properties. In fact, this drug is practically insoluble in water whereas is soluble in 96% ethanol [47]. For this reason, it was chosen to use a 12000 Da cut-off dialysis membrane that allowed to read the adsorption of cyclosporin-A present in the receptor compartment without any kind of possible interference. In particular, the active substance is present in the receptor compartment in the order of 1×10^{-7} g (0.002% of the applied dose) after 30 min. This amount remains almost unchanged in the following time intervals (1 h, 2 h, 4 h, 6 h, 24 h). This excluded the possibility of using SLNs for transdermal drug delivery and therefore the use for systemic absorption. The results are consistent with previous studies reported in the literature describing how particular drug delivery systems (microparticles and nanoparticles) increase the drug's permanence in the skin without transdermal delivery [48,49,5]. This is desirable for topical treatment of skin disorders such as psoriasis as it reduces the possibility of side effects due to systemic absorption of cyclosporin-A and increases drug concentration at the disease level.

3.3.2. Tape stripping test

The tape stripping method was used to determine the drug content in skin layers: SC and dermal tissues (epidermis and dermis; E + D). Table 3 describes the drug content in receptor compartment, SC and dermal tissues (E + D) after *in vitro* skin permeation studies for 24 h. The amount of drug detected in SC and dermal tissues from SLNs release was higher as compared to control formulation (cyclosporin A solution in olive oil). SLNs significantly enhanced the penetration of drug in SC as well as in dermal tissues (E + D). Previous reports suggest the ability of nanoparticulate delivery systems to increase the skin permeation. Labouta et al. suggest that due to their particulate nature, permeation is dependent on the complex microstructure of the stratum corneum with its tortuous aqueous and lipidic channels [50]. Barry proposed several possible mechanisms. They speculate that the large surface area of nanoparticles play an important role in dermal penetration facilitating the contact of the encapsulated molecules with the stratum corneum. Moreover, drug loaded nanoparticles are considered as a super saturated system but in drug solution, the saturation limit is quite below, so the supersaturated state facilitates the partitioning of drug into the SC [51].

3.3.3. CLSM studies

Fluorescence microscopy imaging (CLSM) was used to visualize the distribution and penetration depth of our SLNs through the skin. It is clearly visible in Fig. 3 that the SLNs were found to be concentrated into the superficial layer of the skin as compared to control formulation.

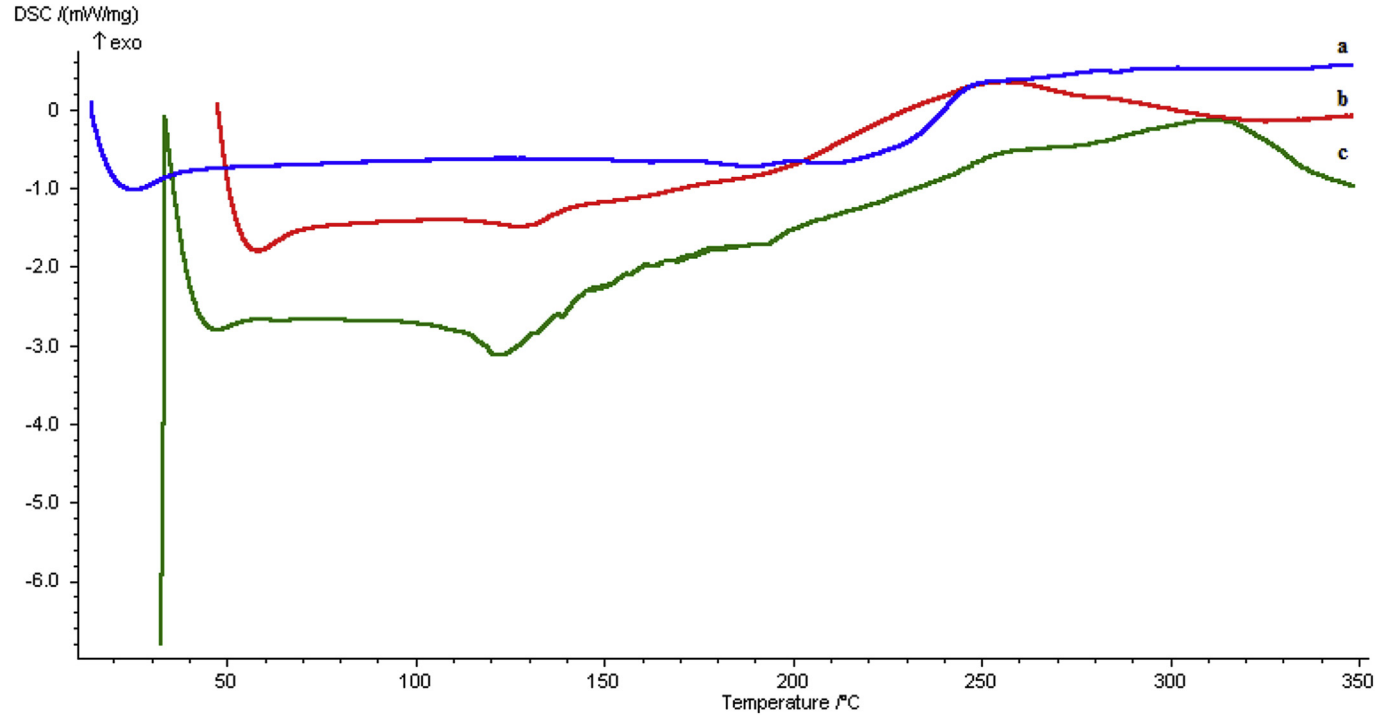


Fig. 2. DSC thermograms of trealose oleate (a), empty SLNs (b) and SLNs containing cyclosporin-A (c).

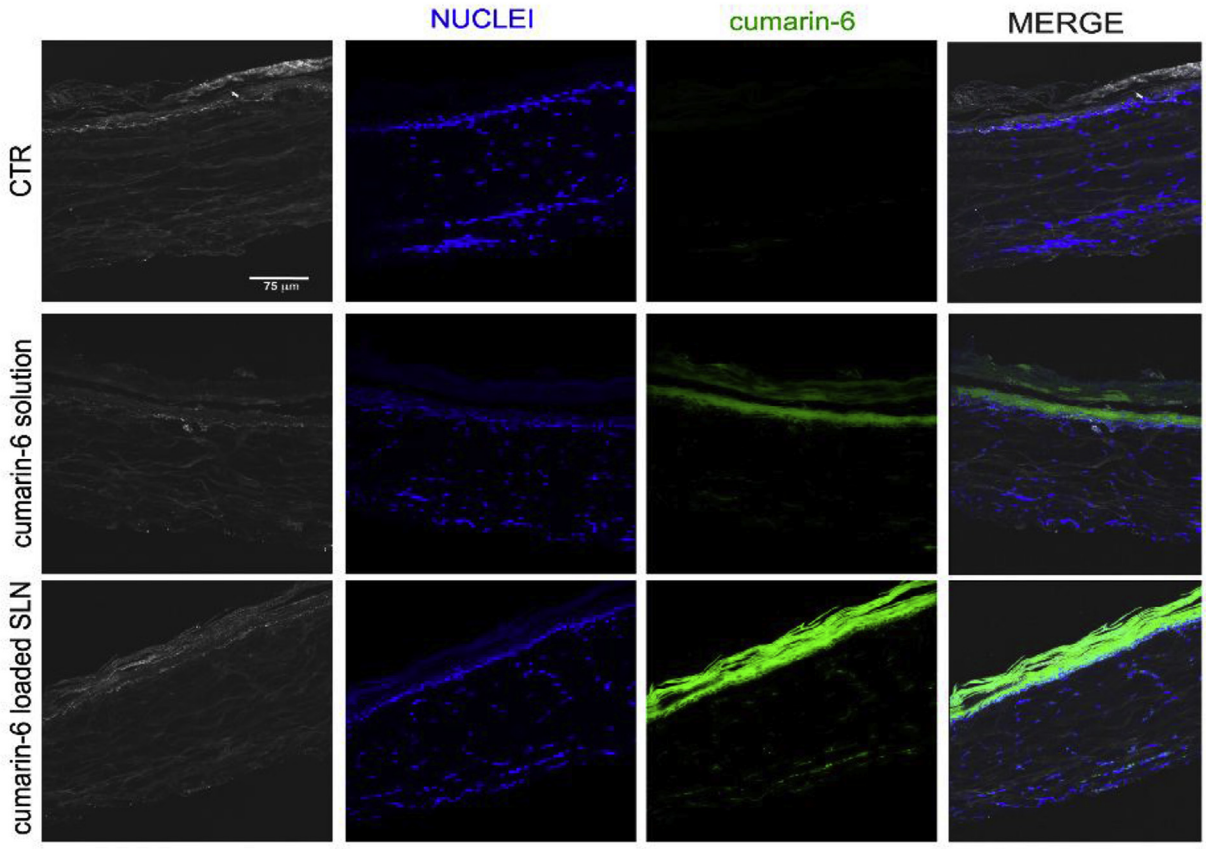


Fig. 3. Confocal imagine of trealose oleate SLNs (CTR), Cumarin-6 solution and SLNs containing cumarin-6.

This was a further confirmation of tape stripping test results. This results are in accordance with previous finding using other nanoparticulate drug carriers for the topical delivery [52,53]. It can be speculated that trehalose monooleate solid lipid nanoparticles, due to their dimensions, mainly followed transfollicular route for their penetration into the skin layers. However, although this study represents a step forward in the knowledge of skin absorption, the mechanism by which such particulate formulations facilitate skin transport remains ambiguous and more experiments are needed to understand the penetration mechanism.

3.4. Topical formulation enriched with trealose-SLNs containing Cyclosporin-A

The ideal formulation that unlike gels and lotions gives maximum permeation and maximum deposition of the skin for the topical formulation of active ingredients used in psoriasis is the cream that was prepared cold and without turboemulsifier to avoid the destruction of the SLNs [54]. The cream appeared white, soft and omogeneous and composed by ingredients that meet the requirements indicated by various European Pharmacopoeia.

The cream was submitted to the same skin permeation experiment performed for SLNs formulation (see 2.6.1 section) and also in this case Cyclosporin-A was not found in acceptor compartment of Franz cells.

This result confirms the potential topical effect of Cyclosporin-A on the skin.

4. Conclusions

The aim of this work was the preparation of trehalose monooleate SLNs for cyclosporin-A encapsulation and its topical release. The obtained results show that these lipid nanoparticles efficiently encapsulate the drug and exhibit suitable sizes for topical administration.

In vitro permeation studies showed that SLNs increase the amount of cyclosporin-A in SC and dermis over control. Furthermore, the localization of SLNs showed that dye loaded nanoparticles are concentrated into superficial skin layers.

Therefore, the obtained nanoparticles could be a potential system for the delivery, on the skin, of cyclosporin-A in patients affected by psoriasis limiting its systemic toxicity.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2018.12.026.

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