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Submicron polymeric particles prepared by vibrational spray-drying: semisolid formulation and skin penetration/permeation studies

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Abstract

Topical glucocorticoids (TG) such as dexamethasone (DEX) have been used for decades for the treatment of skin diseases. However, TG present well-documented side effects and their delivery to the skin is often insufficient. Therefore, many efforts have been undergone to improve the amount of drug delivered to the skin and to reduce side effects at the same time. In this work, the feasibility of DEX-submicron polymeric particles (SP) prepared by vibrational spray-drying as an approach to overcome the challenges associated with the topical administration of this drug class was evaluated. DEX was homogeneously dispersed in the SP matrix, according to confocal Raman microscopy analysis. Drug-loaded SP were incorporated into the oil phase of oil-in-water emulsions (creams). The formulation containing polymeric submicron particles (C-SP) showed controlled drug release kinetics and a significant drug accumulation in skin compared to formulations containing non-polymeric particles or free drug. DEX accumulation in the *stratum corneum* was evaluated by tape stripping and a depot effect over time was observed for C-SP, while the formulation containing the free drug showed a decrease over time. Similarly, C-SP presented higher drug retention in epidermis and dermis in skin penetration studies performed on pig skin in Franz diffusion cells, while drug permeation into the receptor compartment was negligible. It was demonstrated, for the first time, the advantageous application of submicron polymeric particles obtained by vibrational spray-drying in semisolid formulations for cutaneous administration to overcome challenges related to the therapy with TG such as DEX.

Keywords: *submicron particles, vibrational spray-drying, skin delivery, dexamethasone, skin penetration.*

Abbreviations:

SP, dexamethasone-submicron polymeric particles; SNP, dexamethasone-submicron non-polymeric particles; PCL, poly(ε-caprolactone); HPLC, high-performance liquid chromatography; LD, laser diffraction; PDI, polydispersity index; PCS, photon correlation spectroscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy; SC, *stratum corneum*; VE, viable epidermis; DER, dermis.

Introduction

Dexamethasone (DEX) is a synthetic glucocorticoid approved by the FDA in 1958 and classified as mild potency (class 4) or low potency (class 7) glucocorticoid according to the British National Formulary or American Classification, respectively [1]. The potency of glucocorticoids is evaluated by a skin vasoconstriction assay known as the blanching test [2]. DEX has been used in the treatment of several dermatological diseases such as psoriasis, atopic dermatitis and acne due to its anti-inflammatory, immunosuppressive, vasoconstrictive and anti-proliferative properties [1, 3].

However, topical administration of dexamethasone as well as other topical glucocorticoids (TG) can induce local and systemic side effects. The most common topical side effects are skin reactivity, atrophy, hypopigmentation and telangiectasia [4]. These problems can be aggravated when patients have to use TG for long-term treatment [5].

The main challenges in the cutaneous therapy with glucocorticoids are: to increase their topical bioavailability, to deliver them to the skin as target site – epidermis and/or dermis – and to reduce the dose and frequency of administration for better safety [6, 7]. Apart from the development of new molecules, one of the main strategies currently studied to overcome these challenges is the use of drug delivery systems. Liposomes [8], solid lipid nanoparticles [9], nanostructured lipid nanocarriers [7] and polymeric micro [10] and nanoparticles [11-14] have been investigated, as the type of carrier can have a strong impact on release kinetics and pharmacodynamics of an encapsulated drug.

Despite many published studies about nanoparticles containing DEX in the last decade [15-18], there are few reports about *in vitro* skin penetration/permeation after cutaneous administration of these formulations. Cevc, Blume & Schätzlein [19] reported highly deformable lipid carriers known as Transfersomes® containing DEX and others TG. They evaluated its biodistribution and pharmacokinetics after cutaneous administration in mice. Specially, administration at high doses led to a systemic absorption of drugs, similar to a subcutaneous injection. On the other hand, the same group described that Transfersomes® with DEX and hydrocortisone were able to deliver the drug to viable skin layers allowing the reduction of the required dose

to maintain its efficacy compared to conventional vehicles [20]. More recently, Chandra, Sharma & Irchhiaya [21] developed microemulsions prepared with several oils and lecithin as surfactant and isopropyl alcohol as co-surfactant for transdermal delivery of DEX. Formulation based on olive oil showed the best *in vitro* drug permeation, while the microemulsion based on nutmeg oil showed higher *in vivo* anti-inflammatory activity.

In dermatology, polymeric nanoparticles have been used as an interesting and modern approach to improve drug solubility and efficacy, allowing sustainable distribution within the skin besides reducing dose and frequency of administration [13]. However, there is a lack of studies showing the influence of nanoencapsulation of DEX in polymeric nanoparticles on its skin permeation/penetration profile. Marchiori and co-workers [12] prepared hydrogels containing DEX-loaded polymeric nanocapsules intended to treatment of proliferative disorders as psoriasis showing a controlled drug release compared to a hydrogel prepared with the non-encapsulated drug. However, the authors did not perform skin penetration/permeation studies.

Recently, the vibrational atomization spray-drying technology was introduced as an innovative technique for a one step submicron drug particles production. The setup comprises three subunits: (1) an atomization system composed of a piezoelectric actuator driven at an ultrasonic frequency (60 kHz) generating a fine aerosol of droplets with controlled size; (2) a glass dry tower with a laminar air flow promoting fast drying of droplets into dry particles using a gentle heating system; (3) an electrostatic particle collector consisting of a star electrode (cathode) and a cylindrical particle collecting electrode (anode) with an applied high voltage causing the electrostatic deposition of particles on the surface of the cylindrical electrode. In contrast to traditional spray-drying, in which very small particles (< 2 µm) are lost during the drying process, this new equipment allows collecting particles in the submicron scale [22, 23]. Vibrational atomization spray-drying has been studied to produce polymeric or non-polymeric particles with a range from 300 nm to 5 µm intended for several pharmaceutical applications, including cerebral [24], oral [25], ophthalmic [26] and pulmonary administration [27-29].

In 2013, Durli and co-workers reported the influence of surfactants and solvents on the preparation of non-polymeric submicron particles by vibrational

atomization technology using DEX [30]. The type of solvent showed influence on the viscosity of the primary organic solutions, while the nature of surfactant did not show any influence on this parameter. On the other hand, acetone showed the lowest superficial tension among the evaluated organic phases, which is important to facilitate the passage of organic solutions through the membrane and the good formation of the fine droplets during atomization. Regarding the type of surfactant, the addition of ionic surfactants showed higher increase in the process yield (> 60%) compared to the addition of non-ionic surfactants, which was explained by the improved electrostatic attraction of particles. The particles obtained in this study showed size around 1 μm. Moreover, the preparation of nanocrystals of DEX and fluorometholone from ethanolic solutions was studied by Baba & Nishida [26] using the Nano Spray Dryer intended to treatment of ophthalmic diseases. The mean particle sizes varied from 833 and 1344 nm depending on the mesh aperture of the atomization membrane.

In this context, the aim of our study was to evaluate the potential of submicron polymeric particles prepared by vibrational atomization spray-drying for local delivery of DEX to the skin. DEX was chosen as a model drug due to its wide use in treatment of skin diseases, its adverse effects and systemic absorption risks. Polymeric submicron particles containing DEX were produced, characterized and incorporated into semisolid creams (oil-in-water emulsions) to study the *in vitro* drug release as well as the drug skin penetration and permeation. To investigate the influence of the particles as well as of the polymer, the semisolid formulation with submicron polymeric particles was compared with semisolid formulations containing non-encapsulated drug or non-polymeric submicron particles, respectively.

Materials and methods

Materials

Dexamethasone (DEX) was kindly donated by Multilab Industry of Pharmaceutical Products Ltda (São Jerônimo, Brazil). Poly (ε-caprolactone) (PCL) (Mw = 80,000) and sodium deoxicholate were acquired from Sigma-Aldrich (São Paulo, Brazil). Caprylic/capric triglyceride and imidazolidinyl urea

were supplied from Delaware (Porto Alegre, Brazil). Polysorbate 80 and Salcare® SC 91 (BASF) were acquired from Brasquim (Porto Alegre, Brazil) and Henrifarma (São Paulo, Brazil), respectively. The dialysis membrane was obtained from Millipore (São Paulo, Brazil). HPLC grade acetonitrile was purchased from Tedia (São Paulo, Brazil). All chemicals and solvents were of analytical or pharmaceutical grade and were used as received.

Preparation of submicron drug particles

Submicron polymeric particles were prepared according to a protocol established in our research group [31]. An organic solution of acetone:water (20:1, v/v) containing 0.1% polycaprolactone (PCL), 0.1% DEX and 0.02% sodium deoxicholate was fed into the Nano Spray Dryer B-90® (Büchi, Switzerland) linked to an Inert Loop B-295 (Büchi, Switzerland) with a spray rate of 100% keeping the oxygen level below 4%. Inlet temperature and air flow were set to 55 °C and 110L/min, respectively. The Nano Spray Dryer was equipped with a spray mesh of 4.0 mm aperture size and the pump was run in mode number 2. DEX-submicron polymeric particles (SP) were collected from the electrostatic cylinder with a particle scraper as a dry powder. For comparison, a powder consisting of DEX-submicron non-polymeric particles (SNP) was prepared under the same conditions, omitting the PCL.

Physicochemical characterization of submicron particles

Yield, encapsulation rate and drug content

The process yield (expressed in percentage) was calculated as the ratio of the total weight of recovered powder in the collector and the total dry mass used in the organic solution, while the encapsulation rate was calculated based on the recovery of drug in the powder considering theoretical and experimental values.

The drug content (μg/mL) was assayed by high-performance liquid chromatography (HPLC) according to a previously validated method [32]. The chromatographic system consisted of a Discovery® C18 column (150mm x 4.6 mm, 5 μm, Supelco Analytical, Sigma-Aldrich, Brazil) and a Shimadzu LC-20A system (LC-20AT pump, SPD-M20A photodiode-array (PDA) detector, CBM-

20A system controller, SIL-20A auto-sampler (Tokyo, Japan). The mobile phase was a mixture of acetonitrile and water (45:55, v/v). The injected volume was 100 µL at an isocratic flow rate of 1.0 mL/min. For analysis, the powder was dispersed in acetonitrile, followed by a dilution in the mobile phase and subsequent filtration through a membrane (0.45 µm, Millipore®). To increase the sensitivity of the method, dexamethasone was detected at 240 nm instead of 254 nm [33]. The method was linear (r = 0.9999) in the range of 0.25 – 3 µg/mL.

Morphological and particle size analysis

Size and morphology of the submicron particles (shape and surface) were investigated by scanning electron microscopy (SEM) (JEOL JSM-6060, Japan) operating at 10 kV in different magnifications at Centro de Microscopia Eletrônica – UFRGS (Porto Alegre, Brazil). Samples were attached to aluminum stubs and sputter coated with gold. Particle size distribution and mean particle diameter were obtained by analyzing SEM images using the software Image J (version 1.44u, National Institutes of Health). Furthermore, the size distribution of particles in the powders was evaluated by laser diffraction (LD) using the dry powder dispersion unit of the Mastersizer 2000® (Malvern, UK). In this case, the refractive index of DEX (n = 1.5) was used to calculate the mean size [14].

Investigation of drug distribution

The DEX distribution within the powder bed of spray-dried particles was investigated by confocal Raman microscopy. Spectral data sets were acquired with a confocal Raman microscope WITec alpha 300R+ (WITec GmbH, Ulm, Germany) through a Zeiss objective (50x magnification, N.A. 0.8) at an integration time of 0.2 s per spectrum. The microscope was operated with a diode laser emitting an excitation wavelength of 785 nm. The laser power was set to 50 mW before the objective. Raman spectra were recorded every 0.5 µm in x and y direction. Due to the surface roughness of the powder bed a topography profile of the investigated area was acquired with a built-in sensor prior to confocal Raman microscopy analysis. Spectral Raman data sets were background corrected and converted into false color images using the software WITec Project Plus (WITec GmbH, Ulm, Germany).

Preparation of semisolid formulations

Emulsions, here denominated creams due to their consistency, were prepared with mortar and pestle. First, different proportions of medium-chained triglycerides (MCT) and the emulsifier system Salcare® SC 91 were tested to promote adequate dispersion of the submicron particles (data not shown). Salcare® SC 91 is a mixture of sodium acrylates copolymer, mineral oil and PPG-1 trideceth-6. It was chosen due to its ability to form emulsions using a cold process. Prior to preparation of these formulations, a polymer swelling test with a polymeric film (PCL) fully immersed in the pure emulsifier was performed in order to check if the emulsifier system dissolves PCL and consequently disrupt the original structure of the polymeric particles. This test was carried out over 28 days at 25 ± 2 ºC. Each day the polymer film was removed, thoroughly cleaned and weighed. For the preparation of drug-loaded creams, the submicron particles were slowly dispersed in the oil phase. A previously prepared aqueous phase composed of water, citric acid and imidazolidinyl urea (preservative) was slowly added to the oil phase and homogenized using a pestle. According to this procedure creams containing non-encapsulated DEX (free drug) (C-D), creams containing DEX-polymeric submicron particles (C-SP), creams containing DEX-submicron particles (non-polymeric particles) (C-SNP), and blank creams (C-B) were prepared (Table 1). Three independent batches of each formulation were prepared and analyzed.

Please, insert Table 1 about here.

Physicochemical characterization of semisolid formulations

Determination of DEX

DEX content in the semisolid formulations was assayed by HPLC. Approximately 1.0 g of each formulation was placed in a 25 mL volumetric flask. Acetonitrile was added and the flask was subjected to 2 minutes of vortex stirring, 30 minutes of ultrasonic bath and 1 minute of vortex stirring. The resulting dispersion was centrifuged at 2,300 X g for 15 minutes, followed by a dilution with mobile phase, filtration through a 0.45 µm membrane filter (Millipore®, Billeria – MA, USA) and subsequent injection into the HPLC, according to the parameters described previously. The method was linear ($y = 237701x – 2881.1$; $r = 0.9999$) in the range of 0.25-3.0 µg/mL, precise (relative standard deviation of 2.3% for repeatability and 2.8% for intermediate precision) and accurate (103.3%). Specificity was checked and proved, as excipients of the formulations did not alter the DEX assay.

Determination of pH values

The pH values were measured in the dispersions of creams in distilled water (10%, w/v) using a calibrated potentiometer (B474, Micronal, Brazil).

Morphological and particle size distribution analysis

The morphological analysis of semisolid formulations was done by transmission electron microscopy (TEM) (JEM – 1200, ExII, operating at 80 kV, Centro de Microscopia Eletrônica – UFRGS, Porto Alegre, Brazil). Before analysis, appropriate dilutions were prepared in ultrapure water and an aliquot was deposited on carbon support films (grid) and negatively stained with an uranyl acetate solution (2% w/v).

Particle size and particle distribution of creams were analyzed by laser diffraction (LD, Mastersizer 2000®, Malvern, UK) and by photon correlation spectroscopy (PCS) (Zetasizer Nanoseries, Malvern Instruments, Worcestershire, UK). The mean diameter (by volume) was measured ($D_{4.3}$) during LD analysis. For PCS analysis, the *z*-average diameter was measured after the dilution of an aliquot of cream in purified water (2,000x) followed by shaking and filtering through 1.2 mm membrane (Millipore Millex-HP, Billeria MA, USA).

Evaluation of the rheological behavior

Analysis of the rheological properties of formulations was carried out at 25 ± 1°C using a rotational viscosimeter (LVDV II + Pro model, Brookfield, Middleboro, MA USA) and a spindle SC4-25 with a small sample adaptor. A shear stress (τ) ramp was run up and down from 0.40 to 2.00 rpm, registering 18 points.

Rheograms were obtained by plotting the shear stress (τ) as a function of the shear rate (γ). Resulting data were analyzed with the Rheocalc software (v3. 1-1 version, Brookfield Middleboro, MA, USA). The different flow models Bingham (ideal plastic, $\tau = \tau o + \eta\gamma$), Casson (plastic, $\tau^{0,5} = \tau o^{0.5} + \eta^{0.5}\gamma^{0.5}$), Ostwald (pseudoplastic, $\tau = K\gamma^{n}$) and Herschel-Bulkley (yield-pseudoplastic, $\tau = \tau^{o} + K\gamma^{n}$) were used to evaluate the rheograms, where η represents the viscosity (Pa.s), *K* the consistence (Pa.sn), γ_o the yield shear stress (Pa), and *n* is the power law index [34].

In vitro drug release assay

Vertical automated Franz diffusion cells (MicroettePlus Multi-Group®, Hanson Research Corporation, Chatsworth, CA, USA) were used to study the *in vitro* release of DEX from creams at 37 ± 0.5°C carried out in triplicate for each formulation. The diffusion area was 1.766 cm^2 and the receptor chamber volume was 7.0 mL. A dialysis membrane (12 kDa, Sigma-Aldrich), pre-hydrated for 8 h, was fixed between donor and receptor compartment. The receptor medium composed of phosphate buffer (pH 7.4) and polysorbate 80 (0.02%) was constantly stirred (400 rpm) to ensure sink conditions. Approximately 300 mg of formulation was applied on top of the membrane in the donor compartment. This amount corresponds to an infinite dose able to avoid drug depletion from donor compartment and also suitable to determine the steady state flux values. Half of a milliliter of the receptor medium was taken at predetermined time intervals (2, 4, 8, 12 and 16 h) and replaced by an equal volume of fresh medium. Released DEX was determined by HPLC according to the previously described method. However the receptor medium was used for dilution here instead of the mobile phase. Furthermore, the injection volume was set to 50 µL. The method was linear (y = 113995x + 2527; r = 0.9999) in the range of 1.0-20.0 µg/mL, precise (relative standard deviation of 1.2% for repeatability and 2.8% for intermediate precision) and specific. For specificity, the components of the medium were tested and none of them did alter the DEX assay. The Higuchi model was used to evaluate the drug release profiles. The software MicroMath® Scientist® (St. Louis, MO, USA) for Windows™ was used to perform this mathematical modeling.

In vitro skin penetration and permeation assay

Full-thickness skin samples were obtained from the abdominal area of female pigs kindly donated from a local slaughterhouse (Araldi, Nova Roma do Sul, Brazil). Hair from the skin surface was cut and adipose tissue was carefully removed. Pieces of skin were cut into circles (3.0 cm of diameter) and their thickness was measured with a dial thickness gage. Only skin slices with a thickness between 1.8 and 2.2 mm were used. Skin were stored in a freezer (-20°C) wrapped in aluminum foil. The study was carried out using vertical Franz diffusion cells and receptor medium composed of phosphate buffer and

polysorbate 80 (0.02 %), under the same conditions described for the *in vitro* drug release studies. Skin slices were mounted in diffusion cells with the dermal side in contact with the receptor medium. Two independent experiments (n = 3) were performed for each formulation, resulting in a total of six replicates (n = 6).

To analyze the kinetics of skin penetration and permeation of DEX from C-D and C-SP samples were applied for 2, 4, 8, 10 and 12 h on the skin surface. In order to evaluate the influence of the polymer, the same experiment was performed for the formulation containing the non-polymeric submicron particles (C-SNP) after 2 and 12 h of sample application. Approximately 300 mg (infinite dose) of each formulation was applied on the diffusion area (1.766 cm^2) of skin surface.

Stratum corneum (SC), viable epidermis (VE), dermis (DER) and receptor compartment (RC) were analyzed. At the end of each experiment, the total amount of DEX retained in each skin layer was determined by HPLC after appropriated extraction. Formulation excess on the skin surface was removed with cotton. SC was removed by *tape stripping* using 18 tapes (3M tape). After removing the SC, epidermis and dermis were separated by placing the skin in hot water (60 °C) for 45 seconds. DEX was extracted from each layer in the mobile phase (8 mL for SC and 4 mL for the VE and DER) followed by vortex mixing (2 min), sonication (40 min), vortex mixing (1 min) and centrifugation (15 min at 2,300 X g).

DEX was analyzed by HPLC under the same conditions described in the *"In vitro* drug release assay" section above. The method was linear in the range of 0.02 – 20 µg/mL (r = 0.9999). The mean percentage recovery from full skin extraction for the three concentration levels of DEX (0.10, 1.0 and 10.0 µg/mL) were 95.2, 89.1 and 90.5%, respectively, while relative standard deviations (RSD) were 4.82, 1.3 and 0.98%. The resulting mean skin recovery (91.1 ± 3.2 %) is close or higher than observed by other studies. Paturi and co-workers [35] obtained a recovery of 67.5 ± 4% of DEX from hairless rat skin while Li and co-workers [36] found a recovery range from 89.9 to 96.9 % in mouse skin. Moreover, our method was precise (RSD of 2.53% for repeatability and 1.58% for intermediate precision) an accurate (98.43 ± 3.41 %).

Statistical analysis

Results are expressed as mean value ± standard deviation (SD) of a triplicate. Data were evaluated by one-way analysis of variance (ANOVA) for significance at $p \leq 0.05$. The Tukey´s test was used to compare more than two experimental groups. For the *in vitro* penetration/permeation studies, the results were analyzed in sextuplicate and data were assessed by one-way ANOVA (followed by Tukey post-tests) as well considering significance at $p \leq 0.05$. All analyses were performed using the GraphPad Prism® software version 5.00.

Results

All batches of DEX submicron polymeric or non-polymeric particles (SP or SNP, respectively) were characterized for drug content, encapsulation rate, mean size and process yield. Results are shown in Table 2.

Please, insert Table 2 about here.

Regarding the morphological analysis by SEM, submicron particles have a spherical shape, a rough surface and form agglomerates, regardless of the presence of the polymer (Figure 1). Polymeric DEX submicron particles showed a mean size of 0.975 µm with a particle size distribution ranged between 0.446 and 2.252 µm, while SNP had a mean size of 0.523 µm. The size was calculated with the support of the software Image J for more than 300 particles counted in SEM images with a 5,000 x magnification (Figure 1). Figure 2 shows the particle size distribution of the powders obtained from SEM images.

Please, insert figure 1A e 1B about here.

Please, insert figure 2 about here.

To analyze the DEX distribution in the spray-dried particle powder bed, confocal Raman microscopy was performed for SP and SNP. Figure 3A depicts the single Raman spectra of the three components DEX, PCL and deoxycholate contained in the spray-dried particles. As the rough surface of the particle powder hinders accurate spectra acquisition with the confocal microscope, the surface was virtually corrected by recording topography profiles of the respective sample areas prior to Raman analysis [37]. Subsequently, the

topography profile could be overlaid with the false color Raman image for a combined three dimensional visualization. Figure 3B shows the overlay image of the submicron non-polymeric dexamethasone particle (SNP) bed. The small panels on the right hand side depict the Raman images of the two individual substances DEX (red) and deoxycholate (green). Figure 3C shows the three dimensional image of the investigated submicron polymeric particle (SP) bed with the respective two dimensional Raman images for the three individual substances DEX, deoxycholate and PCL in the small panels.

Please, insert figure 3 about here.

To incorporate the produced submicron particles in the oil phase of an emulsion, the emulsifier system was firstly checked for possible negative effects on the structure of PCL. Figure 4 presents the data of the polymer swelling test, where a thin film of PCL was immersed in the emulsifier system and weighted before immersion and after 1, 7, 21, and 28 days. No significant variation in the polymer mass over the whole period was observed.

Please, insert figure 4 about here.

After preparation of semisolid emulsions (creams) their organoleptic and physicochemical characteristics were investigated. Results are summarized in Table 3.

Please, insert Table 3 about here.

Creams were also analyzed regarding the mean particle size, polydispersity index and granulometric profile by LD and PCS after aqueous redispersion. The data are shown in Table 4 and Figure 5. In addition, Figure 6 shows the TEM photomicrographs of formulations C-D and C-SP by SEM.

Please, insert Table 4 about here.

Please, insert figure 5 about here.

Please, insert figure 6 about here.

The rheological data obtained for creams are presented in Figure 7. The rheograms were compiled by plotting shear stress (Pa x 10) against shear rate (s^{-1}) and they were used to model the non-Newtonian flow profiles.

Please, insert figure 7 about here.

The regression coefficients (r^2) for the different flow models of each rheogram are presented in Table 5. The Casson flow model fitted best to the data and was chosen to describe the rheological behavior ($r^2 > 0.99$). The parameters plastic viscosity and yield stress for each cream according to this model are presented in Table 6.

Please, insert Table 5 about here.

Please, insert Table 6 about here.

The *in vitro* drug release profiles of DEX from all formulations using the vertical Franz diffusion cell is depicted in Figure 8. The amount of DEX released after 16 h was 61.5 ± 3.4 $\mu g.cm^{-2}$ for the cream containing the free drug (C-D), 52.2 ± 0.95 $\mu g.cm^{-2}$ for the cream containing the submicron particles without polymer (C-SNP) and 43.1 ± 4.6 $\mu g.cm^{-2}$ for the cream containing polymeric submicron particles (C-SP). The released DEX after 16 h was significantly different among all formulations (ANOVA, $p \leq 0.05$).

Please, insert figure 8 about here.

The DEX flux was calculated from the slope of the linear portion of the curve by plotting the amount of DEX released per cm^2 against the square root of time [38, 39]. Results are presented in Figure 9. Creams containing submicron spray-dried particles showed a slower release rate (C-SP: 11.2 ± 2.2 $\mu g.cm^2.h^{-1/2}$ and C-SNP: 14.5 ± 0.8 $\mu g.cm^2.h^{-1/2}$) compared to the cream containing the non-encapsulated (free) drug (C-D: 19.8 ± 0.9 $\mu g.cm^2.h^{-1/2}$) (ANOVA, $p \leq 0.05$).

Please, insert figure 9 about here.

Furthermore, the drug release data were analyzed the Higuchi square root model (Table 7). C-SP presented the lowest *k* value according to the Higuchi model (11.75 ± 0.24 h^{-1}), followed by C-D (16.56 ± 0.23 h^{-1}) and C-SNP (20.92 ± 0.65 h^{-1}).

Please, insert Table 7 about here.

In order to determine the drug localization and to quantify the amount of DEX delivered to each skin layer after the applying of creams containing the submicron particles or non-encapsulated drug, *in vitro* skin retention and permeation studies using pig skin as membrane were performed in Franz diffusion cells. DEX was analyzed in the *stratum corneum* (SC) and the subjacent layers, viable epidermis (VE) and dermis (DE), according to the tape stripping and skin extraction techniques, respectively. In addition, the receptor compartment was analyzed to determine the amount of permeated DEX.

Figure 10 shows the amount of DEX accumulated in the SC after topical application in a penetration kinetic study. After 2 h there was no difference in the amount of DEX retained in SC among all formulations (C-D: 0.90 ± 0.25 $\mu g.cm^{-2}$; C-SNP 0.89 ± 0.42 $\mu g.cm^{-2}$; C-SP: 0.42 ± 0.08 $\mu g.cm^{-2}$) (ANOVA, Tukey´s test, $p \leq 0.05$). On the other hand, the amount of DEX in the SC was significantly higher after 12 h for C-SP (1.25 ± 0.55 $\mu g.cm^{-2}$) compared to C-D (0.41 ± 0.09 $\mu g.cm^{-2}$). The cream prepared with non-polymeric submicron particles (C-SNP) showed 1.09 ± 0.37 $\mu g.cm^{-2}$ of DEX in the stratum corneum after this time, which was not significantly different from the formulation C-SP (ANOVA, Tukey´s test, $p \leq 0.05$). In addition, analyzing the drug accumulation profile over time, a decrease in the amount of DEX penetrated from the formulation containing the non-encapsulated drug (C-D) was observed after some hours. On the other hand, there was an increase in the amount of DEX accumulated in the SC over time for the cream containing the polymeric submicron particles (C-SP).

Please, insert Figure 10 about here.

In the VE layer the amount of DEX after 2 h was not significantly different among the formulations (C-D: 0.175 ± 0.08 $\mu g.cm^{-2}$; C-SNP: 0.135 ± 0.03 $\mu g.cm^{-2}$; C-SP: 0.152 ± 0.06 $\mu g.cm^{-2}$). For C-D, after 2 and 4 h there was a slight increase of the amount of penetrated DEX, followed by a decrease after 10 and 12 h (Figure 11). However, a significant increase of DEX penetration over the 12 h of the study was observed after the application of the formulation C-SP. After 12 h, the amount of drug retained in the VE was higher (almost twice) for C-SP (0.54 ± 0.07 $\mu g.cm^{-2}$) than for C-D (0.265 ± 0.04 $\mu g.cm^{-2}$) (ANOVA, Tukey´s test, $p \leq 0.05$). C-SNP showed 0.19 ± 0.07 $\mu g.cm^{-2}$ of dexamethasone penetrated to

the viable epidermis, which was lower compared to C-SP (ANOVA, Tukey´s test, $p \leq 0.05$).

Please, insert Figure 11 about here.

Regarding the dermis layer, when both formulations were compared after 2 h, the amount of DEX was similar between them (C-D: 0.40 ± 0.29 and C-SP: 0.41 ± 0.18 µg.cm^{-2}) (ANOVA, Tukey´s test, $p > 0.05$). DEX amount penetrated to the dermis layer from the C-SNP was 0.55 ± 0.23, which was similar to the values obtained for the C-D and C-SP formulations. However, there was a gradual increase in the amount of penetrated DEX for C-D and C-SP over time (Figure 12). So, the amount of penetrated DEX after 12 h was higher for C-SP (2.41 ± 0.33 µg.cm^{-2}) compared to C-D (1.80 ± 0.57 µg.cm^{-2}) (ANOVA, Tukey´s test, $p \leq 0.05$). On the other hand, there was no difference between the DEX penetrated into the dermis after 12 h between C-D and C-SNP (1.35 ± 0.23 µg.cm^{-2}) (ANOVA, Tukey´s test, $p > 0.05$).

Please, insert Figure 12 about here.

The influence of the creams containing free dexamethasone (C-D) or creams containing the submicron polymeric particles (C-SP) on DEX permeation through pig skin after topical application was also assessed *in vitro* using Franz diffusion cells. The amount of DEX was quantified in the receptor compartment of the Franz diffusion cell. Figure 13 shows that formulations containing spray-dried submicron polymeric particles promote a DEX permeation only after 8 h of application. Nevertheless, there was no statistically significant difference to the formulation containing free dexamethasone after 12 h (C-D: 0.47 ± 0.14 µg.cm^{-2}; C-SP: 0.45 ± 0.22 µg.cm^{-2}) (ANOVA, Tukey´s test, $p > 0.05$). In addition, C-SNP showed a permeation of 0.48 ± 0.15 µg.cm^{-2} to the receptor compartment after 12 h, which was also not different from the formulations C-D and C-SP). (ANOVA, Tukey´s test, $p > 0.05$).

Please, insert Figure 13 about here.

In addition, the mean flux (J) of permeated drug was calculated from the slope of the linear portion of the curve plotting the cumulative amount of permeated DEX versus time for the formulations C-D and C-SP. As expected

from the permeation profiles, there was not any difference between the flux for C-D (0.081 ± 0.01 $\mu g.cm^{-2}.h^{-1}$) and C-SP (0.075 ± 0.04 $\mu g.cm^{-2}.h^{-1}$).

Discussion

Our objective in this work was to develop a suitable semisolid formulation (cream) containing a new carrier for topical application in order to control drug release and to deliver the drug to specific skin layers. DEX, a classic glucocorticoid used to treat skin diseases, was chosen as model drug. Thus, polymeric submicron particles containing DEX were prepared by vibrational spray-drying according to a protocol previously optimized by our research group [31]. In order to evaluate the influence of the polymer on the performance of the semisolid formulation, non-polymeric particles (SNP) were also prepared and incorporated in the creams. Polymeric submicron particles (SP) had a mean size of 0.975 ± 0.29 µm while the non-polymeric ones showed a mean size of 0.523 ± 0.18 µm according to SEM image analysis. However, the analysis by laser diffraction demonstrated a mean particle diameter ($D_{3,0}$) of 3.73 ± 0.66 µm for SP by laser diffraction analysis, where 90 % of these polymeric particles had a size lower than 1.51 ± 0.05 µm, while 50 % had a size lower than 0.75 ± 0.05 µm. These slightly different results can be attributed to the different principles of the techniques. The software image J allows determining the mean size considering each individual particle (primary particles) from an image. In this work SEM images with a 5,000 fold magnification were used and more than 300 particles were counted. This method has been employed for size analysis of powders, including those obtained by vibrational spray-drying [24, 28, 40, 41]. On the other hand, laser diffraction analysis is based on light scattering of several particles, leading to the measurement of single particles as well as particle agglomerates, which can increase the final mean size.

Regarding the DEX encapsulation rate, both submicron particles showed values higher than 90%. The production of the powders prepared with PCL was done at higher process yield (> 80%), with a very low loss of powder. This result confirms another notable advantage of vibrational spray-drying over the conventional spray-drying approach (two-fluid nozzle or rotatory atomizer), besides the feasibility of working with very low amount of samples. In the

present work, batches were produced to obtain 100 mg of powder/batch. When powders of SNP were produced, the yield decreased by 50 % probably due to the production of low density particles leading to their losing through the air stream and during the collect.

To evaluate the feasibility of using confocal Raman microscopy for the analysis of DEX distribution within the spray-dried powder, single Raman spectra were recorded for the three incorporated substances DEX, PCL and deoxycholate. For each substance one outstanding peak was identified in the single Raman spectra (Figure 3A). Thus, the components can be discriminated in the spectral data set, making confocal Raman microscopy a suitable technique to investigate drug distribution. For both SP and SNP powders each substance was detected in each acquired spectrum of the spectral data set. Therefore, the small image panels in Figure 3B and 3C show uniform colors each representing one compound. Overlaying these individual Raman images results in a false color image in the mixed color of the individual panels. Thus, the three dimensional image combination of topography and Raman results for the SNP powder is colored orange (red + green), whereas the overlay for SP powder is of pink color (red + green + blue). In fact, we could show that DEX is homogenously distributed within the particle powder bed in both cases. Furthermore, the use of PCL for particle preparation does not influence the drug distribution, which is a valuable information for subsequent release studies from these carrier systems.

After preparation of the carriers in the submicron scale, their incorporation in an adequate vehicle intended for topical use was an essential step. To assure the intact and homogeneous distribution of the particles in the topical skin formulation, a method for their dispersion in a semisolid vehicle was developed. In this study, we proposed the use of emulsions as semisolid formulation to incorporate the developed drug delivery system for dermatological administration. The main semisolid vehicles reported for incorporation of nanoparticles such as solid lipid nanoparticles, nanospheres and nanocapsules for dermatological formulations are hydrogels, since they enable easy incorporation of these colloids structures and, theoretically, less risk of stability problems [42]. So, to the best of our knowledge, there is little information about the incorporation of nanocarriers into emulsions. Creams are

water in oil (w/o) or oil in water (o/w) emulsions and are one of the most used semisolid formulations for dermatological and cosmetic purposes due to their versatility to disperse or dissolve both hydrophilic and lipophilic components and drugs [43]. Moreover, creams are well accepted by patients because they are soft, easily spreadable and have aesthetic properties. Furthermore, they are the most appropriate vehicles to treat acute and subacute dermatitis [44].

The classic method to disperse two immiscible phases is emulsification under very hard heating (70 – 85 °C for each phase), which may cause chemical degradation of the drug and PCL. Here, an emulsifier system to produce o/w emulsions using a cold preparation process was chosen to avoid the exposition of drug and polymer to high temperatures. The components of the oil phase were carefully selected to prepare the creams. Powders and the free (non-encapsulated) drug were successfully dispersed into the oil phase. The risk of dissolving the PCL by the emulsifier and thus altering the properties of the spray-dried particles was investigated using a polymer swelling test. This method was firstly proposed by Guterres and co-workers [45]. According to the polymer swelling experiment, the emulsifier system used to prepare the creams in our work did not dissolve the PCL film within 28 days, suggesting the physical stability of submicron particles after their incorporation in the creams. In addition, it has been previously described that the mixture of caprylic/capric acid triglycerides does not dissolve the PCL [45]. MCT, the main lipid component used in the oily phase of emulsion associated to oil mineral from the emulsifier system facilitated the very easily dispersion of DEX, a lipophilic drug. In addition, it has been reported that MCT is considered as a non skin penetrating oil without influence as penetration enhancer [46], whereas non-ionic surfactants (as PPG1-trideceth-6), may have a small effect in the ability to enhance the skin drug penetration [47].

Therefore, we incorporate the produced submicron polymeric particles in creams, which could be also named emulgel because the emulsifier system is composed of oil, a surfactant and a polymer. Emulgels have recently been considered an emergent technology for topical drug delivery preparations. This term is used for emulsions containing the dispersant phase gelled by a polymer with improved rheology and release properties [48, 49].

Regarding the organoleptic characteristics, all semisolid formulations had a white color as well as a homogeneous appearance being aesthetically acceptable for potential patients. The formulations had a pH value close to neutral which could be explained by the anionic character of the emulsifier system. The decrease in the pH values observed for C-D, C-SNP and C-SP compared to C-B could be attributed to the presence of DEX.

Laser diffraction analysis showed that the semisolid formulations had very similar granulometric profiles. It was not possible to detect only the size profile concerning the submicron particles in the creams. This means that it is difficult to determine the particle size of the nanocarrier in these emulsions, as their oil droplets are sized in the same scale. These results corroborate with results from Jenning and co-workers [50] who described the same limitation to characterize o/w emulsions containing solid lipid nanoparticles due to the coexistence of nanoparticles and droplets in the same nanoscopic range. Nevertheless, it was possible to observe that C-D showed a bimodal profile while C-SP and C-SNP presented a unimodal profile. Furthermore, the particle mean size of C-SP showed that the incorporation of the powder in the oil phase promoted an adequate dispersion, since the raw material (powder) had a higher mean particle size (> 3.7 μm) than the cream (> 0.61 μm) when analyzed by the same technique. PCS analysis has been used to verify the presence of intact nanoparticles after aqueous redispersion of carbomer hydrogels [51]. In our study, the mean size determined by PCS after the aqueous redispersion of creams were lower than those obtained by LD due to the use of a membrane filter with a 1.2 μm pore size, allowing only the passage of small particles. In other words, similar mean particle sizes were observed for all formulations (C-D, C-SP and C-SNP) due to the overlapping of particle size distribution of droplets and particles. Only the blank formulation (C-B) had a different and higher mean size (> 320 nm). In addition, morphological analyses were performed by TEM for C-B and C-SP (Figure 6). In both samples it was observed droplets of very small size.

Rheology analysis is a very important tool to characterize semisolid formulations. Rheological properties of creams as well as other vehicles may influence skin application, the manufacturing process, flow packaging [52], the stability of the formulation [53], controlled drug release [54] as well as skin

permeation/penetration [55]. In our study, all formulations showed non-Newtonian behavior as their viscosities changed as a function of shear rate (data not shown). Mathematical modeling was used to better understanding of the rheological behavior of creams using the shear rate-shear stress data. The Casson model showed the best fit, since the regression coefficients were higher than 0.99. According to the Casson model a semisolid formulation does not flow immediately when a shear stress is applied to it, therefore, presenting a plastic behavior [56]. Plastic as well as pseudplastic flows are described in the literature to be ideal for formulations intended for topical application [34]. The addition of submicron particles and free drug (raw-material) decreased the viscosity of formulations in comparison to the blank cream (C-B). However, according to the rheograms (Figure 6), the flow type was not modified for any formulation. This is in accordance with the results reported by Alves and co-workers [57] showing that the addition of nanocapsules, nanospheres or nanoemulsions does not change the flow properties of hydrogels. In addition, C-D and C-SP lower values necessary for formulations to start flowing (lower yield stress) compared to C-SNP, which suggests its easier skin application.

After the physicochemical and rheological evaluation, the *in vitro* DEX release from all creams was investigated. Analysis of drug release from a topical formulation is important to ensure drug diffusion from the vehicle, affecting its availability to the skin surface. Moreover, the other objective at this point was to evaluate if the submicron particles produced by vibrational spray-drying could promote a controlled drug release from creams. So, according to the *in vitro* drug release profiles, C-D presented the highest amount of released DEX, as expected, since in this formulation the drug was incorporated as raw material (non-encapsulated form). In contrast, creams containing polymeric submicron particles (C-SP) exhibited a controlled DEX release profile during 16 h, which amount of drug released was lower than that obtained for C-SNP. This result shows the positive influence of the presence of the polymer in the submicron particles to control the drug release from the semisolid formulations. Additionally, drugfluxes were in the following order C-D>C-SNP>C-SP confirming the influence of the polymer on the drug released control (Figure 10).

After investigating the *in vitro* controlled DEX release, skin penetration/permeation studies were carried out. At this time, it is important to

keep in mind that one of the most important skin functions is its barrier property against transepidermal water loss and environmental penetration of microorganisms and chemicals [58]. However this is also a limiting factor to transport drugs intended for either topical (local) or transdermal (systemic) therapies [59]. The epidermis, representing the uppermost layer, is responsible for this property due its sophisticated process of differentiation and architecture. It is divided into s*tratum corneum* (SC) and viable epidermis (VE).

SC is the horny layer and consists of highly keratinized dead cells (corneocytes) embedded in a lipid matrix [60]. It is known as a brick and mortar organization represented by the corneocytes and intercellular lipids, respectively. Tight junctions and desmosomes are also responsible for cohesion and intercellular adhesion [61]. Due to its complex composition, SC is the rate limiting barrier to percutaneous absorption of drugs [62]. For topical treatment of dermatological diseases with glucocorticoids (TG), it is necessary to overcome this barrier in order to reach their sites of action, the viable epidermis and/or dermis. The mechanism of action of TG includes their binding in human receptor found in skin keratinocytes and fibroblasts [3, 63]. Some strategies used to increase the skin delivery of topical glucocorticoids include new vehicles such as metered dose aerosol sprays with hydrofluoroalkane [64] and foams [65], chemical enhancers [66], iontophoresis [35] as well as micro and nanoparticles [11, 20, 51, 67, 68].

To prove our initial hypothesis to increase DEX delivery to viable skin, DEX must firstly be released from submicron particles and from the semisolid formulation, as discussed above, followed by its penetration and diffusion through the SC to the viable epidermis and dermis, being available to bind to glucocorticoid receptors [1, 69]. So, the distribution of DEX-associated to submicron particles into skin layers after the application of creams was investigated using Franz diffusion cells and pig skin as biological membrane. Pig skin has great similarity with human skin due to its anatomical and physiological characteristics. It is considered the best choice among animal membranes for *in vitro* skin permeation/penetration studies [70, 71].

Regarding the results from this experiment, the formulation containing free drug (C-D) presented a significant decrease of DEX penetration in the SC as a function of time. The time was the determining factor in the reduction of the

drug amount, when comparing the different time points after application (ANOVA, $p \leq 0.05$). However, the cream containing the submicron polymeric particles (C-SP) led to a significant increase of drug retained in the SC throughout time, especially after 10 h of application (ANOVA, $p \leq 0.05$). Although C-SP retained higher amount of drug in the SC, there was no significant difference compared to the C-SNP. Anyway, these results demonstrated a very pronounced depot effect when the vehicle with submicron particles was applied on the skin.

After stripping the SC off, the underlying epidermis was separated from dermis by heat method. After 12 h, C-SP delivered a higher DEX amount (approximately 50% higher) to the VE compared to the amount delivered by the C-D. These results indicate that the drug depot in the SC previously discussed provides a local skin reservoir to the VE, the main target site of TG treatment. Moreover, C-SP showed higher DEX amounts retained in the VE after 12 h compared to C-SNP, indicating that the presence of the polymer in the submicron particles was significant to increase the localization of drug into the VE. Other authors aimed to increase the accumulation of topical corticoids in the epidermis using nanoparticles. In 2004, Cevc and co-workers [20] compared the skin distribution of DEX-loaded very deformable vesicles and a commercial cream containing the free drug. The nanoencapsulation increased the amount of DEX retained in the VE (from 15 % to 30 % in relation to cream containing its free form). Şenyiğit and co-workers [72] also found significant higher amounts of drug in the epidermis and dermis for clobetasol-17-propionate encapsulated in lecithin/chitosan nanoparticles compared to a chitosan gel and a commercial cream containing the free drug at the same concentration. However, our study is the first report about the increased delivery of dexamethasone to VE using polymeric submicron particles.

A similar DEX accumulation profile in the dermis occurred as observed in the VE. Time had a significant influence in the amount of drug reaching the dermis after 10 h, regardless of the formulation. In addition, after 10 and 12 h the drug showed a higher penetration into the dermis from the formulation containing the polymeric submicron particles compared to the formulation containing the free drug.

Although the penetration of the spray-dried polymeric submicron particles in the skin layers was not investigated in the present study, we could suggest that the higher DEX penetration to the viable skin layers as well as the lack of difference on the receptor compartment from the formulation containing the submicron polymeric particles could be a result of their penetration into the hair follicles. There are several reports showing that solid micro and nanoparticles can penetrate and accumulate into hair follicles [73 - 76]. Patzelt and co-workers [77] prepared PLGA and silica particles ranged from 122 to 1000 nm and evaluated their penetration in pig ear skin. They observed that particles with medium size penetrated deeper in the hair follicles.

Finally, the receptor compartment (RC) was analyzed for DEX content to evaluate the risk of its systemic absorption after topical application. Up to 4 h DEX was not detected in the RC, regardless of the formulation. Only after 8 h it was possible to quantify DEX in this medium. After 12 h of the experiment, the permeated amount of dexamethasone was similar and very low for all formulations. Additionally, the calculated flux values to the receptor medium were also not different between the formulations C-D and C-SP. The low content of the drug found in the RC indicates that the formulations have low risk of systemic absorption and besides the targeting to the viable epidermis and dermis, the developed formulation containing submicron polymeric particles does not increase the risk of systemic absorption compared to those containing the free drug and submicron non-polymeric particles.

Conclusions

The present work demonstrates for the first time the production of semisolid formulations containing polymeric submicron spray-dried particles produced by vibrational atomization as potential topical skin drug delivery systems. An o/w emulsion was prepared using the emulgel technology which allows a very good dispersion of the particles. Adequate rheological properties of the formulations for application and patient compliance were accomplished. The presence of PCL in the particles was the main factor controlling drug release from the formulation. The developed semisolid formulation containing the submicron polymeric particles exhibited a depot effect in the SC and improved the drug accumulation in the viable skin layers (epidermis and dermis)

without increasing the risk of systemic absorption. The recent and the modern technology to produce submicron polymeric particles by vibrational spray-drying combined with the novel approach to develop a pharmaceutical vehicle with appropriate physicochemical characteristics is proposed in this study as a promising new strategy to target DEX in viable skin layers with a potential to reduce its dose.

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Figure Captions:

Figure 1. SEM images of submicron particles. (A) polymeric particles (SP) and (B) submicron non-polymeric particles (SNP). Image acquisition with a 5,000x magnification.

Figure 2. Particle size distribution of submicron particles (SP and SNP) calculated from SEM images.

Figure 3. Drug distribution by confocal Raman microscopy. **A)** Single Raman spectra of individual components DEX (red), deoxycholate (green) and PCL (blue). Outstanding peaks for compound identification are highlighted. **B and C)** Overlay of topography image with false color Raman image for SNP (B) and SP (C) powders. Colors from three dimensional images are a result of the color combination from Raman images depicting the distribution of individual compounds within the particle powder. Scale bars are sized to 10 µm.

Figure 4. Polymeric film weight (mg) after immersion in emulsifier system over 28 days at 25 °C.

Figure 5. Granulometric profiles of creams C-B, C-SP, C-SNP and C-D determined by laser diffraction.

Figure 6. TEM photomicrographs of creams containing free drug (A) and polymeric submicron particles (B) (size bar corresponds to 100 nm).

Figure 7. Rheological profiles of creams. Shear stress (Pa x 10) is plotted against shear rate (s^{-1}) (n = 3).

Figure 8. *In vitro* DEX release profile from the semisolid formulations using vertical Franz diffusion cells (n = 3).

Figure 9. Flux ($\mu g.cm^2.h^{-1/2}$) of DEX from creams calculated from *in vitro* drug release experiments (n = 3). Means with the same letter are not statistically different (ANOVA, Tukey test, * $p \leq 0.05$ e *** $p \leq 0.01$).

Figure 10. Amount of DEX penetrated in the *stratum corneum* (SC) after 2, 4, 8, 10 and 12 h. The values are expressed as mean ± standard deviation (n = 6). (ANOVA, Tukey´s test, * $p \leq 0.05$).

Figure 11. Amount of DEX penetrated in the viable epidermis (VE) after 2, 4, 8, 10 and 12 h. The values reported are expressed as mean ± standard deviation (n = 6). (ANOVA, Tukey´s test, * $p \leq 0.05$).

Figure 12. Percutaneous penetration of DEX into the dermis (DE) after 2, 4, 8, 10 and 12 h. The values reported are expressed as mean ± standard deviation (n = 6). * $p \leq 0.05$ e ** $p \leq 0.01$.

Figure 13. Mean cumulative amount of DEX permeated from semisolid formulations (creams) versus time. The values reported are expressed as mean ± standard deviation (n = 6).