International Journal of Pharmaceutics 524 (2017) 1–8

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Nile red nanosuspensions as investigative model to study the follicular targeting of drug nanocrystals

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ARTICLE INFO

Article history:
Received 13 January 2017
Received in revised form 17 March 2017
Accepted 19 March 2017
Available online 21 March 2017

Keywords:
Nanocrystals
Nanosuspensions
Follicular targeting
Dermal delivery
Nile red

ABSTRACT

The strategy of formulating poorly soluble actives as nanosuspension has been explored by more than a thousand research papers, with some medicinal products for oral and intravenous use having reached the market or advanced clinical trials. Interestingly, there is a limited number of reports of nanosuspensions formulated for dermal and transdermal drug delivery. In the present work, a nanocrystals suspension of the fluorescent, water-insoluble dye Nile Red, is prepared through a media milling technique and exploited to characterize the fate of the nanosuspended drug when applied on the skin. More in detail, the accumulation of Nile Red nanocrystals inside the hair follicles is evidenced by scanning electron microscopy, and the diffusion of drug molecules in the different skin layers is evaluated by confocal microscopy and skin permeation studies. Overall, the combination of the analytical techniques provide a description of the mechanisms underlying dermal accumulation, and transdermal penetration of a drug formulated as a nanosuspension.

1. Introduction

Nanocrystals can be defined as nanoparticles of pure drug without any matrix material with an average diameter below 1 μm (typically in the range of 200–500 nm). The drug nanocrystals can be suspended in an outer liquid phase, usually composed of water and/or water-miscible solvents, and stabilized using an ionic or non-ionic surfactant or polymers, to obtain a nanosuspension. Nanosuspensions can be prepared using two different approaches: the bottom up and the top down techniques or a combination of two (Kocbek et al., 2006; Lai et al., 2015). The reduction of the drug crystal mean diameter below 1 μm dramatically increases the particle surface area and decreases the diffusion layer thickness compared to coarse and micronized drug, as described by the Prandtl equation, thus speeding up the dissolution rate (Mosharraf and Nyström, 1995). In addition, nanocrystals are characterized by an enhanced saturation solubility, according to the Freundlich–Ostwald equation (Müller and Peters, 1998).

Despite different commercial nanosuspension products have become available in the last 10 years, with most of them for oral administration, until now, the skin permeation and accumulation of drugs formulated as nanosuspension has not been intensively studied. However, the few published results (Ghosh and Michniak-Kohn, 2013; Lai et al., 2015, 2013; Pireddu et al., 2016, 2015) clearly indicate that this technology could be very effective in improving dermal bioavailability of actives with poor water solubility.

Indeed, the increased saturation solubility and dissolution rate of the nanocrystals determines an increased concentration gradient between the formulation and the skin, thus enhancing the penetration of the lipophilic nanosuspended drug. Moreover, the ability of nanocrystals to accumulate in the hair follicles, from which the drug can diffuse into the surrounding cells, has been proposed as a further mechanism of the increased dermal penetration (Romero et al., 2014; Zhai et al., 2014). The real functionality of the trans-follicular route has been doubted for a long time because this tiny but important skin accessory shows a low density (about 0.1%) on skin surface (Prow et al., 2011). Furthermore, some authors have supposed that sweat and sebum excretion can negatively affect the opposite movement of a penetrating agent (Lademann et al., 2007). However, recent studies have clearly shown the crucial role played by hair follicles in the trans-dermal absorption of molecules and particles with an average size below follicular openings (about 10–210 μm) and

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http://dx.doi.org/10.1016/j.ijpharm.2017.03.042
0378-5173/

able to avoid interaction with sweat or sebum (Jung et al., 2006; Patzelt et al., 2011; Prow et al., 2011). The localization in the hair follicles has been demonstrated for polymeric nanoparticles covalently labeled with a fluorescent dye (Lademann et al., 2007, 1999; Patzelt et al., 2011) and fluorescent liposomes (Jung et al., 2006). In particular, it has been demonstrated that the application of a massage induces a much deeper penetration of nanoparticles into the hair follicles, while the free dye diffusion only slightly improves with the same treatment (Lademann et al., 2007). In this sense, it seems that hair movement acts as a pumping mechanism pushing the nanocarriers into the hair follicle. Moreover, fluorescent nanoparticles with mean diameter around 600 nm have shown to penetrate deeper into the porcine hair follicles than the smaller or larger ones, thus suggesting that a precise control of nanoparticle size in the preparative process is needed to achieve optimal follicular deposition (Patzelt et al., 2011).

Once penetrated into the hair follicle, substances can cross the follicular epithelium, interact with living tissue, and be taken up by systemic circulation. Conversely, nanoparticles are too large to cross the follicular epithelium, thus remaining entrapped into the hair follicle and acting as a reservoir for the topically applied formulation (Jung et al., 2006; Patzelt et al., 2011).

Owing the nanoparticulate nature of nanocrystals, the ability of nanosuspension to accumulate into the hair follicles has been postulated but, until now, has not been demonstrated.

For these reasons, the aim of the present investigation was to visualize nanosuspension deposition on and within the skin after *in vitro* topical administration. In particular, the ability of nanosuspension to accumulate into the hair follicles was studied. For this purpose, nanocrystals of Nile Red (NR) were prepared using Polysorbate 80 or Poloxamer 188 as stabilizer. NR is an uncharged, completely water insoluble, photostable molecule that strongly fluoresces bright red in hydrophobic environments. NR is currently used as low detection limit dye to stain liposomes and other lipid carriers, allowing their localization on the treated tissue by fluorescence microscopy (Ogiso et al., 2001; Pischon et al., 2008; Teeranachaideekul et al., 2008).

In the present work, a NR nanosuspension was prepared by a top down – media milling method using yttrium stabilized zirconium beads as milling media (Pireddu et al., 2015). The formulation was characterized by photon correlation spectroscopy for mean size and size distribution. Scanning electron microscopy was used for morphological studies and to evaluate nanocrystal accumulation in hair follicles, while confocal microscopy studies were performed to check NR distribution in the skin layers. Moreover, quantification of the NR accumulation in the skin was evaluated by *in vitro* skin penetration and permeation studies using vertical Franz cells and newborn pig skin.

2. Materials and methods

2.1. Materials

Nile Red was obtained from Sigma Aldrich (Italy). Polysorbate 80 (Tween 80) and Poloxamer 188 (Lutrol F68) were purchased from Galeno (Italy) and BASF (Germany) respectively. All the other products were of analytical grade.

2.2. Methods

2.2.1. Nanosuspension preparation

Nanosuspensions were prepared by a Top down–media milling process. In a first step a fixed amount of NR (0.05% w/w) was dispersed in the acqueous stabilizer solution and homogenized using an Ultra Turrax T25 basic for 1 min at 8000 rpm. Two types of stabilizers, Polysorbate 80 or Poloxamer 188, were used at different concentrations: 0.01%, 0.025 and 0.05% w/w (Table 1). The obtained coarse suspensions were divided in 1.5 ml conical microtubes containing about 0.4 g of 0.1–0.2 mm yttrium stabilized zirconium beads (Silibeads ®Typ ZY, Sigmund Lindner, Germany) and shaked at 3000 rpm for five cycles of ten minutes each one using a beads-milling cell disruptor equipment (Disruptor Genie, Scientific Industries, USA). In the last step the obtained nanosuspensions were gathered, separated from beads by a sieve and stored at room temperature.

2.2.2. Nanosuspension characterization

The average diameter and polydispersity index (P.I.) of the samples were determined by Photon Correlation Spectroscopy (PCS) using a Zetasizer nano-ZS (Malvern Instrument, UK). Samples were backscattered by a helium–neon laser (633 nm) at an angle of 173° and a constant temperature of 25 °C. Zeta potential was estimated using the Zetasizer nano-ZS by means of the M3-PALS (Phase Analysis Light Scattering) technique. All the samples were analyzed 24 h after preparation. A medium-term stability study of NR nanosuspension stored at 4 ± 1 °C was performed by monitoring average size, polydispersity, and surface charge for 30 days.

2.2.3. Scanning electron microscopy

NR nanosuspension and NR coarse suspension were placed on aluminum stub and dried under vacuum for 12 h and their morphology was analyzed by a Zeiss ESEM EVO LS 10 (Germany) environmental scanning electron microscope (SEM), operating at 20 KV in high vacuum modality with secondary electron detector. For the visualization of nanocrystals in hair follicles, the nanosuspension was deposed on an one-day-old Goland–Pietrain hybrid pig (<1.2 kg) skin specimen and massaged into the tissue for 3 min. The water was left to evaporate at room temperature. The skin specimens were then cut with a surgical scalpel nearly orthogonally to the surface, dried in an Edwards freeze tissue dryer, Model EPD3 (England), for 48 h, and mounted onto aluminum stubs with conductive silver paint and coated with gold in an Edwards S150A Sputter Coater unit (England). Skin specimens were then analyzed by SEM in the same conditions described above.

2.2.4. Confocal laser scanner microscopy

The extent of penetration and distribution of fluorescent molecules through one-day-old Goland–Pietrain hybrid pigs skin were investigated using Franz diffusion vertical cells.

Nanospension was applied on the skin specimens and the experiment was run for 2, 8 or 24 h. At the given times, the skin surface was washed with water, gently rubbing with cotton to

Table 1
PCS mean diameter, polydispersity index (PI) and zeta potential (ZP) of Nile Red nanocrystal formulations.

Formulation	mean diameter (nm)	PI	ZP
NR bulk	5225 ± 1700	–	–
NANOS T1 (Polysorbate 80 0.01%)	597.6 ± 3.7	0.228	-15.6 ± 2.6
NANOS T2 (Polysorbate 80 0.025%)	229.1 ± 1.2	0.187	-18.2 ± 1.3
NANOS (Polysorbate 80 0.05%)	921.5 ± 7.4	0.344	-18.4 ± 1.9
NANOS P1 (Poloxamer 188 0.01%)	888.4 ± 8.2	0.327	-16.5 ± 2.1
NANOS P2 (Poloxamer 188 0.025%)	741.7 ± 3.3	0.439	-15.5 ± 1.4
NANOS P5 (Ploxamer 188 0.05%)	1276 ± 254	0.465	-15.8 ± 1.3