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Methotrexate loaded lipid nanoparticles for topical management of skin-related diseases: Design, characterization and skin permeation potential

Mara Ferreira, Elisabete Silva, Luísa Barreiros, Marcela A. Segundo, Sofia A. Costa Lima*, Salette Reis

UCIBIO-REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

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ABSTRACT

Methotrexate exhibits poor cutaneous bioavailability when administered topically using conventional vehicles. It is recommended for the treatment of skin-related diseases but its systemic side effects hamper application. The aim of this work was to formulate methotrexate in nanostructured lipid carriers using biocompatible lipids, and to investigate their potential for topical drug delivery. Methotrexate-loaded nanostructured lipid carriers were prepared via a hot ultrasonication method. Nanocarriers were evaluated for size, polydispersity, surface potential and entrapment efficiency. Shape and surface morphology of the produced lipid carriers confirmed their spherical shape. After 10 h ca. 50% of methotrexate was released *in vitro* from the lipid nanocarriers following Peppas–Korsmeyer release kinetics. Moreover, methotrexate-loaded nanocarriers were stable at least for 3 months and less toxic towards fibroblasts and human keratinocytes than the free drug. Methotrexate within lipid nanocarriers was able to pass the experimental keratinocyte epidermal barrier at a flux rate of 2 $\mu g\,cm^{-2}\,h^{-1}$. Results demonstrated that lipid nanocarriers constitute a suitable approach for application of methotrexate in skin-related diseases topical therapy.

1. Introduction

Skin-related diseases affect millions of people every day. Any skin pathology caused by infectious pathogens, inflammatory conditions or even cancer is also a matter of cosmetic concern. As the systemic treatment for dermatological problems comes with potential adverse effects, topical application is the preferred mode due to higher patient compliance and satisfaction. Major challenges in the therapy of these diseases encompass poor drug efficacy, low skin penetration, toxicity related effects and patient compliance. Skin disease patients still wait for safer and more efficient cutaneous delivery therapies.

Human skin functions as a topical barrier, mostly due to the stratum corneum layer of the epidermis, and also provides a unique delivery pathway for therapeutic and other active agents. Topical delivery route has several advantages when compared with other commonly used administration pathways (enteral and parenteral), including: (i) higher patient compliance and medical professionals alike; (ii) smaller amounts of drugs are required to produce a therapeutic effect; (iii) drugs are delivered directly to the diseased site; (iv) reduced frequency of dosing and drug peak in the plasma is avoided (Zhang et al., 2013). The stratum corneum is the major challenge to deliver drugs through the skin due to its architecture with keratin-rich corneocytes surrounded by the mortar of the intercellular lipid lamellae (Elias, 2012). A common strategy to enhance drug permeation across the skin is to disrupt and weaken these highly organized intercellular lipids (Tiwary et al., 2007).

Abbreviations: A, area of exposed skin; CEMUP, Materials Centre of the University of Porto; DLS, dynamic light scattering; DMEM, Dulbecco's modified eagle's medium; DMSO, dimethyl sulfoxide; EE, entrapment efficiency; ELS, electrophoretic light scattering; HC, high calcium; HPLC, high performance liquid chromatography; J, flux; LC, low calcium; MTX, methotrexate; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NLC, nanostructured lipid carriers; PDI, polydispersity index; PBS, phosphate buffer saline; Q, quantity of MTX; SD, standard deviation; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TEER, transepithelial electrical resistance; t, time; TJ, tight junctions.

* Corresponding author.
E-mail address: slima@ff.up.pt (S.A. Costa Lima).

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For dermatologic applications, nanocarriers show relevant properties to improve penetration across the stratum corneum, such as electrical conductivity, hardness, increased active surface area and chemical reactivity (Abramovits et al., 2010). An impressive financial investment growth and exponential patents registration of nanocarriers for dermatologic applications have been observed, confirming nano-dermatology as one emerging area of research (Saraceno et al., 2013). Nanocarriers will allow the development of improved and novel targeted treatment approaches, with an added-value to already commercialized therapeutic agents.

Nanostructured lipid carriers (NLCs) are an improved generation of lipid nanoparticles, composed of solid lipid matrix incorporated with liquid lipids, which result in a less organized crystalline structure and therefore better loading capacity. The nanosized NLCs confer a large surface area in close contact with the stratum corneum and an increased skin hydration effect is described due to the occlusive properties of lipid nanoparticles, thus improving skin drug penetration (Abdullah et al., 2011). Indeed, some nano-dermatological applications of NLCs for skin-related diseases can be found in literature (Agrawal et al., 2010; Cirri et al., 2012; Lin et al., 2010; Pardeike et al., 2011; Stecová et al., 2007).

Methotrexate (MTX) is classified as an antimetabolite drug. It is one of the most effective therapeutic agents in treating diseases associated with abnormally rapid cell growth and exhibits anti-inflammatory and immunosuppressant activities. MTX has been used in clinics for the treatment of different tumors (*e.g.* osteosarcoma, skin and breast cancer) and, autoimmune and inflammatory diseases as psoriasis and rheumatoid arthritis (Shinde et al., 2014). Yet, its clinical use is limited due to severe toxic side effects, short halftime in the blood and cellular efflux (Kay and Westhovens 2009; Montaudié et al., 2011). The aim of this work was to design topical MTX loaded NLCs for management of skin-related diseases, such as atopic dermatitis, psoriasis and skin cancer, thus avoiding systemic toxicity.

2. Materials and methods

2.1. Materials

Cetyl palmitate was kindly provided by Gatefossé (Nanterre, France), Miglyol® 812 was purchased from Acofarma (Madrid, Spain) and polysorbate 80 was obtained from Merck (Darmstadt, Germany). All chemicals and solvents were of analytical grade acquired from Sigma-Aldrich (St Louis, MO, USA) unless stated otherwise. Methotrexate was a kind gift from Excella (Feucht, Germany). Aqueous solutions were prepared with double-deionized water (Arium Pro, Sartorius AG, Göttingen, Germany), which possesses conductivity values lower than 0.1 $\mu S\,cm^{-1}$. Fetal bovine serum, penicillin-streptomycin antibiotics mixture and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Gibco® (Invitrogen Corporation, UK). L929 mouse fibroblast cell line was obtained from ATCC-LGC Standards (Barcelona, Spain) and HaCaT keratinocyte cell line was obtained from CLS (Eppelheim, Germany).

2.2. Preparation and characterization of drug-loaded NLCs

MTX-loaded NLCs (MTX-NLCs) were prepared by hot ultrasonication method. Briefly, the oil phase consisting of approximately 150 mg cetyl palmitate, 45 mg Miglyol® 812 and 18 mg MTX was heated at 60 °C. Aqueous phase containing 4.7 mL polysorbate 80 in 7 mL of water was heated at the same temperature in a water bath and then added to the oil phase. Next, the coarse emulsion was sonicated using a probe sonicator (VCX130, Sonics & Materials, 115 Newtown, CT, USA) with amplitude frequency of 70% during 10 min, in order to obtain a nanoemulsion. Unloaded NLCs were prepared in a similar way, without the drug. The formulations were left to cool down and stored at room temperature.

The particle size, polydispersity index (PDI) and zeta potential of the formulations were assessed using a ZetaPALS zeta potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA). Particle size and PDI were evaluated by dynamic light scattering (DLS) and zeta potential was determined by electrophoretic light scattering (ELS). All samples were diluted (1:400) with double deionized water and analyses were carried out at 25 °C with a fixed light incidence angle of 90 °C. For each sample, the corresponding mean and standard deviation values were obtained from six runs of ten cycles and calculated by multimodal analysis. The determination of MTX entrapment efficiency followed a previous report (Pinto et al., 2014), using spectrophotometry. The morphological examination (surface structure and shape) of MTX-NLCs was performed using transmission electron microscopy (TEM Jeol JEM-1400, Tokyo, Japan) for which a drop of diluted sample was placed on the surface of carbon coated copper grid and stained with uranyl acetate for 30 s, at the accelerating voltage of 60 kV. They were also examined by Cryo-scanning electron microscopy (SEM) at experimental facilities of the Materials Centre of the University of Porto, Portugal (CEMUP). A suitable amount of formulation was dropped on a support and rapidly cooled by plunging into sub-cooled nitrogen and transferred under vacuum to the cold stage of the preparation chamber. Cryo-factures were then performed using an ALTO 2500 (Gatan Alto 2500 (Pleasanton, CA, USA)), with subsequent sublimation for 180 s at −90 °C and coating with Au/Pd by sputtering for 35 s. Then, the samples were transferred to the Cryo-SEM chamber and observed at a temperature of −150 °C using a JSM 6301F microscope (JEOL, Tokyo, Japan).

2.3. Stability studies

Stability studies of unloaded and drug-loaded NLCs were carried out by storing the dispersions after production in closed glass vials at room temperature and monitoring for physical-chemical stability by the following parameters: size, polydispersity, zeta potential and drug content.

2.4. In vitro *drug release assays*

The *in vitro* MTX release from MTX-NLCs was assessed by a dialysis bag diffusion technique as previously described (Ferreira et al., 2015). Briefly, a defined amount of NLCs containing 0.25 mg of MTX was poured into dialysis bags (molecular weight cut off 6000–8000 Da, CelluSep1 T2; Membrane Filtration Products Inc., Frilabo, Portugal) with the two ends fixed by thread and placed into the preheated dissolution media. A receptor compartment containing 80 mL of phosphate buffer saline (PBS) pH 7.4 or acetate buffer pH 5.5 was stirred at 350 rpm using a heating and magnetic stirring plate (IKAMAG1, Staufen, Germany) in adequate temperature (37 or 32 °C). The sink conditions were maintained, as the maximum solubility of MTX in aqueous mediums is of <0.1 g/100 mL, and the concentration of MTX in the receptor medium was always at least 20 times lower than this value. Aliquots of 1 mL were withdrawn and the MTX content determined spectrophotometrically (Jasco V-660 spectrophotometer, USA). These conditions intend to mimic three different environments: physiological (pH 7.4, 37 ± 0.5 °C), inflammatory (pH 5.5, 37 ± 0.5 °C) and topical (pH 5.5, 32 ± 0.5 °C). Mathematical models for evaluation of drug release kinetics (zero order, first order, Higuchi, Peppas-Korsmeyer, and Hixson-Crowell) were fitted to the experimental data and the