Acta Biomaterialia 53 (2017) 439–449

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

# Cellular interactions of a lipid-based nanocarrier model with human keratinocytes: Unravelling transport mechanisms

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ARTICLE INFO

*Article history:*
Received 6 October 2016
Received in revised form 5 January 2017
Accepted 21 January 2017
Available online 22 January 2017

*Keywords:*
Endocytosis
Transcytosis
Exocytosis
Biocompatibility
Cellular uptake
Transdermal drug delivery

ABSTRACT

Knowledge of delivery system transport through epidermal cell monolayer is vital to improve skin permeation and bioavailability. Recently, nanostructured lipid carriers (NLCs) have gained great attention for transdermal delivery due to their biocompatibility, high drug payload, occlusive properties and skin hydration effect. However, the nanocarriers transport related mechanisms in epidermal epithelial cells are not yet understood.

In this research, the internalization and transport pathways of the NLCs across the epidermal epithelial cell monolayer (HaCaT cells) were investigated. The 250 nm sized witepsol/miglyol NLCs, prepared by hot homogenization had reduced cytotoxicity and no effect on the integrity of cell membrane in human HaCaT keratinocytes. The internalization was time-, concentration- and energy-dependent, and the uptake of NLCs was a vesicle-mediated process by macropinocytosis and clathrin-mediated pathways. 3% of NLCs were found at the apical membrane side of the HaCaT monolayer through exocytosis mechanism. Additionally, the endoplasmic reticulum, Golgi apparatus and microtubules played crucial roles in the transport of NLCs out of HaCaT cells. NLCs were transported intact across the human keratinocytes monolayer, without disturbing the tight junction's structure. From the transcytosis data only approximately 12% of the internalized NLCs were passed from the apical to the basolateral side. The transcytosis of NLCs throughout the HaCaT cell monolayer towards the basolateral membrane side requires the involvement of the endoplasmic reticulum, Golgi apparatus and microtubules. Our findings may contribute to a systematic understanding of NLCs transport across epidermal epithelial cell monolayers and their optimization for clinical transdermal application.

**Statement of Significance**

Transdermal drug delivery is a challenging and growing area of clinical application. Lipid nanoparticles such as nanostructured lipid carriers (NLCs) have gained wide interest for transdermal drug delivery. However these nanocarriers' interactions with epidermal epithelial barrier are yet unknown. Unveiling the mechanisms involved in NLCs transport across the epidermal epithelial monolayers will contribute with valuable information to achieve enhanced skin permeability, superior bioavailability and consequently improved therapeutic effect. With our present work we could certainly provide researchers and clinicians guidance for the design of optimized transdermal delivery systems, based on the nanomaterials and biological interactions.



## 1. Introduction

The transdermal drug delivery is a challenging and growing area of research. Delivery of drugs through the skin is the most easily accepted route among the assorted administrations manners, as it benefits of being painless, convenient (large accessible surface) easy self-administration and with less dosing, in opposition to frequent administrations and plasma level peaks related with oral route and injections [1]. Transdermal delivery results in a comparatively high patient compliance, particularly when long-term treatment is needed. Avoidance of hepatic first-pass effect, low cost when compared with other therapies and general good acceptability of transdermal devices by patients have clearly increased the market for these products [2].

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http://dx.doi.org/10.1016/j.actbio.2017.01.057
1742-7061/

In recent years, the application of delivery systems has been one of the main strategies to defeat the epithelial barrier and enhance drug skin permeation [3,4]. Nanocarriers can alter the cell uptake pathways of drugs and improve their transport through the epithelial cell monolayer as compared with free drugs [5]. In the past few years, nanostructured lipid carriers (NLCs) have been widely investigated and applied as a promising nanocarriers for skin delivery [6]. NLCs are second generation of lipid delivery systems having solid matrix at room temperature. These nanocarriers are composed by biodegradable, biocompatible and physiological lipids and surfactants and are accepted by regulatory authorities for application in different drug delivery systems.

Thus, NLCs are attractive colloidal nanocarriers for topical administration due to their several desirable effects on skin, namely the ability to protect from chemical decomposition, the film formation, the skin hydration and controlled occlusion, the enhanced bioavailability, the physical stability and the possibility to modulate drug release [7]. Recently, NLCs have emerged as novel drug-delivery systems for targeting to epidermal layer and studies to understand nanoparticles properties influence are needed [8].

NLCs have been tested for several applications and highlights of their cellular interaction may involve, for oral delivery, transport across intestinal *in vitro* models through both caveolae- and clathrin-mediated transcytosis [9] or even enhancement of the absorption of drugs in gastrointestinal mucus and intestinal epithelium [10]. Active endocytosis and passive diffusion of NLCs was described as main mechanism for improved cellular uptake in lung cancer cells, upon inhalational delivery [11]. Solid lipid nanoparticles have been shown to rapidly cross the keratinocyte monolayer and traffic through the cytoplasm to a final nuclear location [12]. However, few studies on the cellular interactions and transport mechanism of NLCs in epidermal epithelial cells have been disclosed so far [13,14]. The transport mechanisms of nanoparticles is quite complex, and includes internalization into cells mediated by endocytosis from the apical side, then intracellular transport, and finally exit from the apical (exocytosis) and/or from the basolateral (transcytosis) side [15]. A comprehensive investigation of the mechanisms involved in NLCs transport across the epidermal epithelial monolayer will give us guidance for the design of optimized transdermal delivery systems with enhanced skin permeability, superior bioavailability and consequently improved therapeutic effect.

In a previous research, NLCs were designed and optimized as drug delivery for skin delivery of methotrexate [16,17]. To further unravel the transport related mechanisms of NLCs across the epidermal epithelial cell monolayer a systemic investigation was conducted. In this work, the NLCs were prepared and simulated epidermal epithelial monolayer established using human HaCaT cells was employed to study the molecular cellular interactions and transport mechanisms. The effects of exposure time, concentration, and energy on NLCs uptake by HaCaT cells were established, and the uptake pathways into the cells and intracellular trafficking were also demonstrated. Herein, several pharmacological inhibitors were used to study the transport related mechanisms taken by the nanocarriers across human epithelial cell monolayers. As the result, a proposal for NLCs transport in epidermal epithelial cells was obtained.

## 2. Materials and methods

### 2.1. Materials

Lipid nanoparticles were prepared using Witepsol® E85 kindly provided by Gatefossé (Nanterre, France), Miglyol® 812 was purchased from Acofarma (Madrid, Spain) and polyvinyl alcohol (PVA) supplied from Sigma-Aldrich (St Louis, MO, USA). All other chemicals and solvents were of analytical grade acquired from Sigma-Aldrich (St Louis, MO, USA) unless stated otherwise. Methotrexate was a gift from Excella (Feucht, Germany). Aqueous solutions were prepared with double-deionized water (Arium Pro, Sartorius AG, Göttingen, Germany), which possesses conductivity values <0.1 $\mu S\,cm^{-1}$. For cell culture, fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/mL) mixture, Dulbecco's Modified Eagle's Medium (DMEM) and trypsin 0.25% (v/v) were acquired from Gibco® (Invitrogen Corporation, UK). HaCaT human keratinocyte cell line was purchased from Cell Lines Service (CLS, Eppelheim, Germany).

### 2.2. Preparation and characterization of coumarin 6 loaded NLCs

NLCs were produced using the hot ultrasonication method previously optimized [16]. The lipid nanoparticles were made based on Witepsol E85® (150 mg), miglyol-812 (45 mg) and PVA 2% (4.7 mL) dispersed in 7 mL of water. NLCs were labeled with 2 mg of coumarin 6 (C6) upon addition to the lipid phase during the preparation procedure, for the cellular uptake mechanisms assays. Particle size and zeta potential were assessed by DLS in a Brookhaven Instrument (Holtsville, NY, USA). NLCs presented a size around 250 nm, polydispersity index below 0.1 and a zeta potential value of −15 mV, similar to the MTX-loaded NLCs (252 ± 9 nm and −14 ± 1 mV) described elsewhere [16]. The size below 500 nm and their lipophilic nature are suitable for topical administration and permeation through the epidermal barrier [18]. Cryo-scanning electron microscopy (cryo-SEM) was used to observe the morphology of the C6-loaded NLCs at experimental facilities of the Materials Centre of the University of Porto, Portugal (CEMUP). The quantification of C6 was determined at the emission wavelength of 518 nm upon excitation at 444 nm, in a spectrofluorometer (Jasco FP-6500 spectrofluorometer, Easton, Maryland, USA).

### 2.3. HaCaT cell culture

HaCaT cells (human keratinocytes cell) were cultivated in DMEM complemented with 10% (v/v) FBS (fetal bovine serum), and penicillin/streptomycin mixture, in a water-jacketed $CO_2$ incubator at 37 °C. Every 3-days cells were passed using trypsin 0.25% (w/v).

#### 2.3.1. Cell viability assays

The cytotoxicity assays of the NLCs and pharmacological inhibitors towards HaCaT cells were conducted using MTT assay. Briefly, $5.0 \times 10^4$ cells per well were seeded in a 96-well plate. Subsequently, the cells were incubated with different concentrations of the NLCs at 37 °C for 24 h. Pharmacological inhibitors were tested at the same concentration used in the transport studies (Table 1). Cells were treated with 1% Triton X-100 as positive control. Then the cells were incubated with MTT solution (0.5 mg $mL^{-1}$) for 3 h at 37 °C. At the end of the incubation, the culture medium in each well was replaced by 100 μL of DMSO to dissolve the formazan-containing crystals. The plate was shaken for 5 min at room temperature, prior measuring the absorbance of each well at 570 nm in a Synergy™ HT Multi-mode microplate reader (BioTek Instruments Inc., Winooski, VT, USA). All of the experiments were performed in triplicate.

#### 2.3.2. Cellular uptake assays

For studying the uptake process of the witepsol/miglyol NLCs by HaCaT cells, the cells were seeded in a 24-well plate at a concentration of $1 \times 10^5$ cells $mL^{-1}$. After 18 h, cells were incubated with C6-