European Journal of Pharmaceutics and Biopharmaceutics 79 (2011) 76–81

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Modification of the conformational skin structure by treatment with liposomal formulations and its correlation to the penetration depth of aciclovir

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ARTICLE INFO

Article history:
Received 8 November 2010
Accepted in revised form 31 January 2011
Available online 15 February 2011

Keywords:
FTIR
Tape stripping
Aciclovir
DPPC liposomes
Cryo-TEM

ABSTRACT

The stratum corneum (SC), top layer of the epidermis, is comprised mostly of lipids, which are responsible for the permeability properties of the SC and which protect the body from external agents. Changes in these skin micro constituents can be understood by instrumental methods such as attenuated total reflectance Fourier-transform infrared (ATR–FTIR) spectroscopy. The present work shows that different types of analyzed skin, dermatomed abdominal porcine skin, pig ear skin and human heat separated skin, influenced both the shape and the intensity of recorded spectra. The typical FTIR spectral bands of the conformation of the lipid aliphatic chains in the skin samples were altered after treatment with pure DPPC liposomes and chitosan (CS) coated DPPC liposomes, but not with aqueous CS-solution. The conformational change could be the reason for the variable permeability of the skin. This was confirmed by tape stripping on pig ear skin (imitating in vivo studies): the amount of aciclovir penetrating from polymer coated and polymer free liposomes was significantly higher under the skin surface in comparison with the aqueous CS-solution. Moreover, the addition of the polymer to liposomes induced a higher skin penetration than pure liposomes. One explanation might be the CS's stronger adhesion to the skin.

1. Introduction

The stratum corneum (SC) is a distinctive two compartment system, consisting of corneocytes embedded in a lipid matrix. The complex structure of the SC lipid bilayers is important in maintaining the barrier properties of the skin [1–3]. To understand the modification of the lipid organisation within the SC many techniques such as X-ray diffraction, IR spectroscopy, DSC, ^{2}H NMR spectroscopy and electron microscopy are applied [4–6]. Most of the experimental evidence is consistent with the domain mosaic model suggested by Forslind et al., in which the skin lipids are organised in ordered domains (orthorhombic (OR) and hexagonal (HEX)) connected by lipids in a disordered phase (liquid–crystalline (LIQ)) [7–9]. It has been proposed that these domains would have crucial impact on SC permeability: the permeability of a disordered phase is in general greater than that of the ordered one [5]. According to Wertz et al. the greatest flux would be at the boundaries of these two phases [7].

In the present study Fourier-transform infrared spectroscopy (FTIR) with attenuated total reflection (ATR) technique was performed. This is a highly suitable technique for determination of molecular vibrations of the components of the SC at the functional group level [8]. The FTIR method has already been used to characterize the phase transitions of the SC lipids. These transitions were detected by shifting of methylene (CH_2) bands of the lipid aliphatic chains [1,2,8,10]. Therefore, in a first step the characteristic CH_2 bands of dermatomed abdominal porcine skin, pig ear skin and human heat separated skin were compared and analyzed. In our last work the ATR–FTIR method was used to better understand interactions of the model drugs and polymers with DPPC liposomes [11]. Based on these results, our intention was to further understand interactions between skin and different formulations. For this purpose the following formulations were applied: DPPC liposomes, chitosan (CS) coated DPPC liposomes as well as aqueous CS-solution. The liposomal formulations were additionally visualised by cryo-TEM to assess whether the differences between polymer free and polymer containing liposomes could be seen. Because the previous studies showed higher aciclovir skin diffusion in vitro (Franz-cell model) from polymer coated versus polymer free liposomes additional penetration studies using the tape stripping method on pig ear skin were performed [11]. With this method it is possible to investigate the drug distribution and penetration layer by layer of topically applied drugs in the SC [12–14].

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doi:10.1016/j.ejpb.2011.01.018

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphatydilcholine (DPPC) was purchased from Lipoid (Steinhausen, Switzerland). The product was synthetic Lipoid PC 16:0/16:0. The content of phosphatidylcholine was at least 99 % related to the dry weight. Chitosan (CS < 500 kDa) in the powder form was a gift from Syntapharm (Mülheim, Germany). The degree of deacetylation was determined by NMR with 95% [15]. Aciclovir was purchased from Fagron GmbH (Barsbüttel, Germany) All other chemicals used in this study were of analytical reagent grade and were used as received without any further purification.

2.2. Formulations

Skin samples for FTIR experiments were treated with DPPC liposomes and chitosan (CS) coated DPPC liposomes, prepared as previously described [11]. Particle size was measured by a Zetasizer Nano ZS (Malvern Instruments, Malvern, United Kingdom). The aqueous CS-solution was produced by dissolving the polymer in 0.01 M acetate buffer. The amount of CS used in formulations was 0.125%.

For the tape stripping procedure DPPC and CS coated liposomes were loaded with 1% aciclovir as reported [11]. Aciclovir, in aqueous CS-solution, was suspended in the same concentration.

2.2.1. Encapsulation of aciclovir in liposomes

Encapsulation of aciclovir in the liposomes was established by differential centrifugation method [16]. Briefly, liposomes and coated liposomes with aciclovir were centrifuged at 1200*g* for 2 h at 25 °C (Hermle Z323K, Wehingen, Germany). The supernatants were removed and dissolved in 96% ethanol. Aciclovir content in the supernatants and in the precipitates was quantified by HPLC method specified previously ($n = 3$) [11].

The encapsulation efficiency of aciclovir was calculated as described below:

$$\text{Encapsulation efficiency (EE\%)} = \frac{\text{Amount of bound drug}}{\text{Total amount of drug}} \times 100 = 24.38 \pm 0.24. \quad (1)$$

2.3. Skin preparation for FTIR

2.3.1. Abdominal porcine skin

The full thickness abdominal skin without hair was dermatomed to a thickness of 1.2 mm and then stored frozen at −20 °C. Skin samples with a dimension of about 7.5 cm^2 (ZnSe crystal dimension) were adopted for the experiment, which was performed ten times.

The following treatments were applied:

(a) Application of 40 μl distilled water.
(b) Application of 40 μl pure DPPC-liposomes.
(c) Application of 40 μl coated CS-DPPC liposomes.
(d) Application of 40 μl aqueous CS-solution.
(e) Untreated skin (control).

After the treatment, the skin samples were placed in PBS buffer and kept at 37 °C or 45 °C in a closed petri dish for 2 h in each case [5,10].

2.3.2. Pig ear skin

The same experimental procedure was repeated on pig ear skin prepared as described later (see Section 2.6.1).

2.3.3. Human skin

The human skin was obtained when excessive skin was removed from one healthy patient undergoing plastic surgery. The appropriately treated skin was heat separated and wrapped in filter paper. It was stored at −20 °C for not longer than 6 months before use [17].

2.4. Attenuated total reflectance Fourier-transform infrared (ATR–FTIR)

The penetration depth of IR is about 1 μm or less, which includes approximately 1–1.5 sheets of cell layers in the SC [10,18]. Infrared spectra of the prepared skin samples were obtained by using FTIR spectrophotometer (model: Tensor 27, Bruker Optics, Ettlingen, Germany) with a photovoltaic MCT detector at a temperature of 37 °C (physiological temperature) and 45 °C (transition temperature of SC lipids). To collect the spectra, the skin samples were placed stratum corneum down onto the ZnSe ATR crystal (tool: Bio-ATR I). To obtain the same intensity of spectra we put the cover glass on the sample. For data treatment we used the software OPUS 5.5.

2.5. Cryo-transmission electron microscopy (cryo-TEM)

Liposomes for cryo-TEM were frozen with a Leica EM GP immersion freezer (Leica Microsystems, Vienna, Austria) with its environmental chamber a 50 °C and 90% relative humidity. Four microliters of the specimen diluted to 2.5 mg/ml and pre-warmed to 50 °C was applied onto glow discharged EM grid coated with perforated Quantifoil R3.5/1carbon films (Quantifoil, Jena, Germany). After 30 s settling, the suspension was automatically blotted for 0.5–1.0 s with Whatman No. 1 filter paper and immediately plunged from the environmental chamber into liquid ethane. Subsequently, the specimens were handled only in the dehumidified working area and stored in liquid nitrogen prior to microscopy.

The vitrified specimens were visualised on a Tecnai F30 'Helium' (Polara) cryo-TEM (FEI Company, Eindhoven, Netherlands) operated at 300 kV and cooled with liquid nitrogen. Micrographs were acquired at nominal magnification of 31,000× at a defocus of −8.0 μm and captured with a Gatan US4000 CCD camera.

2.6. Tape stripping

2.6.1. Pig ear skin

The pig ears were obtained from local butcher and stored in the refrigerator at −20 °C. The night before the tape stripping procedure, the pig ears were stored in the fridge at 4 °C. On the day of procedure the skin surface was cleaned with distilled water and dried. The hair was carefully removed by scissors as closely as possible to the surface.

2.6.2. Tape stripping

The pig ears were fixed on a polystyrene support covered with aluminium foil, an area of 5 × 4 cm^2 was marked with a permanent marker and 5 μl/cm^2 of the tested formulation was applied. After 1 h tape stripping was performed as described previously using Corneofix® tape strips [12,19,20]. Briefly, each tape strip was pressed on the skin with the roller over the paper for 5 s and then removed in a single quick movement. Every tape strip was placed onto the special slide frame to determine the mass of the removed SC. The pseudo-absorption of the skin corneocytes, which