European Journal of Pharmaceutics and Biopharmaceutics xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

^{31}P solid-state NMR based monitoring of permeation of cell penetrating peptides into skin

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ARTICLE INFO

Article history:
Received 10 November 2012
Accepted in revised form 6 May 2013
Available online xxxx

Keywords:
Topical delivery
Cell penetrating peptides
Solid-state NMR
TAT peptide
Polyarginine peptide
Skin permeation
Confocal laser scanning microscopy

ABSTRACT

The main objective of the current study was to investigate penetration of cell penetrating peptides (CPPs: TAT, R_8, R_{11}, and YKA) through skin intercellular lipids using ^{31}P magic angle spinning (MAS) solid-state NMR. In vitro skin permeation studies were performed on rat skin, and sections (0–60, 61–120, and 121–180 μm) were collected and analyzed for ^{31}P NMR signal. The concentration-dependent shift of 0, 25, 50, 100, and 200 mg/ml of TAT on skin layers, diffusion of TAT, R_8, R_{11}, and YKA in the skin and time dependent permeation of R_{11} was measured on various skin sections using ^{31}P solid-state NMR. Further, CPPs and CPP-tagged fluorescent dye encapsulate liposomes (FLip) in skin layers were tagged using confocal microscopy. The change in ^{31}P NMR chemical shift was found to depend monotonically on the amount of CPP applied on skin, with saturation behavior above 100 mg/ml CPP concentration. R_{11} and TAT caused more shift in solid-state NMR peaks compared to other peptides. Furthermore, NMR spectra showed R_{11} penetration up to 180 μm within 30 min. The results of the solid-state NMR study were in agreement with confocal microscopy studies. Thus, ^{31}P solid-state NMR can be used to track CPP penetration into different skin layers.

1. Introduction

One of the important functions of the skin is to provide a barrier to the penetration of foreign chemicals into the body [1]. The major barrier of transdermal absorption is the exterior skin layer, stratum corneum (SC), which is composed of very densely packed dead corneocyte cells embedded within extracellular lipid lamellae [2]. Among various physicochemical methods proposed to enhance penetration of micro- and macromolecules into the skin, use of cell penetrating peptide (CPP) carriers has emerged as one of the most promising approaches. The CPPs were first discovered in biological systems such as herpes and HIV, and they possess the ability to permeate cell membranes [3]. In recent years, many studies have demonstrated that CPPs can pass through skin barriers to deliver cargo molecules into the skin [4–14]. Growing interest in CPPs is attributed to the simplicity of use, diversity, and potential ability to target cellular subtypes within the skin. Several CPPs, including the well-known TAT [5,10,11,13,15], polyarginines [7,9,12], penetratin [8,9,16], RALA [6,9], and TD-1 [17], are known to penetrate across the SC. Some of the CPPs also have the ability to translocate themselves and cargo molecules within viable epidermis [11,18,19]. Previous studies have suggested that the active sequence of TAT peptide containing six arginine residues is essential for its uptake function. Further, polyarginine can translocate more efficiently over other homopoly-cationic/polybasic peptides in live cells [20]. Similar results were observed for the skin tissue, where TAT and polyarginine peptides (R_n; $n = 8$, 11, and 15) effectively delivered encapsulated dye and/or drug from nanocarriers into the epidermal–dermal junction [11,18,19].

Abbreviations: ATR-FTIR, attenuated total reflectances Fourier transform infrared spectroscopy; CLSM, confocal laser scanning microscopy; CPP, cell penetrating peptide; DOGS-NTA-Ni, 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl) imidodiacetic acid) succinyl nickel salt]; FLip, fluorescent dye containing liposomes; FLip-R_{11}, polyarginine-11 (R_{11}) coated FLip; FLip-YKA, YKA coated FLip; MAS, magic angle spinning; PI, polydispersity index; R_{11}, polyarginine-11; R_8, polyarginine-8; SC, stratum corneum; TAT, trans-acting activator of transcription.

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http://dx.doi.org/10.1016/j.ejpb.2013.05.003

In general, CPPs can translocate into cell cytoplasm by at least two independent mechanisms: (i) energy dependent endocytosis and (ii) rapid crossing of the cell membrane by direct membrane penetration [3]. As SC is a formidable layer, the mechanism of SC penetration may be different than that of the cell membrane. It was suggested that the initial step of CPP uptake into cells and/or skin is through strong ionic interaction between the positively charged amino acid residues and the negatively charged barrier constituents [15]. The electrostatic effects initiate peptide aggregation into lipid bilayers, and these can lead to membrane perforation [8]. The classical mechanism for skin penetration of molecules is proposed to be diffusion through intercellular lipids, and the lipid organization is considered to be very important for the skin barrier function [21]. Therefore, CPPs have been postulated to cause a dynamic structural disorder in the epidermal intercellular lipids for transepidermal penetration enhancement [22]. Recently, it was discovered that topical application of penetratin on the skin surface can cause structural changes within the SC lipids [6,8,9]. However, once partitioned in the microenvironment, the molecular level interactions of the CPPs with skin lipids from skin layers are still unknown. Therefore, our present understanding is insufficient to predict the exact mechanism of skin penetration of CPPs and cargo molecules. To investigate the translocation ability of CPP in cells, several techniques, such as cellular flow cytometry [23] and fluorescence microscopy [13], have been reported. Unfortunately, these techniques are either complicated or cannot be used to investigate the molecular mechanisms involved in the translocation of CPP across the skin. Recently, attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) has been used to study the tissue-specific transport of CPPs [8,22]. ATR-FTIR can provide insights into the vibrational frequencies of the components in the SC, but in these studies, only the effect of CPPs in the SC was studied. For percutaneous delivery, it is very important to study the effects in deep skin layers. Therefore, there is a need for additional techniques to track and understand the interaction of various CPPs with skin lipids.

Solid-state NMR spectroscopy has proven to be a valuable tool for the structural analysis of biomolecules immobilized within lipid bilayers, as well as the bilayers themselves. Molecular structures of cell penetrating peptides within bilayers have been probed at high concentration within artificial constructs via ^{13}C and ^{15}N isotopic labeling of peptide molecules [3,24,25]. Lipid polymorphism is reflected in the ^{31}P NMR spectra of the phospholipid head groups [24–26]. Effects of CPP-lipid interactions on CPP and lipid molecular structures are important because they are closely related to mechanisms of CPP uptake and transport within biological membranes. The high natural abundance of ^{31}P, and the sensitivity of lipid ^{31}P NMR signal to the presence of CPP, constitutes an opportunity to detect the presence of CPP in the in vivo-derived skin samples without the need for isotopic labeling or fluorescent tags.

In the present study, we show that the ^{31}P NMR chemical shift observed under magic angle spinning (MAS) is sensitive to peptide permeation into rat skin at different skin depths. In skin layers, many parameters affect the permeation of CPPs, such as lipid composition, lipid/protein ratio, and water content [3]. These parameters differ when different skin layers are compared to each other or to lipid bilayers of cell membranes mimicked by artificial constructs. Nevertheless, we found that optimized sample preparation and proper control experiments enable the use of ^{31}P chemical shift as a clear indicator of the presence for CPP within skin. Sensitivity to skin depth was achieved by sectioning different depths of skin into separate NMR samples following permeation experiments.

This is the first study involving the use of solid-state NMR to observe the effect of CPP application on ^{31}P solid-state NMR spectra of skin tissue. Using TAT as a model CPP, we investigated the CPP concentration dependence to determine the CPP concentration range which optimizes the sensitivity of ^{31}P chemical shift to CPP penetration. We further explored the effects of different peptides TAT, R_8, R_{11}, and YKA (non-transduction peptide, negative control) on the skin layers using the ^{31}P chemical shift. Confocal laser scanning microscopy (CLSM) is one of the recently developed and widely studied techniques to study the skin distribution of fluorescent probes. To support the NMR observations, CLSM was employed to measure the permeation of CPPs in skin. Finally, this study utilizes the advantage of CPP as a skin penetration enhancer for the development of an improved liposomal formulation for topical delivery into skin. To evaluate the efficacy, CLSM was used to study the bio-distribution of the encapsulated fluorescent dye from fluorescent dye containing liposomes (FLip) and R_{11} or YKA tagged FLip.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(poly-ethylene glycol)-2000] (ammonium salt) (DSPE-PEG_{2000}), cholesterol, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl) imidodiacetic acid) succinyl nickel salt] (DOGS-NTA-Ni) were purchased from Avanti Polar lipids (Alabaster, AL, USA). High performance liquid chromatography (HPLC) grade water and phosphate buffered saline (PBS) were procured from Sigma–Aldrich Chemicals (St. Louis, MO, USA). The histidine tagged and fluorescein isothiocyanate (FITC) labeled peptides TAT (YGRKKRRQRRRHHHHHH-FITC, MW: 2382.67); polyarginine-8 (RRRRRRRRHHHHHH-FITC, MW: 2203.54); polyarginine-11 (RRRRRRRRRRHHHHHH-FITC, MW: 2672.21); polyarginine-15 (RRRRRRRRRRRRRRHHHHHH-FITC, MW: 3296.86); and non-transduction peptide YKA (YKALRISRK-LAKHHHHHH-FITC, MW: 2269.7) were synthesized by GenScript Corporation (Piscataway, NJ, USA). Fluorescent dye DiD oil (excitation 644 nm and emission 665 nm) was procured from Invitrogen Corp (Eugene, OR, USA). All other chemicals used in this research were of analytical grade.

2.2. Animals

CD®(SD) hrBi hairless rats (weighing 250–300 g; male; Charles River Laboratories, Wilmington, MA) were grouped and housed ($n = 3$ per cage) in cages with bedding. The animals were kept under controlled conditions of 12:12 h light: dark cycles, 22 ± 2 °C and 50 ± 15% relative humidity. The mice were fed (Harlan Teklad) and water ad libitum. The animals were housed at Florida A&M University in accordance with the standards of the Guide for the Care and Use of Laboratory Animals and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The animals were acclimatized to laboratory conditions for 1 week prior to experiments. The protocol of animal study was approved by the Institutional Animal Care and Use Committee (IACUC), Florida A&M University, FL, USA.

2.3. In vitro percutaneous permeation

2.3.1. Preparation of skin

For the skin collection, CD(SD) hrBi hairless rats were sacrificed by an overdose of halothane anesthesia. The skin from the dorsal surface was excised, and adherent subcutaneous fat and connective tissues were carefully removed. The collected skin was rinsed

Please cite this article in press as: P.R. Desai et al., ^{31}P solid-state NMR based monitoring of permeation of cell penetrating peptides into skin, Eur. J. Pharm. Biopharm. (2013), http://dx.doi.org/10.1016/j.ejpb.2013.05.003