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³¹P Solid-state NMR based monitoring of permeation of cell penetrating peptides into skin

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Abstract

The main objective of the current study was to investigate penetration of cell penetrating peptides (CPPs: TAT, R₈, R₁₁ and YKA) through skin intercellular lipids using ³¹P magic angle spinning (MAS) solid-state NMR. In vitro skin permeation studies were performed on rat skin, sections (0–60, 61–120 and 121–180 μm) were collected and analyzed for ³¹P NMR signal. The concentration dependent shift of 0, 25, 50, 100 and 200 mg/ml of TAT on skin layers, diffusion of TAT, R₈, R₁₁ and YKA in the skin and time dependent permeation of R₁₁ was measured on various skin sections using ³¹P solid-state NMR. Further, CPPs and CPP-tagged fluorescent dye encapsulate liposomes (FLip) in skin layers were tagged using confocal microscopy. The change in ³¹P 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. R₁₁ and TAT caused more shift in solid-state NMR peaks compared to other peptides. Furthermore, NMR spectra showed R₁₁ penetration up to 180 μm within 30 min. The results of the solid-state NMR study were in agreement with confocal microscopy studies. Thus, ³¹P solid-state NMR can be used to track CPP penetration into different skin layers.

Keywords

Topical delivery; Cell Penetrating Peptides; Solid-state NMR; TAT peptide; Polyarginine peptide

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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 physico-chemical methods proposed to enhance penetration of micro and macro molecules 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 6 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].

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, R₈, R₁₁ 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 R₁₁ 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-PEG₂₀₀₀), 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 (RRRRRRRRRRRRHHHHHH-FITC, MW: 3296.86); and non-transduction peptide YKA (YKALRISRKLAKHHHHHH-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, $22 \pm 2^\circ\text{C}$ and $50 \pm 15\%$ 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 one 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 with physiological saline. The cleaned skin was then soaked in 10% v/v glycerol in normal saline solution for 30 min and then stored at -80°C until used. Prior to use, the skin was rinsed in phosphate buffer solution (pH 7.4) for 30 min. In addition, it is well documented that structural changes in the skin are related to the age [27–29] and to the skin storage conditions [30–31]. Therefore care was taken for consistent skin storage conditions and skin age. Previously, the age-related change in the physiological property of the rat skin was examined after ages of 10 days [28]. Therefore, in the present study, rat skin was collected at the age of 7 days. Furthermore, each dataset was run on the skin collected from only one rat to avoid any biological variations from rat to rat. Therefore, each data-set included a control skin run and which was compared to treatment groups.

2.3.2 In vitro permeation setup—This study has been performed consistently with previously described methods [32–33]. Briefly, the skin was mounted on Franz diffusion cells (Permeagear Inc., Riegelsville, PA) between the donor compartment and receptor compartment with epidermis facing the donor compartment. The receptor compartment was filled with PBS (pH 7.4), stirred at 300 rpm and maintained at $32 \pm 0.5^\circ\text{C}$ using a circulating water bath. 100 μl of the test solution was applied on the diffusional surface of the skin in the donor compartment. The successive skin permeation studies were performed for pre-determined time periods. The skin permeation studies were performed under un-occlusive conditions. The exposed skin area was collected at the end of each study. The donor cell was removed and the excess formulation was removed from the surface of the skin using a cotton swab. The skin was then washed with 50% v/v ethanol and blotted dry with lint-free absorbent wipes. The entire dosing area (0.636 cm^2) was collected with a biopsy punch and subjected to cryo-sectioning. Three thin lateral serial skin sections of 60 μm were collected and labeled as 0–60, 61–120 and 121–180 μm thickness using a cryotome (Shandon Scientific Ltd, England) to represent middle layers of epidermis (including SC), remaining epidermis and initial dermis, respectively. Collected three sections were then analyzed immediately for ^{31}P signal using solid-state NMR.

2.4 ^{31}P solid-state NMR studies

Solid-state NMR spectra were recorded on an 11.7 Tesla Bruker Avance III MAS solid-state NMR system (500 MHz ^1H NMR frequency). Magic angle spinning ^{31}P solid-state NMR spectra were collected at a sample spinning rate of 10 kHz, using a 2.5 mm Bruker probe. To maximize sensitivity, the Ernst angle technique was employed, with a single 45° pulse lasting 2.5 μs prior to acquisition and a recycle delay of 0.1 s [34]. Each NMR spectrum

corresponds to 50,000 scans and an acquisition time of 1.4 hr. The signal averaging time of 1.4 hr was chosen in order to limit the effects of a small (~ 0.1 ppm) broadening and shift of the ^{31}P NMR peak with longer NMR signal averaging times (see Figure S1). We attribute this effects to dehydration caused by the dry nitrogen required for magic angle spinning, as it did not depend systematically on time between skin sectioning and NMR acquisition. This recycle delay was $T_1/3$, which was determined by saturation recovery. NMR spectra were referenced to the ^{31}P NMR resonance of 85% phosphoric acid.

2.4.2 Effect of peptide concentration on NMR spectra—TAT peptide was selected as our model CPP for these studies. The effect of four different concentrations of TAT in water was studied on the skin permeation after 16 h. These concentrations were 25, 50, 100 and 200 mg/ml. For all the different concentrations of TAT, the donor volume was maintained to be 100 μl . As a control, skin treated with only 100 μl HPLC grade water for 16 h was selected. After 16 h of permeation, the dosing area was collected, followed by cryo-sectioning and NMR analysis as explained earlier.

2.4.3 Effect of different cell penetrating peptides on skin—To study the effect of different types of CPPs on the skin selected peptides are: (i) TAT, (ii) R_8 , (iii) R_{11} and (iv) YKA. The effects of different CPPs were studied using two different concentrations, 25 mg/ml and 50 mg/ml. The control experiment was performed similarly to that discussed in section 2.4.2. The in vitro skin permeation was performed for 16 h. At end of the study, the dosing area was collected and subjected to cryo-sectioning before solid-state NMR spectroscopy.

2.4.4 Time dependent effects of cell penetrating peptides on skin—In this study, shorter permeation times were analyzed for 50 mg/ml of R_{11} at a skin depth of 61–120 μm . Time periods of 0.5, 1, 2 and 4 h were selected for R_{11} permeation. As a control, skin permeated with pure water for 0.5 h was used. The skin permeation studies were performed for pre-determined time periods and at the end of the study only the 61–120 μm skin depth was analyzed by solid-state NMR.

2.5 Tracking of different cell penetrating peptides across skin layers using confocal laser scanning microscopy

The imaging was performed using CLSM to study the skin distribution of the FITC labeled CPPs (TAT, R_8 , R_{11}) and YKA. This study was performed as described by Shah et al [19]. Briefly, the skin was mounted between donor and receptor compartments of Franz diffusion cells with the SC facing the donor compartment. 100 μl of 50 mg/ml of FITC labeled CPPs were applied to the epidermal side in the donor compartment. In all subsequent skin permeation studies, similar conditions were used as explained earlier. After 16 h of skin permeation, the skin was taken out from the diffusion cell and blotted dry with Kimwipes® and the entire dosing area was collected using a biopsy punch. The lateral skin sections were collected up to 200 μm using a cryotome, for visualization of skin associated fluorescence. The skin sections were analyzed with CLSM (Leica Microsystems Inc., Buffalo Grove, IL, USA) using 10x objective and the instrument settings were constant for all the samples throughout the study period. Finally, the collected images were analyzed using Digital image software (Museum of Science, Boston, MA, USA) for the skin associated fluorescence.

2.6 Evaluation of the skin permeation of cell penetrating peptides tagged liposomal formulations

2.6.1 Preparation and characterization of surface modified liposomes—To characterize the ability of CPP to carry lipid carriers in to the skin layers, FLip were

prepared by a solvent evaporation and dehydration method. DiD dye (0.5 mg/ml) was encapsulated in FLip consisting of DOPC/cholesterol/DOTAP/DSPE-PEG₂₀₀₀/DOGS-NTA-Ni in a molar ratio of 20/40/15/10/5. The lipophilic fluorescent dye DiD oil, lipids and a spacer (DOGS-NTA-Ni) were dissolved in chloroform and ethanol (9:1) and a thin film was formed by evaporating the organic solvent in a round bottom flask at 60°C under reduced pressure for 3 to 4 h to ensure total removal of organic solvents. The lipid-dye mixture was deposited as a thin film. The film was rehydrated with water and sonicated for 2–3 min using a bath sonicator and allowed to equilibrate. Formed multilamellar vesicles were passed through a high speed homogenizer NanoDebee (BEE International, Inc., South Easton, MA, USA) for 7 min. The liposome surface was modified by incubating liposomes with six histidine tagged R₁₁ and YKA peptides for 30 min at room temperature. The ratio of the linker (DOGS-NTA-Ni) to six-histidine tagged peptides was optimized as 2:1 based on our earlier studies. The mean diameter and polydispersity index (PI) of FLip was measured by using Nicomp 380 ZLS (Agilent Technologies, USA). The Nicomp 380 ZLS analyzer uses dynamic light scattering to obtain the essential features of the particle size distribution. The particle size analysis data were evaluated using volume distribution. Prior to the measurement, the samples were diluted with water to suitable scattering intensity. The zeta potential values of R₁₁ and YKA modified and unmodified FLip were assessed by determining the particle electrophoretic mobility using a Nicomp 380 ZLS. All measurements were performed in triplicate each time.

2.6.2 Confocal laser scanning microscopic studies of modified liposomes

across skin layers—In vitro skin permeation studies were carried out with FLip, FLip-R₁₁ and FLip-YKA formulations on hair less rat skin using Franz diffusion cells. In brief, 100 µl of liposomal formulations were applied to the epidermal side in the donor compartment. After 16 h of permeation, the skin was washed with 50% v/v ethanol and blotted dry with lint-free absorbent wipes and exposed skin area was cut with a biopsy punch and sectioned with a cryotome. Then, vertical skin sections up to depths of 0–200 µm were observed under CLSM as explained earlier. In the end, total percent fluorescence intensity of the collected images was calculated using Digital image software.

2.6 Statistical analysis

Differences between the skin permeation of the peptides TAT, R₈, R₁₁ and YKA were examined using ANOVA and the Tukey multiple comparison test. Mean differences with $p < 0.05$ were considered to be significant.

3. RESULTS

3.1 Effect of peptide concentration for ³¹P NMR spectra

Figure 1 shows the effect of peptide concentration in permeation experiments on the observed ³¹P NMR peak positions. The systematic effect of peptide concentration or skin depth on line width was not observed (mean line width, full width at half maximum: 0.6 ppm; standard deviation: 0.2 ppm). TAT-treated skin showed significant shift in the peak position compared to a control skin sample. It was observed that the peaks were shifted to the right (negative shifts) for TAT treated skin samples compared to the control. Further, TAT-treated skin showed a monotonic effect of TAT concentration on ³¹P NMR peak shift up to 100 mg/ml. Increase of TAT concentration up to 200 mg/ml show no increased effect on the peak position, signifying a saturation effect. The saturation effect was observed at ~100 mg/ml for all three skin depths (Figure 2). These data suggest that 25 to 50 mg/ml is the optimum concentration of CPP to observe concentration-dependent ³¹P NMR shifts in the skin. Hence, for all further studies 25 and/or 50 mg/ml of peptide concentrations were selected.

3.2 Effect of peptide type on skin ^{31}P NMR signal

The results for three different CPPs (R_8 , R_{11} and TAT) and the non-transduction peptide YKA when used at 25 mg/ml are depicted in Figure 3 (a – c). The NMR peaks for 25 mg/ml of TAT and R_{11} treated skin showed larger NMR shifts compared to the control, R_8 and YKA treated skin respectively. Further, the negative control peptide YKA showed less shifts in NMR peak compared to CPPs. Meaning on application of YKA on the skin leads to very less structural changes in skin lipids compared to changes caused after CPPs application. R_8 showed a shift in peak position that was similar compared to the control and YKA treated skin at different skin depths. The skin permeation results for 50 mg/ml peptide concentration are summarized in Figure 3 (d – f). Similar peptide dependence was observed for both the 25 mg/ml and 50 mg/ml concentrations and the order of the peak shifts were: Control < YKA < R_8 < R_{11} ~ TAT. The difference between R_8 and YKA was more pronounced at 50 mg/ml (Figure 3f). These data suggest that CPPs like TAT and R_{11} can permeate into the deeper skin layers.

3.3 Time dependent permeation of R_{11}

The results for time dependent permeation of R_{11} are shown in Figure 4. For this study R_{11} was selected as a model CPP and the depth of 61–120 μm was selected. The results demonstrate that R_{11} shows a monotonic time dependent ^{31}P chemical shift for all time points compared to the control skin. Interestingly, the peptide started reaching the 61–120 μm depth even after only a 0.5 h. This indicated that R_{11} penetrates the SC and upper-layers of the epidermis efficiently and quickly.

3.4 Tracking of different cell penetrating peptides across skin layers using confocal laser scanning microscopy

To support the solid-state NMR observations of CPP on the skin, the permeation of fluorescently labeled CPPs was tracked in the skin using CLSM. Figure 5 shows the skin permeation of TAT, R_8 , R_{11} and YKA after 16 h at different depths. Bright fluorescence signal was observed at 0–20 μm for all peptides. However, with increase in the depth of the skin, decreased fluorescent signal was observed. The permeation was observed to be different for different CPPs, where TAT and R_{11} were able to penetrate up to 200 μm depths, while R_8 was detectable only up to 160 μm and no fluorescence was observed at 200 μm . However, the fluorescence from YKA permeated skin sections was observed only up to 120 μm . The percent fluorescence intensity, calculated from confocal microscopy images, was found to be in the range between 0 and 21 (Figure 6). The YKA was unable to penetrate beyond 120 μm and the total percent intensity was less compared to the CPPs. The fluorescent intensities observed for TAT and R_{11} were similar. The percent intensity of TAT and R_{11} were significantly ($p < 0.05$) higher than YKA at various depths. These observations were in agreement with the results from solid-state NMR studies, where the CPPs like TAT and R_{11} were able to cause more change in the ^{31}P NMR chemical shift.

3.5 Evaluation of the skin permeation of CPP-modified liposome formulations

3.5.1 Physicochemical characterization of surface modified liposomes—The prepared FLip showed particle size in the range of 110 nm – 130 nm with mean particle size of 119 ± 9 nm. The surface modification of FLip with six histidine tagged CPPs showed little effect on particle size. The formulated FLip showed a narrow size distribution with polydispersity index (PI) of 0.19 ± 0.06 . On other hand, the zeta potentials of FLip were highly affected with surface modification. The zeta potential of the FLip in double distilled water (pH 6.4) was found to be 20.63 mV, which was changed to 29.12 mV after surface modification with R_{11} . Further, the surface modification of FLip with YKA resulted in a zeta potential of 22.92 mV. FLip were stable for 3 months with no change in particle size

and PI values at 30°C/65% RH and 40°C/75% RH. However, a gradual increase in particle size and PI values was observed for FLip when stored for 6 months at 30°C/65% RH and 40°C/75% RH.

3.5.2 Confocal laser scanning microscopic studies of permeation of CPP-modified liposomes across skin layers—

The results from skin tracking of surface modified and unmodified FLip are shown in Figure 7. The results from CLSM showed that surface modification of liposomes with R₁₁ peptide can enhance the skin permeation of encapsulated dye. DiD dye from the FLip-R₁₁ formulation was observed in the epidermal-dermal junction at 200 µm suggesting that CPP can translocate the particles into the skin by breaching the SC barrier. CLSM studies demonstrated that most of the fluorescent dye from FLip was present in the SC and upper epidermis up to depth of 120 µm. Also, the fluorescence from FLip modified with YKA was detectable up to 160 µm. The quantification of the fluorescence intensity is illustrated in Figure 8. The fluorescence intensity from R₁₁ is significantly higher ($p < 0.05$) than FLip and FLip-YKA at all the depths. These results suggest that R₁₁ has the ability to enhance the delivery of lipid payloads to the epidermal-dermal junction. This junction is the site of action for most skin related disorders.

4. DISCUSSION

The current understanding of interaction between CPPs and lipid components of epidermis is very limited. Therefore here in this study, ³¹P solid-state NMR was probed to study the change in the local organization of the lipids on application of various CPPs. In the present study we have recorded ³¹P chemical shifts to derive the information on the interaction of CPP with skin lipids as they diffuse from the SC to epidermis and dermis. Further in the present samples, the amount of peptide relative to lipid was low (less than 10%, based on mass of CPP introduced relative to dry mass of skin lyophilized after NMR measurements and assuming complete CPP uptake), suggesting that the effects on ³¹P solid-state NMR signals are the result of long-range effects of CPPs on lipid bilayers, which could include changes in the local organization of the lipids. This interpretation is consistent with the previous explanations for the origin of the effect of CPP permeation on ATR-FTIR signals in the SC [8, 22, 35]. Effects of CPPs on artificial lipid bilayers and vesicles were also previously observed using solid-state NMR by Ulrich et al. [25–26]. In these experiments, CPPs like TAT [26], R₈ [26] and penetratin [25] induced a change in the ³¹P NMR signal from lipid vesicles. On further investigation, Ulrich et al. concluded that TAT can form short inverted rod-like lipid micelles within the artificial lipid vesicles to get translocated [26]. A similar interpretation of CPP effects on present ³¹P chemical shifts in skin samples implies that ³¹P NMR is an indirect probe of the presence of CPP in skin lipid bilayers.

It is important to note that skin lipid composition is highly dependent on depth within skin. The SC is expected to be invisible to ³¹P NMR because SC lipids do not have phosphate groups in their polar heads. In addition, phospholipids degrade into glycerol and free fatty acids while glucosylceramides into ceramides due to enzymatic degradation [21]. Furthermore, the self-renewing nature of the epidermis produces a highly depth dependent composition resulting from apical migration of keratinocytes and lipids through successive epidermal layers from the basal layer to the upper layer of the SC. Depth-dependent lipid composition contributes to depth-dependent ³¹P NMR chemical shifts and highlights the importance of depth-dependent ³¹P NMR control experiments. Therefore in the present study, we selected three different skin depths, 0–60, 61–120 and 121–180 µm, to represent middle epidermis, lower epidermis and initial dermal layers, respectively. The first depth was selected to combine SC (10–15 µm) and upper epidermis, as SC forms the formidable barrier for skin permeation and it is very critical to cross the SC barrier. However,

permeation up to epidermal-dermal junction (61–120 μm) and up to deep dermis (121–180 μm) is very essential for effective topical skin delivery. In our previous studies, six histidine tagged CPPs or YKA were attached on the surface of the nanoparticles and the effect of surface modified nanoparticles on the topical delivery of the encapsulated active drug molecules was studied. Therefore in the current study, six histidine tagged peptides were used as representative peptides for CPPs (TAT, R₈ and R₁₁) or YKA.

There are mainly three possible mechanisms by which permeation enhancers enhance transdermal transport of drug molecules: (i) by structurally disturbing the lipids present in the intercellular region (intercellular pathway); (ii) by alternating the protein domain including the cross-linked keratin and other protein cellular components of keratinocytes along with the covalently bound lipids (non-extractable lipids) (transcellular pathway) and (iii) through hair follicles (transappendageal pathway) [35]. Since cells in the SC are non-living, CPPs must penetrate this skin layer via passive diffusion. Further the penetration mechanism of CPPs into the skin was proposed to be through the intercellular lipid domains in the layers of skin, since most of the molecules applied onto the skin permeate along the lipid domains [8]. Regardless of specific penetration mechanism, the observed shift in ³¹P NMR signal requires a highly localized effect on the organization of lipid molecules within biological membranes, which is likely to happen only with partitioning of CPP into lipid bilayers. In addition to the intercellular pathway, the transappendageal pathway may also play role in the penetration of CPPs into the skin. The role of hair follicles in penetration process is known to be minor based on the fact that orifices of hair follicles occupy only approximately 0.1% of the total skin surface area [3].

The dependences of ³¹P NMR chemical shift on peptide, peptide concentration and time reveal insight into the nature of CPP interactions with skin lipid bilayers. The concentration dependence (Figures 1 and 2) indicates behavior similar to previously observed interactions between CPPs and lipid bilayers on Langmuir troths [36]. Such a saturation isotherm behavior is consistent with both energy dependent transcytosis, implying limited number of receptors, and also energy independent membrane penetration, implying limited surface CPP coverage. In either case, the data suggest that interaction with skin surface is a key step to permeation and may represent the limiting factor in overall skin absorption. The peptide-dependent shift in NMR signal (Figure 3) follows a well-known trend for permeation effectiveness (TAT ~ R₁₁ > R₈ > YKA) [11, 18, 32]. These results are consistent with the interpretation that the effects of CPPs on ³¹P NMR spectrum are due to changes in the organization of skin lipids in response to interactions with CPP molecules. However, caution should be taken when interpreting quantitative differences in ³¹P NMR shifts for different peptides because it is not clear that different peptides have similar effects on skin lipid structures. Interestingly, the time dependent ³¹P chemical shift at 61–120 μm depth for R₁₁ permeation indicated rapid transport with most of the observed effect occurring by 30 min. Cohen-Avrahami et al., also reported the skin permeability enhancement from penetratin using ATR-FTIR. They reported that faster diffusion of penetratin and its cargo molecules into the skin occurs as penetratin affects the hexagonal symmetry of SC lipids with a transition towards a liquid-disorder state [8]. It was assumed that after topical application of the peptide on the skin surface, it may take time to form the reservoir in the SC and then after peptide reaches the critical concentration; it may start the penetration process. Hence, 30 min was selected as the starting point of the measurement and second depth (61–120 μm) was selected for kinetic studies.

The CPPs are likely to interact with lipid components of the cell membrane by various mechanisms depending on the physico-chemical properties of the CPP. Some reports have speculated that CPPs like penetratin can interact with amphiphilic lipids by forming a hairpin structure, where the hydrophobic tryptophan side chains embed into the hydrophobic

lipid area and the polar residues bind to the hydrophilic phosphate groups on the membrane [8]. In addition, polar poly-cationic peptides like TAT, polyarginine and polylysine show some specific interaction with lipid head groups and chains of model membranes [23]. Recently, Kumar et al. showed that the T2 peptide (LVGVFH) can enhance the partitioning of small molecules with different lipophilicities into skin by altering the conformation of acyl chain of skin lipids [22]. Interestingly, disruption of the SC barrier was found to be more profound with TAT and R₁₁, compared to R₈. It is reported that polar polycationic peptides and proteins related to the TAT family may utilize the inverted-micellar mechanism [26]. These reports have speculated that CPPs can interact with the lipids differently depending on the type of CPP and the type of lipid.

We have observed clear correlations between ³¹P MAS solid-state NMR peak positions from skin samples and CPP permeation into the skin. Since ³¹P (spin-1/2) has a 100% natural abundance, all P containing compounds in the skin are expected to contribute to observed signals in a way that is roughly proportional to molecular populations. Compounds found in skin which would contribute to ³¹P NMR signals include macromolecules such as phospholipids, DNA, and phosphorylated proteins, as well as water-soluble compounds such as phosphate ions and adenosine tri-phosphate. The line widths observed in the present data (0.6 ppm full width at half maximum) are considerably larger than typically expected for soluble species homogeneous solutions. In tissue samples, all NMR signals are likely to be broadened by anisotropies and local gradients in magnetic susceptibility. MAS is expected to significantly reduce line broadening by averaging away the effects of magnetic susceptibility gradients, though it will not eliminate all effects of heterogeneous magnetic susceptibilities [37]. We suggest that signals from soluble species are eclipsed by stronger signals from insoluble species. We believe the primary source of our observed NMR signals is skin phospholipids for the following additional reasons: 1) in rat skin epidermis, 20 weight percent (dry weight) is accounted for total lipids and about 35% of that lipid is phospholipids [38]; 2) CPPs are known to interact with and affect ³¹P NMR signals from lipid bilayers as described below, whereas CPPs are not known to interact strongly with other biological macromolecules or soluble species. In the present studies, water was used to dissolve the peptides or FLip to avoid expected interference with observed NMR signals.

To further investigate the ability of CPPs to partition in the skin, the in vitro skin permeation of CPPs was evaluated by CLSM. CLSM results complement the findings based on solid-state NMR, confirming that the permeated skin sections of R₁₁ and TAT showed enhanced diffusion into the skin. CLSM also showed less permeation for R₈ and YKA at tested concentration (50 mg/ml). The close observation of fluorescence from images suggests that R₁₁ penetrates the skin through an intercellular pathway. Further studies were performed to show the effective delivery of FLip using R₁₁ tagging on the surface. FLip without R₁₁ surface modification has limited permeation only up to 120 μm. Similar observations have been reported from our laboratory where R₁₁ delivered encapsulated DiO dye from nanostructured lipid carriers was observed in rat skin at depths up to 240 μm through the intercellular pathway [19]. It was previously postulated that R₁₁ tagged to nanostructured lipid carriers has the ability to deliver encapsulated fluorescent dye [3, 11, 19]; small drugs like celecoxib [11, 18], ketoprofen [19], and peptides like Spantide II [19]. These results also support the interpretation that R₁₁ can enhance the diffusion of these molecules (liposomes and encapsulated DiD dye) into intercellular lipids of the SC.

From the studies already conducted in our laboratory, current experiments and understanding from the literature, the following sequence of events can be hypothesized after topical application of the CPPs consisting of mostly polyarginines (i) intimate complexes may form between the lipid phosphate groups and arginine side chains; (ii) this may cause defects into the lipid layer by influencing the packing of the lipid structures, that

make the hydrophobic bilayer accessible to the external topically applied molecules; (iii) if the concentration of the locally applied linear peptide is sufficient then this may result in significant interaction between skin-lipids and CPPs, therefore finally CPPs can perturb the lipid layers allowing the CPP and/or cargo molecules to translocate across the skin layers. Hence, coating of R₁₁ on the surface of the liposomes helps in delivering encapsulated lipophilic fluorescent dye into deep epidermis or at epidermal-dermal junction.

5. CONCLUSIONS

The present study establishes that a solid-state NMR observable, namely the ³¹P chemical shift, is indicative of the presence of CPPs in rat skin up to depths of 180 μm. The observed effect depends monotonically on peptide concentration. The magnitude of the effect depends on the peptide used in permeation studies, with the largest effects observed for the peptides known to permeate most effectively. Furthermore, the solid-state NMR measurement does not require introduction of isotopic or fluorescent labels or radioactive tags, which could modify the behavior of CPPs. While CPP concentrations in the present sample were too low for conventional NMR detection of peptide signals within rat skin, emerging solid-state NMR signal enhancement techniques based on dynamic nuclear polarization may help overcome this limitation [34]. While direct detection of CPP using NMR signal will require isotopic labeling with ¹³C and ¹⁵N. Future, structural analysis of CPP associated with skin lipids may yield more specific information about the nature of CPP-lipid interactions. These interactions promote effective permeation in the in vivo-derived samples. When CPPs are tethered to cargo-carrying constructs such as vesicles, NMR detection of CPP signal could allow researchers to quantify the integrity of CPP in skin co-localization with cargos.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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- <i>sn</i> -glycero-3-[(<i>N</i> -(5-amino-1-carboxypentyl) imidodiacetic acid) succinyl nickel salt]
FLip	fluorescent dye containing liposomes
FLip-R₁₁	Polyarginine-11 (R ₁₁) coated FLip
FLip-YKA	YKA coated FLip
MAS	Magic angle spinning
PI	Polydispersity index

R₁₁	Polyarginine-11
R₈	Polyarginine-8
SC	Stratum corneum
TAT	Trans-acting activator of transcription

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Figure 1.

The ^{31}P solid-state NMR spectra – Effect of TAT concentration on skin: Spectra correspond to the following TAT concentrations during skin permeation experiments: 0 mg/ml (black, control), 25 mg/ml (red), 50 mg/ml (blue), 100 mg/ml (green) and 200 mg/ml (orange).

Figure 2.

^{31}P NMR maximum peak position versus TAT concentration for spectra in Figure 1: Values are reported relative to the position of the control. Estimated uncertainties in line position are on the order of the symbol size.

Figure 3.

^{31}P NMR spectra – Effect of different peptides on ^{31}P peak position for skin layers at two different peptide concentrations: a – c) ^{31}P NMR spectra for different skin layers obtained through 16 h of permeation using 25 mg/ml solutions of peptide. (d – f) Spectra collected for similar experiments, using 50 mg/ml solutions of peptide. Colored ^{31}P spectra correspond to permeation with no peptide (black, control); YKA (green, negative control); R₈ (orange); R₁₁ (red) and TAT (blue).

Figure 4.

^{31}P NMR spectra – Effect of time on skin penetration of R_{11} (25 mg/ml): Spectra correspond to a skin depth of 61–120 μm and R_{11} permeation times of 0.5 (red), 1 (blue), 2 (green) and 4 h (orange).

Figure 5.

Confocal laser scanning microscopy (CLSM) of lateral sections of fluorescent-CPP permeated skin: In vitro rat skin permeation of fluorescent dye (FITC) labeled CPPs was carried out for 16 h and then collected skin biopsies were subjected to lateral sectioning up to a depth of 200 μm with a cryotome and observed under CLSM for skin associated fluorescence. The rows indicate the depths of the skin sections.

Figure 6.

Comparison of the fluorescence percent intensity of the TAT, R₈, R₁₁ and YKA permeated skin sections: Fluorescence was calculated from confocal images using Digital image software. The percent intensity of the images was plotted against the skin depth for different CPPs. Data represent mean $\pm$ SEM (n=4); A significant effect was observed between TAT and R₁₁ compared to YKA, *p < 0.05. SEM: standard error of mean.

Figure 7. Confocal laser scanning microscopy (CLSM) of lateral sections of surface modified and unmodified FLip permeated skin: After 16 h of in vitro rat skin permeation, skin was collected and lateral skin sections were observed under CLSM. Lateral skin sections of different depths from 0 to 200 μm are represented in each row.

Figure 8.

Comparison of fluorescence percent intensity of FLip, FLip-R₁₁ and FLip-YKA permeated skin sections: Fluorescence was calculated from confocal images using Digital image software. The percent intensity for each image was plotted against the skin depth for surface modified and un-modified FLip. Data represent mean $\pm$ SEM (n=4); significance FLip-R₁₁ against FLip and FLip-YKA, *p < 0.05; SEM: standard error of mean.