Pharmaceutical nanotechnology

Doxorubicin liposomes as an investigative model to study the skin permeation of nanocarriers

Cedar H.A. Boakye, Ketan Patel, Mandip Singh *

College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, FL 32307, USA

A R T I C L E   I N F O

Article history:
Received 2 February 2015
Received in revised form 2 April 2015
Accepted 19 April 2015
Available online 21 April 2015

Pubchem:
Doxorubicin HCL
Polyethylene glycol
Coumarin 6
1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine
1,2-Dioleoyl-3-trimethylammonium-propane (chloride salt)
1,2-Dioleoyl-sn-glycero-3-phosphate
1,2-Distearyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)-2000] (ammonium salt)
1,2-Dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)imidodiacetic acid) succinyl nickel salt]

Keywords:
Topical drug delivery
Doxorubicin liposomes
Skin permeation
Cell penetrating peptides
Tape stripping
Hair follicle blocking

A B S T R A C T

The objectives of this study were to develop an innovative investigative model using doxorubicin as a fluorophore to evaluate the skin permeation of nanocarriers and the impact of size and surface characteristics on their permeability. Different doxorubicin-loaded liposomes with mean particle size <130 nm and different surface chemistry were prepared by ammonium acetate gradient method using DPPC, DOPE, Cholesterol, DSPE-PEG 2000 and 1,1-Di-((Z)-octadec-9-en-1-yl) pyrrolidin-1-ium chloride (CY5)/DOTAP/12-dioleoyl-sn-glycero-3-phosphate (DOPA) as the charge modifier. There was minimal release of doxorubicin from the liposomes up to 8 h; indicating that fluorescence observed within the skin layers was due to the intact liposomes. Liposomes with particle sizes >600 nm were restricted within the stratum corneum. DOTAP (p < 0.01) and CY5 (p < 0.05) liposomes demonstrated significant permeation into the skin than DOPE and PEG liposomes. Tape stripping significantly (p < 0.01) enhanced the skin permeation of doxorubicin liposomes but TAT-decorated doxorubicin liposomes permeated better (p < 0.005). Blockage of the hair follicles resulted in significant reduction in the extent and intensity of fluorescence observed within the skin layers. Overall, doxorubicin liposomes proved to be an ideal fluorophore-based model. The hair follicles were the major route utilized by the liposomes to permeate skin. Surface charge and particle size played vital roles in the extent of permeation.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

The skin presents a superior advantage for the delivery of therapeutic cargos over other conventional routes such as oral administration, intravenous and subcutaneous injections (Guy et al., 1987). The approaches utilized for topical drug delivery are mostly less invasive, widely accepted by patients and do not require specialized training for drug administration. However, the skin puts up a barrier function against the permeation of foreign materials into the viable epidermis (Curdy et al., 2001). This protective measure is to prevent water loss as well as to inhibit the invasion of pathogenic substances that will cause infection to the body (Elias et al., 2014; Feingold and Elias, 2014; van Smeden et al., 2014).

The stratum corneum, which forms the outermost layer of the epidermis, is the main barrier structure of the skin. According to the “brick-and-mortar” model, the stratum corneum comprises of horny corneocytes, which are dead keratinocytes filled largely with keratin, interspersed within a lipid matrix consisting of free fatty acids, ceramides and cholesterol (Gray and Yardley, 1975). The lipid

http://dx.doi.org/10.1016/j.ijpharm.2015.04.059
0378-5173/© 2015 Elsevier B.V. All rights reserved.
lamellae domain is demonstrated as an extended lipid bilayer that has one bilayer stacked on top of the other (Iwai et al., 2012). This hence presents an impermeable barrier that prevents the entry of mostly hydrophilic and macromolecular substances. A lot of research strategies have been exploited to overcome the skin barrier temporarily in order to deliver therapeutic agents into the skin. Diverse permeation enhancers including cell penetrating peptides (CPP), fatty acids, lipids, polymers and surfactants have been employed to either create channels or aqueous pores within the stratum corneum to enhance permeation (Desai et al., 2014; Marepally et al., 2013).

Nanoparticles have gained interest for the enhanced topical delivery of drugs or macromolecules (Alvarez-Roman et al., 2004, b; Shah et al., 2012a,b). Different polymer and lipid based nanosystems including solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), nanoemulsions, polymeric nanoparticles etc. have been utilized to encapsulate these impermeable and macromolecular drugs to enhance their surpassing of the stratum corneum barrier. Further, surface functionalized nanoparticles like TAT surfaced modified NLC and oleic acid modified polymeric nanoparticles have been used to augment the nanoparticle driven enhanced skin permeability (Shah et al., 2012a,b). Although a lot of literature is available on the improvement of the permeation of hydrophilic or macromolecular drugs into skin via these systems, the mechanisms involved have always been a matter of debate. The question is whether the nanoparticles carry the drugs into the deeper layers of the skin or if the solubility/permeability enhancement of the drugs (resulting from their incorporation into the nanoparticles) is responsible for the higher skin permeation observed.

Some of the pathways suggested for the percutaneous entry of nanoparticles include the transcellular, paracellular and transappendageal (i.e., through sweat glands and hair follicles) routes (Tran, 2013). Skin hair follicles have gained a lot of attention for exploitation for the delivery of therapeutics into the skin. They were previously suggested to play a minor role in the topical delivery of drugs due to the fact that they occupy a small surface area of the skin (0.1%). However, current studies have shown that the skin hair follicles penetrate deeply into the dermal layer with rich perifollicular blood supply and provision for a large surface area for permeation (Lademann et al., 2001). They have hence been suggested to play crucial roles in the permeation of particularly hydrophilic and macromolecules as well as nanoparticles by serving as reservoirs of high drug concentration from which the drugs leach out into the skin domain (Dokka et al., 2005).

Further, some studies have illustrated that the stratum corneum serves as a short-term reservoir for storing nanoparticles in contrast to the hair follicles. Researchers have attributed this phenomenon to the continuous depletion of the dead stratum corneum layer through desquamation and replacement with new cells. They have hence been indicated to play less important roles in the permeation of substances compared to the hair follicles (Lademann et al., 2006; Weigmann et al., 2001). In contrast, other studies have indicated that certain lipophilic substances permeate the skin significantly by utilizing the intercellular lipid matrix and diffusing further down into the epidermis whilst few others permeate directly across the corneocytes into the deeper skin layers. In spite of all these views, there still remains speculation about the ultimate route employed for the permeation of nanoparticles across the stratum corneum into the skin.

Many studies have exploited fluorophores such as nile red, rhodamine, dialkylcarbocyanine derivative, DiO (D275), fluorescein, coumarin 6 and fluorescein isothiocyanate (FITC) labeled macromolecules to track nanoparticles in the skin layers. FITC labeled macromolecules and other lipophilic dyes are easily released from nanoparticles and they diffuse readily into the deeper skin layers. Nanoparticle formulations usually contain non-ionic surfactant which helps in breaching the stratum corneum (SC) barrier. As a result, lipophilic fluorophores by virtue of their high lipid solubility themselves cross the SC and permeate deeply into the dermis. Hence, it has been difficult to conclusively state that the fluorescence observed at the various depths of the skin is due to the intact nanoparticles or due to the fluorescent cargos which have diffused out of the nanoparticles. Therefore, the objectives of this study were firstly to develop and evaluate a nanocarrier system encapsulated with a poorly permeable and diffusible hydrophilic fluorophore; and secondly to investigate the impact of size and surface characteristics of nanocarriers on their permeability to the viable deep epidermal and upper dermal layers. Doxorubicin liposome prepared by the pH gradient method was chosen as the model for the studies because of the low diffusivity of the encapsulated drug from the liposomes. DoxIL, which is prepared by the same method, has been shown to be stable for a long period of time with no or very little leakage of doxorubicin from the core of the liposomes (Elbayoumi and Torchilin, 2008, 2009; Lukyanov et al., 2004). Hence, in this study, we purport that any fluorescence observed would be due to the intact doxorubicin liposomes and not due to leaked free drug.

2. Materials and methods

2.1. Materials

1,1-Di-(((Z)-octadeic-9-en-1-yl) pyrroolidin-1-ium chloride (Cy5 lipid) was synthesized as reported (Marepally et al., 2014), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-N-[amino(polyethyleneglycol)-2000] (ammonium salt) (DSPE-PEG 2000), 1,2-dioleoyl-sn-glycero-3-[[N-(5-amino-1-carboxyethyl) imidodiacid acid] succinyl nickel salt] (DOGS-NTA-Ni) and cholesterol were purchased from Avanti Polar lipids (Alabaster, AL, USA), Doxorubicin HCl (DOX), coumarin-6 (C6) dye, sephadex® (G-25), polyethylene glycol (PEG), HPLC grade water and ethanol were procured from Sigma-Aldrich Co (St. Louis, MO, USA). FITC-labeled siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Histidine tagged TAT peptide (YGRKKRRQRRRHHHHH-FITC, MW: 2382.67) was synthesized by GenScript Corporation (Piscataway, NJ, USA). All other chemicals used in this research study were of analytical grade.

2.2. Animals

CD®(SD) hrBi hairless rats (weighing 250–300 g; male; Charles River Laboratories, Wilmington, MA) were grouped (n = 3) in cages with bedding and were kept under controlled conditions of 12:12 h light: dark cycles, 22 ± 2 °C and 50 ± 15% relative humidity. The rats were provided with feed (Harlan Teklad) and water ad libitum. They 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 acclimated to laboratory conditions for a week prior to the start of any experiment. The protocol of animal study was approved by the Institutional Animal Care and Use Committee (IACUC), Florida A&M University, FL, USA.

2.3. Preparation of skin

For rat skin, CD®(SD) hrBi hairless rats were sacrificed by exposure to an overdose of halothane anesthesia. The dorsal skin
was collected, the adherent subcutaneous fat and connective tissue were carefully removed without damaging the skin and then the skin was rinsed in physiological saline.

For porcine skin, porcine ears (Yorkshire marine pigs, male, weighing about 200lb) were obtained from a local slaughterhouse (Limestone Meat House, Monticello, FL, USA). The ears were cleaned under cold running water and then dermatomed carefully to remove the epidermal and upper dermal layers without causing damage.

Both types of skin were soaked in 10% v/v glycerol in normal saline solution for 30 min and then stored at –80 °C until used. Prior to use, each skin was rinsed in phosphate buffer solution (pH 7.4) for 30 min.

2.4. Preparation of liposomes

Doxorubacin liposomes were prepared by the pH gradient method with modifications (Zhu et al., 2014). Briefly, DOTAP/cy5: DPPC:DOPC:Cholesterol:DSPE-PEG 2000 (5:20:5:4:2.5) weight ratio were dissolved in 200 µl of ethanol and heated in a water bath to a temperature range of 50–60 °C to constitute the organic phase. Further, ammonium acetate (300 mM) was dissolved in water to make up the aqueous phase and was then brought up to the same temperature as the organic phase. The lipid phase was injected into the aqueous phase and stirred continuously for 1 h to evaporate the ethanol. The particle sizes of the liposomes were reduced by ultrasonication (Brenson Probe Sonicator, USA) for 2 min and then allowed to cool to room temperature.

The pH gradient system of the liposomes was created by separating unentrapped ammonium acetate by passing the freshly prepared liposomes through sephadex (G-25) column. Doxorubicin was loaded (6.85% drug loading) by incubating with the liposomes at 50–60 °C for 1 h. Free drug was separated using sephadex column. Coumarin-6 and FITC-labeled siRNA liposomes were also prepared by the ethanolic injection method.

Doxorubicin liposomes with different surface charges were prepared by the same method; the weight equivalent of cy5 lipid was replaced with DOTAP, PEG and DOPA lipids in each instance to prepare positively, neutral and negatively charged liposomes, respectively. The prepared liposomes (total of two cationic, one non-ionic and one anionic liposomes) were used for skin permeation studies.

Further, to evaluate the effect of cell penetrating peptides on the skin permeation of the liposomes, surface modification of the liposomes (prepared without cy5 or DOTAP lipids) was carried out with Histidine tagged TAT peptide. The same procedure was followed for the preparation of the liposomes using the linker, DOGS-NTA-Ni in place of cy5 or DOTAP lipids. The liposomes were then incubated with TAT peptide (1:2 ratio with DOGS-NTA-Ni) at room temperature on constant stirring for 30 min and then loaded with doxorubicin. The physical mixture was prepared in the same way as dox TAT liposomes but without the linker, DOGS-NTA-Ni.

2.5. Characterization of liposomes

The particle size and zeta potential of the liposomes were determined using Nicomp 380 ZLS (Particle Sizing Systems, Port Richey, FL), which utilizes dynamic light scattering and particle electrophoretic mobility to measure particle size distribution and zeta potential, respectively.

The entrapment efficiency was determined as reported earlier by (Patilola et al., 2010a). Briefly, the liposomes were put in the donor compartment of a vivaspin centrifuge filter membrane and centrifuged at 5000 rpm for 15 min. The amount of drug present in the receiver compartment (RC) was determined by spectrophotometric analysis at 485/530 nm. The entrapment efficiency was calculated using the formula:

\[
\text{Entrapment efficiency} = \frac{[\text{the total drug content} - \text{amount of drug in RC}] \times 100}{\text{the total drug content}}
\]

2.6. In vitro drug release studies

A semi-permeable dialysis membrane of molecular weight cut off (MWCO) of 25,000 Da (Sigma–Aldrich Co., MO) was used for the release study. The membrane was mounted between the donor and receiver compartments of Franz diffusion cells (PermeGear Inc., Riegelsville, PA). 150 µl of DOX liposomes was applied evenly on the surface of the membrane in the donor compartment. The receiver compartment was filled with PBS (pH 7.4), was stirred at 300 rpm and maintained at 37 ± 0.5 °C using a circulating water bath. At predetermined time intervals (0.5, 1, 2, 3, 4, 6, 8 and 12 h), 0.3 ml samples were collected from the receiver compartment and replaced with fresh PBS buffer. The samples collected from the receiver compartment were then analyzed using spectrophotometry.

2.7. In vitro skin permeation studies

Skin permeation studies were performed using established procedures per (Patilola et al., 2010b). Briefly, the skin (dermatomed porcine or full thickness rat skin) was mounted on Franz diffusion cells with the stratum corneum in contact with the donor compartment. The receiver compartment was maintained the same as for the drug release studies. 150 µl of each formulation was applied evenly on the surface of the skin in the donor compartment. The receiver compartment was maintained at 37 ± 0.5 °C using a circulating water bath to simulate the skin temperature at normal physiological level. The permeation studies were performed under unocclusive conditions for 6 h.

After the set time for permeation studies, 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 in water and blotted dry with lint-free absorbent wipes. For evaluation of drug retention in the skin, the entire dosing area (0.64 cm²) was collected with a biopsy punch, cryosectioned and analyzed using fluorescence microscopy.

To determine the role of skin hair follicles in the topical delivery of drugs, the hair follicles were blocked as proposed by (Desai et al., 2013). Briefly, each hair follicle opening was blocked using a micro drop of varnish wax mixture from a 1 ml syringe containing a 30G needle and a magnifying glass. The varnish wax was then allowed to dry and the skin was mounted between the donor and receiver compartments of the Franz diffusion cell as previously mentioned. Permeation studies were performed as stated previously and analyzed.

To ascertain the importance of the stratum corneum in skin barrier functions, tape stripping was carried out to partially remove the stratum corneum as proposed by (Bronaugh and Stewart, 1985). The method followed is in accordance with (Alberti et al., 2001). Briefly, an adhesive contact tape (Scotch® Book Tape) was pressed evenly unto the surface of the skin. The direction of stripping was changed each time. To validate the number of tape stripplings required, the stripping was performed 5–15 times on blank rat skin and the skin was analyzed through microscopic imaging. For all other experiments performed, 10-tape stripping was done and the skin was subjected to the same permeation studies and analysis as stated above.
2.8. Skin imaging studies

Microscopic imaging was accomplished using a confocal laser-scanning microscope (CLSM) (Leica Microsystems Inc Buffalo Grove, IL, USA) equipped with a Digital image software (Museum of Science, Boston, MA, USA) with a 10× objective and using an Olympus BX40 microscope equipped with computer-controlled digital camera (DP71, Olympus Center Valley, PA, USA) (10× and 20× objectives) to establish the skin distribution of dox liposomes. The CLSM images were obtained as compressed figures of 10 z-stack images across 60-micron thick porcine skin. Full thickness cross-sections (30 μm) and lateral sections (60 μm) were obtained via cryosectioning using a cryomate (Shandon Scientific Ltd., England). The skin sections were visualized and analyzed for fluorescence from dox. The quantity of fluorescence was calculated using Image software to determine the amount of dox present.

2.9. Statistical analysis

The differences between the skin permeation of C6 liposomes, FITC labeled siRNA and DOX liposomes were determined using ANOVA and Student T test for comparison among multiple and between two groups, respectively. Mean differences with **p < 0.005, *p < 0.01 and +p < 0.05 were considered to be significant.

3. Results

3.1. Characterization of liposomes

The particle sizes, polydispersities (P.I.) and zeta potentials of the liposomes prepared with cy5, DOTAP, PEG and DOPA lipids have been represented in Table 1. DOTAP liposomes showed the least particle size of 107.50 ± 9.23 nm whilst DOPA liposomes showed the greatest particle sizes of 254.10 ± 11.53 nm. Both cy5 and PEG liposomes showed relatively similar particle size (127.98 ± 25.44 – 128.35 ± 191 nm). The P.I.s of the different liposomes showed a narrow particle size distribution (0.065 ± 0.0001 – 0.242 ± 0.004).

Further, DOTAP and cy5 lipids produced positively charged liposomes with zeta potentials of +16.05 ± 0.40 and +16.96 ± 1.43 mV, respectively. Surface modification of the liposomes prepared without either cy5 or DOTAP lipids also produced positively charged liposomes but with reduced zeta potentials (+9.55 ± 2.25 mV). PEG and DOPA lipids on the other hand, produced negatively charged liposomes (–6.76 ± 0.35 and –8.74 ± 0.71 mV respectively). All the different liposomes prepared showed >70% entrapment efficiency.

3.2. In vitro drug release studies

The objective of this study was to evaluate the efficiency of the liposomes in encapsulating dox and in preventing the drug’s leakage from the core into the exterior. At 8 h, both cy5 and DOTAP liposomes showed less than 30% release of the free drug into the receiver compartment (Figs. 1 and 2a). Doxorubicin solution showed a quick release of drug, which was later on sustained until the end of the studies. At 12 h, there was about 60% of the drug released from the solution. However, both cy5 and DOTAP liposomes showed delayed but sustained release of the drug up to 12 h.

3.3. In vitro skin permeation studies

3.3.1. Skin permeation of FITC labeled siRNA and C6 liposomes

FITC labeled siRNA solution was mostly accumulated within the stratum corneum layer and upper epidermis of the skin whilst the FITC labeled siRNA liposomes showed significant (p < 0.05) permeation with fluorescence intensity observed within the deeper dermal layers of the skin (Fig. 1a). Further, tape stripping of the skin did not result in any significant difference between the amount of permeation of FITC labeled siRNA liposomes in intact stratum corneum and tape stripped skin. The converse was however observed for FITC labeled siRNA solution; there was a significant difference (p < 0.05) observed between the extent of permeation of the solution in intact stratum and tape stripped skin.

This was similar to the results observed for the skin permeation of C6 (Fig. 1b). The fluorescence was observed mostly within the stratum corneum layer of the skin whilst there was significantly (p < 0.05) increased permeation and fluorescence intensity noted for C6 cy5 liposomes and C6 cy5 + DOTAP liposomes.

3.3.2. Validation of tape stripping

The microscopic images (Fig. 2b) showed a corresponding decrease in the thickness of the stratum corneum with increase in the number of tape stripping. There was no significant difference observed between the thickness of the stratum corneum without tape stripping and with 5× tape stripings. 15× tape stripings however almost removed the total stratum corneum layer. Further, 10× tape stripings was selected for all experiments because it substantially reduced the thickness of the skin barrier without totally ridding off the stratum corneum.

3.3.3. Time-dependent study of skin permeation with DOX liposomes

The analysis showed that there was increase in the extent of skin permeation of both dox solution and dox liposomes with increase in duration of permeation studies. However, the permeation of dox liposomes was significantly (p < 0.05) more than dox solution (2.0 and 2.4-fold more at 3 and 6h, respectively). Further, permeation duration of 6h resulted in 1.5-fold more skin permeation of dox liposomes than 3h. The results have been illustrated in Fig. 3a.

3.3.4. Effect of tape stripping of skin on permeation of DOX liposomes

The results showed that tape stripping increased the permeation of dox liposomes (Fig. 3b) into rat skin. In normal skin, the red fluorescence due to dox was observed mainly within the hair follicles with minimum amount observed in the skin matrix. However, tape stripping resulted in more diffusion of the dox liposomes and was quantified to be about 1.5-fold more. Further, permeation of dox liposomes with or without tape stripping showed significantly (p < 0.05 and p < 0.01) more red fluorescence than dox solution.

Table 1

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle size (nm)</th>
<th>P.I.</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX cy5 liposomes</td>
<td>127.98 ± 25.44</td>
<td>0.18 ± 0.05</td>
<td>+16.96 ± 1.43</td>
</tr>
<tr>
<td>DOX DOTAP liposomes</td>
<td>107.50 ± 9.23</td>
<td>0.18 ± 0.02</td>
<td>+16.05 ± 0.40</td>
</tr>
<tr>
<td>DOX PEG liposomes</td>
<td>128.35 ± 191</td>
<td>0.38 ± 0.16</td>
<td>–6.76 ± 0.35</td>
</tr>
<tr>
<td>DOX DOPA liposomes</td>
<td>254.10 ± 11.53</td>
<td>0.065 ± 0.001</td>
<td>–8.74 ± 0.71</td>
</tr>
<tr>
<td>DOX TAT liposomes</td>
<td>150.70 ± 10.12</td>
<td>0.219 ± 0.01</td>
<td>+9.55 ± 2.25</td>
</tr>
<tr>
<td>DOX TAT physical mixture</td>
<td>116.70 ± 1.84</td>
<td>0.242 ± 0.004</td>
<td>+1.72 ± 0.80</td>
</tr>
</tbody>
</table>
3.3.5. Effect of hair follicle blocking on skin permeation of DOX liposomes

Blocking the hair follicles of porcine skin prior to permeation studies caused significant reduction in the skin permeation of DOX liposomes compared to normal skin (no hair follicle blocking). There was red fluorescence observed up to a depth of 180 μm for normal skin whereas there was very little fluorescence observed even up to 60 μm for skin with hair follicles blocked. Further, tape stripping significantly improved the permeation of DOX liposomes up to a depth of 360 μm. However, there was a reduction in the extent of skin permeation (red fluorescence was observed only up to 180–240 μm) when the hair follicles were blocked even with tape stripping. The results have been illustrated in Fig. 4a and b.

3.3.6. Effect of charge on skin permeation of DOX liposomes

The results showed that the positively charged liposomes permeated into the skin better than the negatively charged liposomes (Fig. 5). Both DOTAP and cy5 liposomes permeated deeper into the skin and showed more significant (p < 0.01 and p < 0.05, respectively) fluorescence intensity than both DOPA and PEG liposomes. However, DOTAP liposomes permeated better than cy5 liposomes into the skin. DOTAP liposomes showed 2.4, 2.0 and 1.3-fold more fluorescence intensity than DOPA, PEG and cy5 liposomes, respectively. For the negatively charged liposomes, red fluorescence was observed only in the stratum corneum layer.

3.3.7. Effect of size on skin permeation of DOX liposomes

The analysis revealed that there was significant (p < 0.005) reduction in the skin permeation of DOX liposomes with increase in particle size (Fig. 6). Dox liposomes with particle size range of 600–1000 nm were mainly retained on the skin surface in the stratum corneum layer whilst those of sizes of ~100 nm permeated more and revealed significantly more fluorescence. The quantified red fluorescence for liposomes of particle size of ~100 nm was 8.4 and 10.4-fold more than liposomes of size ~600 nm and ~1000 nm, respectively.

3.3.8. Effect of surface modification of DOX liposomes with TAT peptide on skin permeation

Surface modification of dox liposomes with TAT peptide significantly (p < 0.005) enhanced the skin permeation of the liposomes (Fig. 7). Dox TAT liposomes showed 7.0 and 4.5-fold more red fluorescence than dox solution and physical mixture, respectively. The fluorescence was observed at the deeper layers of the skin whilst dox solution and the physical mixture showed fluorescence mainly in the stratum corneum and epidermal layers, respectively.

Further, hair follicle blocking reduced the extent of permeation of dox TAT liposomes although there was still substantial fluorescence observed at the deeper layers of the skin. The quantified fluorescence due to permeated dox TAT liposomes in skin with hair follicles blocked was however 2.8 and 1.9-fold more than fluorescence quantified due to permeation of dox solution and physical mixture in normal skin.

4. Discussion

The initial experiments of this study were carried out using C6 liposomes and FITC labeled siRNA liposomes to comprehend the role nanocarriers play in the permeation of these lipophilic cargos into the skin as well as the effect skin tape stripping has on their permeability. As shown in Figs. 2 and 3, C6 and FITC labeled siRNA liposomes both exhibited significant permeation across the skin.
layers into the dermis; and this was more pronounced after tape stripping, especially for their solutions. This indicates that such molecules have a good permeability across the skin layers once their nanocarriers facilitate their entry across the stratum corneum. Further, although the nanocarriers may be confined within the hair follicles, these fluorescent molecules can diffuse into the surrounding skin tissues and deeper layers beneath. Hence, it is impossible to confirm if the observed fluorescence is from the free molecules or from the nanocarriers encapsulating the fluorophore cargos. The observation of fluorescence is therefore not the true reflection of the presence of nanocarriers within the skin layers.

In contrast, such phenomenon was not observed with doxorubicin liposomes. The results of this study demonstrate for the first time that the dox liposomes prepared are a good model for the investigation of the permeation pathways utilized by nanocarriers for entry into the skin as well as of the roles played by surface chemistry, size and permeation enhancers on the extent of permeation of nanoparticles into the skin. This can be attributed to observation that there was low diffusivity and leakage of dox from the pH gradient liposomes into the exterior buffer. The release studies indicated that there was minimal diffusion of the free drug from the core of the liposomes for up to 8 h, which was in agreement with previous studies that have illustrated that dox liposomes prepared by the pH gradient method including Doxil® are stable and show negligible leakage of the drug (Levacheva et al., 2014; Lukyanov et al., 2004). Further, this time period was greater than the duration of the permeation studies; hence, suggestive that all the fluorescence observed within the skin layers was due largely to the intact dox liposomes. The low diffusivity and minimal leakage of dox from the liposomes into the exterior buffer thus assured that fluorescence observed in the skin was mostly due to the liposome instead of free drug.

Also, the physicochemical characteristics of dox including its hydrophilicity and charge (Herai et al., 2007) cause it to be highly impermeable across a lipophilic membrane barrier; hence it is unlikely that the diffused free drug would be able to achieve substantial permeation across the stratum corneum into the dermis. This was illustrated in the permeation studies carried out with dox solution, which revealed fluorescence only at the surface of the skin and within the hair follicles. Also, even after tape stripping (10×), the free drug failed to diffuse out of the hair follicles or skin surface into the viable epidermal layer beneath. However, there was substantially greater staining of the hair follicles with tape stripping than without, which can be due to the removal of the skin barrier. This therefore shows that doxorubicin has no permeability within the skin layers and any fluorescence observed within the skin layers can only be attributed to its entrapment within the core of the liposomes.

Noteworthily, all the different liposomes prepared showed particle sizes less than 130 nm with the exception of those prepared with DOPA, which showed size of about 250 nm. This greater size might have contributed to the retardation of the permeation of the DOPA liposomes additionally to that caused by the negative charges present on the surface of the liposomes. However, PEG liposomes, which also had negative charges, were of smaller size (<130 nm) but they were mostly restricted to the stratum corneum and upper epidermis. This hence suggests that the surface charges of nanocarriers play crucial role in the extent of their permeation into the skin layers and in their accumulation within the stratum corneum.

Fig. 2. (A) Graph illustrating the drug release profile of doxorubicin from solution, DOTAP liposomes and cy5 liposomes. (B) Bright field microscopic illustration of the cross section of untreated rat skin. Images show the corresponding decrease in the thickness of the stratum corneum with increase in the number of tape stripping.
(Gillett et al., 2011). This may be due to the fact that the skin surface together with the hair follicles, serves as a negatively charged membrane due to the relatively higher ratio of negatively charged skin lipids (which produce a net negative charge) (Sinico et al., 2005; Yoo et al., 2008) and hence repels particles of similar charges and prevents their permeation across it.

The contrary was observed for the positively charged liposomes, DOTAP ($p < 0.01$) and cy5 ($p < 0.05$) liposomes, which both showed significant increase in the permeation of dox into the deeper dermal layers of the skin. This has been proposed that positively charged liposomes are absorbed to a greater extent across the stratum corneum than negatively charged ones (Manosroi et al., 2004). This has been suggested to be as a result of the stronger electrostatic interaction between the positively charged liposomes and the skin lipids/skin surface, which further enhances permeation. This was demonstrated by Hasanova et al. (Hasanovic et al., 2010) in a study to show that the addition of cationic polymers such as chitosan to DPPC liposomes increased the skin permeation of the positively charged-coated liposomes in comparison to the uncoated liposomes.

Another important observation made in this study was the effect of size on the permeation of the liposomes. DOTAP liposomes of average particle size of 100 nm showed significant ($p < 0.005$) permeation into the deeper dermal layer whereas 600 nm and 1 μm sizes did not permeate the skin and were restricted to the upper epidermis and stratum corneum, respectively. Many researchers have shown that particles of less than 500 nm have the propensity to cross the stratum corneum whilst larger ones including micron-sized nanocarriers, peptides and proteins are mostly restricted because they cannot utilize any of the permeation pathways available including directly across or between the corneocytes or through the hair follicles.

The hair follicles have been suggested to play very critical roles in the permeation of nanocarriers across the stratum corneum. They have been indicated to possess a large surface that can serve as reservoirs for the collection of nanocarriers from which the entrapped drugs diffuse out farther into the skin layers. However, not many definitive studies have been carried out to conclusively confirm that nanocarriers permeate the skin mostly by the transfollicular pathway. Using this investigative model, the permeation data revealed that the dox liposomes utilized the hair follicles to a great extent to access the skin layers beneath. This was observed in the substantial fluorescence observed within the hair follicles at both 3 and 6 h of permeation studies, which were due to the collection of the dox liposomes within the infundibulum of the hair follicles.

Further, this phenomenon was confirmed with the blocking of the hair follicles, which revealed significant reduction in the amount of fluorescence observed within the skin layers in comparison to control (skin with open hair follicles) after 2 stack confocal microscopic analysis. However, with tape stripping, even with the hair follicles blocked, there was substantial increase in the permeation of the dox liposomes; suggestive of the fact that the tape stripping provided alternative routes of permeation for the liposomes into the skin when their major permeation pathway (hair follicles) was removed. However, the extent of permeation was not comparable to the situation whereby the hair follicles were intact and the stratum corneum was additionally removed by tape stripping. This further supports the proposal that the hair follicles play a very essential role in the overall percutaneous delivery by allowing nanocarriers to accumulate and disperse further into the skin layers. Shim et al. have demonstrated that minoxidil-loaded nanoparticles penetrated into the hair follicles of guinea pigs whilst the particle sizes influenced greatly how efficiently these.
Fig. 4. Effect of tape stripping and hair follicle blocking on the skin permeation of doxorubicin carrier in pigskin. (A) Confocal z-stack microscopic analysis showing red fluorescence due to permeated doxorubicin from Cy5 liposomes at different skin depth. (B) Surface Plot depicting the fluorescence intensity at 61–120 μm for normal skin, tape stripped skin, skin with hair follicles blocked and tape stripped skin with hair follicles blocked, respectively. Results show that the skin hair follicle is the major route of permeation of the doxorubicin liposomes with tape stripping further enhancing permeation of the liposomes via other possible routes.
nanoparticles permeated into the skin (Shim et al., 2004). Others have shown through diverse analytical techniques to visualize the transfollicular penetration and aggregation of microparticles and nanoparticles including the study of Lademann et al., which utilized tape stripping and spectroscopic analysis to confirm the follicular aggregation of coated titanium dioxide microparticles (Alvarez-Roman et al., 2004; Lademann et al., 1999). Removal of the skin barrier function through tape stripping hence just potentiates the permeation process.

Tape stripping has been extensively utilized in numerous studies to remove the stratum corneum. Some researchers employ this as a tool to quantify the amount of drug that has permeated into the various skin layers (Alberti et al., 2001) by sequentially stripping the skin and extracting the drug in each layer for analysis. Others use this as a tool to enhance the permeation of large hydrophilic molecules and nanocarriers into the skin by perturbing the structural barrier of the skin (Lademann et al., 1999; Nelson et al., 1991). The stratum corneum by virtue of its structural composition of dead keratinocytes interspersed within a lipid matrix presents a barrier, which is more impermeable than the cell membrane and is insurmountable for the permeation of nanocarriers. Tape stripping is therefore an alternative strategy to using permeation enhancers, heating and massaging the skin to overcome the skin barrier to enhance topical drug delivery (Knorr et al., 2009). The results of this study supported this by demonstrating that tape stripping significantly \( p < 0.01 \)

\[ \text{Fig. 5. Effect of charge on the permeation of doxorubicin liposomes into pig skin. Results indicate that the charges present on the surface of the liposomes play significant role in the percutaneous penetration of the doxorubicin liposomes in pig skin.} \]

\[ \text{Fig. 6. Effect of size on the permeation of doxorubicin DOTAP liposomes into pig skin. Results indicate that there is limited permeation of doxorubicin for liposomes with sizes } \geq 600 \text{ nm.} \]
increased the permeation of dox liposomes into the deeper dermal layer than both solution and dox liposomes permeation through intact stratum corneum.

However, one of the most exploited approaches for enhancing skin permeation nanocarriers is surface modification or coating of nanocarriers with cationic polymers, lipids or cell penetrating peptides (CPP). Several studies have illustrated that these permeation enhancers interact with the skin lipids, temporarily disrupt the lipid arrangement and create aqueous pores and channels that facilitate the permeation of nanocarriers (Apte et al., 2014). One commonly employed CPP is TAT, which comprises of six arginine residues and has been proposed to cause enhanced diffusion through the intercellular lipid matrix and the corneocytes of the stratum corneum for improved skin permeation (Desai et al., 2010; Shah et al., 2012a). TAT has also been suggested to increase permeation of nanocarriers by interacting with the keratin filaments present in the corneocytes or by accumulating within the hair follicles and allowing the entrapped drug to diffuse out into the surrounding skin tissue (Knorr et al., 2009). Further, the peptide interacts with the negatively charged skin lipids electrostatically and causes membrane destabilization. These proposed alternate mechanisms to transfollicular transport employed by TAT were illustrated in the event when the hair follicles were blocked but there was still substantial permeation of dox liposomes, although the fluorescence intensity was reduced when compared to permeation across skin with open hair follicles. This suggests an important role played by TAT to enhance the permeation of cargos across the skin barrier.

5. Conclusion

This study suggests strongly that the dox liposomes were an appropriate exploratory model for studying the mechanisms involved in the topical delivery of nanocarriers. The results also indicated that particle size and surface charge played essential functions in the permeation of the liposomes into the skin. The stratum corneum is the major barrier to skin permeation by nanocarriers and was surmounted by tape stripping. Additionally, the transfollicular pathway may be the major pathway utilized by nanocarriers for permeation whilst the transcellular, paracellular and other non-specific routes may be utilized to a minor extent because the blockage of the hair follicles significantly reduced the extent of drug accumulation within the skin layers. Lastly, the surface modification of the liposomes with TAT peptide significantly enhanced the permeation of the liposomes, even in situations when the hair follicles were blocked, confirming the fact that the CPP employs alternate routes for skin permeation aside the transfollicular pathway.

Acknowledgements

The authors would want to extend their gratitude to Ms Ruth Didier of the School of Medicine, Florida State University, Tallahassee, FL. This project was supported by the NIH Minority Biomedical Research Support (MBRS)-SC1 program [Grant # SC1 GM092779-01; MS] and the National Institute of Minority Health and Health Disparities (NIMHD) NIMHD P20 [Grant # 1P20 MD006738-03; MS].

References
