Percutaneous Absorption and Antibacterial Activities of Lipid Nanocarriers Loaded with Dual Drugs for Acne Treatment

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The aim of this study was to develop lipid nanocarriers that combine tretinoin and tetracycline for the efficient topical delivery to treat acne vulgaris. Two different nanocarriers, nanoemulsions (NEs) and nanostructured lipid carriers (NLCs), were prepared, and we examined their average size, zeta potential, drug encapsulation percentage, and drug permeation via the skin. The antibacterial activities of the nanosystems against Staphylococcus aureus, Pseudomonas aeruginosa, and Propionibacterium acnes were evaluated by an agar diffusion assay and the amount of total protein. A ca. 200-nm particle size was achieved with the prepared nanoparticles. The size increased when incorporating a cationic surfactant. Dual-drug loading did not largely affect the size of negatively charged nanoparticles, but significantly reduced the particle size of positively charged nanocarriers. NEs and NLCs exhibited high entrapment of tretinoin which ranged 60–100%. Tetracycline mainly resided in the aqueous phase, with ca. 10% of molecules located at the particulate interface. An in vitro skin permeation study showed that NLCs enhanced tetracycline flux by about 2-times over the control solution. Tretinoin permeation was generally unaffected after nanoparticulate encapsulation. There was no significant difference in tretinoin delivery before or after tetracycline incorporation, while tetracycline permeation significantly decreased by 2-fold in the dual-drug system. Nanoparticulate loading mostly maintained the antibacterial activity of tetracycline. Negatively charged NEs and NLCs even strengthened the antibacterial ability against S. aureus compared to the control solution. This is the first report examining skin permeation and antibacterial activities of dual-drug nanocarriers for acne treatment.

Key words nanoemulsion; nanostructured lipid carrier; tretinoin; tetracycline; percutaneous absorption; antibacterial activity

Almost >80% of people suffer from acne vulgaris during their lives, which is the most frequent disorder involving human skin. Acne is a multifactorial disease with various symptoms such as comedones, nodules, papules, cysts, and pilosebaceous inflammation. Topical treatment is employed as first-line therapy for mild and moderate acne. The topical delivery of tretinoin (all-trans retinoic acid) is the most common treatment for acne with promising efficacy. Tretinoin can increase the turnover of follicular epithelial cells and facilitate comedolysis. Topical tretinoin is also advantageous in increasing skin elasticity and moisture, mitigating fine wrinkles, and preventing photoaging. Nevertheless, topical tretinoin administration consistently leads to skin irritation, a burning sensation, and peeling which may cause discontinuation of the therapy. Moreover, tretinoin is very sensitive in water and surfactant solutions, leading to great degradation during storage. Novel delivery systems can play an important role in overcoming such drawbacks of topical tretinoin. Previous studies showed that entrapment in solid lipid nanoparticles (SLNs) can reduce skin irritation produced by tretinoin and protect the drug from photodegradation and the external environment.

SLNs are derived from oil-in-water nanoemulsions (NEs) by replacing liquid oils with solid lipids. SLNs are less toxic than polymeric nanoparticles. Scaling up production is easier for SLNs compared to polymeric nanoparticles and liposomes.

The close contact with the skin surface by nano-range particles should enhance drug penetration into the skin. A problem related to SLNs is the low drug encapsulation and increased drug expulsion during storage because of the recrystallization process. This can be resolved by employing nanostructured lipid carriers (NLCs), which are composed of a blend of liquid and solid lipids as the inner core. The initial aim of this work was to develop NLC formulations for tretinoin. Besides tretinoin, antibiotics have been widely prescribed for acne patients to inhibit bacterial growth and reduce inflammation. Combined therapy with retinoids and antibiotics is recommended. The primary limitation of combined therapy is the inconvenience of administration. A drug vehicle should first be applied to skin, followed by application of another vehicle after a set time. This may cause poor patient compliance, thus reducing healing rates. Dual drugs in one vehicle system may provide improved efficiency.

It is difficult to incorporate two drugs with various physicochemical properties into a single vehicle. A nanocarrier with both non-polar and polar phases can overcome this issue. Although some investigations prepared lipid nanoparticles for topical tretinoin delivery, there is no study on the development of nanocarriers with dual drugs for complete acne treatment. Our aim was to load tretinoin and antibiotics in NEs and NLCs and examine their skin delivery and therapeutic activities. Tetracycline was used as the model antibiotic because of its anti-inflammatory effect and broad spectrum of antibacterial activity on the skin. The physicochemical
characteristics of the nanocarriers, such as the size, zeta potential, and drug loading efficiency, were examined. In vitro Franz cells were used to determine the skin permeation of both drugs from nanoparticles. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Propionibacterium acnes* were chosen as models for the antibacterial assay of the nanoparticles.

**MATERIALS AND METHODS**

**Materials** Tretinoin, tetracycline HCl, squalene, Pluronic F68 (PF68), and polyethylene glycol (PEG) 400 were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Precirol® ATO 5 (a mixture of 8–22% monoaoyl, 40–60% diacyl, and 25–35% triacyl glyceryl palmitate and stearate) was provided by Gattefossé (Gennevilliers, France). Myverol 18-04K® (palmititic acid monoglyceride) was supplied by Quest (Naarden, the Netherlands). Forestall® (FE, soyaethyl morpholinium ethosulfate) was from Croda (East Yorkshire, U.K.).

**Preparation of NEs and NLCs** Squalene/Preirol and Myverol were mixed as the lipid phase, while the aqueous phase consisted of double-distilled water and PF68. FE was added to the lipid phase if necessary. Both phases were heated separately to 85°C for 15 min. The aqueous phase was added to the lipid phase, and then homogenized using Pro 250 (Pro Scientific, Monroe, CT, U.S.A.) at 12000 rpm for 10 min. The dispersion was further mixed using a probe-type sonicator (VCX 600, Sonics and Materials, Newtown, CT, U.S.A.) for 10 min. Tretinoin (0.05%, w/v) and/or tetracycline (0.5%) were respectively incorporated in the lipid and aqueous phases if necessary. The total volume of the dispersions was 10 mL. Table 1 summarizes the compositions and percentages of the prepared formulations.

**Average Size and Zeta Potential** The average particle size (z-average) and zeta potential of the nanoparticles were determined by photon correlation spectroscopy (Nanosizer ZS90, Malvern, Worcestershire, U.K.) using a helium–neon laser at a wavelength of 633 nm. Photon correlations of the spectroscopic measurements were performed at a scattering angle of 90°. Dispersions were diluted 1:100 with double-distilled water before the determination.

**Encapsulation Efficiency of the Drugs** The nanosystems were centrifuged at 100000×g and 4°C for 30 min in a Beckman Optima MAX® ultracentrifuge (Beckman Coulter, Brea, CA, U.S.A.) to separate the incorporated drugs in the lipid phase from the free form. The supernatant was filtered through a polyvinylidene difluoride filter (0.45 μm pore size). Subsequently the filtrate was analyzed by liquid chromatography (HPLC) to measure the encapsulation percentage.

**HPLC Analysis of the Drugs** The HPLC system consisted of a Hitachi L7110 pump (Tokyo, Japan), a Hitachi L7200 autosampler, and a Hitachi L7400 UV/visible detector. A 25-cm-long, 4-mm inner diameter reverse-phase column (Merck, Darmstadt, Germany) was used as the stationary phase. The mobile phase for tretinoin was a mixture of methanol and sodium acetate buffer (pH 4.5) at a ratio of 90:10. The mobile phase consisted of methanol and pH 2.5 citrate–phosphate buffer (40:60) for tetracycline. The flow rate was 1 mL/min. The respective wavelengths for detecting tretinoin and tetracycline were 343 and 270 nm.

**Animals and Skin Preparation** The skin used for the percutaneous absorption experiment was derived from the dorsal region of female nude mice (ICR-Foxn1nu strain, 8 weeks old) and female hairy mice (Balb/c strain, 8 weeks old), which were provided by the National Laboratory Animal Center (Miaoli, Taiwan). The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Chang Gung University. Animals were maintained under standard diurnal conditions and were allowed access to food and water *ad libitum* in accordance with animal protection standards. Delipidized skin was obtained by treating the stratum corneum (SC) of nude mouse skin with chloroform–methanol (2:1) for 1 h.

**In Vitro Percutaneous Absorption** Skin permeation of the drugs was measured by Franz cells. The excised skin was mounted between the donor and receptor compartments. The donor medium included 0.5 mL of vehicle containing drugs. The receptor medium (5.5 mL) was an ethanol–pH7.4 buffer (3:7) mixture to maintain sink conditions. The available permeation area between the compartments was 0.785 cm². The stirring rate of the stir bar on the receptor bottom was kept at 600 rpm and the temperature of the receptor at 37°C. At determined intervals, 300-μL aliquots of the receptor medium were sampled and immediately replaced by equal volumes of fresh buffer. Cumulative amounts of the drugs were quantified by HPLC.

**Bacteria and Growth Conditions** Antibacterial activities of the nanocarriers were tested against three bacteria including *S. aureus*, *P. aeruginosa*, and *Pro. acnes* all from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). To obtain fresh pre-cultures, the strains were first cultivated in 60 mL of medium in 250-mL Erlenmeyer flasks. The media used for cultivation were prepared according to the proposed media shown on the ATCC web site (http://www. atcc.org). Incubation was carried out under aerobic conditions for *S. aureus* and *P. aeruginosa* and anaerobic conditions for *Pro. acnes* with shaking (180 rpm). The growth of the strains was determined by measuring the optical density (OD) at 600 nm. Cultures in the exponential growth phase (OD<sub>600</sub> reached 0.5–0.8) were diluted to an OD<sub>600</sub> of 0.1, and then immediately used for the following antibacterial assay.

**Microtiter Plate Assay** To measure IC<sub>50</sub> values of tretinoin and tetracycline, serial 2-fold dilutions were prepared in the range 0.01–400 μg/mL using a 96-well microtiter plate.

<table>
<thead>
<tr>
<th>Code</th>
<th>Squalene</th>
<th>Preirol</th>
<th>PF68&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Myverol</th>
<th>FE&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE1</td>
<td>12.0</td>
<td>—</td>
<td>2.4</td>
<td>0.2</td>
<td>—</td>
</tr>
<tr>
<td>NE2</td>
<td>12.0</td>
<td>—</td>
<td>2.4</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>NLC1</td>
<td>9.0</td>
<td>3.0</td>
<td>2.4</td>
<td>0.2</td>
<td>—</td>
</tr>
<tr>
<td>NLC2</td>
<td>9.0</td>
<td>3.0</td>
<td>2.4</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> PF68; Pluronic 68. <sup>b</sup> FE, Forestall.
assay as described in a previous study.\textsuperscript{16} The incubation duration of the drugs with \textit{S. aureus} and \textit{Pse. aeruginosa} was 1 d. The duration for \textit{Pro. acnes} was 7 d under anaerobic conditions. IC\textsubscript{50} values were expressed as the concentration (µg/mL) of the drugs required to inhibit 50% of cell growth at OD\textsubscript{600} in 100 µL of inoculated medium. To determine the IC\textsubscript{50}, at least three independent experiments, each with three replicates, were conducted.

**Agar Diffusion Assay** The agar diffusion assay for examining the antibacterial activity was performed by a method described by Finn\textsuperscript{17} with minor modifications. The discs (6 mm in diameter) containing the drugs at the IC\textsubscript{50} concentrations after dilution were placed onto agar plates that had been seeded with the test strains. The diameters of inhibition (diameter of the inhibition zone—the diameter of the disc) were measured in millimeters after incubation at 37°C for 1 d (\textit{S. aureus} and \textit{Pse. aeruginosa}). The incubation time for \textit{Pro. acnes} was 7 d. The test was determined in triplicate.

**Bicinchoninic Acid Assay for Total Protein** Total protein of the bacteria treated by nanocarriers was detected using a micro bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.) after the microtiter plate assay. IC\textsubscript{50} concentrations of various strains were used as the drug dose for bacteria treatment. Briefly, one part of the samples was mixed with another part of the working reagent in a multiwell plate. After incubation at 37°C for 2 h, the absorbance of the solution was measured at 560 nm. Bovine serum albumin was used as the standard to establish the calibration curves.

**Statistical Analysis** The experimental results are expressed as the mean ± standard deviation (S.D.). Statistical analyses were carried out using the non-parametric Kruskal–Wallis test with Dunn’s post-test. Differences were considered significant at \(p<0.05\).

**RESULTS**

**Physicochemical Characterization of the Nanocarriers** The NEs and NLCs but not the SLNs were utilized as the lipid nanoparticulate systems in this study because both formulations exhibited good stability for anti-acne drug storage.\textsuperscript{18} Nanosystems made of squalene/Precirol as the core materials were emulsified with PF68 and Myverol. Squalene is a naturally derived compound found in the skin which has the ability to hydrate and nourish skin. Precirol is a safe, well-studied, solid lipid which is a mixture of glyceryl palmito-stearate. Since tretinoin can form ion pairing with lipophilic amines to improve encapsulation and stability,\textsuperscript{19} FE was added to the systems (NE2 and NLC2) to provide the amine moiety. The different lipid nanoparticles were respectively measured with a Zetasizer. Average sizes are shown in Table 2. With respect to the data measured in the absence of drugs, the composition of the nanoparticulate core and the emulsifier system largely affected the z-average. The size analysis showed respective mean diameters of 218 and 202 nm for NE1 and NLC1. After adding FE, the sizes increased to 322 (NE2) and 244 nm (NLC2). As for data of the formulations in the presence of drugs, a comparable or marginal increase in the particle size was found for systems without FE (NE1 and NLC1) compared to the drug-free dispersions. Contrary to this result, drug addition to FE-containing formulations (NE2 and NLC2) significantly reduced \((p<0.05)\) the particle size. In the case of NLC2, the combination of the two drugs produced a smaller size \((p<0.05)\) than single-drug incorporation. Loading of dual drugs or a single drug did not influence \((p>0.05)\) the average size of the formulations without FE (NE1 and NLC1). NLCs generally demonstrated a smaller size compared to NEs.

Zeta potentials of the nanoparticles are presented in Table 3. In the absence of drugs, NE1 showed a surface charge of −19 mV. FE loading (NE2) reversed the negative charge to a positive charge (+47 mV). The same result was observed for NLCs. NLC1 demonstrated a higher negative zeta potential (−34 mV) compared to NE1. Incorporation of tretinoin alone (in the absence of tetracycline) did not alter the zeta potential \((p>0.05)\) of any formulation. The addition of tetracycline alone significantly diminished \((p<0.05)\) the high negative zeta potentials of NE1 and NLC1. The combination of both drugs also produced a reduction in the negative charge. The positive charges of NE2 and NLC2 slightly but significantly increased \((p<0.05)\) after tetracycline loading.

Table 2. The Particle Size (nm) of NEs and NLCs with or without Tretinoin and Tetracycline

<table>
<thead>
<tr>
<th>Formulation</th>
<th>NE1</th>
<th>NE2</th>
<th>NLC1</th>
<th>NLC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>218 ±7.8</td>
<td>321.6 ±0.4</td>
<td>201.8 ±1.7</td>
<td>243.6 ±3.0</td>
</tr>
<tr>
<td>Tretinoin + tetracycline</td>
<td>223.9 ±8.8</td>
<td>288.6 ±4.2</td>
<td>213.4 ±2.4</td>
<td>201.3 ±1.6</td>
</tr>
<tr>
<td>Tretinoin</td>
<td>210.2 ±10.8</td>
<td>260.6 ±1.7</td>
<td>219.6 ±4.3</td>
<td>231.0 ±2.5</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>226.3 ±4.5</td>
<td>276.8 ±3.7</td>
<td>216.1 ±3.8</td>
<td>227.6 ±0.7</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. \((n=3)\).

Table 3. The Zeta Potential (mV) of NEs and NLCs with or without Tretinoin and Tetracycline

<table>
<thead>
<tr>
<th>Formulation</th>
<th>NE1</th>
<th>NE2</th>
<th>NLC1</th>
<th>NLC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>−19.1 ±1.0</td>
<td>47.2 ±0.5</td>
<td>−33.7 ±1.0</td>
<td>39.9 ±0.5</td>
</tr>
<tr>
<td>Tretinoin + tetracycline</td>
<td>−7.5 ±0.1</td>
<td>40.6 ±0.8</td>
<td>−8.0 ±0.4</td>
<td>46.4 ±0.3</td>
</tr>
<tr>
<td>Tretinoin</td>
<td>−19.2 ±0.4</td>
<td>45.5 ±3.1</td>
<td>−32.2 ±2.6</td>
<td>41.4 ±2.2</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>−0.2 ±0.2</td>
<td>49.9 ±1.0</td>
<td>−6.4 ±0.4</td>
<td>44.3 ±0.3</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. \((n=3)\).
alone in NE1 and NLC1 were 62% and 80%, respectively. FE incorporation in nanoparticles (NE2 and NLC2) increased tretinoin encapsulation to ca. 100%. This complete encapsulation was significantly reduced (p<0.05) to ca. 57% when tetracycline was simultaneously incorporated. The reduction in tretinoin encapsulation after tetracycline loading was not significant for dispersions without FE (NE1 and NLC1). Tetracycline revealed an encapsulation percentage ranging 10–13% in the four nanosystems. No significant difference (p>0.05) was detected in tetracycline entrapment among these formulations. Tretinoin loading decreased tetracycline encapsulation by 1.7–2.6-fold in the various dispersions.

**In Vitro Percutaneous Absorption** In *vitro* skin permeation of tretinoin and tetracycline from dual-drug nanocarriers is depicted in Figs. 1A and B, respectively. Skin permeation was calculated in terms of the cumulative amount that had diffused at different time points over 48h. The vehicle for the control group was 30% PEG400 in water. As shown in Fig. 1A, the control group revealed the highest cumulative amount of tretinoin. However, tretinoin permeation from NEs (NE1 and NE2) was comparable (p>0.05) to that from the control. NLCs (NLC1 and NLC2) exhibited lower permeation (p<0.05) than NEs. Both NLCs showed a longer lag time than the control and NEs. No tretinoin was detected in the receptor until 6h for both NLCs. The period of this lag time for the control and NEs was 2–4h. Figure 1B shows cumulative amount-time profiles of tetracycline from the dual-drug systems. Tetracycline from the free control exhibited very low permeation via nude mouse skin. In all cases, tetracycline permeation from the nanosystems was greater than that from the reference solution. NLC1 produced the highest cumulative amount, followed by NLC2, NE2, and NE1. The slope of the cumulative amount-time curves was calculated as the flux, which is summarized in Fig. 2. Drug fluxes in the presence and absence of another drug are also compared in this figure. Tretinoin fluxes from the free control and all nanoparticles with dual drugs were comparable (p>0.05) to each other as shown in Fig. 2A. Whether or not tetracycline was incorporated did not significantly (p>0.05) affect tretinoin flux except with NLC2. The addition of tetracycline to NLC2 decreased (p<0.05) tretinoin permeation. Figure 2B demonstrates that tetracycline fluxes from the dual-drug systems of NE2, NLC1, and NLC2 were significantly higher (p<0.05) compared to the control. The statistical analysis revealed significant enhancement (p<0.05) of tetracycline flux in the absence of tretinoin in the nanocarriers.

To explore the mechanisms and pathways of nanocarriers transported via skin, hairy mouse skin and delipidized nude mouse skin were utilized as permeation barriers to examine drug delivery. Only the dual-drug systems were studied in this experiment. Figure 3A compares tretinoin flux via intact, hairy, and delipidized skin. The data above the columns of this figure are the ratios of fluxes between hairy mouse skin/nude mouse skin (ratio_hairy/nude) or delipidized skin/nude mouse skin (ratio_delipidized/nude). Hairy mouse skin possesses higher follicle numbers and densities compared to the nude species. Tretinoin flux via hairy mouse skin was slightly lower (p>0.05) than that via nude mouse skin, resulting in a ratio_hairy/nude of <1 (0.77). The incorporation by NLCs achieved higher ratios of 0.98 and 1.04 for NLC1 and NLC2, respectively. Delipidation greatly enhanced tretinoin flux by 19-fold. The nanosystems showed limited enhancement after SC lipid removal.

### Table 4. The Encapsulation Percentage (%) of NEs and NLCs with Tretinoin/Isotretinoin

<table>
<thead>
<tr>
<th>Drug</th>
<th>Combined drug</th>
<th>NE1</th>
<th>NE2</th>
<th>NLC1</th>
<th>NLC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tretinoin</td>
<td>Tetracycline</td>
<td>61.93±1.41</td>
<td>57.05±1.03</td>
<td>76.19±1.44</td>
<td>56.71±2.29</td>
</tr>
<tr>
<td>Tretinoin</td>
<td>—</td>
<td>61.99±1.33</td>
<td>97.52±1.07</td>
<td>80.02±0.51</td>
<td>93.70±2.65</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Tretinoin</td>
<td>5.02±0.27</td>
<td>7.14±0.07</td>
<td>4.91±3.36</td>
<td>7.17±2.75</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>—</td>
<td>12.90±3.60</td>
<td>11.28±2.06</td>
<td>9.78±5.40</td>
<td>12.43±3.51</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.D. (n=4).
ratio_{delipidized/nude} of nanoparticles ranged 4–6, which was much lower than that of the free control. As shown in Fig. 3B, tetracycline flux from the control penetrating via hairy mouse skin was >7-fold that via nude mouse skin. The ratio_{hairy/nude} of tetracycline from the nanocarriers was comparable or lower compared to the value of the control group, with NLCs showing the lowest ratio. The same with tretinoin, the free control demonstrated the highest ratio_{delipidized/nude} level (39.52) of tetracycline delivery.

**Antibacterial Assay**

The present study also aimed to estimate the antibacterial effects of the nanocarriers with dual drugs. To compare different activities of formulations on the growth of bacteria which are present in skin flora, *S. aureus*, *Pse. aeruginosa*, and *Pro. acnes* were cultured to test the activities. IC_{50} values of tretinoin and tetracycline in the free control were first determined using a microtiter plate assay. IC_{50} values of tetracycline against *S. aureus*, *Pse. aeruginosa*, and *Pro. acnes* were 1.64, 128.8, and 1.64 µg/mL, respectively. Tretinoin did not inhibit bacterial growth of any species. The growth-inhibitory activity of the dual-drug systems with the tetracycline concentration at the IC_{50} value was subsequently tested using an agar diffusion assay as shown in Fig. 4. Since the *Pro. acnes* yield after culturing on agar was very low, this bacterium was excluded from the test. Figures 4A and B show the inhibitory diameters of *S. aureus* and *Pse. aeruginosa* after treatment with the nanoparticles. It can be seen that all nanoparticles possessed antibacterial activity against the bacteria. The nanocarriers had widespread and obvious activity similar to that of the control solution (*p* > 0.05).

Figure 5 shows the total protein amount of bacteria after treatment with nanoparticles. The negative control was the group with no drug treatment. With respect to *S. aureus* (Fig. 5A), it is worth mentioning that the nanosystems without FE (NE1 and NLC1) revealed greater antibacterial activity (*p* < 0.05) than did the control solution. The activities of the systems with FE (NE2 and NLC2) were comparable (*p* > 0.05) to that of the free control. Superiority of the lipid nanoparticles was not observed against *Pse. aeruginosa*. Only NLC1 reached the same level (*p* > 0.05) as the control (Fig. 5B). Activities by the other nanocarriers against *Pse. aeruginosa* were significantly lower (*p* < 0.05) than that by the control group.
Fig. 4. Inhibitory Diameters of the Aqueous Control and Lipid Nanocarriers with Dual Drugs against (A) *Staphylococcus aureus* and (B) *Pseudomonas aeruginosa* in an Agar Diffusion Assay

All data are presented as the mean of three experiments ± S.D.

Fig. 5. Total Protein amounts of (A) *Staphylococcus aureus*, (B) *Pseudomonas aeruginosa*, and (C) *Propionibacterium acnes* after Treatment with the Aqueous Control and Lipid Nanocarriers with Dual Drugs

* indicates a lower value (*p* < 0.05) compared to the control solution with dual drugs; ** indicates a higher value (*p* < 0.05) compared to the control solution with dual drugs. All data are presented as the mean of three experiments ± S.D.
For *Pro. acnes* incubated with the control solution, a 2-fold reduction in the total protein was detected (Fig. 5C). There were no significant differences (*p > 0.05*) between antibacterial activities of the free control and the four nanosystems.

**DISCUSSION**

In the present work, we provide evidence that the prepared nanocarriers could increase skin permeation of anti-acne drugs. The lipid nanoparticles showed antimicrobial activity against some microbial species that grow on the skin. The strategy of dual drugs in nanosystems may be advantageous for anti-acne therapy. NLCs generally showed a smaller size than did NEs. Precirol is a mixture of fatty esters with amphiphilic characteristics. The additional activity of emulsification by Precirol was helpful in minimizing particle sizes and formed a rigid lipid/water interface. The increase in surfactant may have contributed to the size reduction. This was not the case for FE, although it is a typical emulsifier (Table 2). FE may be too lipophilic to form stable nanosystems, since a hydrophilic emulsifier is mostly needed to stabilize dispersions with a high percentage of water. The additional FE in the interface did not favor interfacial film curvature, thus increasing the particle size.

It was interesting that drug incorporation resulted in different effects on particle sizes in the presence and absence of FE (Table 2). The loading of tretinoin/tetracycline only contributed to a slight increase in the mean diameter in the case without FE (NE1 and NLC1). On the other hand, sizes of the dispersions with FE (NE2 and NLC2) significantly decreased after drug entrapment. The high solubility of tretinoin in lipids may have produced the high encapsulation efficiency in NEs and NLCs. Tretinoin might be predominantly located in the inner core of the particles. Castro *et al.* demonstrated that ion pairing between tretinoin and amines enhanced drug encapsulation in SLMs. The amine group of FE may favor tretinoin locating in the interface, thus increasing the entrapment percentage. This was confirmed by the nearly 100% tretinoin encapsulation in NE2 and NLC2 in the absence of tetracycline. Since tretinoin possesses some amphiphilic properties, the particle sizes of NE2 and NLC2 were reduced based on an emulsification effect. Because of the lipid matrix of NEs and NLCs, negligible entrapment into the core can be assumed for hydrophilic drugs. Tetracycline is a hydrophilic molecule with a log *p* value of −1.4. Encapsulation of ca. 10% by lipid nanoparticles is not regarded as being low since tetracycline is insoluble in the lipid phase. The possibility of tetracycline residing in the lipid/water interface should be considered. Kächler *et al.* assumed localization of hydrophilic substances within the PF68 layer of lipid nanoparticles because of the formation of intermolecular interactions such as hydrogen bonding. This phenomenon was also expected in our case. It can be verified by the increased zeta potential after tetracycline incorporation (in the absence of tretinoin). Tetracycline has a cationic charge in acidic environments. This molecule must be intercalated in the interface to offer a positive charge to the zeta potential. Similar to tretinoin, tetracycline may exhibit some amphiphilic properties when forming nanoparticles with a more-stable status and of smaller sizes. Tretinoin could potentially be adsorbed by the FE-containing interface; however, the zeta potential did not significantly change with the incorporation of tretinoin alone. This may have been due to the low tretinoin concentration (0.05%, w/v) in the nanosystems, which was insufficient to modulate the surface charge.

The incorporation of tretinoin/tetracycline did not greatly alter the particle sizes of NE1 or NLC1 (Table 2). In the absence of FE, tretinoin was not located within the interface. The emulsification effect was thus attenuated. The location of tetracycline was not affected by FE, since PF68 was the predominant substrate for interaction. According to the zeta potential, the negative charges of NE1 and NLC1 were lessened by tetracycline addition. Particulate aggregation could therefore occur more easily because of an insufficient electrostatic repulsion, leading to a slight increase in the particle size. Another factor was that the sizes of the nanosystems without FE (NE1 and NLC1) were small enough (ca. 200 nm) that any further emulsification could not reduce the size. The emulsification effect of the drugs was emphasized in the case of larger particles such as NE2 and NLC2.

Plain NE1 without drug entrapment showed a zeta potential of −19 mV (Table 3). Since PF68 is non-ionic, the ionization of Myverol was possibly responsible for the negative surface charge. Some fatty acids derived from the hydrolysis of monoesters in Myverol could have occurred, creating the negative charge. The negative charge was also caused by fatty ester hydrolysis of Precirol. This effect resulted in the higher negative zeta potential of NLC1 than NE1. The positive zeta potentials of NE2 and NLC2 suggested the participation of the cationic FE in the particulate surface. Since tretinoin basically did not influence the zeta potential, it is thought that the cationic tetracycline predominated surface charge modulation after the combined incorporation of both drugs. Nevertheless, the positive zeta potential of NE2 significantly decreased from 47 to 41 mV with dual-drug loading. This could have been due to a redistribution and reorientation of interfacial components after incorporation of both drugs. Further study is needed to elucidate the mechanisms.

The good compatibility between tretinoin and lipids and the low concentration of tretinoin contributed to the high encapsulation efficiency of this lipophilic drug (Table 4). The high entrapment by NEs and NLCs was beneficial in reducing skin irritation elicited by the drug because direct contact was avoided. NLC1 showed greater entrapment of tretinoin than did NE1, indicating a higher compatibility of tretinoin to Precirol compared to squalene. The types of inner cores (NEs vs. NLCs) did not influence tetracycline encapsulation, since this drug predominantly resided at the interface. It was obvious that tretinoin entrapment decreased after tetracycline addition, especially in formulations containing FE. A similar trend was observed for tetracycline encapsulation. The decrease in the loading efficiency of both drugs by dual-drug inclusion may have been due to the portion of one drug embedded in the particles being expelled by the other drug. Another possibility is the occurrence of incompatibility between drugs and lipids when preparing dual-drug systems.

Studies with human subjects are costly and time-consuming. Nude mouse skin is preferred as an alternative model because of its ease of topical application, limited variability among individuals, and similar follicle density to that of human skin. Previous studies showed that the nude mouse is an ideal animal model for investigating topically applied tretinoin. Since nude mouse skin is thinner and more
permeable than human skin, drug permeation across the skin in an in vitro condition can be an indicator of drug absorption by targeted skin.\textsuperscript{[13,31]} According to the in vitro skin permeation experiment (Fig. 1), basically none of the nanosystems increased tretinoin flux compared to the free control. The flux was similar for all nanosystems except NLC2 without tetracycline incorporation (Fig. 2). Although comparable fluxes were seen, the cumulative amount of tretinoin in the receptor from NLCs was much lower than those of the control and NEs. This was because of the longer time lag for NLCs to permeate into the receptor. The delayed absorption could be attributed to inclusion of tretinoin in the lipid matrix. The addition of solid lipids to the matrix causes a tight crystalline order to the structure, thus the diffusional mobility of the drug decreases.\textsuperscript{[32,33]} This result was similar to that of a study by Ourique et al.\textsuperscript{[3] which found that nanoencapsulation of tretinoin prolonged the lag time.

The slow tretinoin release cannot explain the comparable fluxes to the control and the higher flux from NLC2. There must be some other mechanisms governing tretinoin's absorption from nanoparticles. The occlusive effect is a possible mechanism for enhancing skin permeation of entrapped drugs from NLCs. Following water evaporation from lipid nanoparticles carried to the skin, particles form a thin layer that occludes the skin surface. Subsequently, SC hydration can result in reduced keratinocyte packing and widened intercellular lacuna.\textsuperscript{[34]} The occlusion level greatly depends upon the crystallinity of the lipid matrix and the particle size. Highly crystalline particles such as NLCs produce greater occlusion compared to NEs.\textsuperscript{[35]} Sizes of NLCs were smaller than those of NEs in our study. The occlusion and skin hydration can increase by decreasing the particle size due to a compact density on the skin surface.\textsuperscript{[10,32]} Tretinoin permeation enhancement was only observed with NLC2 and not with NLC1. Crystallinity and the particle size cannot totally explain this difference. Regarding topical delivery, the use of positively charged nanoparticles is advantageous because of adsorption onto negatively charged SC lipids is promoted.\textsuperscript{[33]} This electrostatic interaction by nanoparticles is especially beneficial for tretinoin permeation such as the case of cationic liposomes and submicron emulsions.\textsuperscript{[36,37]} In addition, the interaction of lipid nanoparticles with SC lipids is considered an important factor increasing drug penetration. Lipid nanoparticles attach themselves to the skin and allow lipid exchange and mixing to the SC.\textsuperscript{[11,38]} SC lipids are found in high amounts within the permeation barrier. Experimental results demonstrated that the ratio of delipidized/nude SC was greatly reduced from 19 to 4—5 after loading tretinoin into the nanocarriers (Fig. 3). This indicates loss of the lipid exchange capability by nanoparticles after removing SC lipids. We concluded that an important role of lipid interactions exists between SC lipids and the developed nanocarriers in the present work.

Enhancement of skin permeation by nanocarriers was more significant in the case of tetracycline than tretinoin. Both NEs and NLCs showed increased tetracycline flux to different extents. The larger molecular weight of tetracycline HCl (481 Da) led to difficulty of skin absorption via the SC layers. SC lipids also prevent the penetration of hydrophilic permeants much more efficiently than lipophilic ones. The results of permeation via delipidized skin showed a higher enhancement ratio between fluxes of delipidized skin and intact skin for tetracycline than for tretinoin. Most of the tetracycline molecules resided in the external phase. Different from tretinoin absorption, it was not necessary for tetracycline to be released from the inner core before it can partition into the SC. This is the reason for the greater enhancement of tetracycline than tretinoin. The mechanisms of occlusion and lipid exchange may explain the enhanced permeation of both drugs. Tetracycline delivery from NLCs was greater compared to that from NEs. The ratio of delipidized/nude of tetracycline flux from NLCs was lower than that from NEs (Fig. 3), indicating a significant lipid interaction with NLCs. The greater enhancement of NLCs over NEs was not observed for tretinoin because of slow or sustained release of tretinoin from nanoparticulate cores, which offset the mechanisms of lipid interaction and occlusion. NLC2 demonstrated the lowest ratio of delipidized/nude value for tetracycline. This suggests a further interaction by positively charged particles. Investigations of NEs and NLCs for hydrophilic drug permeation are few. Our results suggest the possibility of enhancing drug delivery in the external phase of lipid nanoparticles.

All nanocarriers showed reduced tetracycline permeation after tretinoin loading. This reduction was also observed for tretinoin permeation from NLC2 with tetracycline. The possibility of formation of chemical complexes was considered. The –OH groups in the four-ring moiety of tetracycline may interact with –COOH groups in tretinoin’s structure to form ester bonds. Nitrogen molecules in tetracycline’s structure can also donate lone electron pairs to attach to the –OH of tretinoin. The complex may impede skin permeation of both drugs because of the large molecular volume. The flux reduction for tetracycline was more significant than that for tretinoin. The flux value of tetracycline was greatly higher as compared to that of tretinoin (Fig. 2). It may be anticipated that most of tretinoin molecules in the formulations was not absorbed by the skin. These tretinoin molecules may interact with tetracycline, which was largely absorbed by skin. That is, the effect of this interaction could significantly affect tetracycline permeation but not tretinoin permeation. A similar phenomenon was demonstrated in the previous study,\textsuperscript{[13,31]} which combined calcipotriol and methotrexate in one vehicle for skin delivery. Further investigation is necessary to examine this possibility of complex formation. Drug molecules being attached on the surface of lipid nanoparticles should facilitate drug dissolution in lipids of the SC.\textsuperscript{[10]} The loading efficiency of tetracycline decreased by ca. 2-fold after tretinoin incorporation, thus attenuating the dissolution effect. An approximately 2-fold decrease in tetracycline flux was detected in the dual-drug systems compared to single-drug ones. This is another explanation for the lowered tetracycline permeation.

Tretinoin and other retinoids are able to reverse abnormal desquamation by influencing epithelial turnover in hair follicles.\textsuperscript{[39]} Lipid nanoparticles have the potential to deliver drugs via follicles.\textsuperscript{[18,40]} Furthermore, each follicle is associated with a sebaceous gland that releases sebum which creates a lipid-enriched environment.\textsuperscript{[40]} This environment is beneficial for trapping lipid nanoparticles. The ratio of delipidized/nude of tretinoin flux from NLCs was ca. 1, which was higher than that from the free control (0.77). Considering the difference between hairy mouse and nude mouse skin, it is suggested that NLCs could be absorbed by follicles and/or sebaceous glands (shunt routes). This indicates the suitability of NLCs for acne
treatment. Sebum is a mixture of squalene, triglycerides, and waxes. Squalene and Precirol present in the NLCs may accelerate the entrance into follicles/sebaceous glands. NEs with only squalene showed less of an effect on follicular uptake. On the other hand, values of the ratio $\text{hairy/nude}$ of tetracycline from nanocarriers did not exceed that from the control group (Fig. 3). The ratio $\text{hairy/nude}$ of tetracycline from the free control was very high (7.46), suggesting the ease of tetracycline penetration into follicles. Moreover, the different histological structures and morphologies between the skins of these two species contributed to this large discrepancy. It was inferred that nanoparticles would block follicular orifices after their entrance. This may impede follicular transport of tetracycline in the external water phase.

*Pro. acnes* is a Gram-positive anaerobic bacterium which plays a critical role in the development of inflammation with acne vulgaris. Reactive oxygen species (ROS) are produced by keratinocytes upon stimulation by surface proteins of this bacterium.42) *Pro. acnes* mainly resides in follicles and sebaceous glands where it irritates the skin epithelium. Some symptoms of skin inflammation are due to infection by bacteria, principally *S. aureus* and *Pse. aeruginosa*.35,43) *S. aureus* is a Gram-positive bacterium capable of causing skin infections in hair follicles such as sycosis, folliculitis, and furuncles.46) *Pse. aeruginosa* is a Gram-negative species. It occasionally causes dermatitis and deeper soft-tissue infection of the skin.47) According to the $IC_{50}$ values of these three bacteria by incubation with tretinoin and tetracycline, it was suggested that tretinoin exhibited no bacterial activity. Activities of bacterial inhibition of the dual-drug systems were predominantly derived from tetracycline. Most *S. aureus* strains are susceptible to tetracycline.46) Our results also showed that tetracycline potentially decreased the total protein of *S. aureus* (Fig. 5). It was impressive that the negatively charged nanoparticles (NE1 and NLC1) demonstrated higher inhibition against *S. aureus* than the control solution and positively charged nanoparticles. Most bacteria including *S. aureus* have a net negative charge on their cell surface.47) Adhesion of positive nanoparticles can be anticipated due to electrostatic attraction. Since most of the cationic tetracycline was in the outer phase of the nanoparticles, the drug and particles may have competed with each other for cell surface adhesion. This led to lower inhibition by positive nanosystems than negative ones. Xing et al.49) indicated that nanoparticles can bind to and interact with *S. aureus* surfaces. This is followed by pore formation and surface disintegration. The negatively charged nanoparticles developed in this work may possess this ability, thus promoting the antibacterial activities of NE1 and NLC1 against *S. aureus*.

Comparable or lower antibacterial activities of the nanocarriers than the control solution against *Pse. aeruginosa* and *Pro. acnes* suggested that the bacterial disruption effect observed with *S. aureus* did not occur with either bacterium (Figs. 4, 5). A previous study49) suggested that enzymatic degradation of NLCS and SLNs is likely to occur due to microbial flora on the skin surface. The growth of *Pro. acnes* was dose-dependently inhibited by superoxide anions.42) Tretinoin can act as an antioxidant agent against oxidative stress. The presence of tretinoin in NEs and NLCs would offset the antibacterial effect of tetracycline. We believe that the action mode of nanocarriers is more complex than that assumed above. The present work did not attempt to establish the detailed mechanisms of activity by the lipid nanoparticles. Future investigations should aim at elucidating the target of nanoparticles on cell surfaces and the killing process. Our study verified maintenance of the antibacterial activity of tetracycline after loading into NEs and NLCs. Since most of tetracycline molecules were resided in external phase of nanosystems, the nanoparticles did not largely influence the effect of this drug on bacteria as compared to aqueous control solution.

**CONCLUSION**

Acne therapy often requires multiple drug treatment. Development of integrated vehicles by combining drugs is especially important for patient convenience and compliance. Lipid nanocarriers of NEs and NLCs loaded tretinoin and tetracycline were prepared in this work for this purpose. It was hypothesized that encapsulation of tretinoin in nanoparticles will bypass its contact with the skin and consequent irritation. By controlling the components of the nanoparticles, a high loading efficiency of tretinoin of $>$90% was achieved. Most of the tetracycline was located in the aqueous phase. About 10% of the tetracycline was loaded in the nanoparticulate shell. Enhanced skin permeation of tetracycline was obtained by employing lipid nanocarriers, with NLCs showing greater absorption than NEs. We hypothesized that the occlusion effect and lipid exchange with the SC may be the mechanisms for this enhancement. Tetracycline flux could be adjusted by selecting different nanosystems. The presence of tretinoin reduced tetracycline permeation from the nanoparticles because of possible complex formation. Tretinoin permeation from nanoparticles generally did not exceed that from the control solution. NLCs increased tretinoin delivery via follicular routes. The antibacterial activity of tetracycline was preserved after nanoparticulate loading. Negatively charged NEs and NLCs even promoted antibacterial activity compared to the control solution. NLCs may be superior carriers to LEs because of the higher tetracycline permeation, more-significant tretinoin uptake by follicles, and conservation of bacterial growth inhibition. Our data provide evidence of a successful anti-acne drug combination in lipid nanoparticles. It can be used for efficient and safe acne therapy. The present results suggest a novel opportunity for dual-drug nanosystems to be employed as topical formulations.

**REFERENCES**


5) Fu JJJ, Hillebrand GG, Raleigh P, Li J, Marmor MJ, Bertucci V, Grimes PE, Mandy SH, Perez MI, Weinkle SH, Kaczvinsky JR. A


8) Pople PV, Singh KK. Development and evaluation of topical for...


