Tadalafil-loaded nanostructured lipid carriers using permeation enhancers

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Tadalafil-Loaded Nanostructured Lipid Carriers Using Permeation Enhancers

Running Title: Enhanced transdermal drug delivery of tadalafil

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Graphical abstract

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Abstract
Tadalafil is a phosphodiesterase-5 inhibitor indicated for the treatment of erectile dysfunction. In this study, we prepared and evaluated transdermal nanostructured lipid carriers (NLC) to improve the skin permeability of tadalafil. Tadalafil-loaded NLC dispersions were prepared using glycercylyl monostearate as a solid lipid, oleic acid as a liquid lipid, and Tween 80 as a surfactant. We characterized the dispersions according to particle size, polydispersity index, zeta potential, encapsulation efficiency, and transmission electron microscopy. In vitro skin permeation studies were carried out using Franz diffusion cells, and cytotoxicity was examined using HaCaT keratinocyte cell lines. Tadalafil skin permeability increased for all tadalafil-loaded NLC formulations. The tadalafil-loaded NLC dispersion with ethanol and limonene as skin permeation enhancers exhibited the highest flux (~4.8-fold) compared to that observed with tadalafil solution alone. Furthermore, a tadalafil-loaded NLC gel with selected permeation enhancers showed tolerance against toxicity in HaCaT cells. These results suggest that the NLC formulations with ethanol and limonene as skin permeation enhancers could be a promising dermal delivery carrier for tadalafil.

Keywords: Nanostructured lipid carriers; Tadalafil; Transdermal drug delivery; Permeation enhancer.
1. Introduction

Tadalafil (TAD) is the most potent phosphodiesterase (PDE) 5 inhibitor and at least 9000 times more selective than other PDE 5 inhibitors. Furthermore, TAD shows less inhibitory activity for PDE 6 compared to sildenafil and vardenafil and has less than a 0.1% occurrence of vision abnormalities (Brock et al., 2002; Carson et al., 2005; Coward and Carson, 2008). However, TAD has low-solubility and high-permeability, which leads to its classification as a class II drug within the FDA biopharmaceutical classification system (BCS) (Chavda et al., 2010) and poor dissolution in the gastrointestinal tract, resulting in variable bioavailability (Löbenberg and Amidon, 2000). TAD has the slowest absorption, reaching its maximum concentration (C\text{max}) in 2 h compared to ~50 min for sildenafil and vardenafil (Saharan et al., 2009). Poor solubility and low bioavailability often result in limited or irreproducible clinical responses to the drug. Therefore, various techniques such as solid dispersion, microemulsion, lipid-based formulations, and complexation (El Maghraby et al., 2009) have been used to enhance bioavailability.

Transdermal drug delivery systems are an attractive alternative to traditional oral and hypodermic delivery, as they can overcome the limiting hepatic first-pass effect encountered by oral administration and are safe, painless, and easy to use (dos Anjos and Alonso, 2008). The effectiveness of transdermal drug delivery depends on the drug’s ability to reach therapeutic levels by penetrating the skin sufficiently (Müller et al., 2002). For transdermal drug delivery, nanostructured lipid carriers (NLC) are an attractive strategy since the adhesion of an NLC to the skin surface provides an occlusive effect, which can eventually lead to an increase in skin hydration and promote the deposition of drugs by reducing corneocyte packing and widening inter-corneocyte
gaps (Schäfer-Korting et al., 2007; Guo et al., 2012). Furthermore, the lipid and surfactant components can enhance permeation by reducing the barrier properties of the stratum corneum, thereby increasing drug permeation through the skin (Joshi and Patravale, 2006). However, NLC must be combined with topical formulations for easy application. Vitorino had published co-encapsulating of olanzapine and simvastatin nanostructured lipid carriers with permeation enhancers including ethanol and limonene (Vitorino et al., 2013; Vitorino et al., 2014). Therefore, this study aims to prepare and evaluate a novel TAD-loaded NLC gel and establish an optimal formulation for efficient skin permeation of TAD.

2. Materials and methods

2.1. Materials

TAD was obtained from Korea United Pharm., Inc. (Seoul, Korea). Dynasan 118 was purchased from Sigma-Aldrich (Steinheim, Germany). Stearic acid, Miglyol 812, Tween 80, oleic acid, glyceryl monostearate (GMS), and triethanolamine were purchased from Samchun Chemical Co., Ltd. (Pyungtaek, Korea). HPLC-grade acetonitrile was obtained from JT Baker (Phillipsburg, NJ, USA). Carbomer 940 was used as received without further purification. All solvents were analytical grade and used without further purification.

2.2. Solubility of TAD in lipids

To design formulations, the solubility of TAD was determined in stearic acid, GMS, or Dynasan 118 as solid lipids and soybean oil, Miglyol 812, or oleic acid as liquid lipids. For solid lipids, 2 g lipid was melted 5°C above the melting point of each solid
lipid in a water bath, and 2 mg TAD was added to the melted lipid until saturation was achieved (Li et al., 2008). For liquid lipids, excess TAD was dispersed in a tube containing the liquid lipid (5 mL) and stirred for 48 h at room temperature. The samples were then centrifuged at 12,000 rpm for 5 min, and 0.5 mL of the supernatant was suitably diluted with methanol and analyzed by HPLC. Each determination was carried out in triplicate.

2.3. Preparation of TAD-loaded NLC formulations

NLC was prepared by a hot-melted ultrasonic method to enhance the encapsulation of lipophilic drugs in the nanoparticles (Doktorovova and Souto, 2009). GMS was selected as the solid lipid matrix and mixed with oleic acid as the liquid matrix and TAD at 70°C to form a lipid phase. The aqueous phase was composed of distilled water and 1.5% (w/v) or 3% (w/v) Tween 80 (surfactant), which was utilized as an emulsifier for the nanoparticles. After the two phases were separately heated to 70°C, the aqueous phase was added to the lipid phase, and the mixture was homogenized at 70°C in a water bath for 3 min to obtain the coarse emulsion. Then, this emulsion was sonicated with a probe-type sonicator for 20 min and quickly cooled in an ice bath to form NLC, hereafter referred to as the TAD-loaded NLC dispersion.

Subsequently, a gel was introduced to obtain viscosity levels suitable for transdermal application. Briefly, 0.5% (w/v) of Carbomer 940 and 30% (v/v) ethanol were added to the TAD-loaded NLC dispersion and hydrated under gentle stirring for 1 h with triethanolamine, hereafter called the TAD-loaded NLC gel.

The TAD gel was prepared by dispersing Carbomer 940 to a solution of TAD in water containing 30% (v/v) ethanol under stirring followed by neutralization with
triethanolamine. As a control, a blank NLC gel without TAD was prepared using the process described for the TAD-loaded NLC gel.

2.4. *Determination of particle size, polydispersity, zeta potential, and morphology*

Particle size and polydispersity (PDI) were determined by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). All samples were diluted with distilled water to gain optimal intensity, and measurements were performed at room temperature. Each value was measured in triplicate, and the results are presented as mean ± standard deviation.

The zeta potential is a physical property exhibited by particles in suspension and is related to the respective surface charge. It is a useful parameter to predict the physical stability of colloidal systems (de Vringer and de Ronde, 1995). Zeta potential analysis was carried out using a dynamic light scattering analyzer (ELS-8000, Otsuka Electronics, Japan). All samples were diluted with distilled water, and experiments were performed in triplicate to ensure a suitable conductivity for analysis.

The surface morphology of the TAD-loaded NLC dispersion was evaluated by transmission electron microscopy (TEM) (Zeiss 902A, Zeiss, Germany) operating at 80 kV. Before loading into the microscope (direct deposition), the samples were drop-cast onto a carbon-coated copper grid and dried for 1 min at room temperature.

2.5. *Determination of occlusion factor*

The occlusive properties of the TAD-loaded NLC gel, blank NLC gel, and TAD gel were evaluated by an *in vitro* occlusion test (Farthing et al., 2010). Beakers (100 mL) with a diameter of 5.0 cm were filled with 50 mL distilled water and covered with
a filter paper (Whatman number 6, cutoff size: 3 µm, USA). Each formulation (250 mg) was applied uniformly with a spatula on the filter paper surface (18.8 cm$^2$), and the beakers were subsequently stored at 32°C (to mimic the temperature of the skin surface) for 48 h. The water remaining in the beaker was weighed after 6, 12, 24, and 48 h. The occlusion factor (F) was calculated by the following equation:

$$F = \frac{A - B}{A} \times 100$$

where A and B represent the water loss in the absence or presence, respectively, of TAD-loaded NLC gel, blank NLC gel, or TAD gel. An F value of 0 indicates no occlusive effect, whereas an F value of 100 indicates maximum occlusiveness.

2.6. HPLC analysis

HPLC analysis of TAD from buffer and rat plasma was performed as described previously with slight modification (Williams and Barry, 2004). The mobile phase composed of acetonitrile and water (30:70, v/v) was filtered using a 0.45-µm filter, degassed, and delivered at a flow rate of 1.2 mL/min. The column temperature was maintained at 25°C, the injection volume was 20 µL, and TAD was detected at 285 nm.

2.7. In vitro skin permeation study

All animal studies were conducted in accordance with the “Guiding Principles in the Use of Animals in Toxicology” adopted by the Society of Toxicology (USA), and the experimental protocols were approved by the Animal Care Committee of Chungnam National University. Dorsal skin was excised from Sprague-Dawley rats whose hair had been previously removed. All adhering fat and other visceral tissue was removed carefully with forceps and scissors. The excised rat skin was stored at -20°C.
prior to use. Franz diffusion cells with an effective diffusion area of 1.81 cm$^2$, a
diameter of 16 mm, and a receptor volume of 11.8 mL were used to assess in vitro skin
permeation. The receptor compartment was maintained at 37°C, and a surface
temperature of 32°C was assured to mimic human skin conditions. The receptor
medium was selected as pH 7.4 phosphate buffered solution containing 1% Tween 80
and stirred continuously with a magnetic stirrer at 500 rpm. Each TAD-loaded NLC
formulation (500 µL) was placed in the donor compartment. Permeation experiments
were carried out until 48 h after application. At predetermined time points (0, 3, 6, 12,
24, 36, and 48 h), 300-µL aliquots were collected from the receiver compartment and
immediately replaced with the same volume of fresh receptor medium. The amount of
TAD in the removed samples was determined by HPLC.

2.8. Effect of permeation enhancers

Limonene was dissolved in ethanol at 1%, 3%, or 5% (w/v) concentration and
added to the TAD-loaded NLC dispersion or TAD-loaded NLC gel to a final proportion
of 30:70 (v/v). The effect of ethanol without limonene in the TAD-loaded NLC
dispersion or TAD-loaded NLC gel was also investigated. The permeation studies were
conducted as described above. The TAD release rate was evaluated according to
mathematical modeling based on the Higuchi diffusion equation (Oh et al., 2008).

2.9. Viscosity measurement

A Brookfield digital viscometer (DV-II+ Pro, Brookfield Engineering, MA) was
used to measure the viscosity of the TAD-loaded NLC gel or TAD gel. The viscosity
was measured at 25 ± 1°C with a no. 6 spindle.
2.10. Cytotoxicity study

HaCaT cells were grown in Dulbecco modified eagle medium (DMEM) (Gibco, UK) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin in 5% CO₂ at 37°C. The HaCaT cells were exposed to a series of concentrations (0.001–10 µM) of TAD solution or each TAD formulation (TAD-loaded NLC dispersion, TAD-loaded NLC gel, and TAD-loaded NLC with ethanol and limonene), with a final concentration of DMSO in the culture medium below 0.5%. After 72 h incubation (70% confluent) with TAD solution or each TAD formulation, cytotoxicity was determined by MTT assay according to the manufacturer’s protocol. Briefly, after incubation of cells with TAD solution or each TAD formulation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) was added to each well and incubated for 2 h at 37°C. The crystals of viable cells were solubilized in isopropanol. Absorbance was determined at 570 nm in a microplate reader (Sunrise, Tecan, Austria). Cell viability was expressed as the percent absorbance for cells exposed to TAD solution or each TAD formulation relative to absorbance measured for cells without treatment.

2.11. Cellular uptake using confocal microscopy

For qualitative evaluation of cellular uptake, 0.05% (w/w, with regard to the lipid matrix) Nile red-loaded NLC dispersion was prepared using the method described for the TAD-loaded NLC dispersion. Nile red-loaded NLC dispersion, Nile red-loaded NLC gel, or Nile red-loaded NLC with permeation enhancers was applied to HaCaT cells grown on a glass bottom dish (SPL, Pocheon, Korea) for 30 min or 1 h. After incubation,
cells were washed with PBS three times then fixed with 4% (w/v) paraformaldehyde solution for 15 min in the dark. After washing with PBS, cells were treated with fluorescent mounting medium including 4’,6’-diamidine-2’-phenylindole dihydrochloride (DAPI) for nucleus labeling. Thereafter, the cells were imaged using an LSM 5 Live Vario Two VRGB (Zeiss, Jena, Germany).

2.12. Statistical analysis

Student’s t-test was used to compare two different groups of samples. A p-value < 0.05 was considered significant.

3. Results and discussion

3.1. Solubility of TAD in solid and liquid lipids

The solubility of TAD in the lipid matrix is a major factor determining drug loading and encapsulation efficiency in the NLC. Therefore, we investigated the solubility of TAD in solid lipids or liquid lipids to determine conditions for loading the maximum amount of TAD into the NLC. For solid lipids, the solubility of TAD in stearic acid, GMS, or Dynasan 118 was 0.42%, 0.59%, or 0.55% (w/w), respectively. For liquid lipids, the solubility of TAD in soybean oil, Miglyol 812, or oleic acid was 1.12%, 1.51%, or 1.98% (w/w), respectively (Figure 1). Based on these results, GMS and oleic acid were selected to allow the greatest solubility of TAD among tested lipids. Interestingly, oleic acid is a well-known skin permeation enhancer (Trommer and Neubert, 2006), and the penetration enhancing effects arise from intercalation into the structured lipids of the stratum corneum, which disturbs the lipid packing order (Mehnert and Mader, 2001).
3.2. Screening and characterization of the TAD-loaded NLC dispersion

The optimal conditions for the preparation of the TAD-loaded NLC dispersion were screened using a two-level and three-variable strategy with $2^3$ full factorial planning. For this design, ultrasonication time, lipid ratio, and emulsifier concentration were chosen as three critical independent variables. The ultrasonication time is a production condition that impacts the whole system, while the other two affect composition: the solid-to-liquid lipid ratio influences the inner phase behavior, and the emulsifier concentration affects interface stabilization in the external phase. Formulations 1–8 (F1–F8) were therefore defined using these three parameters as follows: sonication time, 10 min (F1–F4) and 20 min (F5–F8); solid-to-liquid lipid ratio, 70:30 (F1, F2, F5, and F6) and 50:50 (F3, F4, F7, and F8); and poloxamer 188 concentration, 1.5% (w/v) (F1, F3, F5, and F7) and 3.0% (w/v) (F2, F4, F6, and F8) (Table 1). For each condition, dependent variables such as particle size, PDI, zeta potential, and encapsulation efficiency (E.E) were examined (Table 2).

All formulations (F1–F8) exhibited similar PDI, zeta potential, and E.E of 0.2, -25.9 to -35.4 mV, and 78.5–87.2%, respectively (Table 2). All TAD-loaded NLC dispersions exhibited PDI values lower than 0.3, which was considered optimal for the dispersion and homogeneity of these nanoparticles (Vitorino et al., 2011). Furthermore, a colloidal suspension is considered stable when its zeta potential is higher than the absolute value of 30 mV (Helgason et al., 2009; Igbal et al., 2012). Consequently, the particle size was mainly used to characterize TAD-loaded NLC dispersions.

Sonication time did not significantly affect the particle size, suggesting that ultrasonication for 10 min was sufficient to obtain small particle sizes. Interestingly,
increasing the proportion of liquid lipid compared to the solid lipid decreased the particle size. This effect might arise from a lower viscosity of the dispersed phase (Wissing et al., 2004). Also, it was suggested that emulsifier concentration did not significantly affect particle size against that particle size generally decreased when the emulsifier concentration was increased, and this effect was thought to arise from increased surface area in increasing emulsifier concentrations (Jacobs et al., 2000; Schäfer et al., 2002).

Based on these results, F4 was chosen for further analyses because the lower ultrasonication time reduces production time and cost with only a marginal difference in size compared to F7 and F8. Some reports have demonstrated phagocytic uptake of nanoparticles by macrophages with a lower cut-off size around 0.5 µm (Teskač and Kristl, 2010). They reported that resveratrol-loaded SLN crossed the keratinocyte membrane in less than one minute (Kuchler et al., 2009). Our optimized formulation (F4) also exhibited particle sizes below 0.5 µm. The E.E (%) of F4 was the highest at 89.6%, associated to a drug loading of 4.48% ± 0.14% (w/w) (data not shown). Importantly, the TEM image of F4 showed a typical spherical shape that correlated with particle size (Figure 2).

3.3. Occlusion effect

The occlusion factor of blank NLC gel or TAD-loaded NLC gel was ~80% at 6, 12, 24, and 48 h. No significant difference in the occlusion factor between blank NLC gel and TAD-loaded NLC gel was observed with increasing time (Figure 3). However, the occlusion factor of TAD gel was 47.5% at 6 h, significantly lower than those for blank
NLC gel and TAD-loaded NLC gel. This result suggested that the occlusion effect of TAD-loaded NLC gel was greater than that for TAD gel.

Occlusion is a key factor for skin hydration and the subsequent controlled penetration of compounds (Jenning et al., 2000; Cua et al., 1990). The stratum corneum contains 10–20% water (Vogel, 1981), and the water and lipid content strongly influence skin resistance (Zhai and Maibach, 2002). Therefore, skin hydration and the occlusion effect are key factors in the cosmetic and dermatology fields. Vehicles may affect the hydration state of the stratum corneum. Occlusion of the skin significantly enhances penetration of the compound (Leveque et al., 1980). NLC could provide better skin hydration and occlusion compared to traditional delivery systems (for example, microemulsion) because it is nanosized. NLC localized on the skin forms a film with an occlusive action to prevent water loss from the skin through evaporation. The degree of the occlusion effect on skin is based on the size of the particles: smaller particles decrease evaporation of water from the skin (Chang and Riviere, 1993). Due to the occlusion of NLC, water evaporation from the skin to the atmosphere is decreased, and water is thus retained within the skin, swelling the stratum corneum and leading to enhancement of drug permeation (Müller et al., 2007).

3.4. In vitro skin permeation studies

We conducted in vitro skin permeation studies with F4, F4 supplemented with ethanol, and F4 with ethanol and limonene (1%, 3%, and 5%) to enhance penetration of TAD from TAD-loaded NLC dispersion. We prepared a TAD solution with 30% ethanol. The flux of TAD from the TAD solution was 0.307 µg/cm²/h, and that from F4 was 0.719 µg/cm²/h. F4 with ethanol showed a flux of 1.066 µg/cm²/h (Table 3). Thus, the
TAD-loaded NLC dispersion could increase the flux of TAD even in the presence of ethanol. Ethanol has been reported to act by several mechanisms: acting as a co-solvent, increasing permeant partitioning and solubility into the stratum corneum, and acting as a permeation enhancer by extracting large amounts of stratum corneum lipids (Barry et al., 1984; Walker and Smith, 1996). Therefore, ethanol alters the barrier properties of the skin, which partly accounts for the high flux values (Benson, 2005). Furthermore, three concentrations of limonene, a well-known permeation enhancer (Sapra et al., 2008), were tested for permeability and found to increase skin permeability compared to the ethanol group (Figure 4).

The enhancement ratio (ER) was calculated to compare the flux between TAD-loaded NLC formulations and TAD solution. The ER was increased to 3.9–4.8 when limonene (1–5%) was added to F4 with ethanol. The addition of both ethanol and limonene increased the flux of TAD in a limonene concentration-dependent manner (Table 3) and showed a synergistic permeation effect. Limonene was previously shown to be a potent skin permeation enhancer (Sinha and Kaur, 2000), which is proposed to arise from modification of intercellular packing and disruption of highly ordered lipid structures (Sinha and Kaur, 2000; Williams and Barry, 1991). These properties appear to involve solvent-mediated modification of the stratum corneum, thus improving drug partitioning into the tissue. The synergistic effect of ethanol and limonene was previously reported to be an effective binary enhancer system (Sapra et al., 2008; Bhaskar et al., 2009).

Next, we added F4 to a gel for skin application. The cumulative permeated amounts of TAD from F4 gel, F4 gel with ethanol, and F4 gel with ethanol and 5% limonene in 48 h were 29.8 μg/cm², 42.3 μg/cm², and 55.1 μg/cm², respectively, and the flux of TAD was 0.619 μg/cm²/h, 0.881 μg/cm²/h, and 1.149 μg/cm²/h, respectively (Table 4).
As we expected, TAD-loaded NLC gel formulations showed a decrease in flux compared to the TAD-loaded NLC dispersion (Figure 5). This behavior might be due to slower release from the polymeric matrix and the increased viscosity promoted by the gelling agent (Bhaskar et al., 2009; Brosin et al., 1997).

3.5. Cytotoxicity study

To determine the potential cytotoxic activity of the TAD-loaded NLC formulation, cell viability was evaluated for human HaCaT keratinocyte cells. The cytotoxicity of each TAD solution and TAD-loaded NLC formulation (F4, F4 gel, or F4 gel with ethanol and 5% limonene) was observed for 72 h for different TAD concentrations (Figure 6). With increasing concentration of TAD solution or TAD-loaded formulation, cell viability decreased. The viability of HaCaT cells at 10 μM TAD from TAD solution, F4, F4 gel, or F4 gel with ethanol and 5% limonene was 64.2 ± 2.4%, 52.3 ± 4.1%, 57.0 ± 2.5%, or 50.0 ± 3.8%, respectively. Incorporation of the TAD-loaded NLC dispersion into the gel exhibited no increase in cytotoxicity. Furthermore, F4 with ethanol and 5% limonene did not exhibit increased cytotoxicity. According to the OECD guideline (Tabata and Ikada, 1990), an irritant substance is defined by mean relative tissue viability below 50% of the mean viability of the negative controls after 15–60 min exposure. In this work, HaCaT cells were used to assess cytotoxicity to the skin (Brosin et al., 1997; Baek et al., 2013) through exposure for 72 h, and the cell viability of all formulations was above 50%, indicating the tested formulations are considered non-irritant.

3.6. Viscosity
F4, F4 gel with ethanol, and F4 gel with ethanol and 5% limonene were diluted with DMEM to determine the viscosity of formulations tested in the cellular uptake study (Figure 7). The viscosities of F4, F4 gel with ethanol, and F4 gel with ethanol and 5% limonene were 2.8 ± 0.7, 11.4 ± 1.8, and 12.2 ± 1.4 cP, respectively, indicating that TAD-loaded NLC gel has a higher viscosity than TAD-loaded NLC dispersion. The addition of ethanol and 5% limonene did not affect viscosity.

3.7. Cellular uptake study

The cellular uptake of Nile red-loaded NLC dispersion, Nile red-loaded NLC gel, and Nile red-loaded NLC gel with ethanol and 5% limonene was assessed by confocal microscopy (Figure 8). After 0.5 h of incubation at 37°C, fluorescence was detected in the cytoplasm of HaCaT cells treated with Nile red-loaded NLC dispersion and accumulated with increasing incubation time. Cells treated with Nile red-loaded NLC gel or Nile red-loaded NLC gel with ethanol and 5% limonene exhibited lower fluorescence intensity than those treated with Nile red-loaded NLC dispersion. For all incubation times, Nile red-loaded NLC dispersion exhibited more fluorescence than Nile red-loaded NLC gel, suggesting no increase in cellular uptake for Nile red-loaded NLC gel.

After incorporation into the gel, nanoparticles were trapped in the gelling polymer, leading to relatively higher viscosity of gel formulations (Figure 5). Furthermore, internalization of Nile red from Nile red-loaded NLC gel was delayed. These results are consistent with previous reports on the sustained delivery of nitrendipine using NLC by gel formulations (Bhaskar et al., 2009).
4. Conclusion

Different TAD-loaded NLC formulations were assessed for their potential to increase skin permeability using penetration enhancers compared to TAD solution. Skin permeation of TAD was increased for TAD-loaded NLC gel in the presence of ethanol and 5% limonene. Furthermore, HaCaT cells tolerated the cytotoxicity of TAD-loaded NLC gel. Together, these results indicate that TAD-loaded NLC gel with ethanol and 5% limonene could be a promising transdermal drug delivery carrier for TAD.

Acknowledgements

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**Figure Captions**

**Fig. 1.** TAD solubility in (A) solid lipids and (B) liquid lipids (n=3).

**Fig. 2.** TEM image of F4 as optimal NLC formulation (scale bar 2 um).

**Fig. 3.** Occlusion factor of unloaded nanostructured lipid carriers gel (blank NLC gel), tadalafil-loaded nanostructured lipid carriers gel (TAD-NLC gel) and tadalafil gel (TAD-gel) at 6, 12, 24 and 48 h after treating formulations, * p<0.05 versus blank NLC gel, * p<0.05 versus TAD-NLC gel (n=3, mean ± SD).

**Fig. 4.** In vitro permeation profiles of NLC formulations up to 48 h (n=3, mean ± SD).

**Fig. 5.** In vitro permeation profiles of NLC gel formulations up to 48 h (n=3, mean ± SD).

**Fig. 6.** In vitro cytotoxicity of different TAD formulations in HaCaT cells for 72 h up to 10 µM (n=3, mean ± SD).

Fig. 7. Viscosity of TAD-NLC, TAD-NLC in EtOH gel and TAD-NLC in EtOH and 5% limonene gel diluted with DMEM medium. * p<0.05 versus TAD.

**Fig. 8.** Fluorescent microphotographies in LSM5 of the nile red-labeled NLCs uptake by HaCaT keratinocyte cell line for 30 min (A, B and C) or 60 min (D, E and F): (A and D) NLC; (B and E) NLC gel; (C and F) NLC gel + skin permeation enhancers.
Fig. 1.

(A) 

![Bar chart showing solubility of different solid lipids: Stearic acid, GMS, and Dynasan 118.](chart1)

(B) 

![Bar chart showing solubility of different liquid lipids: Soybean oil, Miglyol 812, and Oleic acid.](chart2)
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.
Fig. 7.

![Graph showing viscosity (cP) comparison between F4, F4 gel with 30% ethanol, and F4 gel with 30% ethanol and 5% limonene.](image-url)
Tables

**Table 1.** Composition of TAD-loaded NLC dispersions.

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<th>Sonication time</th>
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</tr>
<tr>
<td>F8</td>
<td>20</td>
<td>50:50</td>
<td>3.0</td>
</tr>
</tbody>
</table>
Table 2. Particle size, polydispersity index (PDI), zeta potential, and encapsulation efficiency (E.E) of different TAD-loaded NLC dispersions (n = 3, mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>Particle size (nm)</th>
<th>PI</th>
<th>Zeta potential (mV)</th>
<th>E.E (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>215.4 ± 5.1</td>
<td>0.258 ± 0.031</td>
<td>-25.9 ± 1.2</td>
<td>80.7 ± 5.4</td>
</tr>
<tr>
<td>F2</td>
<td>209.4 ± 6.3</td>
<td>0.269 ± 0.022</td>
<td>-27.1 ± 3.5</td>
<td>83.1 ± 4.1</td>
</tr>
<tr>
<td>F3</td>
<td>195.7 ± 3.9</td>
<td>0.251 ± 0.027</td>
<td>-29.1 ± 2.2</td>
<td>85.3 ± 3.9</td>
</tr>
<tr>
<td>F4</td>
<td>190.6 ± 5.1</td>
<td>0.241 ± 0.035</td>
<td>-35.4 ± 4.5</td>
<td>89.6 ± 2.8</td>
</tr>
<tr>
<td>F5</td>
<td>211.8 ± 3.9</td>
<td>0.236 ± 0.024</td>
<td>-27.4 ± 3.8</td>
<td>78.5 ± 1.2</td>
</tr>
<tr>
<td>F6</td>
<td>201.1 ± 4.2</td>
<td>0.221 ± 0.015</td>
<td>-29.1 ± 2.7</td>
<td>85.7 ± 5.6</td>
</tr>
<tr>
<td>F7</td>
<td>190.5 ± 2.7</td>
<td>0.228 ± 0.009</td>
<td>-35.4 ± 5.2</td>
<td>83.1 ± 5.4</td>
</tr>
<tr>
<td>F8</td>
<td>181.1 ± 2.4</td>
<td>0.214 ± 0.012</td>
<td>-32.9 ± 4.4</td>
<td>87.2 ± 4.3</td>
</tr>
</tbody>
</table>
Table 3. Skin permeation parameters in TAD-loaded NLC dispersions in the absence/presence of permeation enhancers (n = 3, mean ± SD).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Flux (µg/cm²/h)</th>
<th>ER&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Q&lt;sub&gt;24&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (µg/cm²)</th>
<th>Q&lt;sub&gt;48&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt; (µg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAD solution</td>
<td>0.307 (r² = 0.995)</td>
<td>1.0</td>
<td>4.9 ± 2.6</td>
<td>11.2 ± 1.8</td>
</tr>
<tr>
<td>F4</td>
<td>0.719 (r² = 0.987)</td>
<td>2.3</td>
<td>21.6 ± 4.2</td>
<td>34.5 ± 3.3</td>
</tr>
<tr>
<td>F4 with ethanol</td>
<td>1.066 (r² = 0.998)</td>
<td>3.5</td>
<td>27.0 ± 5.7</td>
<td>51.2 ± 6.2</td>
</tr>
<tr>
<td>F4 with ethanol and 1% limonene</td>
<td>1.212 (r² = 0.997)</td>
<td>3.9</td>
<td>35.3 ± 4.1</td>
<td>58.2 ± 3.9</td>
</tr>
<tr>
<td>F4 with ethanol and 3% limonene</td>
<td>1.296 (r² = 0.999)</td>
<td>4.2</td>
<td>38.2 ± 7.4</td>
<td>62.2 ± 6.3</td>
</tr>
<tr>
<td>F4 with ethanol and 5% limonene</td>
<td>1.463 (r² = 0.998)</td>
<td>4.8</td>
<td>44.5 ± 3.1</td>
<td>70.2 ± 6.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>ER: Enhancement ratio. For the calculation of ER, the permeation of TAD solution was taken as reference.

<sup>b</sup>Q<sub>24</sub>: Cumulative permeated amount of TAD after 24 h.

<sup>c</sup>Q<sub>48</sub>: Cumulative permeated amount of TAD after 48 h.
Table 4. Skin permeation parameters in TAD-loaded NLC gel in the absence/presence of permeation enhancers (n = 3, mean ± SD).

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Flux (µg/cm²/h)</th>
<th>ER a</th>
<th>Q₂₄ b (µg/cm²)</th>
<th>Q₄₈ c (µg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAD solution</td>
<td>0.307 (r² = 0.995)</td>
<td>1.0</td>
<td>4.9 ± 2.6</td>
<td>11.2 ± 1.8</td>
</tr>
<tr>
<td>F4 gel</td>
<td>0.619 (r² = 0.981)</td>
<td>2.0</td>
<td>18.3 ± 2.7</td>
<td>29.8 ± 2.4</td>
</tr>
<tr>
<td>F4 gel with ethanol</td>
<td>0.881 (r² = 0.979)</td>
<td>2.9</td>
<td>27.6 ± 5.4</td>
<td>42.3 ± 3.3</td>
</tr>
<tr>
<td>F4 gel with ethanol and 5% limonene</td>
<td>1.149 (r² = 0.976)</td>
<td>3.7</td>
<td>34.9 ± 3.6</td>
<td>55.1 ± 5.6</td>
</tr>
</tbody>
</table>

aER: Enhancement ratio. For the calculation of ER, the permeation of TAD solution was considered as reference.

bQ₂₄: Cumulative permeated amount of TAD after 24 h.

cQ₄₈: Cumulative permeated amount of TAD after 48 h.