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The combination of Nanomicelles with Terpenes for Enhancement of Skin Drug Delivery

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Abstract

The nanomicelles have recently drawn a great deal of attention for drug delivery into the skin. However, these carriers have only deposited in hair follicles and furrows, and drug in the micelles may not therapeutically reach into viable skin layers. The aim of this study was to formulate a combination of nanomicelles with terpenes to overcome this challenge and evaluate their potential for topical drug delivery into the skin. The nanomicelles were characterised with respect to size, size distribution (PDI), zeta potential, morphology and encapsulation efficiency (%). The drug accumulation and penetration were examined by tape stripping method in the skin. The colloidal stability of nanomicelles was followed with respect to size and PDI values. The nanomicelles were about 25-30 nm in size with narrow distribution. All of them had slightly negative surface charge, spherical shapes and high encapsulation efficiency (%). The tape stripping data revealed that nanomicelles consisting of terpinolene led to accumulation of more drug in the stripped skin as compared with commercial product and nanomicelles without terpene. Also, micelle formulations consisting of terpinolene (2.0 %) had the highest colloidal stability. Consequently, combination of nanomicelles with terpinolene could be a feasible approach for enhancement of skin drug delivery.

Keywords: nanomicelle, polymeric micelle, terpene, skin topical delivery, skin penetration enhancement, penetration enhancer

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1. Introduction

The nanomicelles are aggregates with "core-shell" structures, self-assembling from amphiphilic polymers at a specific polymer concentration and temperature in an aqueous medium (1, 2). The "core" formed from hydrophobic polymer units has increased solubility of lipophilic drugs, meanwhile, the "shell" has ensured physical stability of the micelles in an aqueous medium, helping with steric hindrance in polyethylene glycol groups of hydrophilic polymer units (1-3). Although these nano-carriers are mostly investigated for the treatment of cancer and inflammatory diseases in the literature (2), some researchers suggested that an increment in the thermodynamic activity of the compound in consequence of the increased drug solubility *via* the nanomicelles contributed to enhancement of drug delivery into the skin (4-6). Thus, the nanomicelles have recently attracted a great deal of attention for drug delivery into the skin for the treatment of dermatological diseases. These carriers have only accumulated in the hair follicles and furrows, without passing the *stratum corneum* barrier after applied on the skin. Afterward, the drug molecules have been released from the micelles in the hair follicles and reached into the viable skin layers, partly overcoming the *stratum corneum* barrier (4-6). However, drug amount released from the micelles could be inadequate for effective treatment of the skin diseases because the hair follicles are less than 0.1 % of the total skin surface in the human body.

The addition of penetration enhancers into topical formulations is an essential approach which is often used to improve the penetration of drugs into the skin layers (7). The terpenes are generally regarded to be safer in comparison with frequently-used penetration enhancers such as fatty acids/esters, ethanol, urea, dimethyl sulphoxide, and surfactants (8). These excipients disrupt the structure of the intercellular lipid layers in the *stratum corneum* to increase skin penetration of both hydrophilic and lipophilic drugs (9, 10). However, they have been particularly useful for delivery of small molecules (<500 Da) into the skin (11). Based on aforementioned knowledge, we aimed (i) to develop an aqueous formulation for topical delivery of a lipophilic compound, which resulted in high

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patient compliance because of easy application and absence of greasiness, with drug content equivalent to that of a commercial product, and (ii) to enhance delivery of a drug with high lipophilicity and large molecular weight into the deeper layers of skin, utilizing the increase in drug solubility *via* the nanomicelles and impairment of the *stratum corneum* through terpenes

2. Materials and Methods

2.1. Materials

Pluronic® F127 and L61 were kind gifts of BASF (Ludwigshafen, Germany). Tacrolimus monohydrate (TAC) was kindly provided by Bilim Pharmaceutical Company (Turkey). D,l-limonene, d,l-menthol, and nerolidol were obtained from Merck (Darmstadt, Germany). Terpinolene and eucalyptol were purchased from Fluka (St. Gallen, Switzerland). All other chemicals and reagents were of analytical grade and were commercially available.

2.2. Preparation of Nanomicelles

Tacrolimus monohydrate (TAC), which is a model drug, loaded polymeric micelles were prepared by thin film hydration method (12). Briefly, Pluronic® F127 and Pluronic® L61 were dissolved in 1.5 mL of acetonitrile. TAC was dissolved in 1.5 mL of acetonitrile. The solutions were mixed with each other. In order to obtain a thin film layer in a round-bottom flask, acetonitrile was evaporated in a rotary evaporator (IKA HB10 Control, IKA Works GmbH & Co., Germany) at 45°C, 150 mbar and 75 rpm/min. Then, the thin film layer was kept overnight in a desiccator to remove completely the residual acetonitrile. The film layer was hydrated with 3 mL of ultrapure water, stirring at 60°C for 1 hour. After 1 hour, the size and size distribution of clear formulations were measured.

On the preliminary studies, micelle formulations were prepared with using different concentration of polymers (1, 2, 3, 4, 5, 10 or 15 %, w/v of Pluronic® F127 and 0, 0.1, 0.25 or 0.5 %, w/v of Pluronic® L61) and TAC (0.03, 0.05 or 0.1 % w/v) . In order to determine optimum concentration of polymers and drug, the size stability of clear formulations was examined with DLS measurements for 14 days at

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room temperature, then the formulation exhibiting the highest colloidal stability was determined to prepare the micelles consisting of penetration enhancer.

Different types of terpenes namely, d,l-limonene, terpinolene, d,l-menthol, eucalyptol or nerolidol as penetration enhancers at the various of concentration (0.5, 1.0 or 2.0 %, v/v) were added into micellar formulations. For the preparation of polymeric micelles containing penetration enhancer, terpenes were added to mixed acetonitrile solution composed of polymer and drug, and the procedure resumed as aforementioned before. The codes of obtained micellar formulations are presented in Table 1.

Table 1: Formulation codes and terpenes (%, v/v) in the nanomicelles

Formulation Codes*	Type of Terpenes	% Terpenes (v/v)		
TD401	-	-	-	-
LD05	d,l-Limonene	0.5	-	-
LD10	d,l-Limonene	-	1.0	-
LD20	d,l-Limonene	-	-	2.0
TD05	Terpinolene	0.5	-	-
TD10	Terpinolene	-	1.0	-
TD20	Terpinolene	-	-	2.0
MD05**	d,l-Menthol	0.5	-	-

* All formulations contained 0.03 % of TAC, 4 % of Pluronic® F127 and 0.1 % of Pluronic ®L61

** (%, w/v)

2.3. Size and Size Distribution of Nanomicelles

The hydrodynamic size and size distribution (polydispersity index, PDI) of the micelles were measured using dynamic light scattering (DLS) method by a ZetaSizer Nano ZS (Malvern Instruments,

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UK) at 25±0.1°C with angle of 173°. Each measurement was repeated at least three times for all samples.

2.4. Zeta Potential of Nanomicelles

The zeta potential of the micelles was determined by a ZetaSizer Nano ZS (Malvern Instruments, UK) at 25±0.1°C, using electrophoretic light scattering method. The time and voltage parameters were automatically set. The measurement parameters were as follows; refractive index: 1.330; viscosity: 0.8872; dielectric constant of water: 79 and f(ka):1.50 (Smoluchowski value). Each measurement was repeated at least three times.

2.5. Morphology of Nanomicelles

The morphology of the micelles was observed by Atomic Force Microscopy (SPM-9600, Shimadzu, Japan). A sample of the micellar formulation was fixed onto the mica surface after the mica surface was cleaned. The resonance frequency and force constant were set to 320 kHz and 42 N/m, respectively. Then, the sample was scanned in "dynamic mode".

2.6. Encapsulation Efficiency and Drug Loading of Nanomicelles

Vivaspin®6 centrifuge tubes with polyethersulphone membrane (MWCO: 3500 Da, Sartorius AG, Germany) were used to determine encapsulation efficiency and drug loading capacity of the nanomicelles (13). 3 mL of the micellar formulation was placed into the centrifuge tube and centrifuged for 30 minutes at 3000 RCF (g). 100 µL of filtrate was diluted with 0.9 mL of acetonitrile and concentration of TAC in the samples was quantified by validated High Pressure Liquid Chromatography (HPLC) as described below. The encapsulation efficiency (%) and drug loading capacity (%) of the micelles were estimated according to Eqs (1) and Eqs (2), respectively;

$$\text{Encapsulation Efficiency (\%)} = 100-(a \times 100/b) \quad (1)$$

$$\text{Drug Loading Capacity (\%)} = [(b-a)/(b+c+d)] \times 100 \quad (2)$$

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a: The concentration of TAC in filtrate (mg/mL)

b: The concentration of TAC used for micellar formulation (mg/mL)

c: The concentration of Pluronic® F127 (mg/mL)

d: The concentration of Pluronic® L61 (mg/mL)

2.7. Skin Accumulation and Penetration Studies

2.7.1. Skin Preparation

The excised porcine skin was used as a model membrane for the skin accumulation and penetration studies. The porcine dorsal skin was obtained from a local slaughterhouse, washed carefully under cold running water and blotted dry with soft tissue. The hairs were cut with a scissors and subcutaneous fatty tissue was carefully removed using a scalpel from the skin. After that, the skin was packed in aluminium foil and frozen at -20°C until use. The skin samples were thawed at room temperature before the experiments and the skin surface was carefully cleaned with cold phosphate-buffered saline (pH 7.4, PBS).

2.7.2. Skin Permeation Studies

The skin permeation studies were performed using Franz diffusion cells (PermeGear, V6A Stirrer, Hellertown, PA, USA) with an available diffusion area of 1.77 cm^2 and a receptor volume of 12 mL. The receptor chambers were filled with PBS (pH 7.4) containing serum bovine albumin (BSA) of 1 % (w/v) to maintain the sink condition during the experiment (5). The *stratum corneum* layer of full-thickness skin samples was exposed to donor compartments and mounted onto Franz diffusion cells, the donor chambers were clamped. The receptor medium was magnetically stirred at 300 rpm and kept at 37±0.5 °C during the experiment, to reach 32±0.5 °C for the skin temperature. All air bubbles in the receptor medium were carefully removed. After equilibration for 30 min, 1 mL of the micellar formulations and 1 g of commercial ointment (Protopic®, 0.03 %) as control formulations were applied to the skin surface in the donor compartments. At the end of 24 hours, 1 mL of samples were

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withdrawn from the receptor chambers, filtered through 0.45 µm Millex® syringe filters (PTFE, LCR, Merck Millipore, Darmstadt, Germany). The drug concentration in the samples was quantified by LC/MS using the method described below.

2.7.3. Skin Cleaning and Tape-Stripping Prodecures

Upon completion of skin permeation studies, the receptor medium was removed and the diffusion cells were dismantled. The skin surface was washed 5 x 1 with mL ultra pure water in order to remove the remaining formulation and then, was gently dried with a cotton swab (14).

The tape stripping method was used to separate the *stratum* corneum from the viable epidermis and dermis (15, 16). To avoid any furrows which could result in mistaken data of the tape stripping procedure, the skin was stretched and mounted with pins on cork disks. 20 strips (2x2 cm^2) of transparent adhesive tape (3M, Hutchinson, MA, USA) were sequentially fixed on the cleansed skin. In order to ensure a reproducible working procedure, a constant pressure was applied on the skin moving a roller (400 g) for ten seconds and the tape was removed from the skin surface in a single rapid movement at an angle of 45°. This template ensured for all tape stripping procedures at the same skin specimen. The first tape was discarded to represent unabsorbed drug in the skin surface and the other sequential tape strips were placed in four vials (4 strips in the first vial, 5 strips in the other vials) of suitable size for the extraction. The remaining skin samples were also cut into smaller pieces to increase the surface area and placed in a separate vial. To dissolve the drug in the extraction fluid, each of the samples (*stratum corneum* or stripped skin) was soaked in acetonitrile (4 x 6 mL per vial for tape strips, 4 mL for the stripped skin samples) at ambient temperature for 15 hours under constant shaking (Thermo Forma 420 Orbital Shaker, Waltham, MA, USA) and vortexed vigorously for 5 minutes. The samples were filtered through 0.45 µm Millex® syringe filters, 0.5 mL of each tape strip sample was collected and mixed for 2 minutes. Then, all of the samples was submitted to LC/MS analysis for quantification of drug content.

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2.8. Quantification of Drug Amounts by HPLC and LC-MS/MS Analysis

2.8.1. HPLC Analysis

TAC concentration in the samples was quantified by HPLC equipped with PDA detector (LC 20AT, Shimadzu, Japan). C_{18}, 250 mm x 4.6 mm, i.d. 5 µm column (Thermo Scientific, USA) was used as stationary phase. The mobile phase was a mixture of acetonitrile: water: phosphoric acid (750:250:0.2, v/v/v) filtered through membrane filter (0.45 µm, Millex LH, Merck Millipore, Germany). The analysis was performed at 215 nm of detection wavelength and 1.0 mL/min of flow rate. The injection volume was applied as 50 µL. The method was validated for selectivity, linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ). The validation parameters were found to be linear in concentration range of 0.5 µg/mL and 100 µg/mL ($r^2 > 0.999$), accurate (recovery > 98%), precise (intra and inter day variation < 2 %). LOD and LOQ values were 0.094 µg/mL and 0.286 µg/mL, respectively. Any peaks of the polymers and penetration enhancers did not interfere with TAC peak, demonstrating selectivity of the analytical method.

2.8.2. LC-MS/MS Analysis

In order to improve specificity and sensitivity, LC-MS/MS analysis was carried out to quantify the drug in the penetration studies by LC/MS system (Agilent Technologies 6460 Triple Quadrupole, California, USA) comprising Infinity 1260 series binary pump and jet stream electrospray ionization. C_{18}, 150 mm x 4.6 mm, i.d. 5 µm analytical column (Thermo Scientific, USA) was used for isocratic separation at 25°C. The mobile phase consisted of methanol: ultra pure water with 1.0 % of formic acid (99:1, v/v). The flow rate was set at 0.7 mL/min and injection volume was applied as 2 µL. Repaglinide (REP) at a concentration of 50 ng/mL was used as internal standard. Mass spectrometric detection was performed with electrospray ionization in positive ion mode using multiple reaction monitoring. The detection settings for TAC and REP are presented in Table 2. The method was validated for selectivity, linearity, accuracy and precision. The linear range during the measurements for TAC was from 2 to 500 ng/ml ($r^2 > 0.999$).

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Table 2: LC/MS settings for detection of tacrolimus and repaglinide

	Tacrolimus	**Repaglinide**
Source parameters	Value (+)	Value (-)
Polarity	Positive	Positive
Precursor ion (m/z)	826.4	453.4
Product ion (m/z)	616.3	162.3
Collision energy (V)	36	15
Fragmentor voltage (V)	180	120
Cell accelerator voltage (V)	7	7
Capillary voltage (kV)	3.50	3.50
Nozzle Voltage (V)	500	500
Drying gas temperature (°C)	300	300
Drying gas flow (L/min)	5	5
Nebulizer (psi)	45	45
Sheat gas heater (°C)	250	250
Sheat gas flow (L/min)	11	11

2.9. Colloidal Stability of Nanomicelles

The colloidal stability of the micelles was examined at room storage conditions (25±2°C, 60±5% relative humidity (RH)) and, at accelerated stability test conditions (40±2°C, 75±5% RH), in accordance with ICH Q1 (R2) Guideline. The hydrodynamic size and size distribution of polymeric micelles were measured at the end of 15th day, 1st month, 2nd month and 3rd month by ZetaSizer Nano ZS (Malvern Instruments, UK) following macroscopically observation. Each measurement was repeated at least three times.

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2.10. Data Analysis

The obtained data are presented as mean of three experiments ± standard deviation (SD). All of the data was assessed with Unpaired Student's t-test or One-way ANOVA, followed by the Bonferroni multiple comparison test, using GraphPad Prism Software version 6.05VR (La Jolla, USA). *P* value <0.05 was considered as level of statistical significance.

3. Results and Discussion

3.1. Preparation of Nanomicelles

Pluronic® micelles recognizing as safe have been intensively studied for a long time, despite their low physical stability (17-20). However, their colloidal stability and drug loading capacity could be increased with a mixture of Pluronic® copolymers. Alakhov et al. reported that Pluronic® F127 (2 %) and Pluronic® L61 (0.25 %) mixed micelles were stable in the presence of fetal bovine albumin (2.5 %) (21). Based on this information, the mixture of Pluronic® F127 and Pluronic® L61 was also used to maintain colloidal stability of the micelles in our study. On the preliminary studies, Pluronic® F127 was worked in concentration range of 1 % (w/v) and 15 % (w/v), because of 0.8 % (w/w) < critical micelle concentration (22) and 18 % (w/w) < gelation concentration at body temperature (23). However, Pluronic® F127 concentration was selected between 1 % (w/v) and 5 % (w/v) to form the micelles because more than 5 % (w/v) of polymer concentration gelled in the hydration process. Pluronic® L61 was added to the formulations at three different concentration (0.1, 0.25 and 0.5 %, w/v) based on the literature (21).

The drug concentration loading into the micelles has affected their formation, size and colloidal stability (24, 25). At the end of 14 days, some visible aggregates were viewed in the blank formulations (the micelles not loading TAC) and the most of drug loaded micelles. Conversely, the micelles with drug concentration of 300 µg/mL showed higher colloidal stability from blank and more drug loaded micelles. This situation indicated that drug loading in the micelles improved colloidal

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stability, but there has been a saturation limit of the drug as pointed out by Zhang et al (26). Overall, TD401 coded formulation had the smallest size distribution value (0.218 ± 0.085) for 14 days and its content was used for further studies.

To the best of our knowledge, excipients have also affected micelle formation and characterisation (27, 28). Based on this challenge, we need to study with five different types of terpenes for a well-designed formulation. In our study, the presence of nerolidol, eucalyptol and menthol (except of 0.5 %, w/v) in the formulation prevented formation of the micelles, unlike limonene and terpinolene. When considered of log P values (29-31), molecular formulas of terpenes and the other components (Figure 1), it could be claimed that a balance of hydrophilic and lipophilic groups might need for micelle formation and log P values of terpenes should be in a certain range to form the micelles.

"INSERT FIGURE 1"

3.2. Size and Size Distribution of Nanomicelles

The particle size has played an important role for drug delivery into the skin. Campbell et al. (32) reported that polymeric particles accumulated only on the skin surface despite their small size (20 nm), without reaching into the viable layers. Vogt et al. (33) also indicated that 40 nm particles penetrated to follicular epithelium, but larger particles localised in entry of hair follicles. Therefore, the micellar size is a crucial parameter in view of predicting where they deposit in the skin. In the study, all of the micellar formulations were in range of 25 nm and 35 nm (Table 3). The presence of terpinolene or menthol in the formulations decreased micellar size and caused to an unimodal distribution (Table 3) in comparison with the micelles with limonene and the without terpene. Particularly, the formulation containing terpinolene (TD20) exhibited significantly smaller size and narrower size distribution than control formulation without penetration enhancer (TD401) ($p<0.05$). When the concentration of TAC in formulations with terpinolene were increased to 1000 µg/mL, it was observed that both of the size and size distribution was not significantly changed ($p>0.05$) (*data*

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not given). Hence, it could be claimed that terpinolene could reduce micellar size and size distribution, independently of TAC concentration. Furthermore, menthol caused to a significant decrease in size of the micelles only in which the drug concentration was 300 µg/mL ($p<0.05$). In the micelles with limonene, addition of terpenes into the formulation resulted in increased size and size distribution value of the micelles loaded TAC at concentration of 300, 500 or 1000 µg/mL (*data not given*). This could depend on intensity of micelles with increasing of limonene concentration, unlike ones with terpinolene and menthol (Figure 2).

Table 3: The size, size distribution and zeta potential of the nanomicelles

Formulation Codes	Z-Average Size (d. nm) ± SD*	PDI ± SD	Size Range (nm)**	Zeta Potential (mV) ± SD
TD401	28.85 ± 1.36	0.263 ± 0.040 (Bimodal)	10-16	-2.02 ± 0.56
LD05	29.37 ± 1.40	0.259 ± 0.006 (Bimodal)	15-20	-1.73 ± 0.93
LD10	30.28 ± 2.30	0.325 ± 0.093 (Bimodal)	15-24	-2.26 ± 1.19
LD20	34.70 ± 2.18	0.425 ± 0.096 (Bimodal)	15-29	-2.69 ± 0.83
TD05	29.77 ± 7.94	0.225 ± 0.053 (Unimodal)	13-19	-3.92 ± 0.67
TD10	27.73 ± 4.61	0.215 ± 0.052 (Unimodal)	5-39	-3.30 ± 1.00
TD20	25.76 ± 0.84	0.152 ± 0.015 (Unimodal)	14-16	-2.28 ± 1.11
MD05	25.32 ± 0.58	0.187 ± 0.065 (Unimodal)	5-27	-2.93 ± 2.17

*** DLS data**

**** AFM data**

3.3. Zeta Potential of Nanomicelles

The surface charge of nanoparticles may act as a crucial factor in terms of their colloidal stability and deposition in the skin (34, 35). Hence, we measured zeta potential values of the micelles to comment

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the colloidal stability and deposition in the skin against the surface charges. The zeta potential values were in the range of -1.73 ± 0.93 and -3.92 ± 0.67 mV (Table 3), with respect to the nature of the copolymers and no significant differences existed between the micellar formulations comprising terpene and control formulation (TD401) ($p>0.05$). As a result, these findings showed that the colloidal stability and dermal deposition would not be affected by surface charges of the micelles, in the following experiments.

3.4. Morphology of Nanomicelles

AFM images have revealed that all of micellar formulations consisting of terpenes had spherical shape (Figure 2) as generally seen in Pluronic® micelles (36, 37). Therefore, it may be claimed that the monoterpenes such as d,l-limonene, terpinolene *etc*. did not prevent micelle formation and/or disrupt their spherical shapes. The size of micelles consisting of terpenes from AFM images was smaller than those of measured with ZetaSizer (Table 3). In ZetaSizer measurements, this phenomenon could regard to calculation of diffusion layer formed due to H^+ and OH^- ions in the water (38, 39). Interestingly, it was also observed in AFM images that adsorbed the micelles intensified on the mica surface when concentration of the terpene increased in the micellar formulations with limonene, unlike terpinolene.

"INSERT FIGURE 2"

3.5. Encapsulation Efficiency and Drug Loading of Nanomicelles

Pluronic® micelles differing from their terpene content (from 0.5 up to 2 %, v/v) were formulated and characterised (Table 3 and Figure 2). Encapsulation efficiency (%) and drug loading efficiency (%) increased for all micellar formulations containing any terpene (99.0 % < and approximately 0.73) as compared to control micelles without terpene (94.91 % ± 0.88 and 0.69 ± 0.01), respectively. In addition, drug concentration in control formulation (TD401) was 15.27 ± 2.60 µg/mL in the filtrate, whereas no drug was detected in the filtrate of the formulations containing terpene, indicating an

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increase in drug loading capacity of the micelles with the presence of terpenes. The literature also affirmed that TAC is slightly soluble in water (4-18 µg/mL) despite being a lipophilic compound (40, 41). This was attributed to dissolution of the drug in the terpenes which interacted with polypropylene core of Pluronic® polymers and increased the solubility of TAC in the core. Similarly, Badran et al. (42) reported that the terpenes such as cineole increased encapsulation efficiency of a hydrophobic drug (nimesulide) in the liposomes. Charoenputtakun et al. also revealed that limonene increased solubility of all-trans retinoic acids, and then it was resulted in higher drug loading capacity in the lipid nanoparticles (43).

3.6. Skin Accumulation and Penetration Studies

The efficiency of drugs applied topically depends on the concentration in targeted tissues, which is related to penetration ability of drugs into the skin. Therefore, there is a need for targeting of drugs into the skin layers since conventional dosage forms such as ointments could be inefficient to achieve the required drug concentrations in the target cutaneous tissues. Hence, development of novel carriers would have superiorities in terms of enhancement of therapeutic aspect (44).

In order to examine the skin penetration of TAC which has highly lipophilic structure (log P=3.96) with large molecular weight (802.02 g/mol) *via* the nano-micelles consisting of terpenes, the quantification of TAC in the *stratum corneum* was performed by sequential tape stripping method following *in vitro* skin permeation study at infinite dose. For the comparison, the penetration of TAC from its commercial ointment (Protopic®) and the terpene-free micelles was also examined. (Figure 3). After *in vitro* permeation study for 24 hours, no drug was detected in receptor fluids of the diffusion cells applied either micelle formulations or Protopic ointment. When the micelles without terpene (TD401) was compared with Protopic ointment, the micelles showed an increase in the concentration of TAC by 4.2 fold in the *stratum corneum* and 3.2 fold in the total skin. This enhancement could arise from binding of polyethylene glycol (PEG) (which is a hydrophilic polymer

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and generates the shell of the micelles) to the water molecules and increasing hydration of the *stratum corneum*. Similarly, Rangsimawong et al. also (45) reported that PEG could affect the penetration of hydrophilic drug in the liposomes into the skin layers. TAC in the micelles without terpen and conventional formulation accumulated at similar amount in the stripped skin. This may be low diffusion rate of tacrolimus into the epidermis due to its large molecular weight and highly lipophlicity. When applied the micelles containing d,l-limonene or terpinolene into the skin, drug concentration in the *stratum corneum* were detected similar to that of the micelles without terpene (TD401). However, TAC significantly accumulated much more in the stripped skin ($p<0.05$) following the application of LD20, TD05, TD10 and TD20 micellar formulation. This may be due to stronger penetration enhancer effect of d,l-limonene or terpinolene. In this instance, the micelles could have localized in the hair follicles and furrows as reported in previous study (4) when applied onto the skin. Meanwhile, the drug and terpene were released into the hair follicles and furrows, the terpene broke the hydrogen bonds among the lipids in the *stratum corneum* and the drug penetration dually increased. Additionally, the micelles could improve penetrability of the drugs by increasing its thermodynamic activity and acting the penetration enhancer compound (5, 46). Interestingly, when compared with the micelles without terpene (TD401), tacrolimus *via* the micelles with d,l-menthol significantly penetrated more into the *stratum corneum*, conversely in the stripped skin. This may be due to its poor colloidal stability.

Note that the finite dose which described as <10 µl/cm^2 for liquids and 2-5 mg/cm^2 for semi-solid preparations in the donor compartment could not be applied in the penetration studies due to the challenge of developing a bioanalytical method for tacrolimus in the skin studies. When considered into the comparison of finite dose and infinite dose applications in guidance of earlier reported studies (14, 47), it might be remarked that the absolute drug amounts delivered into the skin could be lower from these results for the micellar formulations and commercial product if a finite dose was applied in our study. The relative amounts recovered were conversely able to be higher for finite

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dose studies. Furthermore, a finite dose of micellar formulations applied in the donor compartment might cause to a decrease in hydration of the *stratum corneum* and reducing in drug penetration into the skin as also reported by (14). On the other hand, this phenomena which is more possible for hydrophilic drugs, could not significantly affect or might increase our results in comparison with the commercial product because of difficulty the partition of highly lipophilic molecules into the "hydrated" *stratum corneum*.

"INSERT FIGURE 3"

3.7. Colloidal Stability of Nanomicelles

The colloidal stability of the micelles with terpenes and control micelles storing at 25±2°C, 60±5% RH and 40±2°C, 75±5% RH are presented in Figure 4. The hydrodynamic size and PDI results revealed that terpinolene (especially at the concentration of 2.0 %, v/v) created a great effect on the colloidal stability of the micelles. This increased stability could be explained by two mechanisms. Firstly, the terpenes in liquid form may enable a more fluidic core in the micelles at room temperature when localized in the hydrophobic units of the micelles. Similarly, Adams et al. showed that the presence of the fluid core in the micelles affected on the kinetic and thermodynamic stability of these nanocarriers (48, 49). Secondly, it has well known that an increase of the core lipophilicity has resulted in a decrease of the critical micelle temperature (CMT), and then the improved kinetic stability (50). As seen in our study, highly lipophilic terpinolene or d,l-limonene localizing in the micelle core could reduce CMT of Pluronic® F127 and L61 which are thermo-sensitive polymers and increase the stability. However, it could be suggested considering the similarity of molecular structures of terpinolene and d,l-limonene (Figure 1) that the melting point and indirectly solubility of the terpenes in the water affected the colloidal stability of the micelles. As seen in Figure 1, the melting point of d,l-limonene is much lower than that of terpinolene. According to our knowledge, the melting point of compounds is inversely proportional with the solubility. In this situation, it could be asserted that the solubility of d,l-limonene in the water is higher than that of terpinolene and

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hence, d,l-limonene could resulted in a higher affinity into the water phase than terpinolene, leakage of d,l-limonene from the core in course of time and shorter colloidal stability period than the micelles with terpinolene (Figure 4).

"INSERT FIGURE 4"

4. Conclusion

The combination of nanomicelles with terpenes was efficiently formulated and characterised in terms of hydrodynamic size, size distribution, zeta potential and morhology. The spherical micelles had slightly negative surface charge and high encapsulation efficiency (%) owing to terpene molecules in the micelle core. The penetration studies revealed that the nanomicelles consisting of terpinolene significantly increased the accumulation of a highly lipophilic and relatively large molecule (TAC) into the viable epidermis and dermis as compared to the nanomicelles without terpene and the commercial product. Depending on the terpene concentration, terpinolene improved the colloidal stability of Pluronic® micelles at room storage conditions and at accelerated stability test conditions. This study confirms that the combination of nanomicelles with terpenes are an innovative carrier able to enhance topical drug delivery into the skin. Based on these positive results, the penetration mechanism of these nanomicelles into the skin, cytotoxicity and biological activity in the cell culture studies will be examined in the future studies.

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References

1. Torchilin VP. Block copolymer micelles as a solution for drug delivery problems. Expert Opin Ther Pat. 2005;15:63–75.

2. Torchilin VP. Structure and design of polymeric surfactant-based drug delivery systems. J Control Release. 2001;73(2):137-72.

ACCEPTED MANUSCRIPT

3. Jones M. and Leroux J. Polymeric micelles - a new generation of colloidal drug carriers. Eur J Pharm Biopharm. 1999;48(2):101-11.

4. Bachhav YG, Mondon K, Kalia YN, Gurny R, Moller M. Novel micelle formulations to increase cutaneous bioavailability of azole antifungals. J Control Release. 2011;153(2):126-32.

5. Lapteva M, Mondon K, Moller M, Gurny R, Kalia YN. Polymeric micelle nanocarriers for the cutaneous delivery of tacrolimus: a targeted approach for the treatment of psoriasis. Mol Pharm. 2014;11(9):2989-3001.

6. Lapteva M, Santer V, Mondon K, Patmanidis I, Chiriano G, Scapozza L, et al. Targeted cutaneous delivery of ciclosporin A using micellar nanocarriers and the possible role of inter-cluster regions as molecular transport pathways. J Control Release. 2014;196:9-18.

7. Barry BW. Lipid-protein-partitationing theory of skin penetration enhancement. J Control Release. 1991;15:237-48.

8. Chen J, Jiang Q, Chai Y, Zhang H, Peng P, Yang X. Natural terpenes as penetration enhancers for transdermal drug delivery. Molecules. 2016;21(12):1709.

9. Moghimi H, Willams A, Barry B. A lamellar matrix model for stratum corneum intercellular lipids. V. Effects of terpene penetration enhancers on the structure and thermal behaviour of the matrix. Int J Pharm. 1997;146:41-54.

10. Williams AC. Transdermal and Topical Drug Delivery: From Theory to Clinical Practice. England: Pharmaceutical Press; 2003.

11. Asbill C. and Michniak B. Percutaneous penetration enhancers: local versus transdermal delivery. Pharm Sci Technol Today. 2000;3:26-40.

ACCEPTED MANUSCRIPT

12. Chen L, Sha X, Jiang X, Chen Y, Ren Q, Fang X. Pluronic P105/F127 mixed micelles for the delivery of docetaxel against Taxol-resistant non-small cell lung cancer: optimization and in vitro, in vivo evaluation. Int J Nanomedicine. 2013;8:73-84.

13. Bahadori F, Topcu G, Eroglu MS, Onyuksel H. A new lipid-based nano formulation of vinorelbine. AAPS PharmSciTech. 2014;15(5):1138-48.

14. Chen M, Liu X, Fahr A. Skin penetration and deposition of carboxyfluorescein and temoporfin from different lipid vesicular systems: In vitro study with finite and infinite dosage application. Int J Pharm. 2011;408:223–34.

15. Trebilcock K, Heylings J, Wilks M. In vitro tape stripping as a model for in vivo skin stripping. Toxicology in Vitro. 1994;8(4):665-7.

16. Erdal M, Yıldız Peköz A, Aksu B, Araman A. Impacts of chemical enhancers on skin permeation and deposition of terbinafine. Pharm Dev Technol. 2014;19(5):565-70.

17. Nagarajan R. Solubilization of hydrocarbons and resulting aggregate shape transitions in aqueous solutions of Pluronic (PEO–PPO–PEO) block copolymers. Colloids Surf B: Biointerfaces. 1999; 16:55–72.

18. Sezgin Z, Yuksel N, Baykara T. Preparation and characterization of polymeric micelles for solubilization of poorly soluble anticancer drugs. Eur J Pharm Biopharm. 2006;64(3):261-8.

19. Pitto-Barry A, Barry NPE. Pluronic® block-copolymers in medicine: from chemical and biological versatility to rationalisation and clinical advances. Polym Chem. 2014;5(10):3291-7.

20. Biswas S, Kumari P, Lakhani PM, Ghosh B. Recent advances in polymeric micelles for anti-cancer drug delivery. Eur J Pharm Sci. 2016;83:184-202.

ACCEPTED MANUSCRIPT

21. Alakhov V, Klinski E, Li S, Pietrzynski G, Venne A, Batrakova E, et al. Block copolymer-based formulation of doxorubicin. From cell screen to clinical trials. Colloids Surf B: Biointerfaces 1999;16:113–34.

22. Gao Q, Liang Q, Yu F, Xu J, Zhao Q, Sun B. Synthesis and characterization of novel amphiphilic copolymer stearic acid-coupled F127 nanoparticles for nano-technology based drug delivery system. Colloids Surf B Biointerfaces. 2011;88(2):741-8.

23. Cunha-Filho MSS, Alvarez-Lorenzo C, Mart´ınez-Pacheco R, Landin M. Temperature-Sensitive Gels for Intratumoral Delivery of β-Lapachone: Effect of Cyclodextrins and Ethanol. The Scientific World Journal. 2012;2012:Article ID 126723, 8 pages.

24. Basak R, Bandyopadhyay R. Encapsulation of hydrophobic drugs in Pluronic F127 micelles: effects of drug hydrophobicity, solution temperature, and pH. Langmuir. 2013;29(13):4350-6.

25. Kahraman E, Ozhan G, Ozsoy Y, Gungor S. Polymeric micellar nanocarriers of benzoyl peroxide as potential follicular targeting approach for acne treatment. Colloids Surf B Biointerfaces. 2016;146:692-9.

26. Zhang Y, Ren T, Gou J, Zhang L, Tao X, Tian B, et al. Strategies for improving the payload of small molecular drugs in polymeric micelles. J Control Release. 2017.

27. Sharp MA, Washington C, Cosgrove T. Solubilisation of model adjuvants by Pluronic block copolymers. J Colloid Interface Sci. 2010;344(2):438-46.

28. Khimani M, Ganguly R, Aswal VK, Nath S, Bahadur P. Solubilization of parabens in aqueous Pluronic solutions: investigating the micellar growth and interaction as a function of paraben composition. J Phys Chem B. 2012;116(51):14943-50.

29. Prasanthi D. and Lakshmi PK. Terpenes: Effect of lipophilicity in enhancing transdermal delivery of alfuzosin hydrochloride. J Adv Pharm Technol Res. 2012;3(4):216-23.

ACCEPTED MANUSCRIPT

30. Aktar B, Erdal M, Sagirli O, Güngör S, Özsoy Y. Optimization of Biopolymer Based Transdermal Films of Metoclopramide as an Alternative Delivery Approach. Polymers. 2014;6(5):1350-65.

31. Lei W, Yu C, Lin H, Zhou X. Development of tacrolimus-loaded transfersomes for deeper skin penetration enhancement and therapeutic effect improvement in vivo. Asian J Pharm Sci. 2013;8(6):336-45.

32. Campbell CS, Contreras-Rojas LR, Delgado-Charro MB, Guy RH. Objective assessment of nanoparticle disposition in mammalian skin after topical exposure. J Control Release. 2012;162(1):201-7.

33. Vogt A, Combadiere B, Hadam S, Stieler KM, Lademann J, Schaefer H, et al. 40 nm, but not 750 or 1,500 nm, nanoparticles enter epidermal CD1a+ cells after transcutaneous application on human skin. J Invest Dermatol. 2006;126(6):1316-22.

34. Gillet A, Compère P, Lecomte F, Hubert P, Ducat E, Evrard B, et al. Liposome surface charge influence on skin penetration behaviour. Int J Pharm. 2011;411:223–31.

35. Bhattacharjee S. DLS and zeta potential – What they are and what they are not? J Control Release. 2016;235:337–51.

36. Han LM, Guo J, Zhang LJ, Wang QS, Fang XL. Pharmacokinetics and biodistribution of polymeric micelles of paclitaxel with Pluronic P123. Acta Pharmacol Sin. 2006;27(6):747-53.

37. Sahu A, Kasoju N, Goswami P, Bora U. Encapsulation of curcumin in Pluronic block copolymer micelles for drug delivery applications. J Biomater Appl. 2011;25(6):619-39.

38. Remant Bahadur KC, Bhattarai SR, Aryal S, Khil MS, Dharmaraj N, Kim HY. Novel amphiphilic triblock copolymer based on PPDO, PCL, and PEG: Synthesis, characterization, and aqueous dispersion. Colloids Surf A: Physicochem Eng Asp. 2007;292(1):69-78.

ACCEPTED MANUSCRIPT

39. Zhao A, Zhou S, Zhou Q, Chen T. Thermosensitive micelles from PEG-based ether-anhydride triblock copolymers. Pharm Res. 2010;27(8):1627-43.

40. Lapteva M, Moller M, Gurny R, Kalia YN. Self-assembled polymeric nanocarriers for the targeted delivery of retinoic acid to the hair follicle. Nanoscale. 2015;7(44):18651-62.

41. Patel P, Patel H, Panchal S, Mehta T. Formulation strategies for drug delivery of tacrolimus: An overview. Int J Pharm Investig. 2012;2(4):169-75.

42. Badran M. Effect of terpene liposomes on the transdermal delivery of hydrophobic model drug, nimesulide: Characterization, stability and in vitro skin permeation. Afr J Pharm Pharmacol. 2012;6(43):3018-26.

43. Charoenputtakun P, Pamornpathomkul B, Opanasopit P, Rojanarata T, Ngawhirunpat T. Terpene composited lipid nanoparticles for enhanced dermal delivery of all-trans-retinoic acids. Biol Pharm Bull. 2014;37(7):1139-48.

44. Güngör S, Kahraman E, Özsoy Y. Polymeric Micelles for Cutaneous Drug Delivery. In: Naik J, editor. Nano Based Drug Delivery. Croatia: IAPC Open Book and Monograph Platform (OBP); 2015.

45. Rangsimawong W, Opanasopit P, Rojanarata T, Ngawhirunpat T. Terpene-Containing PEGylated Liposomes as Transdermal Carriers of a Hydrophilic Compound. Biol Pharm Bull. 2014;37(12):1936–43.

46. Yang Y, Bugno J, Hong S. Nanoscale polymeric penetration enhancers in topical drug delivery. Polymer Chemistry. 2013;4(9):2651-7.

47. Paz-Alvarez M, Pudney P, Hadgraft J, Lane M. Topical delivery of climbazole to mammalian skin. Int J Pharm. 2018;549:317–24.

ACCEPTED MANUSCRIPT

48. Adams ML, Andes DR, Kwon GS. Amphotericin B encapsulated in micelles based on poly(ethylene oxide)-block-poly(L-amino acid) derivatives exerts reduced in vitro hemolysis but maintains potent in vivo antifungal activity. Biomacromolecules. 2003;4(3):750-7.

49. Adams ML, Kwon GS. Relative aggregation state and hemolytic activity of amphotericin B encapsulated by poly(ethylene oxide)-block–poly(N-hexyl-l-aspartamide)-acyl conjugate micelles: effects of acyl chain length. J Control Release. 2003;87:23-32.

50. Owen SC, Chan DPY, Shoichet MS. Polymeric micelle stability. Nano Today. 2012;7(1):53-65.

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Legends for Figures

Figure 1: The chemical structures and physicochemical properties of tacrolimus (A) and the terpenes, eucalyptol (B), nerolidol (C), d,l-limonene (E), terpinolene (E) and d,l-menthol (F)

Figure 2: AFM imagines of the nano-micelles A) without terpene (TD401), B) with 0.5 % of d,l-limonene (LD05), C) 1.0 % of d,l-limonene (LD10), D) 2.0 % of d,l-limonene (LD20), E) 0.5 % of terpinolene (TD05), F) 1.0 % of terpinolene (TD10), G) 2.0 % of terpinolene (TD20) and H) 0.5 % of d,l-menthol (MD05)

Figure 3: Tacrolimus accumulation in the *stratum corneum*, the stripped skin and total skin through commercial formulation (Protopic® Ointment) and the micelles

Figure 4: Change of hydrodynamic size (Z-average) and polydispersity index (PDI) of the nano-micelles stored a) at 25◦C and b) 40◦C over 3 months

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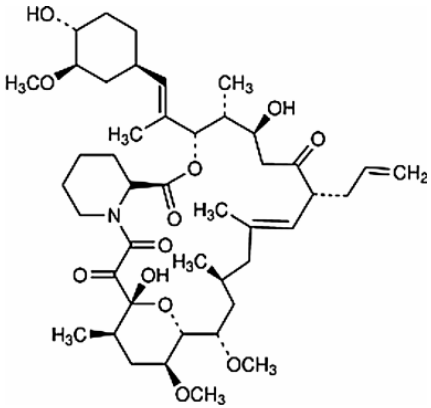
TACROLIMUS



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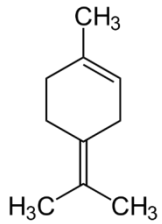

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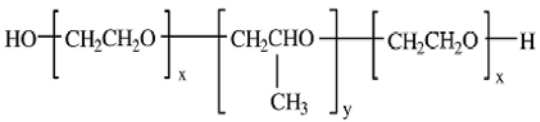
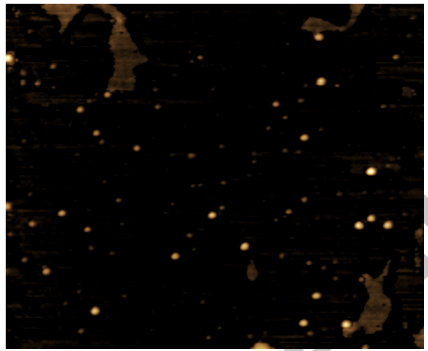
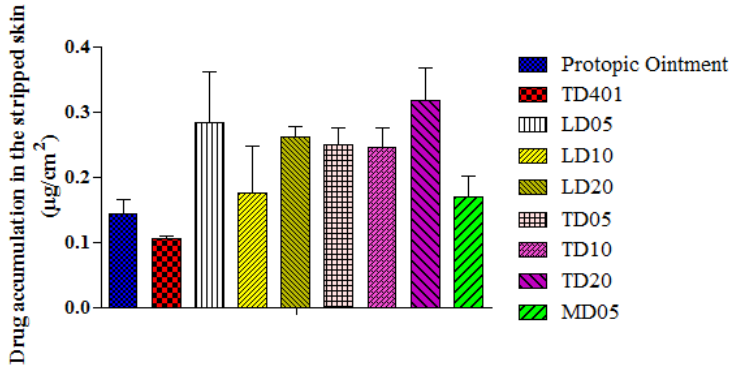


PLURONIC

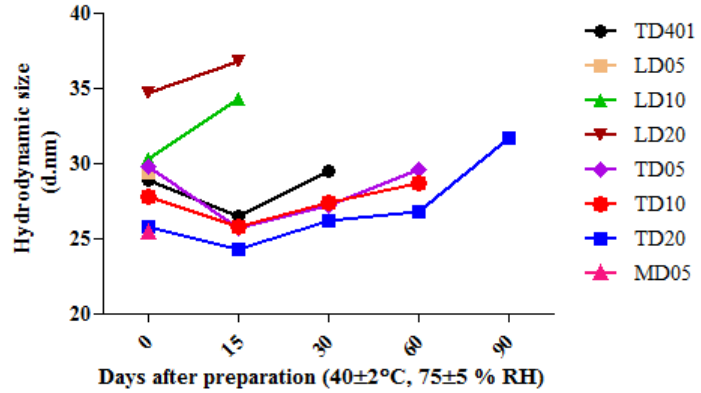
MICELLE FORMATION



Increased Accumulation and Penetration



Improved Colloidal Stability



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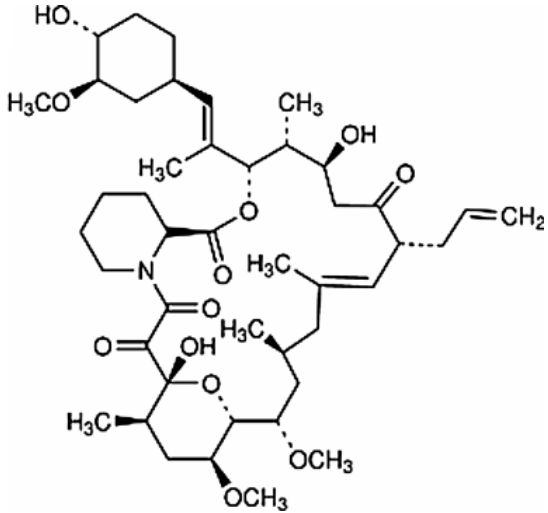
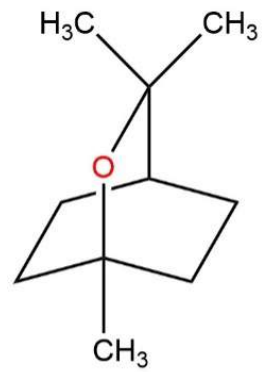
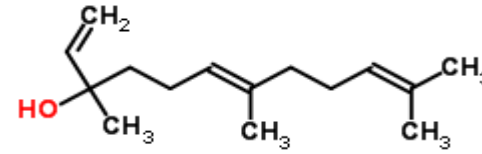
A



B

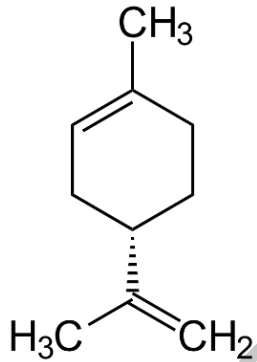


C

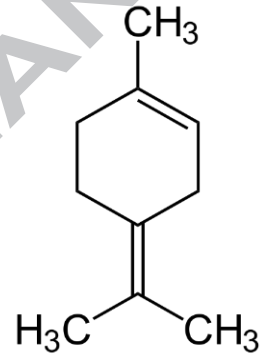


	A	B	C
Log P	3.96	2.82	5.32
Molecular Weight	804.02 g/mol	154.3 g/mol	222.4 g/mol
Melting Point	27-129°C	1.5°C	- 75°C

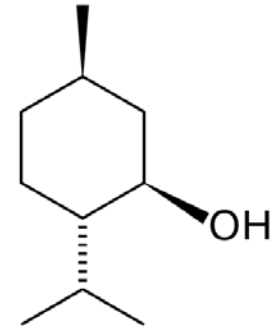
D



E



F



	D	E	F
Log P	4.58	4.52	3.20
Molecular Weight	136.2 g/mol	136.2 g/mol	156.3 g/mol
Melting Point	-74°C (d-limonene), -90°C (l-limonene)	< 25°C	- 8 °C (d-menthol), 41-43°C (l-menthol)

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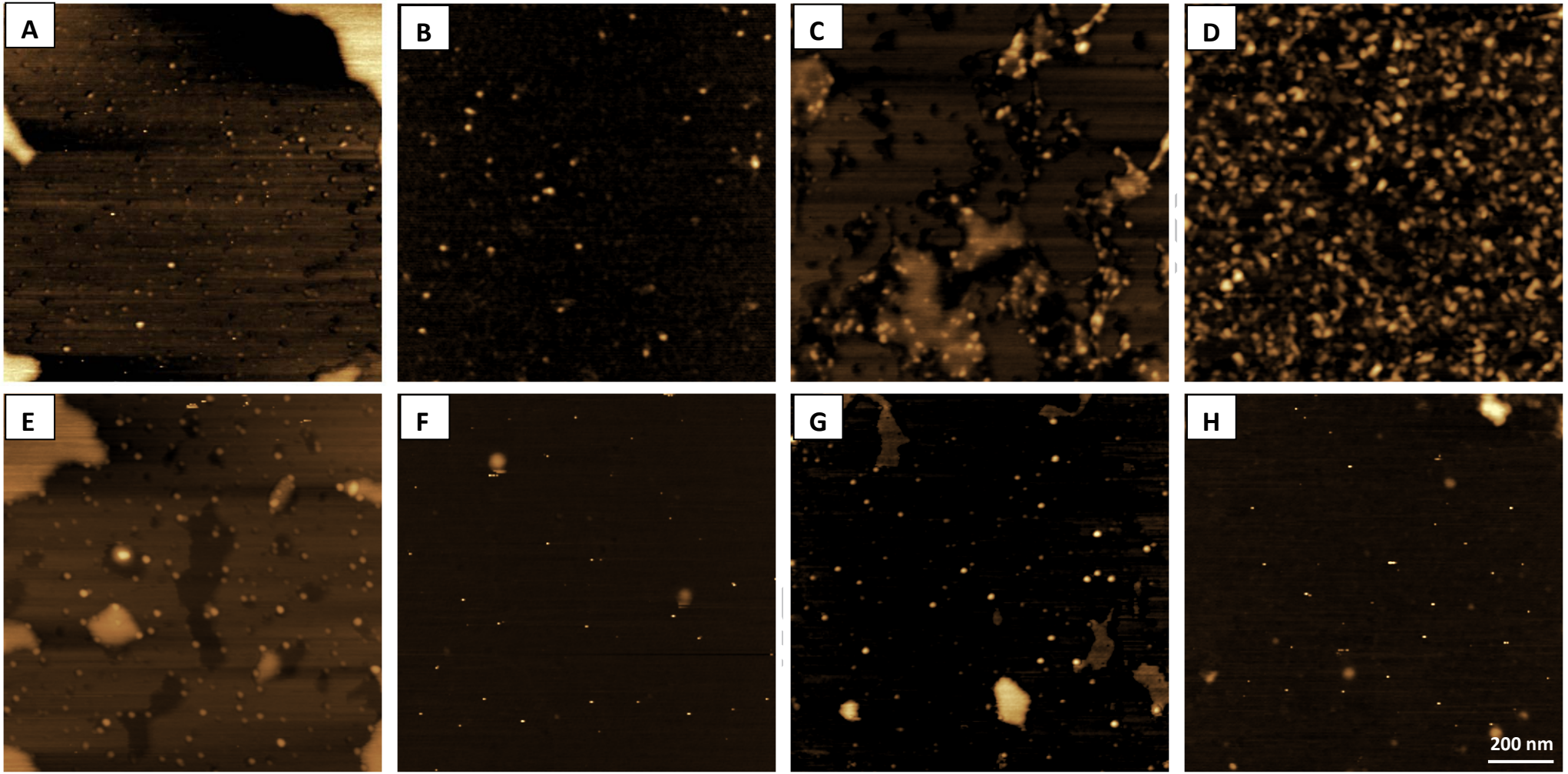
A
B
C
D
E
F
G
H
200 nm

Figure 3

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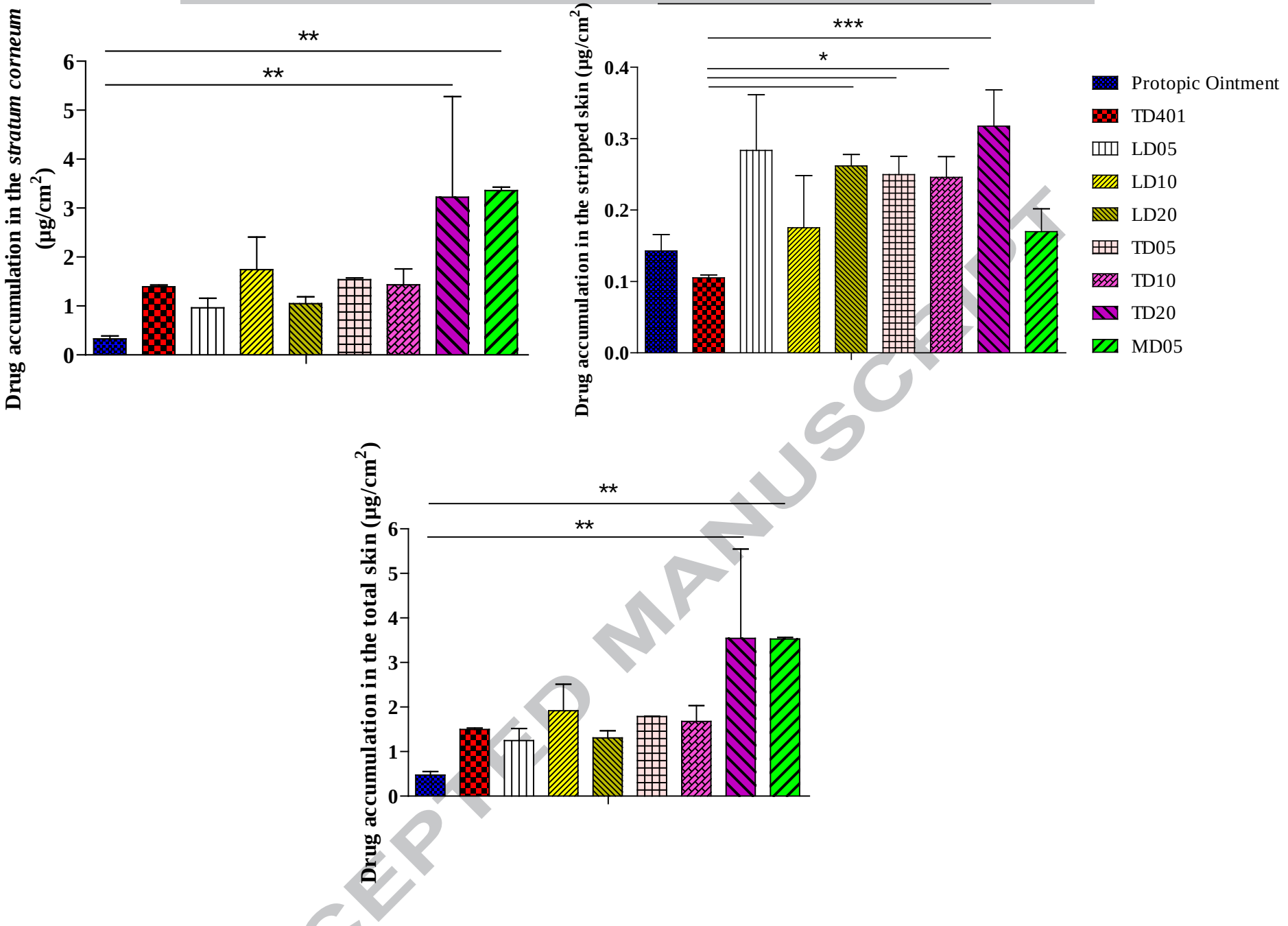

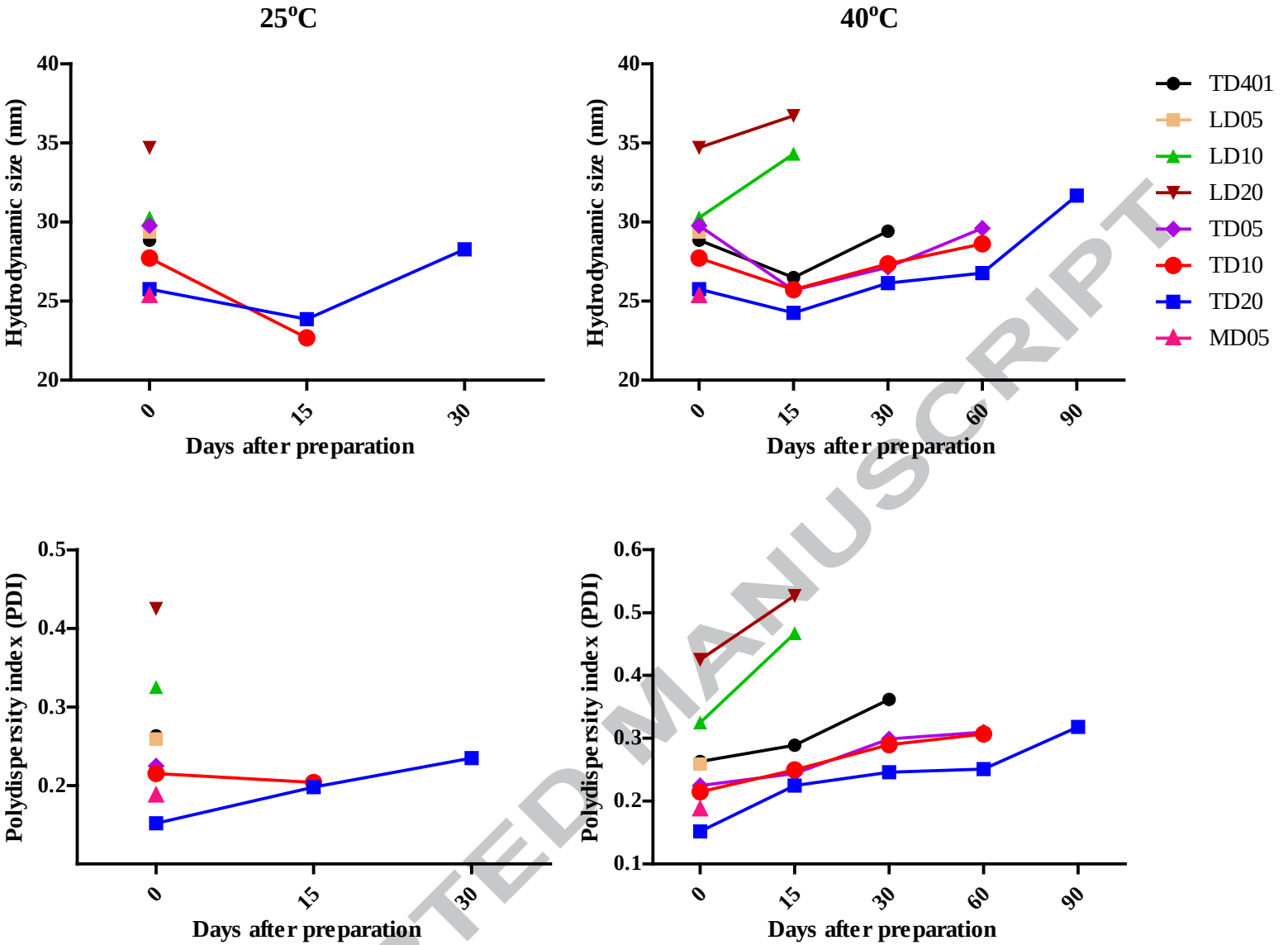

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