International Journal of Pharmaceutics 423 (2012) 303–311

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Physicochemical properties and skin permeation of Span 60/Tween 60 niosomes of ellagic acid

Varaporn Buraphacheep Junyaprasert*, Pratyawadee Singhsa, Jiraphong Suksiriworapong, Doungdaw Chantasart

Department of Pharmacy, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand

ARTICLE INFO

Article history:
Received 13 August 2011
Received in revised form 1 November 2011
Accepted 23 November 2011
Available online 2 December 2011

Keywords:
Ellagic acid
Niosomes
Non-ionic surfactants
Skin permeation
Skin distribution

ABSTRACT

Ellagic acid (EA) is a potent antioxidant phytochemical substance which has limitation to use due to its poor biopharmaceutical properties, low solubility and low permeability. The aim of the present study was to develop niosomal formulations obtained from the mixture of Span 60 and Tween 60 that could encapsulate EA for dermal delivery. The EA-loaded niosomes were prepared with 1:0, 2:1, 1:1, 0:1 Span 60 and Tween 60, using polyethylene glycol 400 (PEG 400), propylene glycol (PG) or methanol (MeOH) as a solubilizer. The influence of formulations on vesicle size, entrapment efficiency and stability of EA-loaded niosomes was investigated. It was found that all ratios of surfactants could produce EA-loaded niosomes when using 15% (v/v) PG, 15% (v/v) PEG 400 or 20% (v/v) MeOH. The niosomes were spherical multilamellar vesicles showing the localization of EA in the vesicles. The vesicle sizes of the niosomes after extrusion were 124–752 nm with PI less than 0.4. The percentages of entrapment efficiency (% E.E.) of all EA-loaded niosomes varied between 1.35% and 26.75% while PEG 400 niosomes gave the highest % E.E. The most stable and highest entrapped formulation was 2:1 Span 60 and Tween 60 niosomes. Additionally, the *in vitro* skin permeation revealed that penetration of EA from the niosomes depended on vesicle size, the amount of EA entrapped and the added solubilizers which could act as a permeation enhancer. From skin distribution study, the EA-loaded niosomes showed more efficiency in the delivery of EA through human epidermis and dermis than EA solution. The results indicated that the Span 60 and Tween 60 niosomes may be a potential carrier for dermal delivery of EA.

1. Introduction

Niosomes are self assembly of non-ionic surfactants which are resemble liposomes in their architecture and can be used as an effective alternative to liposomal drug carriers (Uchegbu and Vyas, 1998). Niosomes are biodegradable, biocompatible, and nontoxic and are capable of encapsulating large quantities of material in a relatively small volume of vesicles (Khandare et al., 1994). In addition, niosomes are versatile carrier systems that can be administered through various routes including intramuscular route (Bal et al., 2010), intravenous injection (Dufes et al., 2004), peroral delivery (Rentel et al., 1999), ocular delivery (Guinedi et al., 2005), pulmonary delivery (Marianecci et al., 2010) and transdermal delivery (Alsarra et al., 2005; Muzzalupo et al., 2011). Particular efforts have been aimed at using niosomes as effective dermal and transdermal drug delivery systems (Manconi et al., 2011; Mura et al., 2009; Muzzalupo et al., 2011). In particular, niosomes are considered as an interesting drug delivery system in the treatment of dermatological disorders. In fact, topical application of niosomes can increase the residence time of drugs in the stratum corneum and epidermis, while reducing the systemic absorption of the drug. They are thought to improve the horny layer properties, both by reducing transepidermal water loss and by increasing smoothness via replenishing lost skin lipids (Junginger et al., 1991). Moreover, non-ionic surfactants normally show favorable dermatological properties, being very mild to the skin and eye (Guinedi et al., 2005; Schreier and Bouwstra, 1994). Therefore, niosomes are interesting to explore the possibility of using as carriers for the topical delivery of active compounds.

Niosomes can be made up of a variety of amphiphiles bearing sugar, polyoxyethylene, polyglycerol, crown ether and amino acid hydrophilic head groups and these amphiphiles typically possess one to two hydrophobic alkyl or steroidal groups (Arunothayanun et al., 2000; Manconi et al., 2002). Among various non-ionic surfactants, niosomes prepared with Tween 61 in the combination with cholesterol at 1:1 mole ratio were found to have the high entrapment efficiency of water soluble substances (Manosroi et al., 2003). In few studies, the niosomes obtained from the mixture Tween 80

* Corresponding author. Tel.: +66 26448677x5730; fax: +66 26448694.
E-mail address: pyvbp@mahidol.ac.th (V.B. Junyaprasert).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.11.032

and Span 80 were seldom investigated (Liu and Guo, 2007; Liu et al., 2007; Tavano et al., 2011) although they were reported to be an effective carrier for topical delivery of drug (Tavano et al., 2011).

Ellagic acid (EA) is a naturally occurring polyphenol, phytochemical substance, and found in wide varieties of fruits and plants. EA has been already proven its antioxidant potential both *in vitro* and *in vivo*. Recent studies have indicated that EA possesses antimutagenic, antioxidant and anti-inflammatory activity in bacterial and mammalian systems (Kaur et al., 1997; Khanduja et al., 1999; Loarca-Piña et al., 1998). In addition, *in vitro* studies found that EA could inhibit mushroom-derived tyrosinase competitively and in a dose-dependent manner; whose inhibition constant (ki) is 81.6 μM (Shimogaki et al., 2000). According to the results of the studies using the brownish guinea pig, EA is more efficient skin whitener and suppressor of pigmentation than arbutin or kojic acid, other active skin whiteners, at the same dose level (1%). Furthermore, the efficacy of EA is almost the same as that of hydroquinone without causing injurious to melanocytes. As a result, EA can prevent the build-up of skin pigmentation after sunburn. It can also be expected to improve the appearance of pigmented skin such as melasma, freckles or postinflammatory pigmentation. Therefore, EA is a promising skin-whitening active agent with photoprotective effect. However, the use of EA is limited due to its poor biopharmaceutical properties. The low oral bioavailability of EA is attributed to its low aqueous solubility, metabolism in GIT (Seeram et al., 2004), irreversible binding to cellular DNA and proteins and first pass effect. In addition, it is poorly soluble in water (~9.7 μg/mL) (Bala et al., 2006) and not soluble in many organic solvents, leading to difficulties in the design of pharmaceutical formulations including niosomes.

In this study, the niosomes obtained from the mixture of Tween 60 and Span 60 were developed to encapsulate EA for dermal delivery. Due to the low solubility of EA, methanol (MeOH), propylene glycol (PG) and polyethylene glycol 400 (PEG 400) were used as solubilizers. The influences of ratios of surfactants and EA loading on the physicochemical properties of the EA-loaded niosomes were investigated in terms of vesicle size, entrapment efficiency and stability. Furthermore, *in vitro* skin permeation study and skin distribution in human skin were studied using static Franz diffusion cells and confocal laser scanning microscopy to evaluate the potential of niosomes as a dermal delivery system.

2. Materials and methods

2.1. Materials

Sorbitan monostearate (Span 60) and polyethylene glycol sorbitan monosterarate (Tween 60) were obtained from Uniqema (Chicago, USA). EA and PEG 400 were purchased from Fluka (West St. Paul, USA). PG was obtained from Kyowa Hakko (Tokyo, Japan). Cholesteryl poly-24-oxyethylene ether (Solulan C24) was received as a gift sample from Amerchol (NJ, USA). Cholesterol was purchased from Sigma–Aldrich (St. Louis, MO, USA). All other solvents were of HPLC grade and triple distilled water was used.

2.2. Preparation of EA-loaded niosomes

The vesicles were prepared by reverse phase evaporation (REV) method (Junyaprasert et al., 2008) using 1:1 mole ratio of surfactants and cholesterol as vesicle forming agents. The surfactants (1:0, 2:1, 1:1 and 0:1 Span 60 and Tween 60), cholesterol, Solulan C24 and EA (1, 3 and 5 mol%) were dissolved in diethyl ether (organic phase). To solubilize EA, 20 and 30% (v/v) MeOH was added into the organic phase containing EA, or 10 and 15% (v/v) PEG 400 or PG was added into an aqueous phase containing EA. At 1.5:1 the organic phase: the aqueous phase, the aqueous phase was added to the lipid mixture and mixed until a homogeneous w/o emulsion was obtained. The w/o emulsion was sonicated at 7 °C for 10 min. Afterwards, diethyl ether was slowly removed under reduced pressure using a rotary evaporator (60 rpm) until niosomal suspension was completely formed. The dispersion was evaporated about 15 min and then nitrogen gas was purged into dispersion for substitution of diethyl ether. The vesicle forming ability of all surfactants was investigated in the presence of different mole ratio of EA. In order to obtain niosomes with homogeneous size, REV vesicles were sonicated for 15 min and submitted to extrusion process for 3 times by an extruder device (Lipex™, Northern Lipids, BC, Canada), equipped with a 400 nm pore size polyester membrane (Cyclopore®, Whatman, NJ, USA). The maximum pressure was set at 800 psi as well as the temperature was controlled at 45 °C during the extrusion process.

2.3. Characterization of niosomes

Niosomes were characterized by optical microscopy and transmission electron microscopy (TEM) for vesicle formation and morphology, by confocal laser scanning microscopy (CLSM) for EA localization in vesicles and by photo correlation spectroscopy (PCS) for mean vesicle size (*z*-ave) and polydispersity index (PI). Optical micrographs were obtained with a Nikon TE-2000 inverted light microscope (magnification 900×) and CLSM images were also obtained by this microscope equipped with laser light sources and PMT detector. For TEM, a drop of vesicle dispersion was applied to a 200 mesh formvar copper grid and was stained with a 1% phosphotungstic acid. Then samples were observed under a transmission electron microscope (Hitachi Model H-7000, Tokyo, Japan).

Size and size distribution of niosomes were determined by PCS with a Malvern Zetasizer ZS (Malvern Instruments, UK). The *z*-ave and PI values were obtained by averaging of 10 measurements at an angle of 90° in 10 mm diameter cells at 25 °C. The real refractive index and the imaginary refractive index were set at 1.456 and 0.01, respectively.

2.4. Determination of entrapment efficiency

The percentage of EA-loaded niosomes (% E.E.) was determined by ultrafiltration using centrifugal filter tubes with a molecular weight cut-off of 30 kDa which were centrifuged (Optima™ Model LE-80K, Beckman, USA) at 10,000 rpm for 15 min. Niosomal pellets were dissolved in MeOH and then diluted 10 folds with MeOH and 0.1% (v/v) phosphoric acid in water (1:1, v/v). The concentrations of EA in EA-loaded niosomes and ultrafiltrate (free drug) were analyzed using HPLC method. Each formulation was performed in duplicate and each replicate sample was diluted and analyzed for three times. The percentage of EA entrapment efficiency was calculated using the following equation:

$$\%\ \text{E.E.} = \frac{\text{Amount of loaded EA in niosomes}}{\text{Total amount of added EA}} \times 100$$

2.5. HPLC analysis

EA concentrations were measured by HPLC (Agilent 1200 series, Waldbronn, Germany). The stationary phase was Hypersil® ODS C18 reversed-phase column (250 mm × 4.6 mm, 5 μm) used in isocratic mode at ambient temperature. The mobile phase was a mixture of 0.1% phosphoric acid, MeOH and acetronitrile (42:48:10, v/v). The UV detection was performed at a wavelength of 254 nm, the flow rate was 0.8 mL/min and the injection volume was 20 μL. Prior to the analysis, validation of the HPLC method was performed to ensure linearity of the calibration curve between 0.05 and 10 μg/mL with a correlation coefficient value of 0.9999. The high

precision for repeatability and reproducibility with coefficients of variation of less than 2% was found. The accuracy was determined by three replicate injections of three different concentrations of EA, and the percent recovery was found to be 100.15%. Therefore, the HPLC method used in this study demonstrated good linearity, precision and accuracy for determination of EA concentration.

2.6. Physical stability of EA-loaded niosomes

The niosomal dispersions were stored at 4, 25 and 40 °C. The physical stability was evaluated by determining mean vesicle size, size distribution and % E.E. over 4-month periods.

2.7. Skin permeation study

2.7.1. Skin preparation

Human skin samples were obtained by abdominoplasty surgeries from female patient ranging in age from 25 to 60 years supported by Yanhee Hospital, Bangkok, Thailand. The study was carried out with the approval of the Committee on Human Rights Related to Human Experimentation, Mahidol University, Bangkok, Thailand. The excess adipose layer was sectioned off from the received tissues by dissecting with the surgical scissors. Then, the skin composed of epidermis and underlying dermis was further used for *in vitro* skin distribution study. For *in vitro* permeation study, the epidermis was separated from the dermis using the heat separation technique. The skin was immersed into hot water controlled temperature at 60 °C for 1 min. Then, the epidermal layer was carefully separated from the dermis using blunt forceps to produce intact sheets ready for mounting on diffusion cells. The obtained epidermis was wrapped with aluminum foil and stored at −20 °C until used. The stored epidermis was allowed to thaw, cut into 4.5 cm × 4.5 cm pieces and hydrated by placing in isotonic phosphate buffer in a refrigerator (at about 4 °C) overnight before used. To ensure that the acceptor medium and the blank niosomes did not affect the integrity of the skin, the skin resistance was measured before and after the experiments. It was found that the skin resistance before and after the experiments was comparable.

2.7.2. In vitro permeation study

In vitro permeation studies through human epidermis were investigated using Franz diffusion cells. The diffusion cells were thermo-regulated with a water jacket at 37 °C. The epidermis was excised from human skins and mounted on Franz diffusion cells. A mixture of isotonic phosphate buffer pH 5.5 and isopropyl alcohol at the ratios of 90 and 10 (%, v/v) was used as an acceptor medium to provide the sink condition during the experiment and because of an appropriate solubility of EA in isopropyl alcohol. After 24 h, the amounts of EA in the acceptor fluid and in the epidermis were analyzed by HPLC spectrophotometry.

To determine EA in the epidermis, the epidermis was removed and rinsed with water and isopropanol and subsequently dried with a cotton swab. This procedure was done in triplicate. Briefly, the skins were cut into small pieces and soaked in 1 mL of MeOH for 12 h in a closed tube. Subsequently, it was subjected to an ultrasonic for three cycles of 15 min to avoid the raise in the temperature during extraction process. To ensure sufficient extraction of EA from skin, the residual extracted skin was performed in the same manner as mentioned above. It was found that no HPLC peak signal of EA was detected (LOD = 10 ng/mL) indicating a complete extraction at the first extraction.

2.8. Skin distribution study

To investigate the distribution of EA from niosomal formulations after being applied on the human skin, confocal laser scanning microscopy (CLSM) was performed. Fortunately, EA molecule can be detected by fluorescence detector. Therefore, EA distribution in the different skin layers was investigated using CLSM (FluoView FV1000 with IX2 inverted microscope: Multi-line Argon laser exciting wavelengths of 457 and 488 nm, and Helium–Neon green laser exciting wavelength of 543 nm). The emission wavelengths were set at 488 and 547 nm. The images were recorded by setting the camera integration time to 10 ms. Sample speed was 4.0 μm/pixel. The objective lens was in the magnification of 20× (Olympus, microscope, Japan).

In this study, the mixture of phosphate buffer pH 5.5 and isopropyl alcohol at the ratio of 90 and 10 (%, v/v) was used as an acceptor medium. After applying EA-loaded niosomes and EA solution on the skin surface, at 24 h, the skin was rinsed with distilled water. Then, the treated skins were removed from the Franz diffusion cells. The skin was sectioned into the pieces of 1 cm^2 size and evaluated for depth of EA penetration. The full skin thickness (epidermis and underlying dermis) was optically scanned at different increments through the *z*-axis of a CLS microscope. The skin slices were investigated under both normal light and fluorescence microscopes. The images from the two techniques were overlaid to obtain the information of the EA distribution in the different skin layers.

2.9. Statistical analysis

Statistical analysis of difference in the physicochemical properties among predetermined intervals in the some formulation and between formulations was performed by using paired *t*-test and one-way analysis of variance (one-way ANOVA), respectively. The level of significance was taken at p value of 0.05.

3. Results and discussion

Niosomal dispersions composed of 1500 μmol lipid phase (surfactants and cholesterol) were prepared using different ratios of surfactants and type of solubilizers. The results showed that all ratios of the surfactant systems (Span 60:Tween 60) were able to form vesicles. In general, surfactants with a single alkyl tail normally form micelles in diluted aqueous solutions and need additives such as cholesterol to achieve suitable molecular geometry and hydrophobicity for bilayer vesicle formation. However, a non-ionic surfactant with single alkyl tail such as Span 60 can form vesicular structure since it has relatively large hydrophobic moiety (HLB = 4.7) with low water solubility. In contrast, some surfactants cannot form vesicle without cholesterol. This must be attributed to the higher HLB (hydrophilicity) of the surfactant molecules. Moreover, these surfactants can form vesicle only in the presence of suitable amounts of cholesterol (Bayindir and Yuksel, 2010; Pardakhty et al., 2007). Tween 60 has a large hydrophilic head group with high HLB (14.9) which cannot form vesicle without cholesterol. Therefore, addition of cholesterol to Tween 60 makes the entire critical packing parameters (CPP) value achieve suitable value 0.5–1 for vesicle formation (Manosroi et al., 2003).

3.1. Effect of solubilizer

To study the effect of the solubilizers on the niosomes properties, a series of formulations were prepared by varying the type and amount of solubilizers at fixed amount of EA (1 mol%). From the results, EA-loaded niosomes were unable to form without the addition of solubilizer because EA is insoluble both in diethyl ether and water. When using 10% (v/v) PG or 10% (v/v) PEG 400 as a solubilizer, EA was not completely dissolved, leading to precipitation of EA and being failure in vesicular formation. On the other hand, 15% (v/v) PG or 15% (v/v) PEG 400 could dissolve the entire amount of added EA,

and EA-loaded niosomes could be formed. The vesicle sizes of all niosomes prepared at different ratios of the surfactants using the two solubilizers were comparable. In contrast, at a high concentration of MeOH (30%, v/v), EA-loaded niosomes could be formed but were not stable. At 20% (v/v) MeOH, EA was dissolved and the formed EA-loaded niosomes had high stability. However, the remaining amount of MeOH in organic phase which were unable to completely removed during evaporation process could interfere thermodynamic properties of niosomal system resulted in aggregation of niosome vesicles after storage.

3.2. Effect of EA concentration

The influence of drug concentration on niosomal formation and encapsulation was examined. The niosomes of 1, 3, 5 mol% EA were prepared using the 1500 μmol total amount of lipid and a constant amount of each solubilizer (20%, v/v MeOH of organic phase, or 15%, v/v PG or PEG 400 of aqueous phase). The results showed that the amounts of EA higher than 1 mol% were not completely dissolved by these solubilizers due to the limitation of EA solubility. Therefore, only 1 mol% EA was used for the preparation of the EA-loaded niosomes for the further study.

3.3. Vesicle characterization

3.3.1. Appearance and morphology

All EA-loaded niosomes using 15% (v/v) PG or 15% (v/v) PEG 400 or 20% (v/v) MeOH appeared in translucent white dispersions without sedimentation after extrusion. These appearances indicated the physical stability of all formulations. This may be due to the fact that the submission to the extrusion process resulted in niosomes of smaller vesicles and more homogeneity. Additionally, the microscopic appearances of all formulations after extrusion showed spherical vesicles with uniform distribution, as seen in Fig. 1A. The mean vesicle sizes were ranged below 1 μm. The TEM image (Fig. 1B) reveals the niosomal structures which were multilamellar vesicles with narrow size distribution.

The localization of EA in niosomal vesicles can be detected by CLSM technique because EA is a fluorescent molecule which can be excited at wavelength 200–400 nm and emit fluorescent light at wavelength 300–550 nm (Wang et al., 2010; Wolfbeis and Hochmuth, 1986). As compared the confocal image (Fig. 1D) to the bright-field image (Fig. 1C) of the niosomes collected simultaneously, it was found that EA localized in the niosome vesicles.

3.3.2. Vesicle size and size distribution

Table 1 shows the mean vesicle size (*z*-ave) and polydispersity index (PI) of EA-loaded niosomes evaluated by PCS after production. The size distribution of niosomes before extrusion was bimodal (data not shown). The vesicle size and PI values of EA-loaded niosomes before extrusion were 130–1150 nm and 0.228–0.912, respectively, and reduced to 124–752 nm and less than 0.4, respectively, after extrusion. In general, the PI values of some EA-free niosomes were higher than 0.4 indicating large size distributions, and tendency to aggregate. Therefore, extrusion process used in the preparation could produce small niosomes with homogeneous size.

The smallest *z*-ave values were observed in Tween 60 niosomes of all solubilizers (S_0T_1-Me, S_0T_1-PG and S_0T_1-PEG). The Span 60 formulations (S_1T_0-Me, S_1T_0-PG and S_1T_0-PEG) gave the largest vesicle sizes when compared to the other surfactant mixtures. Therefore, average sizes of niosomes increased when the HLB values of surfactant decreased from Tween 60 (HLB 14.9) to Span 60 (HLB 4.7). Similar results were reported for paclitaxel niosomes (Bayindir and Yuksel, 2010) and estradiol proniosomes (Fang et al., 2001). In addition, the niosomes of 2:1 Span 60 and Tween 60 (S_2T_1) had significantly larger vesicle sizes than those of 1:1 ratio (S_1T_1) ($p < 0.05$), supposing that the smaller head group of Span 60 as compared to Tween 60 requiring larger aggregation number (Liu and Guo, 2005).

3.3.3. Entrapment efficiency

The entrapment efficiency of the EA-loaded niosomes after extrusion was determined by analysis of entrapped drugs presented in the niosomal pellets after separation by ultrafiltration. The drug content in the vesicles was analyzed by HPLC spectrophotometry using the conditions as previously described. In general, REV method can produce two types of vesicles: a large unilamellar vesicle with a large internal aqueous core or multilamellar vesicles in which multiple lipid bilayers (Weiner et al., 1989). It is suitable for encapsulating water soluble compounds in the core and also lipid soluble compounds in bilayers. However, all prepared EA niosomes in this study were multilamellar vesicles which successfully entrapped EA in the vesicle structure. The encapsulation efficiency (% E.E.) of EA in the niosomes varied between 1.35% and 26.75% as shown in Table 1. The results indicated that the entrapment efficiencies for niosomes prepared using the mixtures of Span 60 and Tween 60 were superior to those prepared using solely Span 60 or Tween 60. This can be explained by many facts: (a) the hydrophilic head group (polyoxyethylene groups) of Tween 60 was larger than Span 60 which could solubilize higher EA due to the presence of phenolic groups and lactone moiety in the structure of EA suggesting higher chances of the substance to form hydrogen bonds (Bala et al., 2006), (b) amphiphilic molecules bearing both highly hydrophilic group and highly hydrophobic group can form rigid membrane with good barrier function against water soluble and insoluble compounds. Therefore, mixtures of Span 60 and Tween 60 gave suitable hydrophobic and high hydrophilic properties providing these systems were excellent to entrap solubilized forms of EA. When comparing three different solubilizers, the highest entrapment efficiency was found in all niosomes prepared with 15% (v/v) PEG 400. This may be resulted from the high solubility of EA in PEG 400 (9.65 mg/mL), and localization of PEG 400 molecules between double layers. The PG niosomes gave a slightly less entrapment ability than the PEG 400 niosomes due to the lower solubility of EA in PG (752.7 μg/mL). Conversely, the niosomes prepared with MeOH gave the lowest entrapping efficiency (% E.E = 1.35–3.48) although its solubility in MeOH (973.9 μg/mL) is higher than that in PG, probably due to precipitation of EA resulted from the vaporization of MeOH during evaporation process. For the PEG 400 niosomes, the niosomes of 2:1 Span 60 and Tween 60 using 15% (v/v) PEG 400 (S_2T_1-PEG) gave the maximum entrapment efficiency (% E.E. = 26.75 ± 0.58) followed by the niosomes of S_1T_1-PEG (% E.E. = 24.90 ± 0.87), S_1T_0-PEG (% E.E. = 23.81 ± 1.55) and S_0T_1-PEG (% E.E. = 21.27 ± 1.96), respectively. Additionally, the entrapment efficiencies correlated to average size of niosomes as seen that, when vesicle size increased, % E.E. also increased except for the niosomes of Span 60 (S_1T_0-PEG). The similar results were also observed in the niosomes prepared with PG or MeOH.

3.4. Stability of EA-loaded niosomes

Niosomes which were not subjected to extrusion process were found partial sedimentation over 4 months period at 4 °C storage temperature and completely aggregated at 40 °C storage temperature. This may be due to their large size and PI values contributing to the low stability of these systems (Uchegbu and Florence, 1995). Conversely, the niosomes submitted to extrusion process showed no sedimentation after stored at 4 °C for 4 months because of the small size and high homogeneity with low PI values of vesicles, as seen in Fig. 2. However, these systems were not stable at high temperatures of 25 and 40 °C as observed by the appearance of

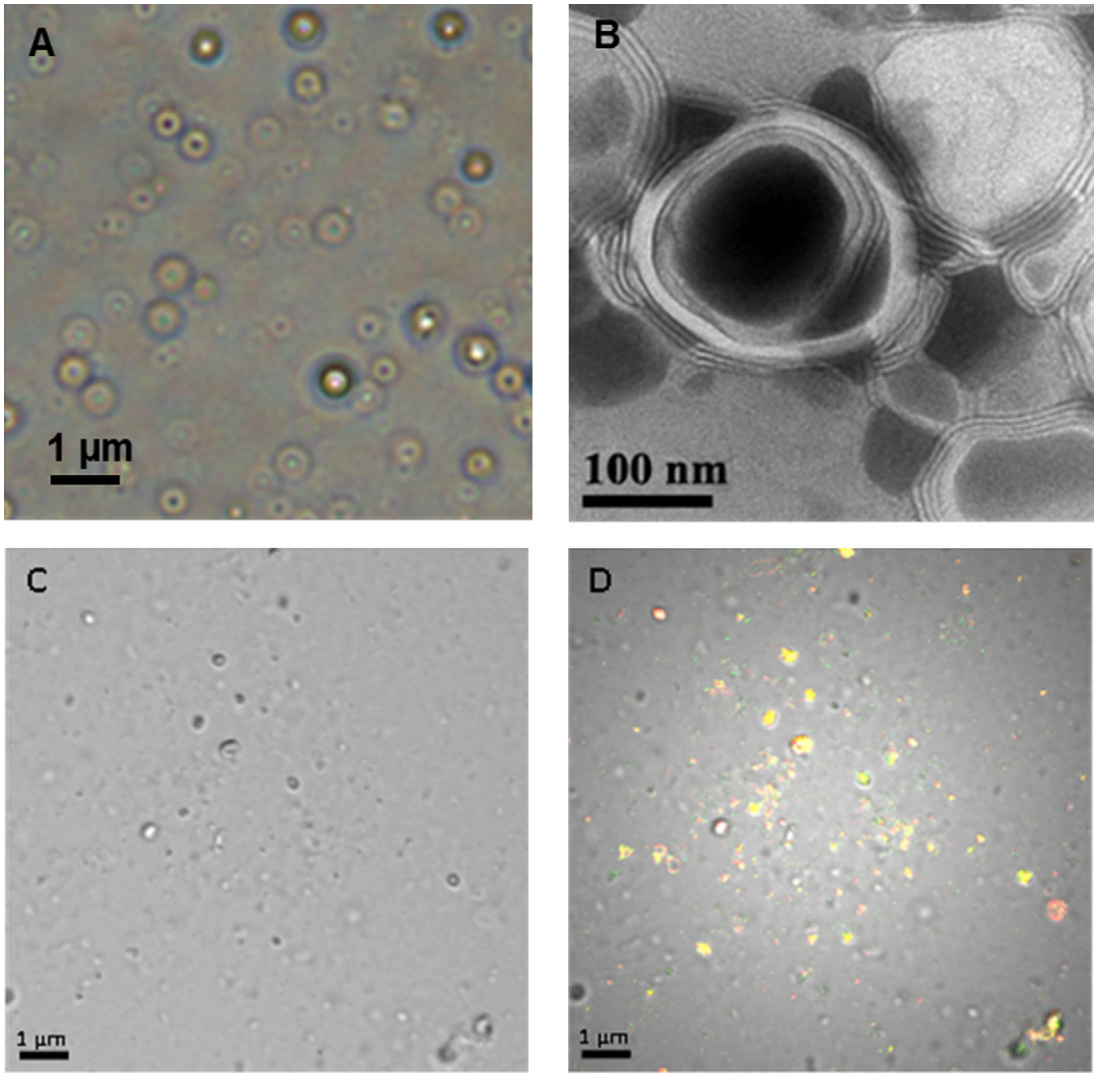

Fig. 1. Photomicrographs of EA-loaded niosomes from; (A) optical microscope (magnification 900×), (B) transmission electron microscopy (TEM), (C) bright-field microscopy (BF), and (D) confocal laser scanning microscopy (CLSM).

sedimentation. In addition, it was found that the mean vesicle sizes slightly changed at 25 °C, but dramatically increased at 40 °C over 4-month period (Fig. 2). With respect to the effect of storage temperature on the physical stability of niosomes, the results demonstrated that most niosome formulations exhibited an increase in mean diameter with broader distribution profiles ($p < 0.05$) during 4-month incubation period when stored at 25 and 40 °C which were correlated to aggregation behavior of the

Table 1
Compositions, mean vesicle size (*z*-ave) and polydispersity index (PI) of EA-loaded niosome formulations.

Formulation (Span 60:Tween 60)	Compositions[a]			Before extrusion		After extrusion		% Entrapment efficiency[b]
	Span 60 (μmol)	Tween 60 (μmol)	Solubilizer	*z*-ave (nm)	PI	*z*-ave (nm)	PI	
S_1T_0-PG (1:0)	750	–	15% PG	723 ± 32	0.605 ± 0.089	418 ± 5	0.341 ± 0.041	18.56 ± 1.30
S_2T_1-PG (2:1)	500	250	15% PG	447 ± 19	0.531 ± 0.046	406 ± 4	0.341 ± 0.025	22.37 ± 0.65
S_1T_1-PG (1:1)	375	375	15% PG	438 ± 6	0.453 ± 0.023	398 ± 5	0.285 ± 0.011	21.78 ± 1.24
S_0T_1-PG (0:1)	–	750	15% PG	255 ± 2	0.550 ± 0.019	179 ± 1	0.173 ± 0.017	16.42 ± 2.11
S_1T_0-PEG (1:0)	750	–	15% PEG 400	1776 ± 82	0.912 ± 0.147	752 ± 6	0.484 ± 0.037	23.81 ± 1.55
S_2T_1-PEG (2:1)	500	250	15% PEG 400	501 ± 13	0.491 ± 0.066	457 ± 6	0.335 ± 0.067	26.75 ± 0.58
S_1T_1-PEG (1:1)	375	375	15% PEG 400	376 ± 9	0.404 ± 0.030	328 ± 4	0.277 ± 0.009	24.90 ± 0.87
S_0T_1-PEG (0:1)	–	750	15% PEG 400	385 ± 8	0.361 ± 0.013	237 ± 2	0.145 ± 0.017	21.27 ± 1.96
S_1T_0-Me (1:0)	750	–	20% methanol	749 ± 44	0.621 ± 0.138	206 ± 2	0.234 ± 0.011	1.35 ± 0.89
S_2T_1-Me (2:1)	500	250	20% methanol	289 ± 20	0.598 ± 0.041	203 ± 8	0.343 ± 0.023	3.48 ± 0.39
S_1T_1-Me (1:1)	375	375	20% methanol	159 ± 5	0.454 ± 0.082	136 ± 1	0.265 ± 0.031	3.23 ± 1.33
S_0T_1-Me (0:1)	–	750	20% methanol	161 ± 3	0.228 ± 0.014	124 ± 1	0.194 ± 0.015	2.19 ± 0.84

[a] Total amount of lipid phase was 1500 μmol and composed of cholesterol and surfactants at 1:1 mole ratio. Each formulation was added with 5 mol% of Solulan C24 and 1 mol% of EA.
[b] The values are expressed as the mean ± SD from at least three batches.

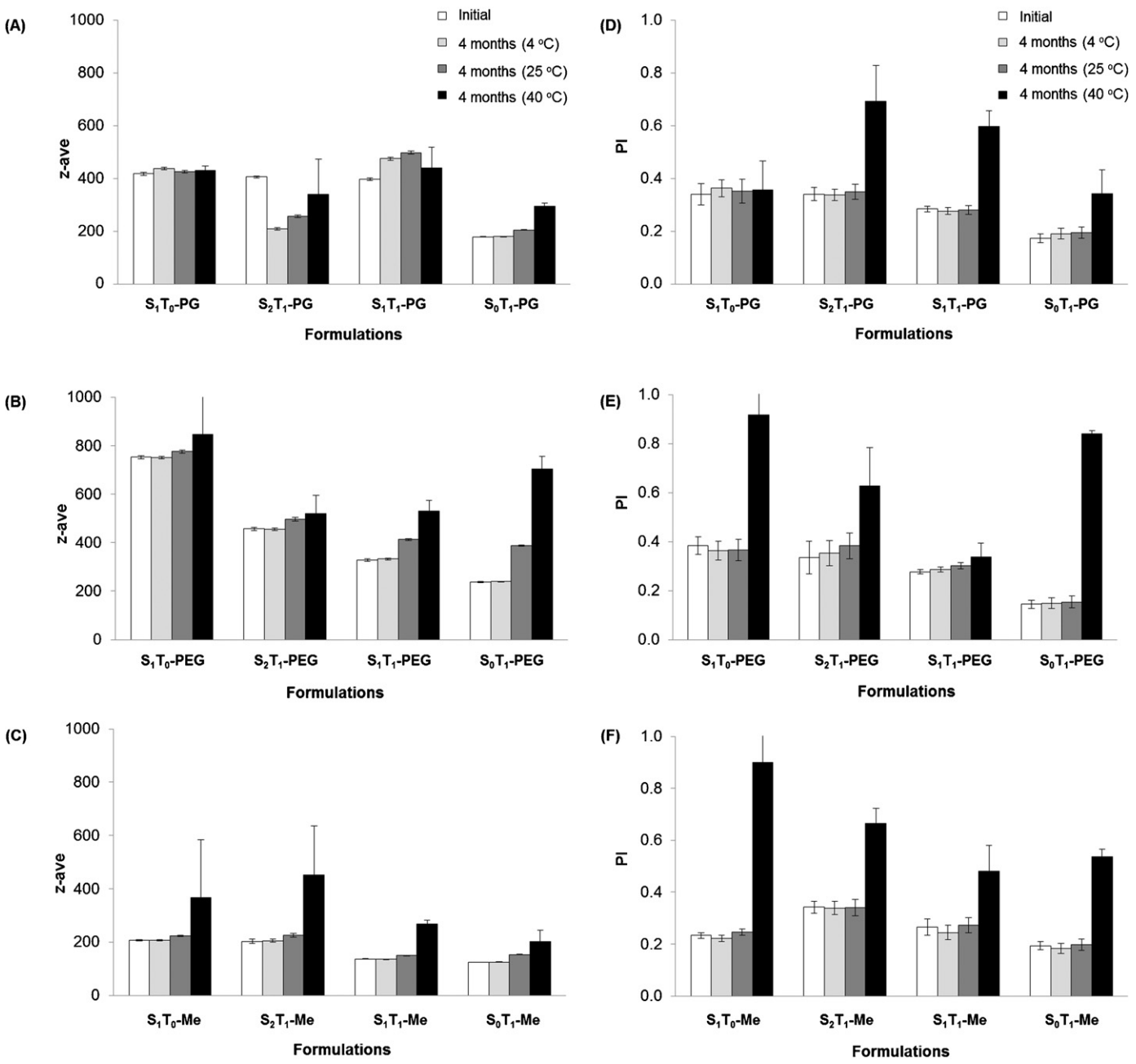

Fig. 2. Mean vesicle size (*z*-ave, right panel) and polydispersity index (PI, left panel) after production and 4 months storage at 4, 25, 40 °C for EA-loaded niosomes formulations prepared with various solubilizers; (A and D) PG, (B and E) PEG 400, and (C and F) MeOH.

vesicles. In contrast, at 4 °C storage temperature, the mean vesicle sizes of most formulations did not change significantly ($p > 0.05$) which indicated a good physical stability of these systems.

The changes of % E.E. were observed to investigate the possibility of EA leaching from the niosomes during storage at three different temperatures as shown in Fig. 3. A direct relationship between a decrease in % E.E. and the storage time as increasing the temperature indicated the high degree of leaching. The higher EA leakage at higher temperature may be due to the higher fluidity of lipid bilayers at high temperature (Hofland et al., 1992). It was observed that the niosomes of 2:1 Span 60 and Tween 60 (S_2T_1) and three solubilizers showed higher stability than the other formulations. The percentages of EA remaining after 4 months at 4 °C in S_2T_1-PG, S_2T_1-PEG and S_2T_1-Me niosomes were 99.91 ± 4.77%, 95.07 ± 4.04% and 101.72 ± 12.64%, respectively, while those in S_1T_1-PG, S_1T_1-PEG and S_1T_1-Me niosomes (1:1 Span 60 and Tween 60) were 90.56 ± 4.26%, 90.11 ± 4.09% and 98.76 ± 22.32%, respectively. Although the % E.E. of S_2T_1 and S_1T_1 niosome formulations was comparable, the S_2T_1 niosomes showed higher stability which may be resulted from the more rigidity of their vesicle membrane. The lowest drug stability was observed in S_0T_1-PG, S_0T_1-PEG and S_0T_1-Me niosomes which had 74.18 ± 16.14%, 68.41 ± 10.16% and 51.14 ± 14.16% of EA remaining at the end of 4 months at 4 °C, respectively. The results indicated that Tween 60 niosomes (S_0T_1) had the lowest stability.

The contents of Tween 60 and Span 60 affected the size and the stability of the niosomes. The effect of Tween 60 and Span 60 on the niosome stability may be explained as the following discussion. The rigidity of the Tween 60 niosome membrane is weak due to the high hydrophilicity of Tween 60. Addition of Span 60 with

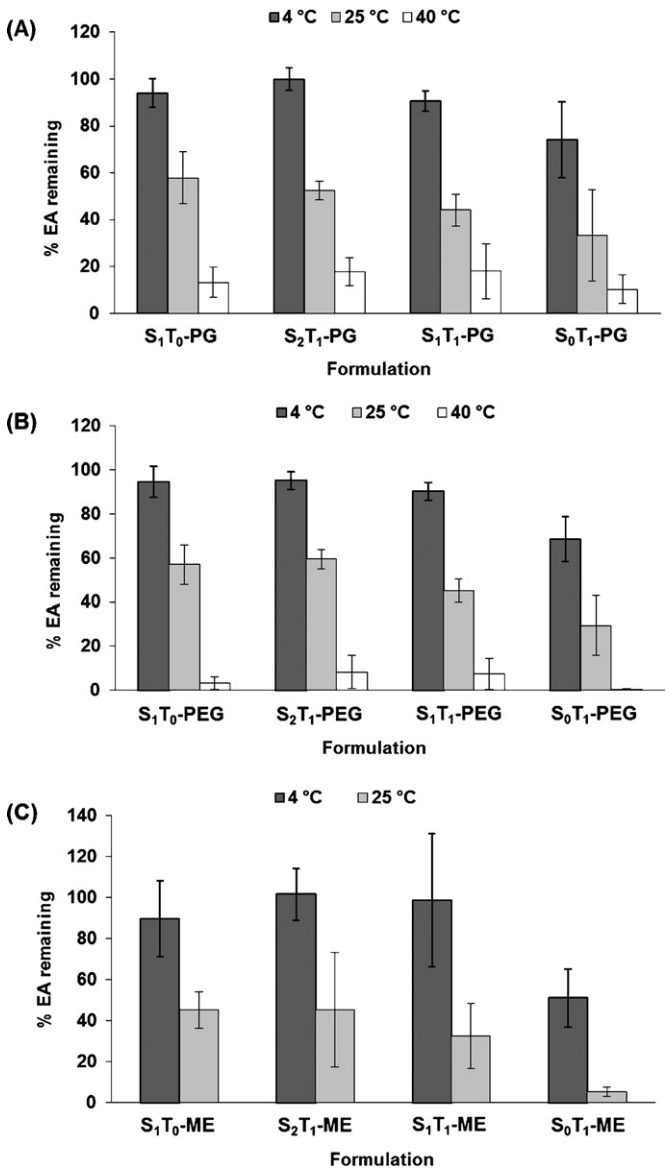

Fig. 3. Percentage of EA remaining after 4 months of storage at 4, 25, 40 °C for EA-loaded niosomal formulations prepared with various solubilizers; (A) PG, (B) PEG 400, and (C) MeOH.

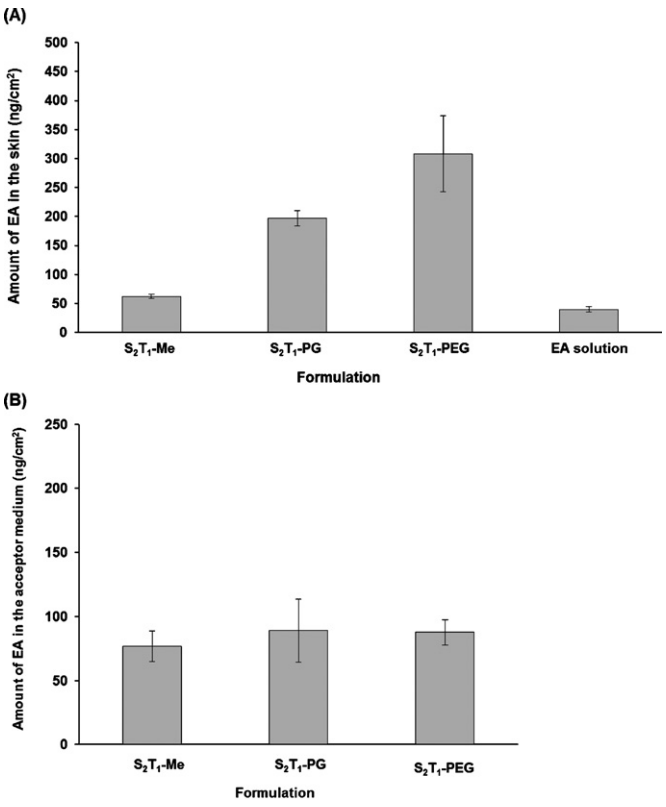

Fig. 4. *In vitro* skin permeation study of EA-loaded niosomes after applying for 24 h; (A) amount of EA in the skin and (B) amount of EA in the acceptor medium ($n = 3$).

more hydrophobicity can improve the hydrophobic capability of the membrane leading to the condensed niosomal films. Therefore, existing of Span 60 in the interphase of the films decreases the interaction between polar heads of the amphiphilic molecules, and thus stabilizes the niosomes. The hydrophobic interaction results in the insertion of the alkyl chain of the surfactants into the hydrophobic domain of the niosomes (Manosroi et al., 2003).

Based on the physicochemical properties and stability of all investigated EA-loaded niosome formulations, it can be concluded that the niosomes of 2:1 Span 60 and Tween 60 (S_2T_1) and the three solubilizers (15%, v/v PG, 15%, v/v PEG or 20%, v/v MeOH) were suitable for further investigation.

3.5. *In vitro* permeation of EA-loaded niosomes

In this study, the S_2T_1 formulations of EA-loaded niosomes prepared by three different solubilizers were selected for the *in vitro* permeation study due to the physical and chemical stability reasons as compared to the other niosomal formulations. Fig. 4 shows the amount of EA in the epidermis and the acceptor medium after applying EA-loaded niosomes or EA solution for 24 h. The permeated EA from EA solution could be detected only in the epidermis, indicating that EA could not penetrate through the epidermis into the dermis. Meanwhile, the permeated EA from all EA-loaded niosomes was detected both in the epidermis and in the acceptor medium. In addition, the amount of EA permeated in the epidermis from EA-loaded niosomes was higher than that from EA solution (40 ng/cm^2), as seen in Fig. 4A. In particular, PG and PEG 400 niosomes gave significantly higher EA amount in the epidermis than MeOH niosomes which may be resulted from higher % E.E. and the penetration enhancement property of PG and PEG 400, increasing the partition and permeation by solvating the keratin of stratum corneum and occupying the hydrogen bonding sites. The amount of EA in the acceptor medium from all EA-loaded niosomes was comparable (Fig. 4B). This result may be explained by the effect of vesicle size and % E.E. of each niosome formulation. The MeOH niosomes were the smallest size, providing that they could more penetrate through the epidermis layer than the PG niosomes and PEG 400 niosomes which were larger in size. Incidentally, the % E.E. of MeOH niosomes was lower than that of PG and PEG 400 resulted in the similarity of EA amount in the acceptor medium from all niosome formulations.

3.6. Skin distribution of EA-loaded niosomes

CLSM studies were conducted to evaluate the extent of penetration and transdermal potential of the permeated system as depicted an increase in both the depth of penetration and fluorescence intensity. Fig. 5A shows the fluorescence images of the epidermis and dermis layers of human skin after being treated with EA solution. The images displayed low fluorescence intensity of EA up to 30 μm depth (stratum corneum layer), indicating that EA solution had low

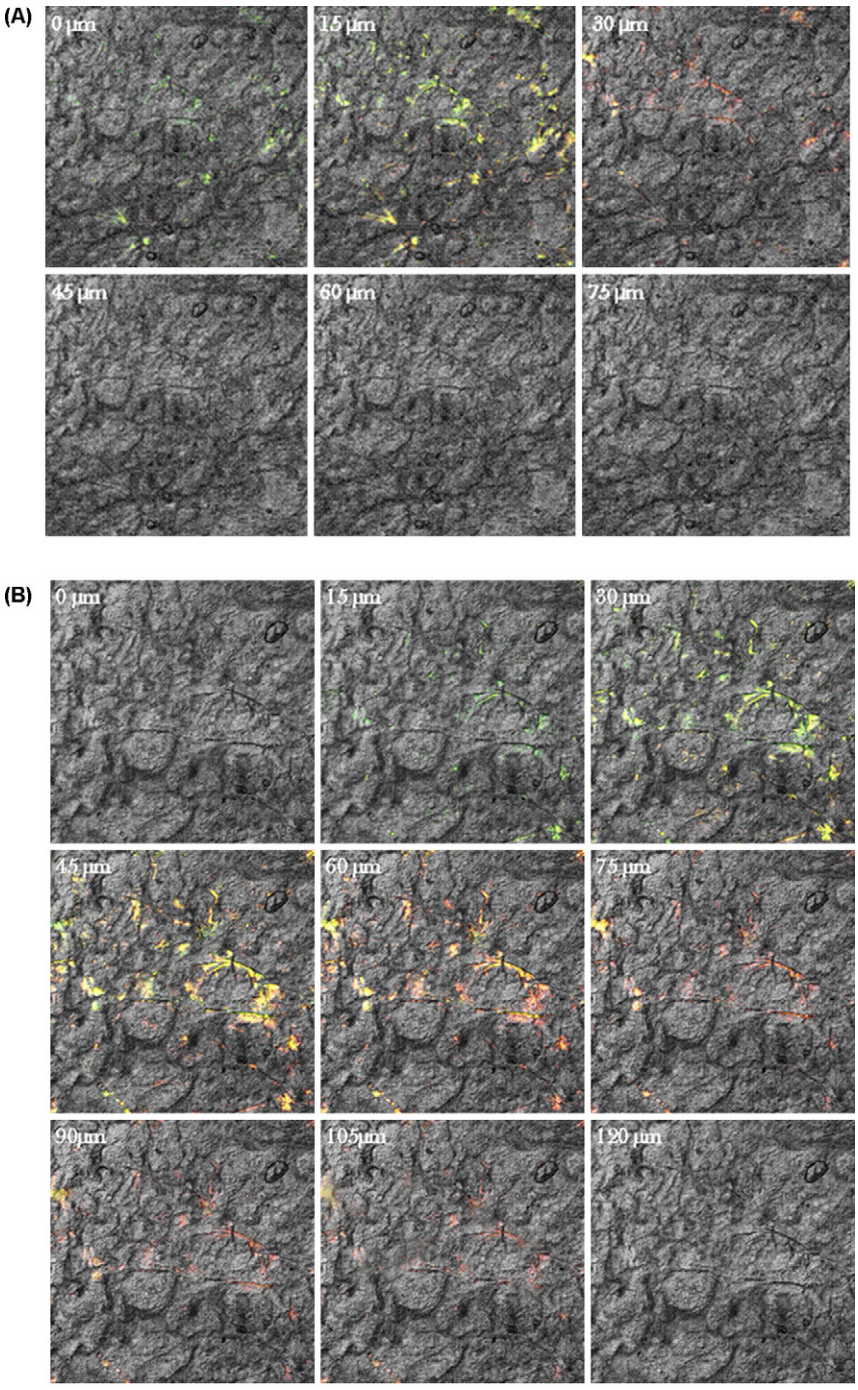

Fig. 5. CLSM images revealing the penetration and distribution of EA within human skin when treated with; (A) EA solution and (B) S_2T_1-PEG niosomes after being applied for 24 h.

penetration ability and could not penetrate to viable epidermis and dermis layer. In contrary, the CLSM images of PEG 400 niosomes (Fig. 5B) showed effective permeation of EA up to 120 μm as seen by high fluorescence intensity in skin depth between 30 and 90 μm (epidermis layer) and lower fluorescence intensity in the depth up to 120 μm (underlying dermis). The similar results were observed from the CLSM images of PG and MeOH niosomes but in lower fluorescence intensity than PEG 400 niosomes, respectively (data not shown). According to the obtained results, it was confirmed that the EA-loaded niosomes provided higher permeation of EA into the epidermis or dermis than EA solution and the presence of the solubilizers (PG, PEG 400, MeOH) affected their penetration efficiency as supported by the results from *in vitro* permeation study.

4. Conclusions

The Span 60 and Tween 60 niosomes of EA were successfully developed with the use of the solubilizers (PG, PEG 400, MeOH). The results from this study indicated that the type and amount of surfactants and solubilizers altered the entrapment efficiency and stability of EA-loaded niosomes. The highest entrapment efficiency and stability were obtained from the niosomes prepared with the mixture of Span 60 and Tween 60 at 2:1 mole ratio using

the three solubilizers (PG, PEG 400 and MeOH). The results from *in vitro* skin permeation and distribution showed that EA-loaded niosomes potentially delivered the higher amount of EA into the deeper layer of the skin as compared to EA solution. Therefore, EA-loaded niosomes could be an effective carrier for the dermal delivery of EA.

Acknowledgements

This project is supported by the Office of the High Education Commission and Mahidol University under the National Research Universities Initiative. The authors wish to thank the National Institute of Health, Department of Medical Sciences (NIH, Nontaburi, Thailand) for the use of CLSM.

References

Alsarra, I.A., Bosela, A.A., Ahmed, S.M., Mahrous, G.M., 2005. Proniosomes as a drug carrier for transdermal delivery of ketorolac. Eur. J. Pharm. Biopharm. 59, 485–490.

Arunothayanun, P., Bernard, M.S., Craig, D.Q.M., Uchegbu, I.F., Florence, A.T., 2000. The effect of processing variables on the physical characteristics of non-ionic surfactant vesicles (niosomes) formed from a hexadecyl diglycerol ether. Int. J. Pharm. 201, 7–14.

Bal, S.M., Ding, Z., van Riet, E., Jiskoot, W., Bouwstra, J.A., 2010. Advances in transcutaneous vaccine delivery: do all ways lead to Rome? J. Control. Release 148, 266–282.

Bala, I., Bhardwaj, V., Hariharan, S., Kumar, M.N.V.R., 2006. Analytical methods for assay of ellagic acid and its solubility studies. J. Pharm. Biomed. Anal. 40, 206–210.

Bayindir, Z.S., Yuksel, N., 2010. Characterization of niosomes prepared with various nonionic surfactants for paclitaxel oral delivery. J. Pharm. Sci. 99, 2049–2060.

Dufes, C., Gaillard, F., Uchegbu, I.F., Schätzlein, A.G., Olivier, J.-C., Muller, J.-M., 2004. Glucose-targeted niosomes deliver vasoactive intestinal peptide (VIP) to the brain. Int. J. Pharm. 285, 77–85.

Fang, J.-Y., Yu, S.-Y., Wu, P.-C., Huang, Y.-B., Tsai, Y.-H., 2001. In vitro skin permeation of estradiol from various proniosome formulations. Int. J. Pharm. 215, 91–99.

Guinedi, A.S., Mortada, N.D., Mansour, S., Hathout, R.M., 2005. Preparation and evaluation of reverse-phase evaporation and multilamellar niosomes as ophthalmic carriers of acetazolamide. Int. J. Pharm. 306, 71–82.

Hofland, H.E.J., Bouwstra, J.A., Verhoef, J.C., Buckton, G., Chowdry, B.Z., Ponec, M., Junginger, H.E., 1992. Safety aspects of non-ionic surfactant vesicles: a toxicity study related to the physicochemical characteristics of non-ionic surfactants. J. Pharm. Pharmacol. 44, 287–294.

Junginger, H.E., Hofland, H.E.J., Bouwstra, J.A., 1991. Liposomes and niosomes: interaction with human skin. Cosmet. Toiletries 106, 45–50.

Junyaprasert, V.B., Teeranachaideekul, V., Supaperm, T., 2008. Effect of charged and non-ionic membrane additives on physicochemical properties and stability of niosomes. AAPS PharmSciTech 9, 851–859.

Kaur, S., Grover, I.S., Kumar, S., 1997. Antimutagenic potential of ellagic acid isolated from *Terminalia arjuna*. Indian J. Exp. Biol. 35, 478–482.

Khandare, J.N., Madhavi, G., Tamhankar, B.M., 1994. Niosomes novel drug delivery system. East. Pharm. 37, 61–64.

Khanduja, K.L., Gandhi, R.K., Pathania, V., Syal, N., 1999. Prevention of N-nitrosodiethylamine-induced lung tumorigenesis by ellagic acid and quercetin in mice. Food Chem. Toxicol. 37, 313–318.

Liu, T., Guo, R., 2005. Preparation of a highly stable niosome and its hydrotrope-solubilization action to drugs. Langmuir 21, 11034–11039.

Liu, T., Guo, R., 2007. Structure and transformation of the niosome prepared from PEG 6000/Tween 80/Span 80/H_2O lamellar liquid crystal. Colloids Surf. A: Physicochem. Eng. Aspects 295, 130–134.

Liu, T., Guo, R., Hua, W., Qiu, J., 2007. Structure behaviors of hemoglobin in PEG 6000/Tween 80/Span 80/H_2O niosome system. Colloids Surf. A: Physicochem. Eng. Aspects 293, 255–261.

Loarca-Piña, G., Kuzmicky, P.A., González de Mejía, E., Kado, N.Y., 1998. Inhibitory effects of ellagic acid on the direct-acting mutagenicity of aflatoxin B1 in the Salmonella microsuspension assay. Mutat. Res. Fundam. Mol. Mech. Mutagen. 398, 183–187.

Manconi, M., Caddeo, C., Sinico, C., Valenti, D., Mostallino, M.C., Biggio, G., Fadda, A.M., 2011. Ex vivo skin delivery of diclofenac by transcutol containing liposomes and suggested mechanism of vesicle–skin interaction. Eur. J. Pharm. Biopharm. 78, 27–35.

Manconi, M., Sinico, C., Valenti, D., Loy, G., Fadda, A.M., 2002. Niosomes as carriers for tretinoin. I. Preparation and properties. Int. J. Pharm. 234, 237–248.

Manosroi, A., Wongtrakul, P., Manosroi, J., Sakai, H., Sugawara, F., Yuasa, M., Abe, M., 2003. Characterization of vesicles prepared with various non-ionic surfactants mixed with cholesterol. Colloids Surf. B: Biointerfaces 30, 129–138.

Marianecci, C., Paolino, D., Celia, C., Fresta, M., Carafa, M., Alhaique, F., 2010. Non-ionic surfactant vesicles in pulmonary glucocorticoid delivery: characterization and interaction with human lung fibroblasts. J. Control. Release 147, 127–135.

Mura, S., Manconi, M., Sinico, C., Valenti, D., Fadda, A.M., 2009. Penetration enhancer-containing vesicles (PEVs) as carriers for cutaneous delivery of minoxidil. Int. J. Pharm. 380, 72–79.

Muzzalupo, R., Tavano, L., Cassano, R., Trombino, S., Ferrarelli, T., Picci, N., 2011. A new approach for the evaluation of niosomes as effective transdermal drug delivery systems. Eur. J. Pharm. Biopharm. 79, 28–35.

Pardakhty, A., Varshosaz, J., Rouholamini, A., 2007. In vitro study of polyoxyethylene alkyl ether niosomes for delivery of insulin. Int. J. Pharm. 328, 130–141.

Rentel, C.O., Bouwstra, J.A., Naisbett, B., Junginger, H.E., 1999. Niosomes as a novel peroral vaccine delivery system. Int. J. Pharm. 186, 161–167.

Schreier, H., Bouwstra, J., 1994. Liposomes and niosomes as topical drug carriers: dermal and transdermal drug delivery. J. Control. Release 30, 1–15.

Seeram, N.P., Lee, R., Heber, D., 2004. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice. Clin. Chim. Acta 348, 63–68.

Shimogaki, H., Tanaka, Y., Tamai, H., Masuda, M., 2000. In vitro and in vivo evaluation of ellagic acid on melanogenesis inhibition. Int. J. Cosmet. Sci. 22, 291–303.

Tavano, L., Alfano, P., Muzzalupo, R., de Cindio, B., 2011. Niosomes vs microemulsions: new carriers for topical delivery of Capsaicin. Colloids Surf. B: Biointerfaces 87, 333–339.

Uchegbu, I.F., Florence, A.T., 1995. Non-ionic surfactant vesicles (niosomes): physical and pharmaceutical chemistry. Adv. Colloid Interface Sci. 58, 1–55.

Uchegbu, I.F., Vyas, S.P., 1998. Non-ionic surfactant based vesicles (niosomes) in drug delivery. Int. J. Pharm. 172, 33–70.

Wang, F., Huang, W., Wang, Y., Tang, B., 2010. Determination of nucleic acid by its enhancement effect on the fluorescence of Ellagic acid – cationic surfactant system. J. Mol. Struct. 967, 183–188.

Weiner, N., Martin, F., Riaz, M., 1989. Liposomes as a drug delivery system. Drug Dev. Ind. Pharm. 15, 1523–1554.

Wolfbeis, O.S., Hochmuth, P., 1986. The fluorescence of ellagic acid and its borax complex. Monatsh. Chem. 117, 369–374.