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Influence of chemical penetration enhancers on skin permeability of ellagic acid-loaded niosomes

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ABSTRACT

This study aimed to develop niosomes of ellagic acid (EA), a potent antioxidant phytochemical substance, for dermal delivery and to investigate the influence of chemical penetration enhancers on the physicochemical properties of EA-loaded niosomes. The EA niosomes were prepared by reverse phase evaporation method using Span 60, Tween 60 and cholesterol as vesicle forming agents and Solulan C24 as a steric stabilizer. Polyethylene glycol 400 (PEG) was used as a solubilizer while dimethylsulfoxide (DMSO) or N-methyl-2-pyrrolidone (NMP) was used as a skin penetration enhancer. It was found that the mean particle sizes of EA-loaded niosomes were in the range of 312–402 nm with PI values of lower than 0.4. The niosomes were determined to be spherical multilamellar vesicles as observed by transmission electron microscope and optical microscopy. All niosomes were stable after 4 months storage at 4 °C.

In vitro skin permeation through human epidermis revealed that the skin enhancers affected the penetration of EA from the niosomes at 24 h. The DMSO niosomes showed the highest EA amount in epidermis; whereas the NMP niosomes had the highest EA amount in the acceptor medium. Concomitantly, the skin distribution by confocal laser scanning microscopy showed the high fluorescence intensity of the DMSO niosomes and NMP niosomes at a penetration depth of between 30–90 μm (the epidermis layer) and 90–120 μm (the dermis layer) under the skin, respectively. From the results, it can be concluded that the DMSO niosomes are suitable for epidermis delivery of EA while the NMP niosomes can be used for dermis delivery of EA.

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1. Introduction

The self-assembly of non-ionic surfactants into vesicles represents an interesting opportunity to achieve vesicular colloidal drug carriers, so-called niosomes, which resemble liposomes in their architecture and can be used as an effective alternative to liposomal drug carriers [1]. Niosomes have been proposed for a number of potential therapeutic applications. In particular, they have been intensively used as effective dermal and transdermal drug delivery systems [2–5]. Ellagic acid (EA) is a potent antioxidant phytochemical substance which has skin-whitening property with photoprotective effect [6–7]. However, it is poorly soluble in water (≈9.7 µg/ml) [8] and not soluble in many organic solvents, leading to difficulties in the design of pharmaceutical formulations. In our previous study [9], we demonstrated that the 2:1 Span 60 and Tween 60 niosomes were able to encapsulate and deliver EA through human epidermises and dermis.

One generalized approach to overcome the barrier properties of the skin for drugs and biomolecules is an incorporation of suitable vehicles or other chemical compounds into transdermal delivery systems. Substances that promote the penetration of topically applied drugs through stratum corneum and epidermis are commonly referred to as skin permeation enhancers, accelerants, adjuvants, or sorption promoters [10]. Chemical skin permeation enhancers have been generally used to promote the drug permeation through the skin [11,12]. Dimethylsulfoxide (DMSO) is one of the earliest and most widely studied penetration enhancers and often used in many areas of pharmaceutical sciences as a “universal solvent”. It has been postulated that DMSO de-natures the intercellular structural protein of the stratum corneum [13], or promote lipid fluidity by disruption of the ordered structure of the lipid chains [14]. Although DMSO at high concentration of >60% was reported to induce erythema and wheals of the stratum corneum, it has been used as nonirritant skin penetration enhancer at 10% in many skin formulations [15]. N-methyl-2-pyrrolidone (NMP) is a polar aprotic solvent and miscible with most common solvents including water and alcohols. NMP can partition well into water was used.

2. Materials and methods

2.1. Materials

Sorbitan monostearate (Span 60) and polyethylene glycol sorbitan monostearate (Tween 60) were obtained from Uni-gema (Chicago, USA). Ellagic acid (EA) and polyethylene glycol 400 (PEG) were purchased from Fluka (West St. Paul, USA). Cholesteryl poly-24-oxyethylene ether (Solulan C24) was received as a gift sample from Amerchol (New Jersey, USA). Cholesterol and N-methyl-2-pyrrrolidone (NMP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) was obtained from Amresco (Ohio, 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 [21] using 1:1 mole ratio of surfactants and cholesterol as vesicle forming agents. The surfactants (2:1 Span 60 and Tween 60), cholesterol, 5 mol% and Solulan C24 and were dissolved in diethyl ether (organic phase) with and without addition of 10% v/v DMSO or 1% v/v NMP. To solubilize EA, 15% v/v PEG was added into an aqueous phase containing 1 mol% 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. 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. Optical micrographs were obtained with a Nikon TE-2000 inverted light microscope (magnification 900×). 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 photon correlation spectroscopy (PCS) with a Malvern...
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 methanol and then diluted 10 folds with methanol 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 duplicates 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 x 4.6 mm x 5 mm) used in isocratic mode at ambient temperature. The mobile phase was a mixture of 0.1% phosphoric acid, methanol and acetonitrile (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, the HPLC validation parameters including precision, accuracy and linearity were determined according to USP guideline [22]. Linearity was observed in concentration range of 0.05–10 ng/ml with a correlation coefficient value of 0.9999. The values of coefficients of variation for intraday and interday were less than 2% and the percent recovery was 100.15%.

2.6. Stability of EA-loaded niosomes

The niosomal dispersions were stored at 4, 25 and 40 °C. The remaining of EA in the niosomes was evaluated by HPLC spectrophotometry over 4-month periods.

2.7. Skin permeation study

2.7.1. Skin preparation

Human skin samples were obtained by abdominoplasty surgeries from female patients 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 aluminium foil and stored at ~20 °C until used. The stored epidermis was allowed to thaw, cut into 4.5 x 4.5 cm² pieces and hydrated by placing in isotonic phosphate buffer in a refrigerator (at about 4 °C) overnight before used.

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 acceptor fluids and in the epidermis were analyzed by HPLC spectroscopy.

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 methanol 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, microscopy, 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 cm² 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 same 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

3.1. Morphology, vesicle size and % entrapment efficiency

Vesicle formation in the presence of the enhancers was confirmed by optical microscopy and TEM. Fig. 1 (A1, A2) shows the obtained niosomes were spherical shape and multilamellar vesicles under optical microscope which the mean vesicle sizes in submicron range. In addition, the similar results were displayed by TEM images as shown in Fig. 1 (A2, B2). In general, an addition of chemical penetration enhancers (10% v/v DMSO or 1% v/v NMP) into the niosomal formulations did not alter vesicle formation. The vesicles could normally
form with the presence of enhancers, and homogeneous white dispersion of niosomes without large dispersed particles or curd was obtained.

The vesicle sizes of all EA-free niosomes were in the range of 396–511 nm, as seen in Table 1. After EA was entrapped into the niosomes, the vesicle size of the niosomes significantly increased (p > 0.05). In addition, after submitted to extrusion process, the niosomes of small and homogeneous size were obtained with the mean vesicle size of 312–402 nm and PI values lower than 0.4 (Table 1). Interestingly, after extrusion the vesicle sizes of the DMSO niosomes were larger than the NMP niosomes which may be due to the fact that NMP could have more effect on increasing fluidity of vesicular membrane resulting in ease of size reduction when subjected to extrusion process. The change of niosome size is an important parameter which can influence both vesicular properties such as entrapment efficiency and stability, as well as skin-vesicle interactions.

The % E.E. of all niosomes was in between 25.63% and 38.73% (Table 1). In particular, the addition of DMSO and NMP increased % E.E. of the niosomes. This may be resulted from the high solubility of EA in the enhancers (6.15 mg/ml in DMSO and 55.42 mg/ml in NMP). The DMSO niosomes had the lower entrapment efficiency than the NMP niosomes which may be described as the lower solubility of EA in DMSO. The higher % E.E. of NMP niosomes could be explained from the high solubility of EA in both PEG and NMP, and PEG molecules can localize between double vesicular layers which may also carry associated NMP with them. In addition, it was reported that NMP acts as penetration enhancer by fluidizing lipid domain (alkyl chains) of the stratum corneum [23,24]. The presence of NMP in the niosome bilayer could produce the same effect, fluidizing lipid domain of vesicle membrane, leading to an increased space in the bilayer for the EA incorporation.

### 3.2. Stability of EA-loaded niosomes

The results of the stability study of EA entrapped in various niosomes stored at 4, 25 and 40 °C for 4 months are shown in Fig. 2. At 4 °C, the EA in all niosomes exhibited a good stability, which the percentages of EA remaining were almost 100. At higher temperature (25 °C), the percentages of EA remaining decreased to 40%–60%. Likewise, at 40 °C, they dramatically decreased to below 30%. 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 [25].

It is well known that the gel to liquid phase transition temperature of the vesicular bilayers played an important role in niosome formation and stability which was influenced by types of non-ionic surfactants, mole ratio of surfactant and cholesterol [1]. The stability of niosomes would be improved by increasing the phase transition temperature of the system by changing surfactant type and the surfactant/lipid level.

### 3.3. In vitro permeation of EA-loaded niosomes

Fig. 3 shows the amount of EA in the epidermis and the acceptor medium after applying EA-loaded niosomes or EA solution for 24 h. The amount of EA in epidermis after applying all niosomes was much higher than the EA solution. In addition, the DMSO niosomes gave significantly higher amount of EA in epidermis than the NMP niosomes. Conversely, the NMP niosomes gave significantly higher amount of EA in acceptor medium than the DMSO niosomes. This indicated that the NMP niosomes could penetrate through epidermis into dermis higher than the DMSO niosomes which may be resulted from the higher penetration enhancement efficiency of NMP [26] which could promote

### Table 1 – Mean vesicle size (z-ave), polydispersity index (PI) and % entrapment efficiency of EA-loaded niosomes.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Before extrusion</th>
<th>After extrusion</th>
<th>Entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EA-free niosomes</td>
<td>EA-loaded niosomes</td>
<td>EA-loaded niosomes</td>
</tr>
<tr>
<td></td>
<td>z-ave (nm)</td>
<td>PI</td>
<td>z-ave (nm)</td>
</tr>
<tr>
<td>E-PEG</td>
<td>408 ± 5</td>
<td>0.387 ± 0.021</td>
<td>427 ± 4</td>
</tr>
<tr>
<td>E-DPEG</td>
<td>511 ± 3</td>
<td>0.273 ± 0.006</td>
<td>515 ± 17</td>
</tr>
<tr>
<td>E-NPEG</td>
<td>396 ± 4</td>
<td>0.423 ± 0.023</td>
<td>560 ± 28</td>
</tr>
</tbody>
</table>

* The 2:1 Span 60:Tween 60 niosomes contained 15% v/v PEG without skin enhancers.
* The 2:1 Span 60:Tween 60 niosomes contained 15% v/v PEG with 10% v/v DMSO.
* The 2:1 Span 60:Tween 60 niosomes contained 15% v/v PEG with 1% v/v NMP.
* The values are expressed as the mean ± SD from at least three batches.

Fig. 3 – In vitro skin permeation study of EA solution, EA-loaded niosomes containing 15% v/v PEG without chemical enhancers (E-PEG) and with 10% v/v DMSO (E-DPEG) or 1% v/v NMP (E-NPEG) after applying for 24 h (n = 3).
lipid fluidity by disruption of the ordered structure of the lipid chains [27]. Therefore, the DMSO niosomes gave an advantage on the delivery to the epidermis layer, while the NMP niosomes were better for delivering EA to the dermis layer.

3.4. 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 in fluorescence intensity. The CLSM images were obtained from overlaying the bright field images and fluorescence images of the same area. Each image was one of the z-stack at 15 μm depth. Fig. 4 shows the fluorescence images of the epidermis and dermis layers of human skin after being treated with the EA-loaded niosomes. The CLSM images of the DMSO niosomes (Fig. 4A) showed effectiveness in permeating EA up to 120 μm which showed high fluorescence intensity at the penetration depth between 30–90 μm (epidermis layer depth) and lower fluorescence intensity at the depth up to 120 μm (underlying dermis depth). Thus, the DMSO niosomes had high ability to deliver EA to the viable epidermis layer which small amount of EA penetrated into dermis layer. Meanwhile, the penetration of the NMP niosomes to dermis layer was better than the DMSO niosomes as revealed by the higher fluorescence intensity of EA at the deeper skin layer (90–120 μm depth), as shown in Fig. 4B. According to the obtained results, it confirmed that the skin enhancers affected the permeation of EA to the epidermis or dermis in accordance with the results from in vitro permeation study.

4. Conclusion

The niosomes of 2:1 Span 60 and Tween 60 using PEG as the solubilizer could be formed in the presence of chemical enhancers (DMSO and NMP). The vesicle shape was spherical multilamellar vesicles as assessed by optical microscopy and TEM. The mean particle sizes were in range of 312–402 nm and PI values were lower than 0.4. An addition of enhancers increased the entrapment efficiency of the EA-loaded niosomes. The results of the skin distribution of EA-loaded

Fig. 4 – CLSM images revealing the penetration and distribution of EA within the human skin after treated with (A) the DMSO niosomes and (B) the NMP niosomes for 24 h.
niosomes by CLSM were in agreement with the results of in vitro permeation which showed that the DMSO niosomes had the higher fluorescence intensity in the epidermis layer whereas the NMP niosomes had the higher fluorescence intensity in the dermis layer. Therefore, the chemical enhancers influenced the permeation of EA from the EA-loaded niosomes through the human skin.

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