Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 356 (2008) 144–152
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Comparison of 5-aminolevulinic acid-encapsulated liposome versus ethosome for skin delivery for photodynamic therapy

Yi-Ping Fang [a], Yi-Hung Tsai [a], Pao-Chu Wu [a], Yaw-Bin Huang [a,b,*]

[a] *Graduate Institute of Pharmaceutical Sciences, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan*
[b] *Graduate Institute of Clinical Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan*

Received 21 August 2007; received in revised form 31 December 2007; accepted 4 January 2008
Available online 19 January 2008

Abstract

Topical photodynamic therapy (PDT) with 5-aminolevulinic acid (ALA) is an alternative therapy for many non-melanoma skin cancers. The major limitation of this therapy, however, is the low permeability of ALA through the stratum corneum (SC) of the skin. The objective of the present work was to characterize ethosomes containing ALA and to enhance the skin production of protoporphyrin IX (PpIX), compared to traditional liposomes. Results showed that the average particle sizes of the ethosomes were less than those of liposomes. Moreover, the entrapment efficiency of ALA in the ethosome formulations was 8–66% depending on the surfactant added. The particle size of the ethosomes was still approximately <200 nm after 32 days of storage. An *in vivo* animal study observed the presence of PpIX in the skin by confocal laser scanning microscopy (CLSM). The results indicated that the penetration ability of ethosomes was greater than that of liposomes. The enhancements of all the formulations were ranging from 11- to 15-fold in contrast to that of control (ALA in an aqueous solution) in terms of PpIX intensity. In addition, colorimetry detected no erythema in the irradiated skin. The results demonstrated that the enhancement ratio of ethosome formulations did not significantly differ between the non-irradiated and irradiated groups except for PE/CH/SS, which may have been due to a photobleaching effect of the PDT-irradiation process.

Keywords: 5-Aminolevulinic acid; Liposomes; Ethosomes; Penetration; Photodynamic therapy; Confocal laser scanning microscopy

1. Introduction

Skin cancer is the most common cancer of all pathologies related to cancerous disease (Lopez et al., 2004), which is divided into melanoma and non-melanoma skin cancers (NMSCs). In fact, NMSCs include basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs) (McGillis and Fein, 2004). Traditionally, several therapeutic approaches have been used, those of surgery, radiation therapy, and topical chemotherapy. However, these approaches generally entail problems including scarring of sensitive areas, producing painful lesions, and not being amenable to or suitable for elderly patients (Chakrabarty and Geisse, 2004). Over the past decade, topical photodynamic therapy (PDT) with 5-aminolevulinic acid (ALA) has been an alternative option for dermatologists treating NMSCs. PDT is a non-invasive treatment and has proven to have several benefits involving low morbidity, minimum functional disturbance, better cosmetic outcomes, good tolerance, and the ability to repeatedly be used many times at the same site (Hopper, 2000).

Fundamentally, PDT is a composite technique which requires three basic elements: a photosensitizer, light irradiation, and singlet oxygen. The mechanism of ALA-PDT employs a photosensitizer which is activated by a suitable wavelength, and consequently singlet oxygen is generated by a cascade of reactions. The cytotoxic activity occurs by two pathways: destruction of tumor cells by necrosis or apoptosis and the failure of tumor vascularization by a decline in oxygen-carrying blood (Henderson and Dougherty, 1992; Szeimies et al., 2005). Indeed, ALA is a precursor of the photosensitizer, protoporphyrin IX (PpIX), formed *in vivo* after the exogenous application of ALA (De Rosa and Bentley, 2000). In addition, ALA molecules are zwitterions which carry both a positive charge at the amine terminal and a negative charge at the carboxylic terminal. These characteristic compounds have limited capacities to reach and

* Corresponding author at: Graduate Institute of Clinical Pharmacy, College of Pharmacy, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, 807 Kaohsiung, Taiwan. Tel.: +886 7 3121101x2166; fax: +886 7 3210683.
E-mail address: yabihu@kmu.edu.tw (Y.-B. Huang).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.01.020

ultimately enter target cells within a biological environment (Fotinos et al., 2006). Thus, the major limitation of this therapy is the poor penetration of ALA through biological barriers like cell membranes or the skin, due to its hydrophilic characteristic and charge (Peng et al., 1997a,b).

In the last two decades, a considerable number of studies have been conducted on the development of carriers, the synthesis or modification of valuable photosensitizers, and the enhancement of ALA by physical methods (Lopez et al., 2004). To date, many carriers have been used to deliver ALA including emulsions, liposomes, a lipid sponge form, and a nanocolloid lotion (Casas et al., 2002; Hürlimann et al., 1998; Merclin et al., 2004). Liposome carriers are generally accepted in various delivery strategies for the systemic or topical administration of drugs. Liposomal delivery systems can enhance the capillary permeability of hydrophilic drugs and localize them to target tissues (Loan Honeywell-Nguyen and Bouwstra, 2005). Phospholipids are recognized as being non-toxic and biodegradable, and they can prolong the half-life of a drug to attain a sustained-release effect (Oku, 1999). On the other hand, previous studies demonstrated that phospholipids can exhibit their enhancing effect on the skin in the presence of organic solvents such as propylene glycol, tertraglycol, and ethanol (Mahjour et al., 1990; Valjakka-Koskela et al., 1998). Ethosome carriers, which were invented by Touitou et al., are a modified form of liposomes that contain a relatively high concentration of ethanol. Moreover, that team discovered that ethosomes are permeation-enhancing carriers, which significantly promote drug delivery into the skin (Touitou et al., 1997; Dayan and Touitou, 2000). To put it more concretely, ethosomes are more efficient at delivering topical agents to the skin, in terms of quantity and depth, than either liposomes or hydroalcoholic solutions (Ting et al., 2004). The unique properties of ethosomes allow their easier penetration into deeper layers of the skin due to the soft, flexible characteristics of the vesicles. By contrast, there are differences in the depths of penetration of the skin between liposomes and ethosomes (Touitou et al., 2000a).

To date, several studies have been performed to create liposomal formulations in attempts to overcome the poor penetration of ALA; however, very few attempts have been made to optimize the formulations. Hence, this study focused on modification formulations so that ALA can be delivered into the skin more efficiently by liposomes/ethosomes. An *in vivo* animal study was carried out to evaluate the relationship of the depth of the skin with the concentration of PpIX by confocal laser scanning microscopy (CLSM). Colorimetry was used to confirm the status of the skin when exposed to PDT.

2. Materials and methods

2.1. Materials

ALA (approximately 98% pure), phosphatidylethanolamine (PE, commercial grade), and cholesterol (CH) were purchased from Sigma Chemical (St. Louis, MO, USA). Sodium stearate (SS) was obtained from Nippon Shinyaku Kogyo (Osaka, Japan). Other chemicals used in the study were of reagent grade.

2.2. Preparation of vesicles (ethosomes vs. liposomes)

Ethosomes and liposomes were prepared according to the thin-film hydration method. Phosphatidylethanolamine, cholesterol, and a surfactant (sodium stearate) were dissolved in chloroform and methanol (2:1, v/v) in a 250-ml round-bottomed flask. The mixture was evaporated in a rotary evaporator above the transition temperature of the phospholipids (60 °C for PE), and solvent traces were removed under vacuum overnight. The film was hydrated with 0.1% ALA dissolved in 15% (v/v) ethanol solution or aqueous solution above the lipid transition temperature for 30 min. The vesicle suspension was dispersed by a probe sonicator (UP50H, Dr. Hielscher, Germany) at 50% amplitude for 1 min.

2.3. Vesicle size and zeta potential

The vesicle size and zeta potential were measured by laser light-scattering (LLS) with a helium–neon laser at 630 nm (Zetasizer 3000HS$_A$, Malvern, UK). The polydispersity index (PI) was used as a parameter of the size distribution. All vesicles were diluted 100-fold with deionized water before the size and zeta potential measurements. The determination was repeated three times per sample for three samples. All particle sizes and zeta potentials were measured at 25 °C. In order to evaluate the stability of ethosomes during storage, the size and zeta potential of vesicles were also monitored after being stored at 4 °C for 32 days.

2.4. Determination of the ALA entrapment efficiency

The entrapment efficiency of ALA in vesicles was determined by high-performance liquid chromatography (HPLC) (Fang et al., 2006a). A centrifugation method was used to separate the incorporated drug from the free form. Vesicle suspensions were centrifuged at 48,000 × *g* and 4 °C for 30 min. Following centrifugation, the supernatant and pellet were separated, and the concentration of ALA was analyzed by HPLC.

2.5. HPLC analytical conditions for ALA determinations

The fluorescence derivation of ALA samples was based on a modification of the Hantzsch reaction, in which amine compounds react with acetylacetone and formaldehyde (Oishi et al., 1996). The acetylacetone reagent was prepared by the addition of 15 ml acetylacetone, 10 ml ethanol, and 75 ml deionized water. The acetylacetone reagent at 3.5 ml, 0.45 ml of a 10% formaldehyde solution, and 50 μl of the ALA sample were vortex-mixed for about 3 s. The mixture was heated in an aluminum-block heater to 100 °C for 15 min, and then cooled in an ice bath.

The ALA content of the various samples was analyzed with an HPLC system consisting of a Waters 515 HPLC pump, a Waters 715 sample processor, and a Waters 474 fluorescence detector (Waters Co. MA, USA). A reverse-phase column (Lichrospher RP-18, 250 × 4 mm, 5 μm, (Merck, Darmstadt, Germany)) was used, with the column oven set to 40 °C. The mobile phase consisting of methanol–water–acetic acid (50:50:1) was used

at a flow rate of 0.7 ml/min. The wavelength of the fluorescence detector was set to excitation at 378 nm and emission at 467 nm. All samples were allowed to stand in the dark.

2.6. In vivo topical delivery of ethosomes and liposomes

Female nude mice (ICR-Foxnl) at 7–8 weeks old were used for the experiments. A glass cylinder with an available area of 0.785 cm^2 was placed on the dorsal skin of a mouse with glue. Ethosomes or liposomes (100 μl) were added to each cylinder, which was covered with parafilm. After being applied for 4 h, each skin site was wiped 10 times using a cotton-tipped stick with distilled water, and the mice were divided into two groups: irradiated and non-irradiated groups. At the end of the irradiative protocol, the treated area was excised. The amount of ALA-induced PpIX retained in the skin was measured by CLSM. All procedures were carried out in the dark to prevent the influence of ambient light.

2.7. PDT instrumentation

A PDT instrument (Omnilux-Red, Photo Therapeutics, UK) was used for the skin irradiative protocol. The device includes dimensions of light active area ($L \times W$: 225 mm × 210 mm) with a light-emitting diode (LED) a red light source at 633 ± 3 nm. A light dose of 120 J/cm^2 was delivered with an output intensity of 80 mW/cm^2 over 25 min. The energy intensity of the LED was pre-measured with a light meter (TES 1339, TES Corp., Taiwan).

2.8. Confocal laser scanning microscopy

Skin samples obtained following *in vivo* treatment were examined for PpIX fluorescence images by CLSM. Each full skin sample was sliced in sections of 6–13 μm in thickness through the *Z*-axis and examined with CLSM (FV 500, Olympus, Japan). Optical excitation was carried out with a 488 nm argon laser beam, and the fluorescence emission was detected at 590 nm. According to the CLSM image of the skin, we used fluoview software to quantify PpIX fluorescence. The enhancement ratio (ER) = ratio of (PpIX intensity of the modified formulation)/(PpIX intensity of ALA in aqueous solution).

2.9. Skin irritation evaluation by colorimetry

The skin irritation level in terms of erythema color was measured by colorimetry (Chroma Meter-CR 221, Minolta, Japan). The instrument records three-dimensional color reflectance (including L^*, a^*, and b^*), as recommended by the Commission International del'Eclairage (CIE). The definitions are as follows: L^* is the relative brightness ranging from total black (0) to total white (+100), a^* is the balance between red (+100) and green (−100), and b^* is the balance between yellow (+100) and blue (−100). The total difference in color between the light-treated site and untreated site as the control is defined as follows: $\Delta C = (\Delta a^2 + \Delta b^2)^{1/2}$ and $\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$.

Table 1
Physicochemical characteristics of ethosome-encapsulated ALA

Ethosome composition (molar ratio)	Entrapment (%)	Size (nm)	Zeta (mV)
PE	8.57 ± 2.2	163.5 ± 0.9	−53.5 ± 1.9
PE/CH (2:1)	9.09 ± 4.02	132.6 ± 1.9	−53 ± 0.8
PE/CH/SS (2:1:2.5)	66.42 ± 0.34	126.4 ± 2.8	−70.06 ± 6.9

Each data represents the mean ± S.D. ($n = 3$). PE, phosphatidylethanolamine; CH, cholesterol; SS, sodium stearate.

2.10. Statistical analysis

Data were analyzed by WINKS software. Statistical comparisons between data were made using analysis of variance (ANOVA). Subgroups were compared using the Newman–Keuls multiple comparisons. A 0.05 level of probability was taken as the level of significance.

3. Results

3.1. Characterization of vesicles (liposomes vs. ethosomes)

3.1.1. Preparation and characterization of vesicles

The particle size and zeta potential of ethosomes were measured by LLS, and the results are shown in Table 1. Data on the particle size showed corresponding decreases depending on the additives, and the results obtained were in the order of PE > PE/CH > PE/CH/SS ($p < 0.05$). Moreover, results of the polydispersity index (PI; representing the distribution of particle size) were <0.48. Incidentally, the average particle sizes of ethosomes were less than those of the liposomal group. The zeta potentials of the PE and PE/CH vesicles were about −53.5 ± 1.9 and −53.0 ± 0.8 mV, respectively. The addition of sodium stearate caused a higher negative surface charge to a value of −70.06 ± 1.9 mV.

3.1.2. Entrapment efficiency of ALA in vesicles

Table 1 shows that the entrapment efficiency did not significantly differ ($p > 0.05$) regardless of whether or not cholesterol was entrapped. The results also showed that adding both cholesterol and sodium stearate to PE vesicles significantly increased the entrapment efficiency. This was revealed by the entrapment efficiency of PE/CH/SS being raised about sevenfold in comparison with PE or PE/CH. Additionally, the influence of additives

Table 2
Stability test by measuring both ethosomal size and zeta potential after 32 days

Formulation	0 Day		32nd Day	
	Size (nm)	Zeta (mV)	Size (nm)	Zeta (mV)
PE	163.5 ± 0.9	−53.5 ± 1.9	158.9 ± 4.1	−47.1 ± 1.8
PE/CH	132.6 ± 1.9	−53.0 ± 0.8	205.0 ± 0.9	−48.6 ± 2.9
PE/CH/SS	126.4 ± 2.8	−70.6 ± 6.9	233.5 ± 4.8	−70.9 ± 9.3

Each data represents the mean ± S.D. ($n = 3$). PE, phosphatidylethanolamine; CH, cholesterol; SS, sodium stearate.

Table 3
Skin irritant evaluation of PDT-irradiated by colorimeter

	Control[a]	Light		
L^*	55.41 ± 2.99	55.04 ± 1.65	ΔL^*	−0.37
a^*	4.16 ± 1.04	3.66 ± 1.08	Δa^*	−0.50
b^*	4.81 ± 1.25	3.22 ± 0.49	Δb^*	−1.59
			ΔC^*	1.67
			ΔE^*	1.71

Each data represents the mean ± S.D. ($n = 4$).
[a] Control group is the neighboring non-irradiated site.

Table 4
The enhancement ratio of the formulation to PpIX in the skin with or without PDT-irradiated

	Enhancement ratio (ER)[a]	
	Non-light	PDT-irradiated
15% EtOH	9.00 ± 2.42	8.51 ± 0.87
PE	15.36 ± 6.51	12.51 ± 3.50
PE/CH	14.68 ± 9.14	14.90 ± 2.37
PE/CH/SS	11.20 ± 1.15	9.80 ± 1.29

Each data represents the mean ± S.D. ($n = 3$–4).
[a] Enhancement ratio (ER) = ratio of PpIX intensity of the modified formulation to PpIX intensity of ALA in aqueous solution.

to ethosomes produced the same effects in liposomes. However, the incorporation of cholesterol and sodium stearate into liposomes resulted in two to fourfold gains in entrapment efficiency. Taken individually, the entrapment efficiencies of ethosomes and liposomes prepared with PE/CH/SS were 66.42 ± 0.34 and 29.37 ± 1.21, respectively.

3.1.3. Stability of ALA in vesicles

The stability of ethosomes was tested using the LLS method. Table 2 shows that during 32 days of storage at 4 °C, PE alone in ethosomal system resulted in a slight change of particle size and an increase of PI after storage. In addition, our previous study showed that the physical stability of liposomes could be maintained by adding cholesterol (Fang et al., 2008). By contrast, the addition of cholesterol significantly increased the vesicle size of ethosomes after storage. Moreover, incorporation of an anion surfactant (sodium stearate) increased the vesicle sizes of both the liposomal and ethosomal systems by 35.32 and 45.87%, respectively (Table 2).

3.2. Penetration behavior by CLSM studies

To elucidate differences in the skin penetration behavior between liposomes and ethosomes, we observed the PpIX level using the CLSM method. Confocal images were acquired after both liposomes and ethosomes had been applied to the back skin of nude mice for 4 h. The skin thickness was optically scanned at 6–13 μm increments for nine fragments from the surface of the skin (left to right, top to bottom).

Fig. 1 shows the results for liposomes in the CLSM studies. It was found that the control group (ALA dissolved in H_2O) presented a weak PpIX signal. By contrast, PE/CH/SS showed a significantly higher intensity of PpIX than the control group ($p < 0.05$); however, the delivery depth was only about 60 μm. On the other hand, the results for ethosomes showed that a higher amount of ALA was delivered when dissolved in a 15% ethanol solution (Fig. 2A) than when dissolved in H_2O (Fig. 1A), and the result was similar to that of PE/CH/SS (Fig. 1B). Moreover, PE in 15% ethanol (Fig. 2B) clearly resulted in a broad distribution of PpIX intensity that extended from the stratum corneum to upper epidermis in comparison with ALA dissolved in either H_2O or 15% ethanol. Because the PpIX intensity of penetration in the PE/CH/SS (ethosome) was lower than that of PE (ethosome), the figure of PE was cited. Overall, the penetration behavior of liposomes differed from that of ethosomes. The intensities of PpIX delivered by of liposome were in the increasing order of H_2O < PE < PE/CH/SS (Fig. 1). In contrast, the delivery of ALA by ethosomes revealed that PE/CH/SS reduced the PpIX found in the skin.

3.3. Effect of light

After the free form of ALA or the various formulations were removed from the treated site on mice skin, the skin was irradiated by Omnilux-red with a fluence rate of 80 mW/cm^2 for 25 min, with a total light dose of 120 J/cm^2. Fig. 3 shows the influence of light on the fluorescence of PpIX. As shown by the results of ALA dissolved in 15% ethanol, no difference was apparent between the non-irradiated (Fig. 2A) and irradiated groups (Fig. 3A); moreover, the intensity of PpIX of the ethosomal formulation (PE) was obviously higher than that in 15% ethanol.

Table 4 summarizes the results of PpIX intensity in the skin under different conditions. The results show that the enhancement ratios of the modified formulations increased from 8.51- to 15.36-fold in contrast to ALA in an aqueous solution. In the irradiated group, the enhancement ratios of different formulations decreased in the order of PE ≅ PE/CH < 15% ethanol ≅ PE/CH/SS. Moreover, the data also showed that the enhancement ratio of the non-irradiated PE/CH/SS group was significantly higher than that of the irradiated group ($p < 0.05$).

3.4. Skin irritant evaluation by colorimetry

For the colorimetric evaluation, there were many parameters including ΔL^*, Δa^*, Δb^*, ΔC^*, and ΔE^*. As shown in Table 3, the data revealed that the indicators did not significantly differ between the control and PDT-irradiated groups ($p > 0.05$). Taken individually, the value of L^*, the luminance of the relative brightness, was about 55 in both the control and PDT-irradiated groups. The values of a^*, the balance between red and green, were 3.66 in the control and 4.66 in the PDT-irradiated groups. The values of b^*, the balance between yellow and blue, were 4.81 in the control and 3.22 in the PDT-irradiated group. In general, differences in color between the control and experimental groups were expressed as ΔE^*, which had a value of 1.71.

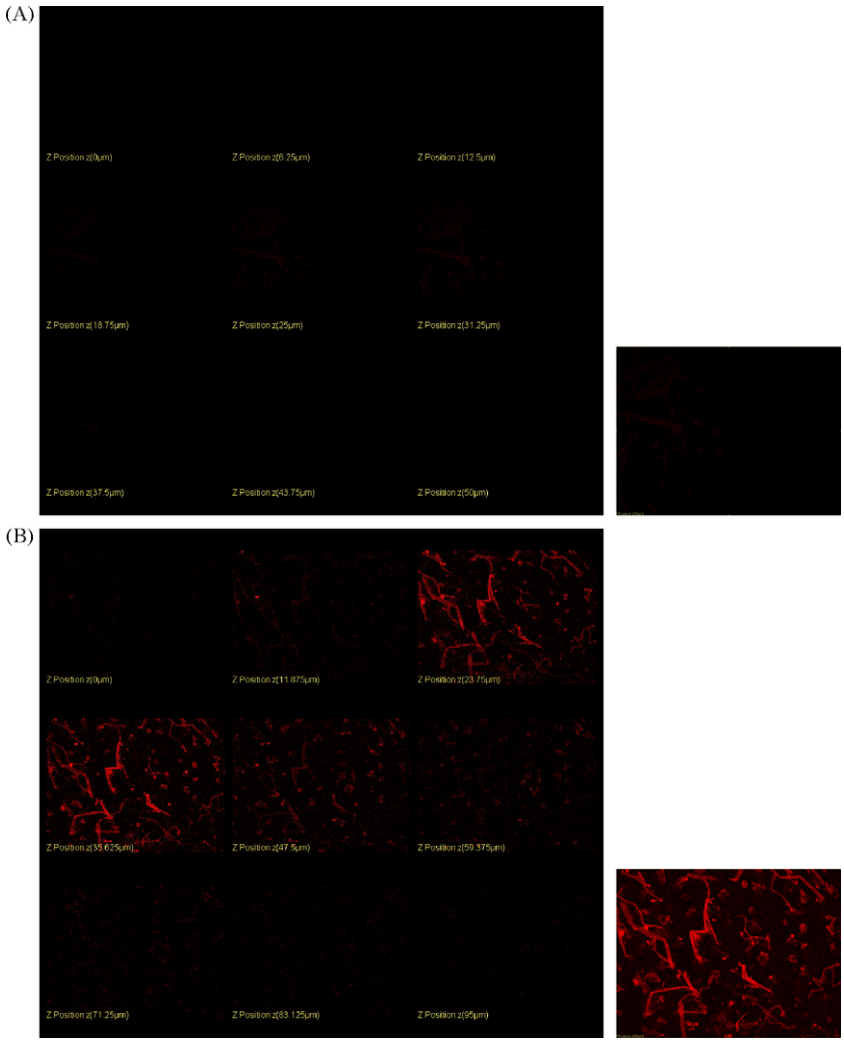

Fig. 1. CLSM micrographs of nude mouse skin after *in vivo* application of liposomal formulation for 4 h. (A) liposome-free (ALA in aqueous solution); (B) liposome (PE/CH/SS) in aqueous solution. The skin was visualized by CLSM at 6–13 μm increments through the *Z*-axis. The full thickness was divided to nine fragments from the surface of the skin (left to right, top to bottom). The right figure is the sum of above fragments.

4. Discussion

4.1. Comparison of the physicochemical properties between liposomes and ethosomes

In past research, a considerable number of studies have attempted to improve ALA skin permeation. Because of the special structure of the skin and the hydrophilic properties of ALA, a major limitation of PDT is drug delivery. Several approaches have been used for transdermal drug delivery (Barry, 2001), one of which uses vesicle formulation (Loan Honeywell-Nguyen and Bouwstra, 2005). Due to the hydrophilic properties of ALA, it might be difficult to entrap ALA by either the liposomal or ethosomal system (Yatvin and Lelkes, 1982; Choi and Maibach, 2005). We attempted to characterize ethosome containing ALA and to compare the physicochemical properties and penetration behaviors of PpIX delivery between conventional liposomal and deformable ethosomal systems.

Vesicle size influences topical drug delivery; vesicles smaller than 300 nm are able to deliver their contents to some extent into deeper layers of the skin (Verma et al., 2003; du Plessis et al., 1994). In the cases of ethosomes and liposomes with average diameters of <200 nm, there is the potential for delivery through the skin. Our study showed that the vesicle size of the ethosomal system was significantly smaller than that of the liposomal system ($p < 0.05$), with sizes ranging from 126.4 to 163.5 nm and 133.6 to 196.0 nm, respectively. This phenomenon was reported in previous studies, and it suggests that ethanol has a fluidizing effect on phospholipid bilayers (Touitou et al., 2000a,b). In other words, ethanol has a predominant role in ethosomal systems, in that adding ethanol to conventional liposomes produces compact, soft, deformable vesicles (Touitou et al., 2000b). On the other hand, the evidence indicated that ethanol probably has a steric stabilization effect due to modification of the net charge of the ethosomal system which is relevant to a decrease in vesicle size (López-Pinto et al., 2005). In the present study, there was no significant difference between liposomal and ethosomal systems in terms of the zeta potential, except for the PE/CH/SS group. However, the notion of steric stabilization might not correspond with most of the present data. It was assumed that

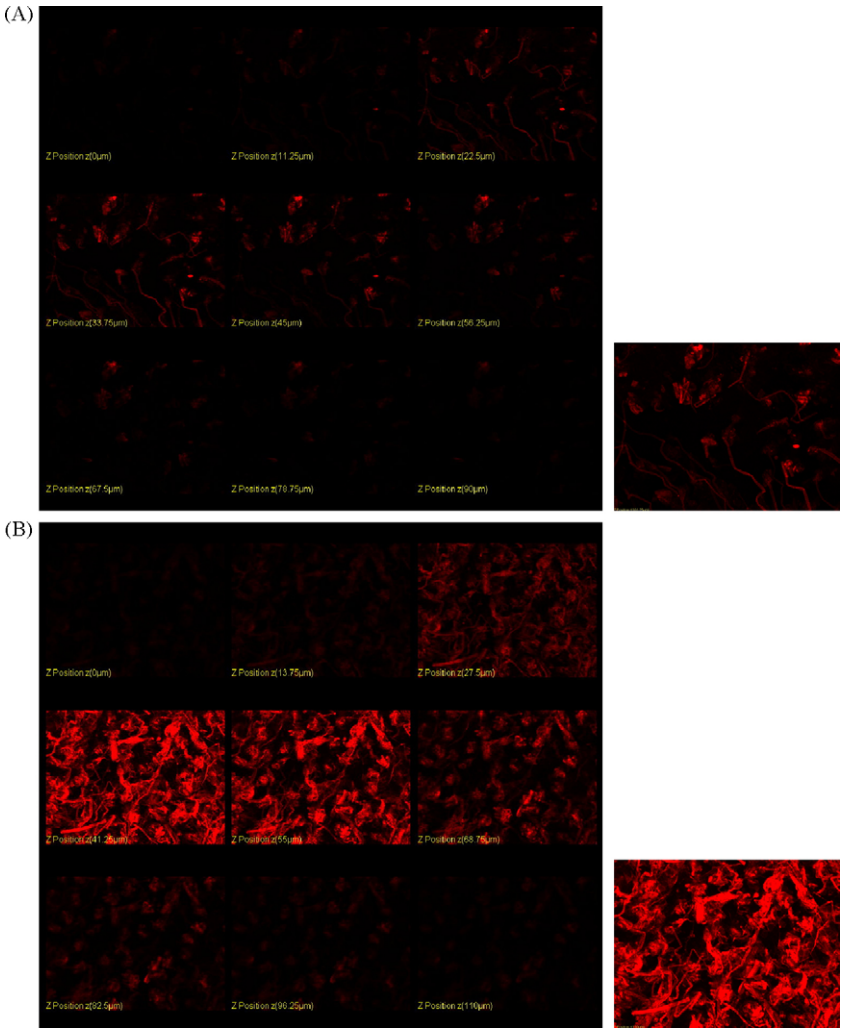

Fig. 2. CLSM micrographs of nude mouse skin after *in vivo* application of ethosomal formulation for 4 h. (A) ethosome-free (ALA in 15% ethanol solution); (B) ethosome (PE) in 15% ethanol solution. The right figure is the sum of above fragments.

steric stabilization distinguished between phosphocholine and phosphoethanolamine due to the different head groups of phospholipids.

Particle size showed a corresponding decrease with the addition of additives, and results were in the order of PE > PE/CH > PE/CH/SS ($p < 0.05$). Here, we discuss the aspects of the effects of CH and SS. First, the size after adding CH with PE was smaller than that with PE alone, and this was apparently due to the influence of CH. It is now generally accepted that the addition of CH to phospholipid films causes the membrane to be more compact (Vemuri and Rhodes, 1995). The results of our study corresponded to this concept not only for the liposomal but also for the ethosomal system. Second, adding SS to PE/CH showed a tendency for the vesicle size to be smaller than other formulations in both the liposomal and ethosomal systems. This phenomenon presumes that the carbon chain of stearate was inserted into the lipid bilayer (Fang et al., 2008).

Theoretically, liposomes and ethosomes can entrap both hydrophobic and hydrophilic drugs (Vemuri and Rhodes, 1995; Touitou et al., 2000a). However, evidence from a previous study demonstrated that the entrapment of hydrophilic molecules was less efficient than that of hydrophobic molecules (Touitou et al., 2000b). Due to its hydrophilic properties, ALA limits the entrapment efficiencies of liposomes and ethosomes (Yatvin and Lelkes, 1982). Table 1 shows that regardless of whether cholesterol was incorporated, no significant difference was noted in terms of entrapment efficiency ($p > 0.05$). Moreover, our findings indicated that PE/CH/SS had high entrapment efficiencies in both the ethosomal and liposomal systems. In our previous study (Fang et al., 2008), the enriched entrapment efficiency may be attributed to interactions between SS and ALA, when the complex was inserted in the lipid bilayer. Overall, the results showed that the entrapment efficiency of PE/CH/SS in ethosome system (with 15% ethanol) was about two orders of magnitude higher than that of the PE/CH/SS group in liposome system (without 15% ethanol). This tendency can be explained by the influence of the surface potential. Yamaguchi showed that high zeta potentials in ethosomes increase the interbilayer distance owing to electrostatic repulsion (Yamaguchi, 1996). Moreover,

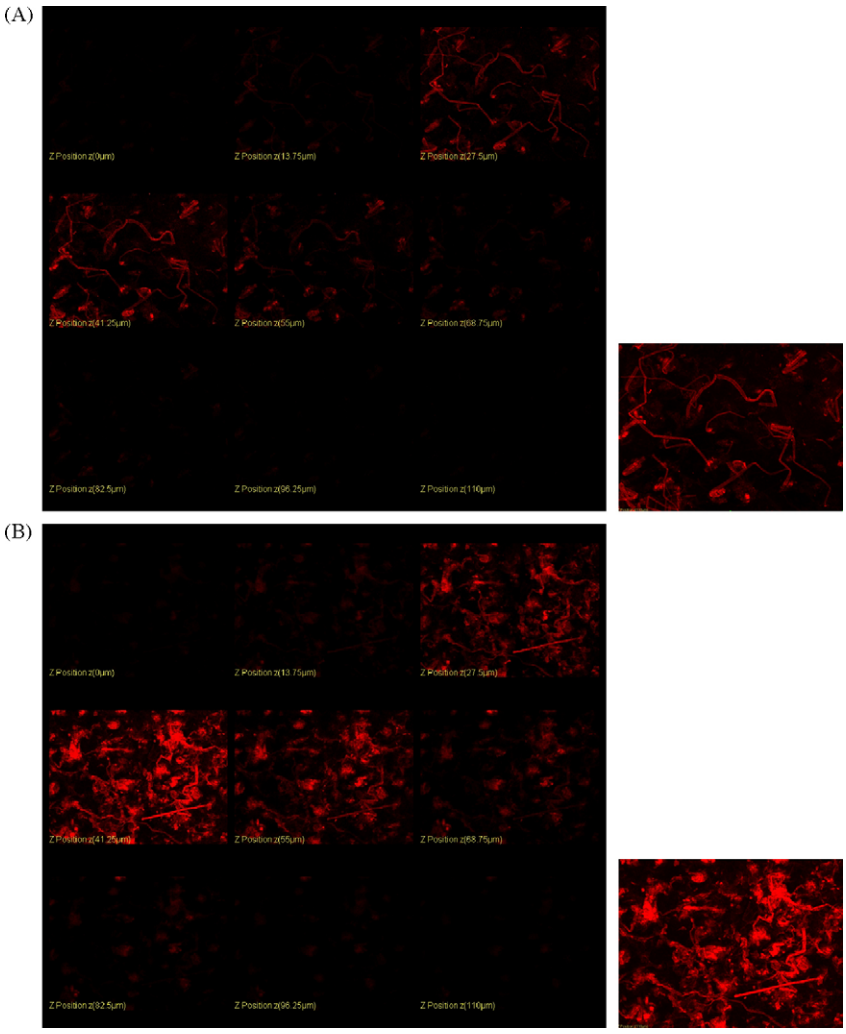

Fig. 3. CLSM micrographs of nude mouse skin after *in vivo* application of ethosomal formulation for 4 h and irradiated with the fluence rate of 80 mW/cm^2 for 25 min, and the total light dose of 120 J/cm^2. (A) ethosome-free (ALA in 15% ethanol solution); (B) ethosome (PE) in 15% ethanol solution. The right figure is the sum of above fragments.

Barry (2001) investigated the detailed mechanisms to clarify the role of ethanol by using ^{2}H and ^{31}P nuclear magnetic resonance (NMR) spectroscopy. They demonstrated that ethanol binds strongly to the bilayers in the region of the lipid–water interface with little or no interaction with the hydrocarbon interior. Several studies also reported the same tendency of ethosomes to load significantly more than liposomes with the same components but without ethanol (Dayan and Touitou, 2000; Maurer et al., 2001).

In the stability study, PE alone in ethosomal system resulted in a slight change of particle size and an increase of PI after storage. On the other hand, the result also showed a decreased in entrapment efficiency (data not shown). In terms of chemical properties of ALA, it may be stable in strongly acidic solution (pH 3). However, we couldn't employ acidic solution to animals in clinical application which may cause more side effects. Briefly, the pH problem might partially contribute to the phenomenon (Elfsson et al., 1999). In order to retain the ALA loaded, the technique of freeze drying should be used in the stored process.

4.2. Penetration behavior by CLSM studies

Visualization of PpIX by CLSM images showed differences in the skin penetration behavior between liposomes and ethosomes (Figs. 1 and 2). The drug penetration behavior was diverse due to the physicochemical properties including particle size, zeta potential, thermodynamic phase, lamellarity, and bilayer elasticity of vesicles; these characteristics are influenced by the components (Loan Honeywell-Nguyen and Bouwstra, 2005).

Overall, our findings indicated that the penetration ability of ethosomes was grater than that of liposomes, and that both ethanol and the surfactant were key factors. Basically, ethanol is an enhancer that interacts with the skin and extracts lipids of the SC, and fluidized SC lipids create channels which allow the increased delivery of a drug (Berner and Liu, 1995). In order to enhance the transdermal delivery efficiency, many strategies attempt to both disrupt/weaken the structure of the skin and increase the driving force for delivering a drug through the skin barrier (Loan Honeywell-Nguyen and Bouwstra, 2005). Touitou

et al. developed an ethosomal system that can transport several drugs across the skin more efficiently. Ethosomes, a unique carrier, simultaneously influence the vesicle characteristics and skin. The team suggested a model of ethanol which exerts a fluidizing effect on both the ethosomal lipids and on the lipid bilayer of the SC (Touitou et al., 2000b).

The result demonstrated that the penetration behavior of PpIX was similar between PE and PE/CH formulation and showed that the enhancement ratios were increased 14- to 15-fold in comparison with that of ALA dissolved in H_2O. However, this phenomenon did not occur when ALA was dissolved in 15% ethanol alone. Actually, the ethosomal suspension used in this study contained less than 10% entrapped and 90% untrapped drug. Hence, the high deposition in the skin might be attributed to the properties of ethosomes and the effect of phospholipids. By the characteristics of ethosome, it may be assumed that the entrapped can be transport to the skin by its flexible properties and the other free form (unentrapped part) might be facilitated by the ethosome itself.

On the other hand, the data also showed that the PE/CH/SS composition was the best at achieving a high entrapment efficiency of about 66%; however, the penetration studies indicated that the PpIX intensity of PE/CH/SS was similar to that in 15% ethanol alone. On the basis of the heme synthesis pathway, free heme can inhibit the synthesis of ALA by a negative freedback mechanism. Indeed, ALA is a precursor of the photosensitizer, protoporphyrin IX (PpIX), formed *in vivo* after the exogenous application of ALA. After exogenous ALA topical applied to skin, PpIX is converted within mitochondria. The process may accumulate due to the limited capacity of ferrochelatase to convert it to heme. Here, we discussed two sets of penetration behaviors of PpIX: first, how drugs were released from the vesicles; and second, how PpIX was transformed from ALA. The phenomenon implies that the amount of PpIX in the skin was significantly lower and might be confined to the release stage from the ethosomal system. The phenomenon could be interpreted as follows: first, phospholipids form an extra lipid barrier at the skin surface; second, the drug is slowly released from the liposomes, or interactions exist between the drug and lipid molecules (Fang et al., 2006b). Also, evidence supports there being no correlation between the entrapment efficiency and drug delivery (Barry, 2001). The present data support that viewpoint.

4.3. Effect of PDT irradiation

Irradiation plays a particular role in PDT, which involves activation at a certain wavelength and suitable fluence rate, and singlet oxygen is generated by a cascade of reactions. PDT efficiency is related to the process of light irradiation (Henderson and Dougherty, 1992; Szeimies et al., 2005). In brief, light sources are divided into three types: (a) lasers, such as the gold vapor laser; (b) the incoherent type, such as a slide projector or arc lamp; and (c) LED systems. While laser systems are widely used, the instrument requires proper staff and sufficient space, and it is more expensive. Incoherent light sources offer such advantages as being cheaper and simpler, and having similar efficacies as laser systems, although they use broadband excitation (De Rosa and Bentley, 2000). LED systems have been developed to overcome the disadvantages of other light sources. These offer significant benefits including a narrowband light source, less hyperthermia, and simple, inexpensive handling (Babilas et al., 2006).

Our work was performed with an LED light delivery system; however, little information is available on the effects of the light dose from LED sources. Previous studies suggested that the light dose is within a range of 60–250 J/cm^2 for laser sources and 30–540 J/cm^2 for non-laser sources (Peng et al., 1997a,b). Omnilux-red, the PDT instrument, is a product of Photo Therapeutics, and its overall safety is thought to be good. In order to confirm the safety in the present circumstances, we used a colorimeter to evaluate irritation to the skin. Overall, there were no significant differences between the non-irradiated and irradiated groups in terms of the indicators of erythema ($p > 0.05$). In other words, skin erythema was not significantly influenced by irradiation in this study.

In the data of the irradiated group, the enhancement ratio with different formulations decreased in the order of PE $\cong$ PE/CH > 15% ethanol $\cong$ PE/CH/SS. The tendency was similar to that of the non-irradiated group. This implies that the enhancement behavior was related to the formulation. The enhancement ratios of PpIX in the skin with 15% ethanol and PE/CH/SS were equivalent because the release of PpIX might have been restricted in the step from the ethosomal system to the skin. On the other hand, PE and PE/CH showed marked PpIX intensities in the skin in that phospholipids as a carrier increase the penetration ability into the skin. This is in accordance with previous studies which demonstrated that ethosomes can deliver drugs deep into the SC layer of the skin (Choi and Maibach, 2005).

In particular, the enhancement ratios showed significant differences between the non-irradiated and irradiated groups with the PE/CH/SS formulation ($p < 0.05$). It is possible that a photobleaching effect occurred during the PDT-irradiation process. Photobleaching, or photochemical degradation, causes a loss of fluorescence intensity due to photon-induced chemical damage and irreversible covalent modifications by light exposure. Our results are in agreement with previous studies on the concept of photobleaching in animal experiments (Ericson et al., 2003). The phenomenon assumes that the absorption spectrum of PpIX fluorescence is similar to that of the PDT irradiation (Nadeau et al., 2004).

5. Conclusions

In summary, ethosomal formulations containing ALA were characterized in this study. The results indicated that there was no correlation between the entrapment efficiency and penetration of PpIX into the skin. This phenomenon implies that PpIX is retarded in the skin possibly due to restrictions at the release stage from the ethosomal system. Results of CLSM indicated that the penetration ability of ethosomes was greater than that of liposomes in terms of PpIX deposition in the skin, and ethanol played a particular role in the ethosomal system. Ethanol exist a fluidizing effect on both lipids of the ethosomal and lipid bilayer

of the SC. In the evaluation of erythema due to the irradiation process, no redness was observed in the skin using a colorimetric method. The penetration behavior of the irradiated group with the ethosomal formulation showed no significant difference except with PE/CH/SS, and we assumed that this was associated with a photobleaching effect.

Acknowledgement

This work was supported by National Science Council of Taiwan (NSC 95-2320-B-037-025).

References

Babilas, P., Kohl, E., Maisch, T., Backer, H., Gross, B., Branzan, A.L., Baumler, W., Landthaler, M., Karrer, S., Szeimies, R.M., 2006. In vitro and in vivo comparison of two different light sources for topical photodynamic therapy. Br. J. Dermatol. 154, 712–718.

Barry, B.W., 2001. Novel mechanisms and devices to enable successful transdermal drug delivery. Eur. J. Pharm. Sci. 14, 101–114.

Berner, B., Liu, P., 1995. In: Smith, E.W., Maibach, H.I. (Eds.), Alcohols. CRC Press, Boca Raton, Fl.

Casas, A., Perotti, C., Saccoliti, M., Sacca, P., Fukuda, H., Batlle, A.M.d.C., 2002. ALA and ALA hexyl ester in free and liposomal formulations for the photosensitisation of tumour organ cultures. Br. J. Cancer 86, 837–842.

Chakrabarty, A., Geisse, J.K., 2004. Medical therapies for non-melanoma skin cancer. Clin. Dermatol. 22, 183–188.

Choi, M.J., Maibach, H.I., 2005. Elastic vesicles as topical/transdermal drug delivery systems. Int. J. Cosmet. Sci. 27, 211–221.

Dayan, N., Touitou, E., 2000. Carriers for skin delivery of trihexyphenidyl HCl: ethosomes vs. liposomes. Biomaterials 21, 1879–1885.

De Rosa, F.S., Bentley, M.V., 2000. Photodynamic therapy of skin cancers: sensitizers, clinical studies and future directives. Pharm. Res. 17, 1447–1455.

du Plessis, J., Ramachandran, C., Weiner, N., Müller, D.G., 1994. The influence of particle size of liposomes on the deposition of drug into skin. Int. J. Pharm. 103, 277–282.

Elfsson, B., Wallin, I., Eksborg, S., Rudaeus, K., Ros, A.M., Ehrsson, H., 1999. Stability of 5-aminolevulinic acid in aqueous solution. Eur. J. Pharm. Sci. 7, 87–91.

Ericson, M.B., Grapengiesser, S., Gudmundson, F., Wennberg, A.M., Larko, O., Moan, J., Rosen, A., 2003. A spectroscopic study of the photobleaching of protoporphyrin IX in solution. Lasers Med. Sci. 18, 56–62.

Fang, J.Y., Hwang, T.L., Huang, Y.L., Fang, C.L., 2006a. Enhancement of the transdermal delivery of catechins by liposomes incorporating anionic surfactants and ethanol. Int. J. Pharm. 9, 131–138.

Fang, J.Y., Hwang, T.L., Huang, Y.L., 2006b. Liposomes as vehicles for enhancing drug delivery via skin routes. Curr. Nanosci. 2, 55–70.

Fang, Y.P., Huang, Y.B., Wu, P.C., Tsai, Y.H., 2008. Physicochemical and safety evaluation of 5-aminolevulinic acid in novel liposomes as carrier for skin delivery. J. Liposome Res. 18, 1–15.

Fotinos, N., Campo, M.A., Popowycz, F., Gurny, R., Lange, N., 2006. 5-Aminolevulinic acid derivatives in photomedicine: characteristics, application and perspectives. Photochem. Photobiol. 82, 994–1015.

Henderson, B.W., Dougherty, T.J., 1992. How does photodynamic therapy work? Photochem. Photobiol. 55, 145–157.

Hopper, C., 2000. Photodynamic therapy: a clinical reality in the treatment of cancer. Lancet Oncol. 1, 212–219.

Hürlimann, A.F., Hänggi, G., Panizzon, R.G., 1998. Photodynamic therapy of superficial basal cell carcinomas using topical 5-aminolevulinic acid in a nanocolloid lotion. Dermatology 197, 248–254.

Loan Honeywell-Nguyen, P., Bouwstra, J.A., 2005. Vesicles as a tool for transdermal and dermal delivery. Drug Discov. Today: Technol. 2, 67–74.

Lopez, R.F.V., Lange, N., Guy, R., Bentley, M.V.L.B., 2004. Photodynamic therapy of skin cancer: controlled drug delivery of 5-ALA and its esters. Adv. Drug. Deliv. Rev. 56, 77–94.

López-Pinto, J.M., González-Rodríguez, M.L., Rabasco, A.M., 2005. Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. Int. J. Pharm. 298, 1–12.

Mahjour, M., Mauser, B., Rashidbaigi, Z., Fawzi, M.B., 1990. Effect of egg yolk lecithins and commercial soybean lecithins on in vitro skin permeation of drugs. J. Control. Release 14, 243–252.

Maurer, N., Wong, K.F., Stark, H., Louie, L., McIntosh, D., Wong, T., Scherrer, P., Semple, S.C., Cullis, P.R., 2001. Spontaneous entrapment of polynucleotides upon electrostatic interaction with ethanol-destabilized cationic liposomes. Biophys. J. 80, 2310–2326.

McGillis, S.T., Fein, H., 2004. Topical treatment strategies for non-melanoma skin cancer and precursor lesions. Semin. Cutan. Med. Surg. 23, 174–183.

Merclin, N., Bender, J., Sparr, E., Guy, R.H., Ehrsson, H., Engstrom, S., 2004. Transdermal delivery from a lipid sponge phase—iontophoretic and passive transport in vitro of 5-aminolevulinic acid and its methyl ester. J. Control. Release 100, 191–198.

Nadeau, V., O'Dwyer, M., Hamdan, K., Tait, I., Padgett, M., 2004. In vivo measurement of 5-aminolaevulinic acid-induced protoporphyrin IX photobleaching: a comparison of red and blue light of various intensities. Photodermatol. Photoimmunol. Photomed. 20, 170–174.

Oku, N., 1999. Anticancer therapy using glucuronate modified long-circulating liposomes. Adv. Drug. Deliv. Rev. 40, 63–73.

Oishi, H., Nomiyama, H., Nomiyama, K., Tomokuni, K., 1996. Fluorometric HPLC determination of delta-aminolevulinic acid (ALA) in the plasma and urine of lead workers: biological indicators of lead exposure. J. Anal. Toxicol. 20, 106–110.

Peng, Q., Berg, K., Moan, J., Kongshaug, M., Nesland, J.M., 1997a. 5-Aminolevulinic acid-based photodynamic therapy: principles and experimental research. Photochem. Photobiol. 65, 235–251.

Peng, Q., Warloe, T., Berg, K., Moan, J., Kongshaug, M., Giercksky, K.E., Nesland, J.M., 1997b. 5-Aminolevulinic acid-based photodynamic therapy. Cancer 79, 2282–2308.

Szeimies, R.M., Morton, C.A., Sidoroff, A., Braathen, L.R., 2005. Photodynamic therapy for non-melanoma skin cancer. Acta Derm. Venereol. 85, 483–490.

Ting, W.W., Vest, C.D., Sontheimer, R.D., 2004. Review of traditional and novel modalities that enhance the permeability of local therapeutics across the stratum corneum. Int. J. Dermatol. 43, 538–547.

Touitou, E., Alkabes, M., Dayan, N., Eliaz, M., 1997. Ethosomes: novel vesicular carriers for enhanced skin delivery. Pharm. Res. 14, S-305.

Touitou, E., Dayan, N., Bergelson, L., Godin, B., Eliaz, M., 2000a. Ethosomes - novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. J. Control. Release 65, 403–418.

Touitou, E., Godin, B., Weiss, C., 2000b. Enhanced delivery of drugs into and across the skin by ethosomal carriers. Drug Dev. Res. 50, 406–415.

Valjakka-Koskela, R., Kirjavainen, M., Mönkkönen, J., Urtti, A., Kiesvaara, J., 1998. Enhancement of percutaneous absorption of naproxen by phospholipids. Int. J. Pharm. 175, 225–230.

Vemuri, S., Rhodes, C.T., 1995. Preparation and characterization of liposomes as therapeutic delivery systems: a review. Pharm. Acta Helv. 70, 95–111.

Verma, D.D., Verma, S., Blume, G., Fahr, A., 2003. Particle size of liposomes influences dermal delivery of substances into skin. Int. J. Pharm. 258, 141–151.

Yamaguchi, T., 1996. Lipid microspheres as drug carriers: a pharmaceutical point of view. Adv. Drug Deliv. Rev. 20, 117–130.

Yatvin, M.B., Lelkes, P.I., 1982. Clinical prospects for liposomes. Med. Phys. 9, 149–175.