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Preparation of curcuminoid niosomes for enhancement of skin permeation

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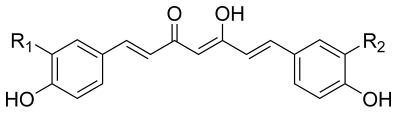
Curcuminoids (curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin) are major bioactive substances found in turmeric (*Curcuma longa* L.) extracts and possess antioxidant, anti-inflammatory, antimicrobial and anticancer properties. In this study, curcuminoid niosomes prepared with a series of Span non-ionic surfactants were developed to enhance the skin permeation of curcuminoids. Formulations were evaluated based on aggregation of niosomes, curcuminoid loading, % entrapment efficiency and *in vitro* permeation of curcuminoids through shed snake skin. Optimal formulations of curcuminoid niosomes including sorbitan monooleate, cholesterol, and Solulan C-24 at a mole ratio of 47.5:47.5:5 were obtained. Up to 11 μmoles of curcuminoids could be loaded in the niosome with a % entrapment efficiency of 83%. About 90% of the niosomes had a diameter of 12.25 ± 5.00 μm. The niosomes significantly enhanced permeation of curcuminoids compared with a methanolic solution of curcuminoids: 4% of entrapped curcuminoids traversed the shed snake skin, whereas permeation from the methanolic solution was undetectable. The fluxes of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin were 1.117, 0.263, and 0.057 μg/(cm^{2}h), respectively, consistent with the relative hydrophobicity of curcumin > desmethoxycurcumin > bisdesmethoxycurcumin. In conclusion, our data show that curcuminoids can be successfully formulated as niosomes and that such formulations have improved properties for transdermal delivery.

1. Introduction

Turmeric (*Curcuma longa* L.) is a rhizomatous herbaceous plant in the family of Zingiberaceae originating from South and Southeast Asia. The rhizome of turmeric is commonly used as a spice and coloring agent in food due to its yellowish-orange color and specific odor. It is also used as a skin care agent for medicinal and cosmetic purposes. Commercially available turmeric extracts contain bioactive substances referred to as curcuminoids, including curcumin (MW = 368.39), desmethoxycurcumin (MW = 338.36) and bisdesmethoxycurcumin (MW = 308.34) (Fig. 1). These molecules have antioxidant, anti-inflammatory, antimicrobial and anticancer activities (Zhou et al. 2011; Haukvik et al. 2010; Buadonpri et al. 2009; Shehzad et al. 2010; Itokawa et al. 2008; Anand et al. 2008). However, curcuminoids are hydrophobic compounds that are insoluble in acidic to neutral pH aqueous solution, but soluble in organic solvents (Tomren et al. 2007; Jayaprakasha et al. 2005). In addition, these compounds are unstable at neutral and basic pH. For example, curcumin is easily degraded to vanillin, ferulic acid (4-hydroxy-3-methoxycinnamic acid) and feruloyl methane (4-hydroxy-3-methoxycinamoyl-methane) under alkaline conditions (Tomren et al. 2007; Wang et al. 1997). The hydrophobicity and instability of curcuminoids results in low bioavailability and reduced transdermal delivery; therefore, various formulations are needed for improvement of curcuminoid bioavailability and biological activities (Mourtas et al. 2011; Gao et al. 2011; Ganta et al. 2010; Patel et al. 2009a, b; Chen et al. 2009; Hegge et al. 2008; Ma et al. 2008).

In recent years, niosomes have received considerable attention as carriers to deliver active ingredients to the skin or through the stratum corneum (Neubert 2011; He et al. 2010; Barakat et al. 2009; Balakrishnan et al. 2009; Khazaeli et al. 2007). Structurally, niosomes are submicron spherical or lamellar vesicles consisting of non-ionic surfactants and membrane additives (Barry 2001; Drummond and Fong 1999; Liu and Guo 2007; Uchegbu and Florence 1995; Uchegbu and Vyas 1998). Various non-ionic surfactants have been developed for niosome formulations using non-ionic surfactants with hydrophilic-lipophilic balance (HLB) values from four to eight (Uchegbu and Florence 1995; Uchegbu and Vyas 1998; Muzzalupo et al. 2008; Yoshioka and Florence 1994; Uchegbu et al. 1995; Hua and Liu 2007; Liu et al. 2007). Similarly to liposomes, hydrophilic substances can be encapsulated inside the aqueous compartment and hydrophobic compounds can be incorporated in the bilayer structure. However, niosomes have advantages over liposomes because non-ionic surfactants are more chemically stable and less expensive than the phospholipids used in preparation of liposomes (Hua and Liu 2007; Schreier and Bouwstra 1994).



(1) $R_1 = R_2 = OCH_3$
(2) $R_1 = H$; $R_2 = OCH_3$
(3) $R_1 = R_2 = H$

Fig. 1: Structures of curcumin (1), desmethoxycurcumin (2), and bisdesmethoxycurcumin (3)

Vesicles are used as topical drug carriers to obtain a higher local drug concentration on skin, to create a sustained release profile, to control diffusion properties of the drug, and to take advantage of the property of non-ionic surfactants as skin penetration enhancers. Niosomes penetrate deeply into the stratum corneum, but whether they migrate as intact vesicles or as a part of curved lamellar structures is unclear (Uchegbu and Vyas 1998; Schreier and Bouwstra 1994; Hofland et al. 1994). One proposed mechanism is fusion of vesicles with cell membranes, leading to a high local drug concentration and resulting in a driving force for the permeation of lipophilic drugs (Fang et al. 2001).

In this study, a series of Span non-ionic surfactants were investigated to select a suitable surfactant for preparation of curcuminoid niosomes. Formulations were evaluated based on aggregation of niosomes, curcuminoid loading and % entrapment efficiency. In addition, *in vitro* permeation of curcuminoids from niosomes through shed snake skin was examined. We show that the formulated niosomes have good physical characteristics and that they enhance curcuminoid skin permeation.

2. Investigations, results and discussion

2.1. Validation of the HPLC analytical method

A typical chromatogram of the curcuminoids showed three peaks at retention times of 16.2, 18.1 and 20.2 min, corresponding to curcumin, desmethoxycurcumin and bisdesmethoxycurcumin, respectively (Fig. 2). Excipient peaks (surfactants, cholesterol and Solulan C-24) did not interfere with the curcuminoid peaks, confirming the specificity of the assay method. The limits of detection of curcumin, desmethoxycurcumin and bisdesmethoxycurcumin were calculated to be 3.36, 0.90, and 0.25 μg/mL, respectively; and the limits of quantification were estimated to be 10.18, 2.73, and 0.75 μg/mL, respectively. The % RSD of the assay for the curcuminoids was less than 0.5%. Linear plots with correlation coefficients > 0.999 were obtained over calibration ranges of 16 to 95 μg/mL for curcumin, 4 to 24 μg/mL for desmethoxycurcumin, and 0.5 to 3 μg/mL for bisdesmethoxycurcumin. The % recovery of curcumin, desmethoxycurcumin and bisdesmethoxycurcumin ranged from 97.2 to 100.9%, 97.7 to 100.4%, and 98.1 to 100.4%, respectively. These results demonstrate that the HPLC method is suitable for analysis of curcuminoids in niosome matrices.

2.2. Preparation of curcuminoid niosomes

Curcuminoid niosomes were prepared using Span 40, 60 or 80 at a mole ratio of non-ionic surfactant:cholesterol:Solulan C-24 of 47.5:47.5:5. Span 40, 60 and 80 were chosen as non-ionic surfactants for the niosome formulations because they possess HLB values in the range of 4–6, which are suitable values for niosome formation (Drummond and Fong 1999; Uchegbu and Florence 1995; Yoshioka and Florence 1994; Uchegbu et al. 1995; Hua and Liu 2007; Liu et al. 2007). Spans are alkyl ester surfactants that are hydrolyzable *in vivo*, resulting in low toxicity and allowing Spans to be used as common pharmaceutical excipients. Cholesterol and Solulan C-24 were added to increase the stability of the niosomes (Uchegbu and Vyas 1998). Typical niosome formulations include a non-ionic surfactant and cholesterol at a molar ratio of 1:1 with a total solid concentration of 10–30 mM (Uchegbu and Vyas 1998; Uchegbu and Duncan 1997; Chandraprakash et al. 1990). This molar ratio is reported to facilitate entrapment of both hydrophilic and hydrophobic compounds (Uchegbu and Duncan 1997; Chandraprakash et al. 1990). Solulan C-24, a steric stabilizer, was added to the formulation to prevent niosome aggregation. Since a hemotoxicity effect has been reported at Solulan C-24 concentrations of higher than 10% by mol, only 5% Solulan C-24 was added. Therefore, a final mole ratio of non-ionic surfactant:cholesterol:Solulan C-24 of 47.5:47.5:5 with a total solid concentration of 30 mM was chosen in this study.

2.3. Characterization of curcuminoid niosomes

The morphology of the curcuminoid niosomes was investigated using optical microscopy and SEM. Use of Span 40, 60 or 80 produced niosomes as smooth-surfaced spherical vesicles of variable size (Fig. 3). Formulations with Span 40 and Span 60 yielded a lower number of niosomes vesicles and these niosomes also tended to aggregate. Since the Span 80 niosomes were less aggregated, we focused on the Span 80 formulation for further examination of the properties of the curcuminoid niosomes.

Various amounts of curcuminoids (5.63, 11.31, 16.92, 22.56, 28.20, and 33.84 μmol) were loaded in 150-μmol Span 80 niosomes to determine the maximum loading amount of curcuminoids. Under optical microscopy, co-precipitation of curcuminoids with curcuminoid niosomes was observed for loading amounts of curcuminoids ≥ 16.92 μmol. Therefore, % entrapment efficiency, % recovery, particle size and size distribution were determined at loading amounts of 5.63 and

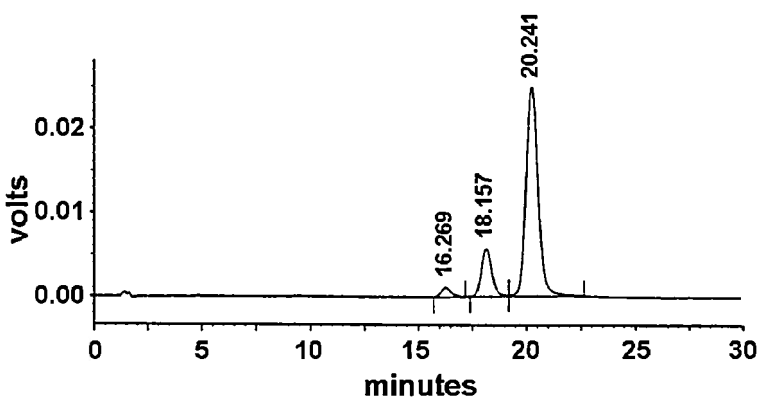


Fig. 2: A typical HPLC chromatogram of the curcuminoids. Curcumin, desmethoxycurcumin and bisdesmethoxycurcumin were eluted at 16.2, 18.1, and 20.2 minutes, respectively

Table 1: Entrapment efficiency and recovery of curcuminoids in niosome formulations

Substance	Amount of curcuminoids (μmoles)				% Entrapment efficiency (E/L) * 100	% Recovery (N + E)/L) * 100
	Loaded [a] (L)	Non-entrapped (N)	Entrapped (E)	Total (N + E)		
Curcumin						
L1 [b]	4.29	2.22	1.30	3.53	30.30	82.28
L2 [b]	8.60	0.41	7.71	8.12	89.65	94.42
Desmethoxycurcumin						
L1	1.18	0.59	0.30	0.89	25.42	75.42
L2	2.39	0.47	1.62	2.10	66.78	87.87
Bisdesmethoxycurcumin						
L1	0.16	0.06	0.03	0.09	18.75	56.25
L2	0.32	0.13	0.10	0.23	31.25	71.88
Total curcuminoids [c]						
L1	5.63	2.17	1.63	4.51	28.95	80.11
L2	11.31	1.01	9.43	10.45	83.38	92.40

[a] Percentages of curcumin, desmethoxycurcumin and bisdesmethoxycurcumin in the curcuminoids are 79.24%, 20.16%, and 2.57%, respectively
[b] L1 indicates loading at 5.63 μmoles of curcuminoids and L2 indicates loading at 11.31 μmoles of curcuminoids
[c] Total = summation of amounts for all compounds

11.31 μmol (Table 1). The % entrapment efficiency increased from 28.95 to 83.38% and the % recovery increased from 80.11 to 92.40% with an increase in the loading amount from 5.63 to 11.31 μmol. About 90% of the Span 80 niosomes loaded with 11.31 μmol of curcuminoids had an average diameter (± SD) of 12.25 ± 5.00 μm. A small number of niosomes (about 10% of the sample) were much smaller, with an average diameter of 0.22 ± 2.33 μm.

Curcuminoids are hydrophobic substances and are likely to be entrapped in the bilayer of the niosomes. The % entrapment efficiency increased with curcuminoid amounts up to 11.31 μmol, with precipitation of curcuminoids observed at higher loading amounts. This supports the hypothesis that curcuminoids are entrapped in the bilayer. As the curcuminoid loading amount is increased, the bilayer is filled and saturated with curcuminoids. The poor aqueous solubility of the excess curcuminoids then results in curcuminoid precipitation. The % entrapment varied among the three curcuminoids, with the highest value obtained for curcumin, followed by desmethoxycurcumin, and then bisdesmethoxycurcumin.

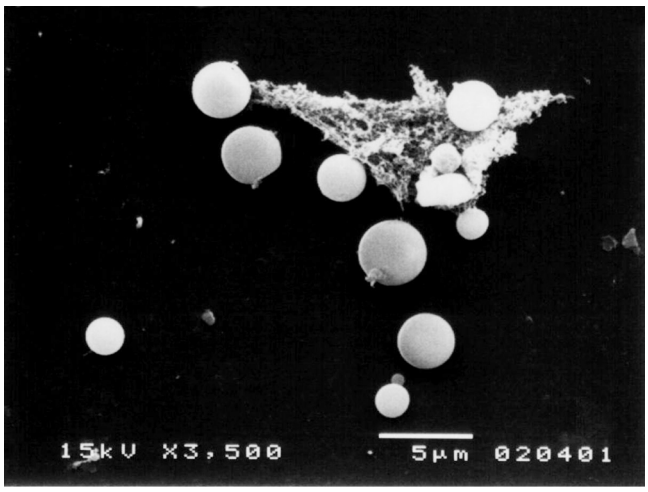
2.4. In Vitro skin permeation study

Shed snake skin consists of three layers: a beta-keratin-rich outer layer, an alpha-keratin layer, and a lipid-rich inner alpha layer (Itoh et al. 1990). The structure and composition of the alpha-keratin layer is similar to that of the human stratum corneum, making shed snake skin a useful model membrane for permeation studies. Furthermore, shed snake skin is more uniform in terms of thickness, composition and structure compared to other animal skins, and also lacks hair follicles; therefore, errors introduced by permeation via the transfollicular route are eliminated. Curcuminoids are insoluble in water, which results in very low skin permeation of curcuminoids from a suspension. Ethanol was also unable to dissolve the curcuminoids completely at the concentration used in this study. Methanol was thus used to dissolve the curcuminoids in the control experiment. To establish the release profiles of hydrophobic drugs from sustained release formulations, the receptor medium typically contains an alcohol, which makes the drug highly soluble in the medium. Examples include 50% methanol (Parikh et al. 1993), 27.5% ethanol (Beck et al. 1980), and 25% *n*-propanol (Yang and Owusu-Ababio 2006). In this study, a 50% methanolic solution was used as the receptor medium to ensure pseudo-sink conditions and prevent precipitation of curcuminoids.

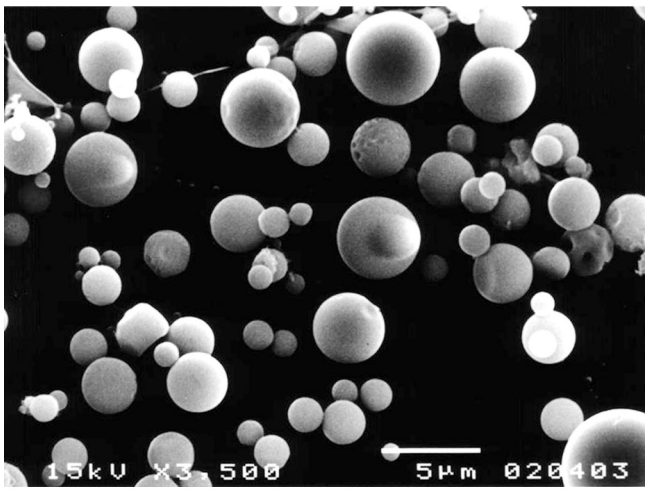
Using 11.31-μmol curcuminoid Span 80 niosomes, the amount of curcuminoids permeating through shed snake skin increased with time, and about 4% of curcuminoids entrapped in niosomes crossed the membrane over 72 h (Fig. 4). In contrast, permeation from a methanolic solution of 11.28 μmol of curcuminoids was undetectable over 72 h. In the preliminary permeation studies, the amounts of the three curcuminoids that permeated through the membrane were undetectable from 0 to 24 h (data not shown). Therefore, the time point at 48 h was designated as the first sampling time in this study. The relatively low permeability of curcuminoids from the solution and the niosomes is probably due to hydrophobicity of the compounds. Curcuminoids may be entrapped in the shed snake skin membrane and the hydrophobicity may cause curcuminoid accumulation in the inner lipid-rich alpha layer, rather than diffusion across the membrane (Itoh 1990).

For the curcuminoid niosomes, the cumulative amount of curcuminoids that diffused across the membrane increased linearly with time, and plots of these data were used to determine the flux and lag time for each curcuminoid from the slope and x-intercept, respectively (Fig. 4). The fluxes of curcumin, desmethoxycurcumin and bisdesmethoxycurcumin were calculated to be 1.117 ± 0.008, 0.263 ± 0.003, and 0.057 ± 0.001 μg/(cm^{2}h), and the lag times were 31.7 ± 0.5, 30.5 ± 0.5, and 37.0 ± 0.2 h, respectively. A fixed-effects one-way ANOVA followed by a Tukey test showed that the fluxes differed significantly among the three curcuminoids ($P < 0.05$). A similar analysis indicated that the lag times of the three compounds were also significantly different from each other ($P < 0.05$). A similar trend was seen for the % entrapment efficiency, as discussed in the previous section. These trends are consistent with the number of methoxy groups (2, 1 and 0, respectively) in the compounds, and this suggests that the relative hydrophobicity of the molecules controls both the % entrapment efficiency and the permeability.

Our data show that curcuminoids can be successfully formulated as niosomes with excellent % entrapment efficiency. The niosomes also significantly enhanced skin permeation of curcuminoids compared with a methanolic solution of curcuminoids. We conclude that the niosomes formulated in this work have improved properties and provide a new approach for transdermal delivery of curcuminoids, and optimization of the formulation may further improve the physical and permeability characteristics of the niosomes.



(A)



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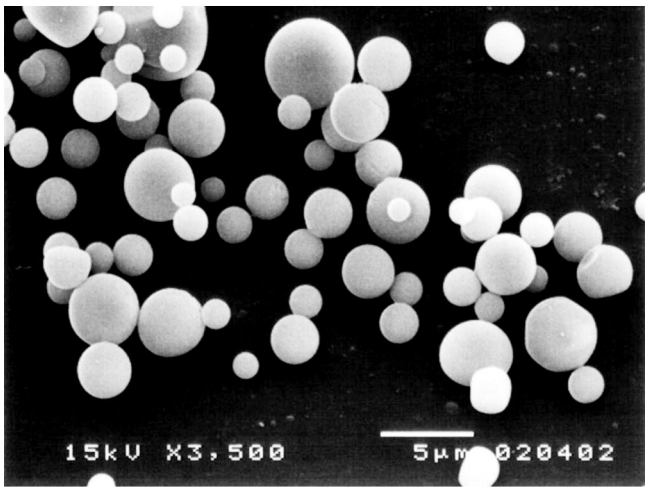

(C)

Fig. 3: Scanning electron micrographs of curcuminoid niosomes prepared using Span 40 (a), Span 60 (b), and Span 80 (c) as non-ionic surfactants

3. Experimental

3.1. Materials

Sorbitan monopalmitate (Span 40), sorbitan monostearate (Span 60), and sorbitan monooleate (Span 80) were obtained from the East Asiatic Public Co. (Bangkok, Thailand). Cholesterol was purchased from Sigma (St. Louis, MO, USA). Solulan C-24 was obtained from Amerchol Corp. (Edison, NJ, USA). Methanol, ethanol, acetonitrile, and acetic acid were obtained from Lab-Scan (Bangkok, Thailand) and were of either HPLC or analytical grade. Commercially available curcuminoids consisting of 76.24%w/w curcumin, 20.92%w/w desmethoxycurcumin and 2.84%w/w bisdesmethoxycurcumin was purchased from Thai-China Flavours and Fragrances Co. (Nonthaburi, Thailand) and used as received.

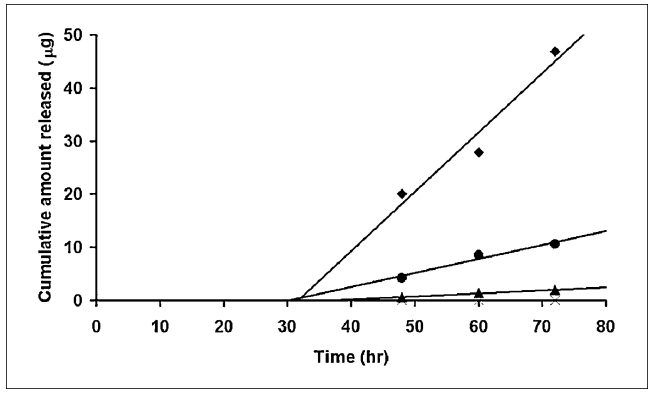

Fig. 4: Time courses of penetration of curcuminoids through shed snake skin. Lines represent linear regression equations used to estimate fluxes and lag times for curcumin (♦), desmethoxycurcumin (●), and bisdesmethoxycurcumin (▲) in niosomes and for a control methanolic solution (×). Data are averages from four independent experiments

3.2. HPLC instrument and conditions

The HPLC system (Shimadzu) consisted of a degasser (DGU-14-A), pump (LC-10AT VP), system controller (SCL-10A VP), UV detector (SPD-10A VP), autoinjector (SIL-10AD VP), and software (Class VP Version 4.20). Concentrations of curcumin, desmethoxycurcumin and bisdesmethoxycurcumin were determined using a previously reported reverse-phase HPLC method with some modifications (Wichitnithad et al. 2009). Chromatography was performed using a HiQ-sil C18 column (4.6 mm × 150 mm, i.d., 5 µm) with isocratic elution of acetonitrile and 2% v/v acetic acid (40:60, v/v) at a flow rate of 1.5 ml/min. UV detection was set at 425 nm. The injection volume was 20 µL and the run time was 30 min.

3.3. Validation of the HPLC analytical method

The HPLC analytical method for curcuminoids was validated in compliance with the International Conference on Harmonization (ICH) guidelines (ICH-Q2 (R1) 2005). The specificity of the method was determined by analyzing a sample containing curcuminoids in a methanolic mixture of excipients (surfactant, cholesterol, and Solulan C-24) to determine if the curcuminoids co-eluted with the excipients. By injecting a series of dilute solutions of known concentrations, LOD and LOQ for curcuminoids were calculated from the Y-intercept and the standard deviation of the blank. Precision was evaluated by carrying out six injections of a curcuminoid solution and calculating the % RSD among the assays. Linearity was determined using six concentrations of curcuminoids, with the peak areas for each concentration subjected to least-squares linear regression analysis. Accuracy was evaluated in triplicate for each of the six concentrations. The % recoveries were estimated by dividing the predicted concentration (obtained from the calibration curve) by the theoretical concentration and multiplying by 100.

3.4. Preparation of curcuminoid niosomes

Curcuminoid niosomes were prepared by the thin film hydration method. A solution of curcuminoids in methanol was added to a mixture of surfactant (Span 40, Span 60, or Span 80), cholesterol, and Solulan C-24 at a mole ratio of 47.5:47.5:5 (Table 2). The final volume was adjusted to 20 mL with methanol. The mixture was transferred to a 100-mL round bottom flask and evaporated using a rotary evaporator at 60 °C until a thin film was formed. The dried lipid film was subsequently hydrated with 5 mL of distilled water and the mixture was shaken in a water bath at 80 °C for 1 h until niosome vesicles were formed. The curcuminoid niosome suspension was centrifuged at 50000 rpm at 4 °C for 30 min, after which the supernatant was decanted and the niosomes were collected for further characterization.

3.5. Formation and morphology of curcuminoid niosomes

Niosome vesicle formation was confirmed using an optical microscope. The morphology of curcuminoid-loaded niosomes was then determined using scanning electron microscopy (SEM, model JSM-5410LV, JEOL, Japan). The curcuminoid niosomes were resuspended in water and deposited onto a glass substrate and fixed with 1% osmium tetroxide vapor. The specimens were then dehydrated with ethanol and dried with a critical-point

Table 2: Composition of curcuminoid niosome formulations containing surfactant (Span 40, Span 60, or Span 80), cholesterol, and Solulan C-24 at a mole ratio of 47.5:47.5:5. The total lipid content was 150 μmoles in each formulation

Formulation	Surfactant	Curcuminoids (μmol)
1	Span 40	2.82
2	Span 60	2.82
3	Span 80	2.82
4	Span 80	5.63
5	Span 80	11.31
6	Span 80	16.92
7	Span 80	22.56
8	Span 80	28.20
9	Span 80	33.84

dryer before being coated with gold. The scanning electron micrographs were taken at 15.0 kV. The effects of different non-ionic surfactants on the formation and morphology of the curcuminoid niosomes were investigated.

3.6. Vesicle size and size distribution analysis

The vesicle size and size distribution of the curcuminoid niosomes were evaluated within 72 h after preparation by a laser light scattering method using a particle size analyzer (model Mastersizer S, Malvern Instruments, UK). The measurement was carried out using a reverse Fourier lens (range lens of 300RF: 0.05–880 μm) and a helium-neon laser as the transmitter. The vesicle size and size distribution were calculated using Mastersizer S software ver. 2.19.

3.7. Determination of entrapment efficiency

The ability of the niosome vesicles to entrap curcuminoids was examined by HPLC. The % entrapment efficiency was expressed as a percentage of the amount of curcuminoids added initially. The amounts of non-entrapped (N) and entrapped (E) curcuminoids were determined in the supernatant and niosome vesicles, respectively. The curcuminoid niosomes were lyzed with methanol prior to HPLC analysis. The % entrapment efficiency and % recovery were calculated using the following equations.

$$\%\text{entrapment efficiency} = \frac{E}{L} \times 100 \quad (1)$$

$$\%\text{recovery} = \frac{N + E}{L} \times 100 \quad (2)$$

where L is the total amount of curcuminoids added (the loading amount).

3.8. In Vitro skin permeation study

In vitro permeation of curcuminoids was studied using Franz cell diffusion. Cobra shed snake skin (*Naja Naja Khoatia*) was mounted on Franz cells with the dorsal region of the skin facing upwards and in contact with 2 mL of a curcuminoid niosome suspension containing 0.8 mg of curcuminoids/mL. Each Franz cell had a diffusion area of 2.01 cm^2 and was equipped with a magnetic stirrer bar. The receptor compartments were filled with 10 mL of 50% methanol and the temperature of the receptor fluid was kept at 32 °C by circulating water through a double-vessel jacket surrounding each receptor compartment. After 48, 60 and 72 h, 2-mL samples were withdrawn from the receptor compartment and immediately replaced with fresh receptor fluid. The withdrawn samples were assayed by HPLC to determine the concentrations of curcumin, desmethoxycurcumin and bisdesmethoxycurcumin that had permeated through the shed snake skin. The permeation data were plotted as the cumulative amount of curcuminoids that penetrated the membrane over time, and the curcuminoid flux was calculated. Permeation was determined in four separate experiments for selected formulations. Permeation of 2 mL of curcuminoids in methanol (0.8 mg/mL) was performed as a control. Statistical analysis of the permeation data was performed using SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA). A fixed-effects one-way ANOVA followed by a Tukey test (post hoc multiple comparison) was carried out to compare the means of flux and lag time of the three curcuminoids. A confidence level of $p < 0.05$ was considered to indicate a significant difference.

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