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Preparation and characterization of cosmeceutical liposomes loaded with avobenzone and arbutin

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Synopsis

The objective of this study was to develop and characterize a liposome delivery system coencapsulating two cosmeceutical ingredients, avobenzone (AVO) and arbutin (AR). Two different liposome preparation methods, that is, thin film hydration and reverse-phase evaporation, were evaluated. To obtain the optimal formulation, various ratios of lipid to AVO or AR were tested. The effects of liposome formulation and preparation method on particle size, entrapment efficiency (EE), and skin permeation rate were studied. The mean particle size of the liposome formulations obtained by the thin film hydration method was smaller than that obtained by the reverse-phase evaporation method. The EE of AR and AVO in liposomes prepared by the thin film method, however, was lower than that prepared by the reverse-phase evaporation method. No differences in membrane permeation were observed between the two preparation methods. A large portion of AR permeated through the membrane into the receptor chamber. On the other hand, AVO remained in the donor chamber or accumulated in the membrane. The results of this study revealed that liposomes are a promising delivery system for coencapsulated AR and AVO. Liposomes may aid in retaining the sunscreen (AVO) at the surface of the skin for sun protection meanwhile facilitating the penetration of the whitening agent (AR) into the deeper layers of the skin for whitening effect.

INTRODUCTION

Ultraviolet radiation (UVR) emitted by sun can damage collagen fibers and accelerate skin aging and is a major cause of skin cancer. To prevent skin damage by sunlight, physical coverage, such as clothing and sunglasses, is the most effective way to prevent sunburn and skin cancer caused by UVR. Applying sunscreens is also helpful in protecting the skin. An efficient sunscreen product, however, should provide effective broad-spectrum protection across the UVR range (280–400 nm). An example of a highly effective sunscreen ingredient with broad-spectrum protection is butyl methoxydibenzoylmethane or avobenzone

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(AVO) (trade name Parsol 1789), which is one of the most frequently used ingredients in sunscreens products. Sunscreens have also been used along with whitening agents to maintain light skin tone. Whitening agents protect the skin against the negative effects of UVR including skin blemishes and brown spots. Arbutin (AR), a potent tyrosinase inhibitor and an antioxidant (1), has been widely used as a skin-whitening agent. It can effectively restrain the activity of tyrosinase and the formation of melanin in the skin (2,3).

Although AVO and AR are widely used as sunscreen and whitening agents, respectively, their physicochemical properties limit their effectiveness, especially when used concurrently. AVO, for example, undergoes photodegradation under sunlight illumination and therefore loses part of its protection capacity during usage (4,5). AR, on the other hand, is very hydrophilic and therefore has limited permeability across the outermost epidermis through the hydrophobic stratum corneum into the deeper layers of the skin (6).

Several strategies have been reported to address some of these limitations. The addition of photostabilizing agents, complexation with cyclodextrins, and encapsulation in polymeric or lipid microparticles were used to enhance the stability of AVO (7,8). Studies have shown that inclusion of a carrier in sunscreen formulations may enhance photoprotection by reducing both skin penetration and photodecomposition of UV absorbers (9). Carriers such as liposomes have also been used to facilitate the penetration of hydrophilic compounds such as AR through the skin. Liposomes are spherical, bilayered structures composed mainly of phospholipids and have been widely used in drug delivery and cosmetic applications (10). Liposomal formulations emerged as attractive alternatives for the topical delivery of drugs and cosmetic ingredients not only because of their biocompatibility, nontoxicity, and suitability for encapsulation of both hydrophilic and lipophilic compounds but also because of their ability to enhance skin penetration. The objective of this study was, therefore, to address the physical properties of AVO and AR that limits their use in cosmetic applications by coencapsulating the two ingredients into liposomal formulations. Due to their differences in lipophilicity, two liposome preparation methods, that is, thin film hydration and reverse-phase evaporation, were used to coencapsulate AVO and AR. The physical properties of liposomes prepared by each method were compared, and their *in vitro* permeation was evaluated using the Franz diffusion cells.

MATERIAL AND METHODS

MATERIALS

AR was purchased from Alfa Aesar (Ward Hill, MA), AVO was purchased from Merck KGaA (Darmstadt, Germany), and L-α-phosphatidylcholine (EPC) was purchased from Avanti (Alabaster, AL). Sephadex cartridges prepacked with G-50 gel were obtained from Pharmacia Biotech (Uppsala, Sweden). Cellulose acetate membranes (pore diameter 2 μm) were purchased from Millipore (Bedford, MA).

SPECTRAL ANALYSIS OF AR AND AVO

The absorption spectra of AR and AVO alone or in combination were carried out in 95% ethanol or methanol. Absorption spectra were measured using Hitachi U-3300

spectrophotometer (Hitachi, Japan). Calibration curves using 95% ethanol or methanol as the solvent were subsequently used for quantifying AR and AVO in the liposomes and the compartments of the *in vitro* permeation assembly, respectively.

PREPARATION OF LIPOSOMES

AR and AVO were encapsulated in the liposomes by the thin film hydration and/or reversed phase evaporation method. The initial step for either method is similar and involves the formation of layered lipid film.

Thin film hydration method. (a) Lipo-AVO: 10–20 μmol EPC was first dissolved in 100–200 μl chloroform containing 125 μg/ml AVO. After evaporating chloroform, the mixture formed a thin film at the bottom of the test tube. The film was then hydrated in 1 ml of 0.9% NaCl by vortexing the dispersion at 37°C for 3 min. (b) Lipo-AR-AVO: 10–20 μmol EPC was first dissolved in 100–200 μl chloroform containing 125 μg/ml AVO. After evaporating chloroform, the mixture formed a thin film at the bottom of the test tube. The film was then hydrated in 1 ml of AR stock solution in 0.9% NaCl by vortexing the dispersion at 37°C for 3 min. (c) Lipo-AR: 10–20 μmol EPC was first dissolved in 100–200 μl chloroform. After evaporating chloroform, the mixture formed a thin film at the bottom of the test tube. The film was then hydrated in 1 ml of AR stock solution in 0.9% NaCl by vortexing the dispersion at 37°C for 3 min.

Reverse-phase evaporation method. (a) Lipo-AVO: 10–20 μmol EPC was first dissolved in 100–200 μl chloroform containing 125 μg/ml AVO. After evaporating chloroform, the mixture formed a thin film at the bottom of the test tube. The dry lipid film was then redissolved in 1 ml of diethyl ether to which 1 ml of 0.9% NaCl solution was added. A stable emulsion was created by vortexing the mixture for 5 min at 37°C. The liposome solution was subsequently formed by slowly removing diethyl ether using a rotary evaporator. (b) Lipo-AR-AVO: 10–20 μmol EPC was first dissolved in 100–200 μl chloroform containing 125 μg/ml AVO. After evaporating chloroform, the mixture formed a thin film at the bottom of the test tube. The dry lipid film was then redissolved in 1 ml of diethyl ether to which 1 ml of AR stock solution in 0.9% NaCl solution was added. A stable emulsion was created by vortexing the mixture for 5 min at 37°C. The liposome solution was subsequently formed by slowly removing diethyl ether using a rotary evaporator. (c) Lipo-AR: 10–20 μmol EPC was first dissolved in 100–200 μl chloroform. After evaporating chloroform, the mixture formed a thin film at the bottom of the test tube. The dry lipid film was then redissolved in 1 ml of diethyl ether to which 1 ml of AR stock solution in 0.9% NaCl solution was added. A stable emulsion was created by vortexing the mixture for 5 min at 37°C. The liposome solution was subsequently formed by slowly removing diethyl ether using a rotary evaporator.

For all liposome preparations, untrapped AR and AVO were removed by size-exclusion chromatography using Sephadex G-50 column. The final liposome preparations were stored at 4°C, unless otherwise specified.

CHARACTERIZATION OF LIPOSOMES

Vesicle size and distribution was measured by dynamic light scattering using Coulter N4 Plus submicron particle size analyzer (Beckman Coulter, Irvine, CA). The amount of AR

and/or AVO encapsulated in the liposomes was determined by monitoring the absorbance of AR and AVO after disrupting the liposomes with 95% ethanol. Reference standards (free AVO and AR) were measured using the same processing conditions. The final concentration of liposomes was determined using the phosphate assay (11), which was subsequently used to calculate the EE(%) as follows:

$$\mathrm{EE}(\%) = \frac{P_f / L_f}{P_i / L_i} \times 100$$

where EE is the entrapment efficiency, L_i and L_f are the initial and recovery concentrations of phosphate, respectively, and P_i and P_f are the initial and recovery concentrations of drugs (AR or AVO, measured in micrograms), respectively.

IN VITRO PERMEATION STUDY

Franz diffusion cells were used to evaluate the *in vitro* permeability of AVO and AR across a cellulose acetate membrane with an average pore size of 2.0 µm. The donor chamber was loaded with 500 µl of the liposome formulations, whereas the receptor chamber contained 0.9% NaCl solution. The receptor chamber was stirred using a magnetic bar at 500 rpm and maintained at 37 ± 0.5°C using a circulating water bath system. At predetermined time intervals, 600 µl samples were withdrawn from the receptor chamber, diluted with fresh media, and analyzed spectrophotometrically for AVO and AR.

RESULTS AND DISCUSSION

SPECTRAL ANALYSIS OF AR AND AVO

The absorption spectra of various concentrations of AR and AVO solutions are shown in Figure 1. Maximal absorption was found to be at 285 and 358 nm for AR and AVO, respectively. A linear correlation between absorbance and concentration was obtained with an r^2 = 0.9998 and 0.9995 for AR and AVO, respectively. To identify potential interferences between AVO and AR after their coencapsulation in the liposomes, the absorption spectra of various concentrations of AR in the presence of AVO and EPC were evaluated (Figure 1C). Although a general increase in AR absorbance was observed in the presence of AVO and EPC, a linear relationship between the concentration and absorbance was maintained and used for the quantification of AR.

CHARACTERIZATION OF THE FORMULATIONS

The entrapment and EE of AR and AVO in the liposomes that were prepared by either the thin film hydration or the reverse-phase evaporation methods are given in Table I. In the thin film hydration method, increasing the concentration of EPC in the liposomes resulted in a decrease in the entrapment of AR and AVO. The highest EE of AR and AVO were only 1.0%

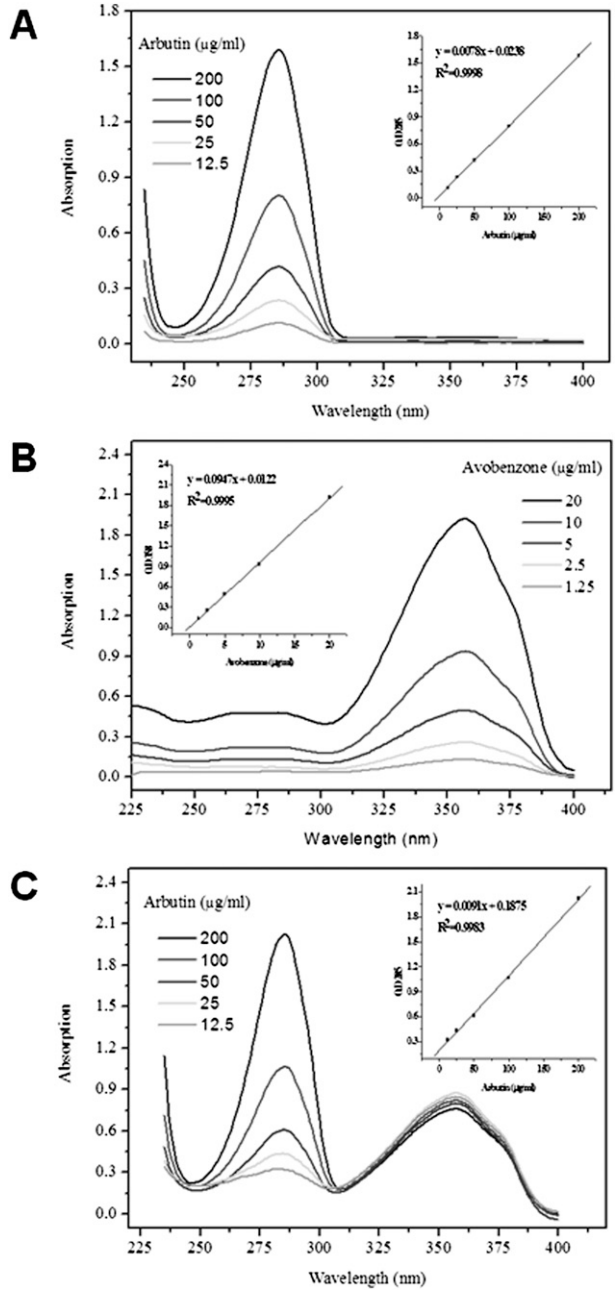

Figure 1. (A) Absorption spectra of AR in 95% ethanol with AR concentrations ranging from 12.5 to 200 μg/ml. The linear relationship between the absorbance at 285 nm versus concentration is shown in the inset. (B) Absorption spectra of AVO in 95% ethanol, with AVO concentrations ranged from 1.25 to 20 μg/ml. The linear relationship between the absorbance at 358 nm versus concentration is shown in the inset. (C) Absorption spectra of AR in 95% ethanol in the presence of AVO and EPC. Here, 7.5 μg/ml AVO and 0.5 μmol EPC were added to the AR solutions. AR concentrations ranged from 12.5 to 200 μg/ml. The linear relationship between the absorbance at 285 nm versus concentration is shown in the inset.

Table I
Properties of the Liposomes Coencapsulating AR and AVO

	Initial loading			AR		AVO			
Method	EPC (μmol)	AR (μg)	AVO (μg)	Entrapment (μg/μmol)	EE (%)	Entrapment (μg/μmol)	EE (%)	Size (nm)	PI
Thin film	10	40,000	125	26.34 ± 12.65	0.65 ± 0.32	7.47 ± 0.73	59.72 ± 5.82	148.5 ± 6.7	0.62 ± 0.08
	20	40,000	125	20.61 ± 1.52	1.03 ± 0.08	3.29 ± 0.14	52.68 ± 2.29	124.4 ± 12.0	0.55 ± 0.07
Reverse-phase evaporation	10	40,000	125	66.52 ± 21.60	1.66 ± 0.54	7.90 ± 2.85	76.39 ± 1.31	235.1 ± 33.3	0.47 ± 0.19
	20	40,000	125	143.14 ± 27.76	7.16 ± 1.39	4.26 ± 1.09	68.16 ± 17.50	198.9 ± 33.5	0.43 ± 0.17

Data represent mean ± standard deviation (N = 3).

and 59.7%, respectively. It was difficult to obtain high EE for AR, a highly hydrophilic compound, by simply using the thin film hydration method for liposome preparation. It is well known that liposomes prepared by the thin film hydration method generate multilamellar vesicles (MLVs). In MLVs, most of the lipids participate in forming the internal lamellae with tight lipid bilayers, which limits their internal aqueous space. Although applying sonication and high-pressure extrusion would yield single unilamellar vesicles with larger encapsulation volume, these techniques, nonetheless, will result in low aqueous space to lipid ratio and thereby low EE for hydrophilic drugs in liposomes (12). In contrast, reverse-phase evaporation method has unique advantages for encapsulating water-soluble materials such as proteins, nucleic acids, and other biochemical reagents (12). With reverse-phase evaporation method, the organic solvent is simply removed from the inverted micelles resulting in vesicles with larger aqueous space to lipid ratio and consequently higher EE.

The EE of AR and AVO increased to 7.2% and 76.4%, respectively, in liposomes made by the reverse-phase evaporation method. Entrapment was depended on EPC whereby the entrapment of AR increased by up to 2.2-folds when the concentration of EPC increased to 20 µmol. These results indicated that the reverse-phase evaporation method is more efficient for preparing liposomes co-encapsulating AR and AVO with high EE. The particle size of the liposomes prepared by either the thin film hydration or the reverse-phase evaporation methods is given in Table I. Data show that the particle size of liposomes was in the range of 150–400 nm.

The stability of the coencapsulated AR and AVO liposomes was evaluated by monitoring the change in particle size and polydispersity index (PI) of liposomes stored for up to 30 days at 4°C and controlled room temperature. The average sizes of the liposomes increased within 1 day and remained relatively constant throughout the 30 days of storage at either 4°C or 25°C (Table II). Upon visual inspection, no signs of drug precipitation were observed. Furthermore, in preliminary studies, it was seen that expulsion of drugs from the liposomes would result in significant change in the size of the particles. Therefore, results shown in Table II indirectly indicate that the liposomes were stable throughout the storage period. Nonetheless, additional studies are planned to confirm the entrapment of AR and AVO within the liposome.

IN VITRO PERMEATION STUDY

In this study, the permeation of individual and coencapsulated AR and AVO liposomes made by the reverse-phase evaporation method was evaluated by measuring the

Table II
Effect of Storage Temperature and Time on the Particle Size Distribution of Lipo-AR-AVO

Storage condition duration (day)	4°C		25°C	
	Size (nm)	PI	Size (nm)	PI
1	228.6 ± 17.1	0.37 ± 0.09	231.9 ± 25.4	0.40 ± 0.12
3	226.2 ± 8.6	0.41 ± 0.09	233.8 ± 13.2	0.40 ± 0.08
10	229.6 ± 16.0	0.44 ± 0.11	225.0 ± 12.3	0.36 ± 0.09
30	229.2 ± 10.7	0.55 ± 0.10	212.8 ± 13.5	0.40 ± 0.13

Data represent mean ± standard deviation (N = 3).

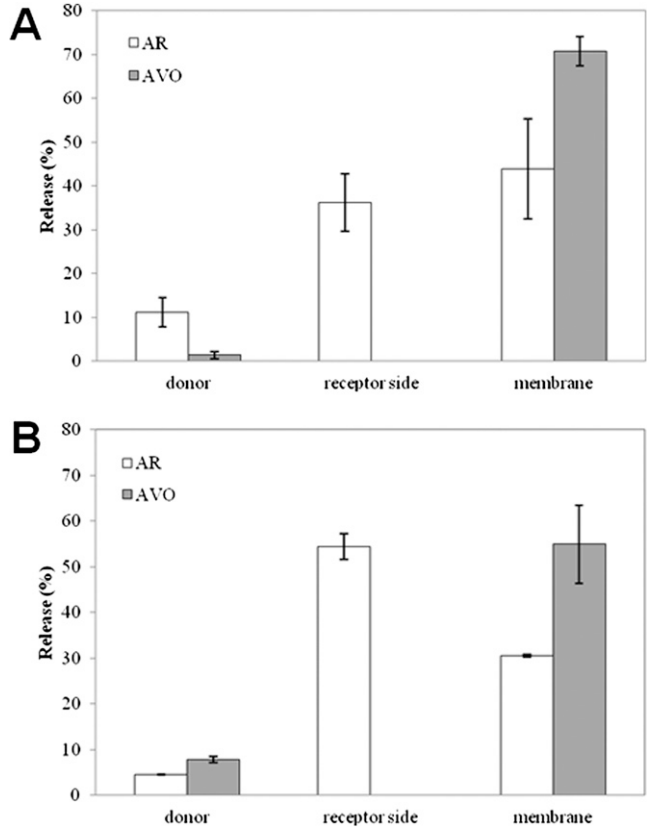

Figure 2. Results from the *in vitro* permeability study showing (A) the distribution of AR and AVO in the donor side, receptor side, and the membrane when administered individually as lipo-AR and lipo-AVO (N = 3), and (B) the distribution of AR and AVO in the donor side, receptor side, and the membrane when administered coencapsulated within a liposomal formulation (lipo-AR-AVO, N = 3).

concentration of the compounds that have permeated through the cellulose acetate membrane after 6 h of treatment. The distribution of individual AR and AVO in the donor and receptor compartments and their accumulation on the membrane is shown in Figure 2A. Only approximately 15% of AR was left in the donor side. Nonetheless, the percentage of AR retained by the membrane was similar to the percentage of AR that permeated into the receptor compartment. A significantly larger percentage of AR permeated into the receptor compartment when AR was formulated into lipo-AR-AVO (Figure 2B). With regard to AVO, insignificant amount of the compound remained in the donor side, and no AVO was detected in the receptor side. The majority of AVO, either in lipo-AVO or in lipo-AR-AVO formulations, was retained by the membrane. The fact that lipo-AR-AVO resulted in the accumulation of AVO and approximately 30–40% of AR on the membrane while allowing the remainder of AR to permeate through the membrane demonstrated the potential benefits of liposomes in the topical delivery of AVO and AR. The accumulation of the compounds on the membrane mimics their accumulation on stratum corneum. In topical applications, it is ideal to prevent the permeation of chemical sunscreens through the stratum corneum into the dermis layer for subsequent systemic circulation. The retention of some AR on the membrane could also help stabilize the

sunscreen (AVO). The majority of AR in the lipo-AR-AVO, however, permeated through the membrane, which is essential for it to exhibit skin whitening effects after topical application. These results indicated that coencapsulated AR and AVO liposomes are a promising delivery system that could help retain the sunscreen on the surface of the skin for sun protection meanwhile aiding in the penetration of the whitening agent into the deeper layers of the skin for whitening effect.

CONCLUSIONS

Liposomal formulation of AR prepared by the reverse-phase evaporation method can effectively enhance the entrapment of this highly hydrophilic compound in the hydrophilic core of the liposome. It may also increase the entrapment of other coencapsulated hydrophobic compounds such as AVO into the lipid bilayer. Besides, coencapsulating AR and AVO has potential cosmetic applications in enhancing the stability and efficacy of the compounds by retaining AVO at the surface meanwhile enhancing the penetration of AR into the deeper layers of the skin. This study provided a proof of concept that liposomal delivery system is a promising technical platform for coencapsulating cosmeceutical agents.

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