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The entrapment of kojic oleate in bilayer vesicles

A. Manosroi [a,b,*], P. Wongtrakul [c], J. Manosroi [a,b], U. Midorikawa [d], Y. Hanyu [d], M. Yuasa [d,e], F. Sugawara [d], H. Sakai [d,e], M. Abe [d,e]

[a] *Pharmaceutical Cosmetic Raw Materials and Natural Products Research and Development Center (PCRNC), Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand*
[b] *Institute for Science and Technology Research and Development, Chiang Mai University, Chiang Mai, Thailand*
[c] *Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand*
[d] *Faculty of Science and Technology, Tokyo University of Science, Noda, Chiba, Japan*
[e] *Institute of Colloid and Interface Science, Tokyo University of Science, Tokyo, Japan*

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Abstract

The entrapment of kojic acid and its newly synthesized ester (kojic oleate) has been evaluated. Kojic oleate was synthesized by DCC (*N*,*N*′-dicyclohexylcarbodiimide, DCC)/(4-(*N*,*N*-dimethylamino)pyridine, DMAP) esterification method and identified by FAB–MS and ^{1}H NMR. The synthesized product was mainly 7-*O*-kojic oleate with more than 80% yield. It was entrapped in vesicular membrane prepared from 9.5:9.5:1.0 molar ratio of amphiphiles (Span 60, Tween 61 or DPPC), cholesterol and dicetyl phosphate. Kojic acid was encapsulated in the water compartment of these vesicles in order to confirm the vesicle formation. The morphology and particle size of the vesicles were characterized by an optical microscope and transmission electron microscope (TEM). The entrapment efficiencies of kojic acid and kojic oleate in the vesicles were investigated by dialysis and column chromatography, respectively. The contents of the entrapped kojic acid and kojic oleate were assayed by HPLC. The entrapment efficiency of kojic acid was 0.01–0.04 mol, whereas kojic oleate gave higher entrapment efficiency of 0.25–0.35 mol/mol of the total compositions of amphiphile/cholesterol/dicetyl phosphate. Structural modification of kojic acid improved its entrapment in the vesicles. Tween 61 vesicles could entrap kojic oleate more than did Span 60 vesicles. The π–*A* isotherms revealed the lower area per molecule of Span 60, which formed a more rigid pack of its molecule on air/water interface than that of Tween 61. This implied the high rigidity of vesicular membrane prepared with Span 60 led to the lower amount of kojic oleate entrapped in the vesicles. From the release study of kojic acid through the dialysis membrane, it indicated that the intercalation of kojic oleate in the vesicular membranes did not significantly affect the release of kojic acid from the vesicles.

Keywords: Kojic acid; Kojic oleate; Tween 61; Span 60; DPPC; Bilayer vesicles

1. Introduction

Vesicles prepared with phospholipids (liposomes) as well as a variety of non-ionic surfactants (niosomes)

* Corresponding author. Tel.: +66 53 944338/894806; fax: +66 53 894169.
E-mail address: pmpti005@chiangmai.ac.th (A. Manosroi).

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have been extensively studied as drug carriers, including their applications to topical delivery (Yoshioka et al., 1994; Van Hal et al., 1996a; Uchegbu and Vyas, 1998; Hao et al., 2002). They have been reported to increase drug stability, enhance therapeutic effects, prolong circulation time and promote uptake of the entrapped drugs into target site while drug toxicity is diminished (Gabizon et al., 1998; Bandak et al., 1999; Tsuchihashi et al., 1999). The development of topical formulations of the bilayer vesicles aims to improve the delivery of the applied drug through the skin (Junginger et al., 1991). Many drugs such as estradiol (Fang et al., 2001), tretinoin (Montenegro et al., 1996; Manconi et al., 2002), dithranol (Agarwal et al., 2001), lorazepam (Nokhodchi et al., 2003), ketoprofen (Wu et al., 2001), lidocaine (Van Hal et al., 1996b), enoxacin (Fang et al., 2001), tranexamic (Manosroi et al., 2002) and amphotericin B (Manosroi et al., 2004) have been successfully encapsulated in liposomes or niosomes for topical application. The vesicles were reported to serve as a solubilization matrix as local depot for sustained release, permeation enhancers of dermally active compounds or as a rate-limiting membrane barrier for the modulation of systemic absorption of drugs via the skin (Touitou et al., 1994; Schreier and Bouwstra, 1994; Van der Bergh et al., 1998). In aqueous solution, phospholipids or non-ionic surfactants are arranged in bilayer structure. The non-polar side chains are located in the membrane's interior and the polar heads are exposed to water. The vesicles can carry both hydrophilic drugs by encapsulation in water phase and hydrophobic drugs by intercalation into hydrophobic domains.

Kojic acid, 5-hydroxy-2-hydroxymethyl-4H-pyran-4-one, was first isolated by Yabuta in 1924. Its chemical structure was determined by Takahashi et al. (Kobayashi et al., 1995). It has been widely used in topical preparations because of its inhibition to melanin synthesis. The tyrosinase enzyme activity is diminished by the removal of its associated copper ion by chelation between the ketone group at position 4 and the hydroxyl group at position 5 of kojic acid (Cabanes et al., 1994). Genotoxicity studies of kojic acid on mouse bone marrow micronucleus tests and an in vivo/in vitro unscheduled DNA synthesis (UDS) tests were negative. The systemic exposure of 1% kojic acid at a concentration of $2.00 \pm 0.16\ mg/cm^2$ in human was estimated to be in the range of 0.03–0.06 mg/kg/day; which was 16,000–26,000-fold lower than the doses that was negative for micronuclei, UDS and gene mutations in vivo (Nohynek et al., 2004). Patch testing for 1 year with kojic acid in about 200 patients, who had not previously used skin care products containing it, showed no evidence of contact allergy (Nakagawa et al., 1995). Therefore, topical use of kojic acid as a skin-lightening agent has a negligible risk of genotoxicity or toxicity to the consumers (Nohynek et al., 2004). However, it has not only high hydrophilicity and low sustained action on the skin but also because of its small molecule, it is hardly absorbed through the lipid membrane of its target sites, the melanocytes (Curto et al., 1991). It is likely absorbed through voids between cells on the skin (Nakayama et al., 2000). An amino acid derivative of kojic acid exhibited stronger tyrosinase inhibitory activity than did kojic acid (Kobayashi et al., 1996, 2001). The current available preparations of kojic acid are cream or gel formulations. There is none in vesicular preparation. Kojic acid entrapped in bilayer vesicles and its structure modification may potentially alter the permeability through the skin. The present study reports the development of vesicular formulations for kojic acid and its ester compound. Oleic acid was selected to synthesize kojic oleate, since it has been widely used as a skin moisturizer and topical enhancer (Escribano et al., 2003; Thomas and Panchagnula, 2003; Dimas et al., 2004). Both phospholipid and non-ionic surfactant based vesicles were evaluated in terms of entrapment efficiency, size and membrane properties.

2. Materials and methods

2.1. Materials

N,*N*′-dicyclohexylcarbodiimide (DCC), 4-(*N*,*N*-dimethylamino)pyridine (DMAP), tetrahydrofuran and kojic acid were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Cholesterol, dicetyl phosphate, sorbitan monostearate (Span 60) and polyoxyethylene sorbitan monostearate (Tween 61) were purchased from Sigma Chemical Company (St. Louis, MO). Dipalmitoyl phosphatidylcholine (DPPC) was from Nikko Company (Tokyo, Japan). All reagents and solvents were of analytical grade.

2.2. Synthesis of kojic oleate

Kojic oleate was synthesized by DCC/DMAP esterification modified from the method of Ammazzalorso et al. (2002). DCC and DMAP acting as catalysts were added to oleic acid solution in dichloromethane. The mixture was then stirred at room temperature ($28 \pm 2\,^{\circ}C$) until a white precipitate was formed. Kojic acid was added to the suspension to obtain a molar ratio of kojic acid per oleic acid of 1:2. Acetonitrile was added to the suspension to improve the solubility of kojic acid. The suspension was continuously stirred overnight at $28 \pm 2\,^{\circ}C$. The reaction mixture was filtered and the filtrate was evaporated in a rotary evaporator (Eyela Co., Tokyo, Japan) at 45 °C. The resulting oily residue was purified by column chromatography using silica gel as a packing material. A mixture of hexane and ethyl acetate (10:1, v/v) was used as an eluent. The synthesized product was identified by TLC using hexane and ethyl acetate (3:1, v/v) as a developing solvent. The TLC plates were visualized under UV light.

Fractions containing the synthesized product were evaporated and identified by ^{1}H NMR (JNM AL-300, JEOL Hightech Ltd., Tokyo, Japan) and FAB mass spectroscopy (JMS-SX102A, JEOL Hightech Ltd., Tokyo, Japan). Peak absorbance of product was determined by UV spectrophotometer (U-3310, Hitachi, Tokyo, Japan). Melting point was measured by a differential scanning calorimeter (type 8240B, Rigaku Denki Co., Tokyo, Japan).

2.3. Surface pressure measurement

The pressure-area isotherms were recorded by a Wilhelmy plate method using a surface pressure meter type HBM-A (Kyowa Interface Science Co. Ltd., Tokyo, Japan) with a Teflon-coated trough and a movable barrier with an initial area of 950 cm^2. The subphase (at 25 °C) was ultra-purified water. The amphiphiles (Span 60, Tween 61 or DPPC) and their mixture with kojic oleate (1:1 molar ratio) were dispersed in chloroform to a final concentration of 1 mM. One hundred microliters of the solutions were dropped at the air–water interface using microsyringe. Twenty minutes after spreading, the film was compressed at a rate of 20 mm/min. The surface pressure was measured with a precision of 0.1 mN/m using a Wilhelmy balance and a platinum plate. Each π–A measurement was repeated for three times.

2.4. Preparation of kojic oleate entrapped in vesicles

The multi-lamellar vesicles were prepared by Bangham method (Bangham et al., 1965). The non-ionic surfactants (Span 60 and Tween 61) or DPPC, cholesterol and negatively charged lipid (dicetyl phosphate) at a molar ratio of 9.5:9.5:1.0 were dissolved together with kojic oleate in chloroform. The total concentration of the substances forming vesicles was adjusted to 20 mM. The organic solvent was vacuum evaporated by a rotary evaporator to get a thin film. The resulting film was dried overnight in a desiccator under vacuum at room temperature. The film was then hydrated to obtain the multi-lamellar vesicles with purified water or 20 mM kojic acid solution under mechanical agitation for about half an hour at $60 \pm 2\,^{\circ}C$. Small vesicles were prepared by sonication of the multi-lamellar vesicle dispersion, using probe type ultrasonic generator (Vibra Cell^{TM}, Sonics & Materials Inc., Newtown, CT, USA) operating at 20 kHz with an amplitude of 3 mm for 5 min.

2.5. Physical structure of the vesicles

The morphology of the bilayer vesicles containing kojic oleate was characterized by an optical microscope with a transmitted light differential interference contrast attachment (type IMT2-NIC2, Olympus Optical Co. Ltd., Tokyo, Japan).

The lamellarity of the bilayer vesicles was observed by a TEM (80 kV, TEM1200SJEOL, JEOL Ltd., Tokyo, Japan) using negative staining technique employing 1% (w/v) of uranyl acetate solution.

The particle size of the multi-lamellar vesicles was detected under the optical microscope while that of the oligo-lamellar vesicles was measured by zeta potential/particle sizer (Nicomp^{TM} 380 ZLS, Santa, Barbara, California, USA). The time-dependent correlation function on the scattered light intensity was measured at a scattering angle of 90°. This measurement is based on a dynamic light scattering method. The vesicles dispersions were diluted about 30 times with purified water before the measurement.

2.6. Measurement of entrapment efficiency of the vesicles

The unentrapped kojic acid was removed by dialysis. The oligo-lamellar vesicles were dialyzed through a seamless cellulose tube (UC8-32-25, size 8/32, Viskase Companies Inc., Japan) against 10 mM of sodium chloride solution for 10 h at $8 \pm 2\,^{\circ}C$. Kojic oleate loaded vesicles were separated from the unentrapped kojic oleate by gel filtration, using the Sepharose CL 6 B (Fluka Chemicals, Gillingham, Dorset, UK) as the packing material. Since the vesicles were dispersed in water, purified water (pH 6.0) was employed as the eluent. Kojic oleate and kojic acid contents entrapped in the vesicles were determined by HPLC (HP, Series 1100, Hewlett-Packard, Waldbronn, Germany). A reverse phase C_{18} column (Phonomenex®, 250 mm × 4.60 mm i.d., 10 μm particle size) was used to analyze kojic acid, whereas a normal phase column (Spherisorb®, 250 mm × 4.60 mm i.d., 5 μm particle size) was used for kojic oleate. Mobile phases employed for kojic acid and kojic oleate were a mixture of 10 mM phosphoric acid/acetonitrile (3:7, v/v), and chloroform/methanol (98:2, v/v), respectively. Butylated hydroxyanisole and propyl paraben were used as internal standards for the determination of kojic acid and kojic oleate, respectively. The UV detections of kojic acid and kojic oleate were performed at 280 and 250 nm, respectively. The purified vesicles were disrupted by each mobile phase. The percent entrapment efficiencies were calculated from the ratio of the amount of kojic acid or kojic oleate entrapped in the vesicles to the total initial amount of kojic acid or kojic oleate.

2.7. Release study of kojic acid from the vesicles

The release of kojic acid from the multi-lamellar vesicles was performed through dialysis tube (UC8-32-25, size 8/32, Viskase Companies Inc., Tokyo, Japan). Two milliliters of the vesicular dispersion was filled in the tube. Both sides of the tube were tightly sealed by the tube sealers. The tube was then placed in 200 ml of 10 mM sodium chloride solution. The bulk solution was continuously stirred by a magnetic stirrer at room temperature ($28 \pm 2\,^{\circ}C$). The 20 mM of kojic acid solution was also studied as a reference. Aliquots of the dialysate were taken at predetermined time intervals for 8 h and replaced immediately with the same volume of sodium chloride solution. The withdrawn samples were assayed for kojic acid by HPLC. Flux was determined from the slope of the linear part of a plot of the cumulative amount released versus time$^{1/2}$. The data was obtained from three determinations.

3. Results

3.1. Synthesis of kojic oleate

The percentage yield of the synthesis was approximately 80% at 1–2 molar ratio of kojic acid to oleic acid. The maximum UV–vis absorbance peaks of the product in methanol were 251.50 and 212.0 nm. FAB–MS found peak at 407, which was a molecular weight of $C_{24}H_{38}O_5$ (cal. 406.57). The ^{1}H NMR (300 MHz, $CDCl_3$) information was δ: 0.90 (3H, br), 1.26 (2H, d), 1.68 (2H, m), 2.01 (2H, br), 2.37 (2H, t), 4.91 (2H, s), 5.36 (2H, t), 6.47 (1H, s), 7.85 (1H, s). The melting point determined by DSC was $34.6\,^{\circ}C$.

3.2. Surface pressure measurement

Fig. 1 was the π–A isotherm ($25\,^{\circ}C$) of pure kojic oleate, pure Span 60, Tween 61 and DPPC monolayers and the binary mixtures of the studied amphiphiles and kojic oleate (1:1 molar ratio) at the air/water interface. The isotherms for the pure Span 60 and DPPC resembled those previously published (Wheatley and Singhal, 1995; Sanchez and Badia, 2003), while there was no publication for the π–A isotherm of Tween 61 so far. The π–A isotherm of pure kojic oleate revealed that kojic oleate deposited at air/water interface. The π–A isotherms of the pure amphiphiles were different from those of the binary mixtures. The area per molecules of pure Span 60, Tween 61, DPPC and their mixtures with kojic oleate were 28.0 ± 1.0, 38.7 ± 1.2, 51.7 ± 2.9 Å^2 and 15.5 ± 1.3, 46.5 ± 1.5, 40.3 ± 0.6 Å^2, respectively.

3.3. Physical structure of the vesicles

The bilayer vesicles were prepared with the mixture of the amphiphilic substances (Span 60, Tween 61 or DPPC) and cholesterol at 1:1 molar ratio. The total concentration of the non-ionic surfactants or

DPPC, cholesterol and dicetyl phosphate was adjusted to 20 mM. The particle size of the vesicles observed under the optical microscope was large multi-lamellar vesicles with the diameter range of about 1–20 μm (Table 1). The vesicles containing kojic acid were larger than those containing kojic oleate (Fig. 2). The particle size of Span 60 and Tween 61 vesicles was smaller than DPPC vesicles. The spherical vesicles (20 mM) could be prepared when 7 mM of kojic oleate was incorporated into the vesicular bilayers with no microscopic detection of the insoluble kojic oleate particles in the suspensions. Fig. 3 showed the negative staining TEM images of the oligo-lamellar vesicles containing kojic oleate (7 mM) prepared by sonication. The particle

Table 1
Particle size (μm) of the vesicles containing kojic acid or kojic oleate (7 mM) prepared by Bangham method without sonication observed under the optical microscope[a]

Formulations	Mean (μm)	S.D.
Kojic acid		
Span 60	2.95	0.84
Tween 61	16.40	5.84
DPPC	18.93	5.35
Kojic oleate		
Span 60	3.95	0.84
Tween 61	3.38	1.72
DPPC	5.32	2.09

[a] Experimental data represented the measurement of 100 particles.

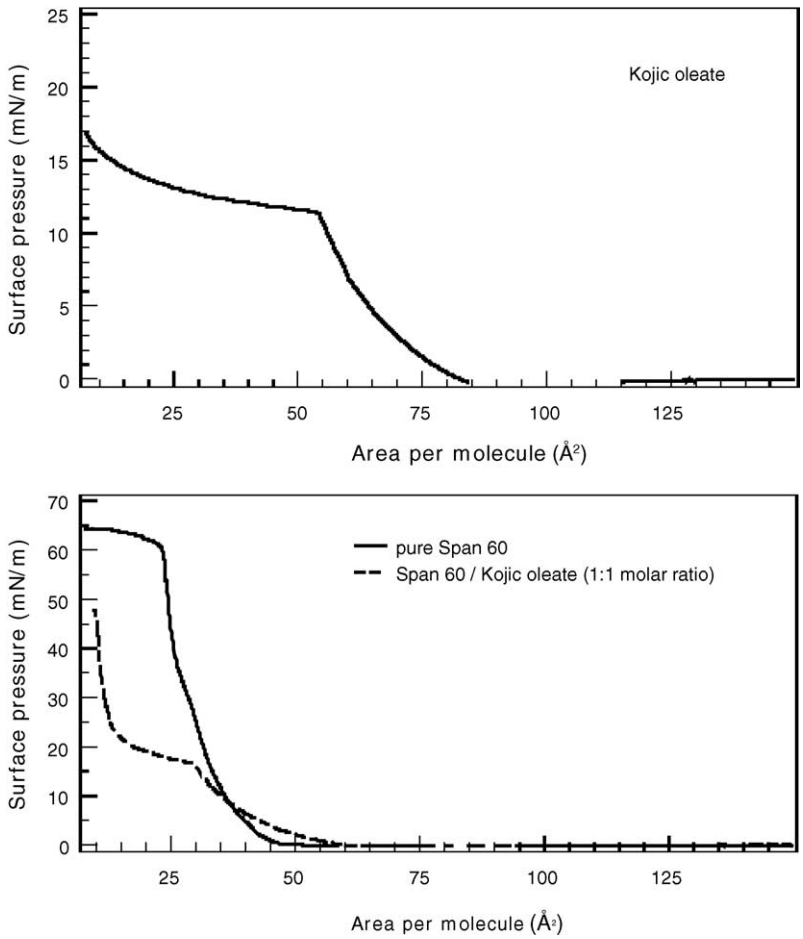

Fig. 1. The π–A isotherm (25 °C) at air/water interface of pure kojic oleate and Span 60, Tween 61, DPPC monolayers and their binary mixtures with kojic oleate at 1:1 molar ratio.

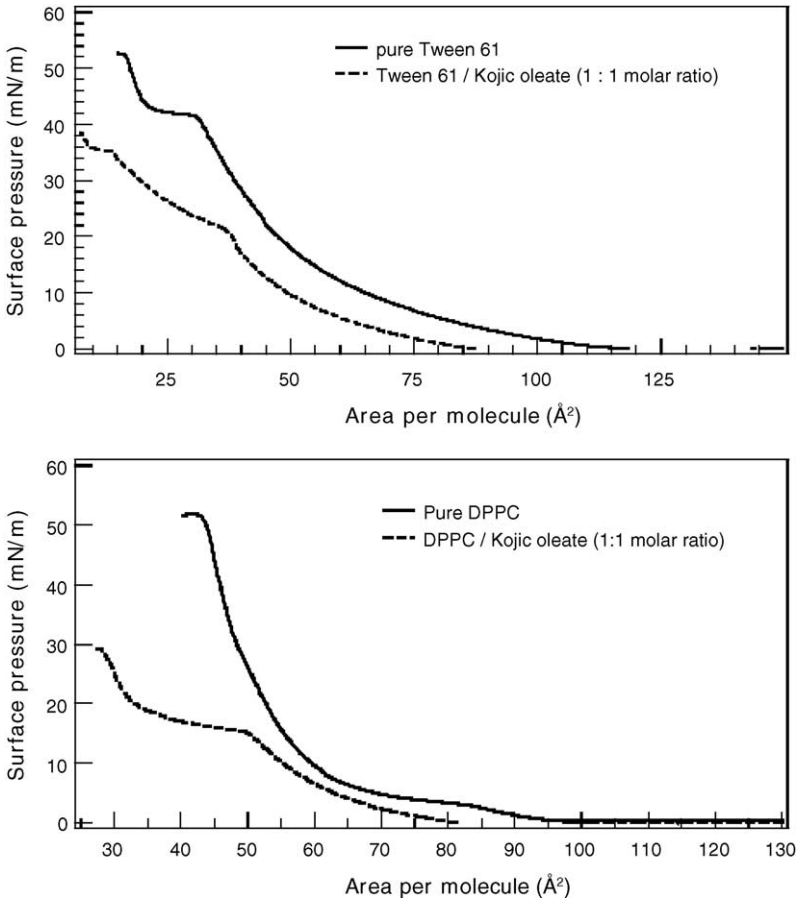

Fig. 1. (*Continued*).

size of the oligo-lamellar vesicles was in the range of 0.1–1 μm (Fig. 4).

3.4. Entrapment efficiencies of the vesicles

The entrapment efficiencies of kojic oleate in the oligo-lamellar vesicles were presented in Table 2. The entrapment efficiencies of kojic oleate per mole of the vesicle forming substances (amphiphiles/cholesterol/dicetyl phosphate) were broadly comparable. At 20 mM of Span 60, Tween 61 and DPPC vesicles, kojic oleate entrapments were about 0.22, 0.33 and 0.35 mol/mol of the total vesicle forming substances, respectively. The bilayer vesicles prepared from DPPC and Tween 61 seemed to have higher ability to encapsulate kojic oleate than did Span 60 vesicles.

The entrapment efficiencies of kojic acid (20 mM) in the Tween 61, Span 60 and DPPC vesicles were quite low of 1.14 ± 0.5, 0.4 ± 0.8 and 0.85 ± 0.9 mol/mol of amphiphile/cholesterol/dicetyl phosphate, respectively. Fig. 5 showed the entrapment efficiencies of kojic acid at various amounts of kojic oleate introduced to vesicular bilayers. The intercalation of kojic oleate into the vesicular membrane slightly affected the trapping efficiencies of kojic acid in the vesicles. The entrapment efficiencies of kojic acid in the vesicles with and without kojic oleate were not significantly different. The vesicles could not be formed with a mixture of kojic oleate and cholesterol, as this system could not entrap any kojic acid and clear solutions with the large insoluble particles of kojic oleate and cholesterol were observed.

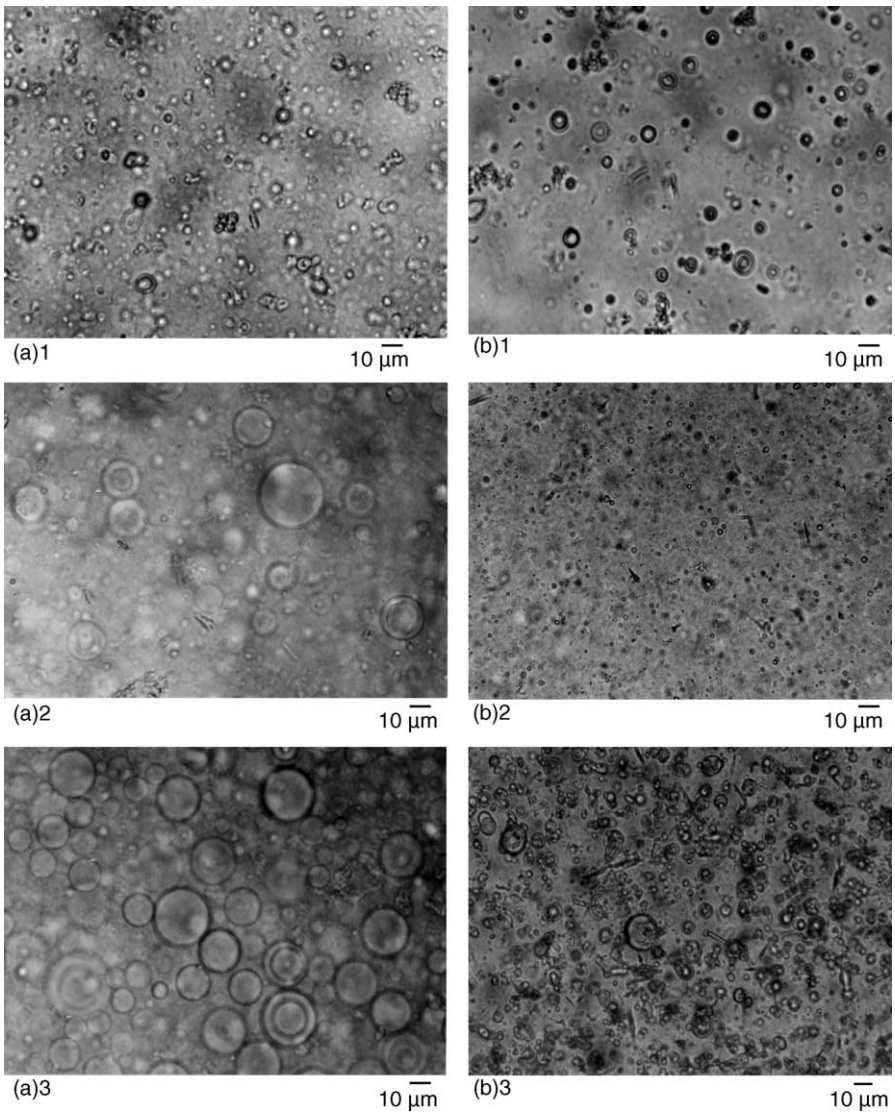

Fig. 2. Microscopic images of the vesicles containing (a) kojic acid and (b) kojic oleate prepared by Bangham method without sonication (1) Span 60; (2) Tween 61; (3) DPPC.

3.5. Release study of kojic acid from the vesicles

The release rates of kojic acid associated with the multi-lamellar vesicles across the dialysis membrane were slightly slower than that of free kojic acid solution. Kojic acid flux from the free solution increased over the first 2 h and then became plateau, whereas the release of kojic acid from the vesicles required longer time for the equilibrium. The slopes or the release rates (k) of kojic acid from Span 60, Tween 61 and DPPC vesicles with and without kojic oleate were 15.75 ± 1.81, $14.34^{*} \pm 1.42$, $14.24^{*} \pm 0.73\ \mu M/min^{1/2}$ and 15.51 ± 0.69, $14.33^{*} \pm 0.93$, $13.23^{*} \pm 0.63\ \mu M/min^{1/2}$, respectively, whereas k from kojic acid solution was $16.49 \pm 0.41\ \mu M/min^{1/2}$. The release rates of kojic acid from Tween 61 and

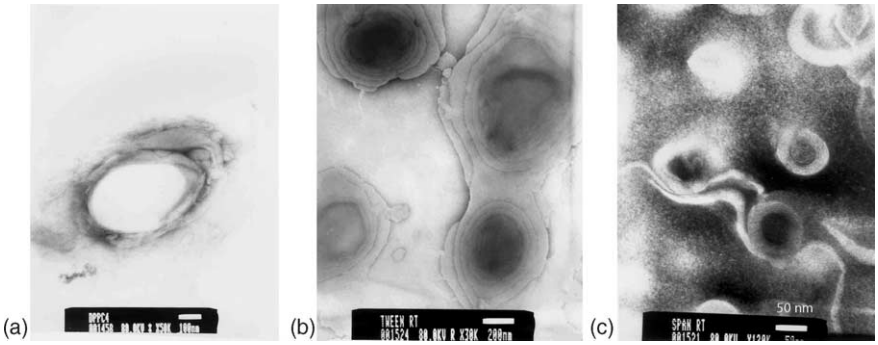

Fig. 3. The negative staining TEM images of the vesicles containing 7 mM of kojic oleate prepared by Bangham method with sonication (a) DPPC/cholesterol/dicetyl phosphate (9.5:9.5:1.0 molar ratio) vesicles (×50 K); (b) Tween 61/cholesterol/dicetyl phosphate (9.5:9.5:1.0 molar ratio) vesicles (×30 K); (c) Span 60/cholesterol/dicetyl phosphate (9.5:9.5:1.0 molar ratio) vesicles (×120 K).

DPPC vesicles seemed to be significantly slower than those from kojic acid solution and Span 60 vesicles ($P < 0.05$).

4. Discussion

For the synthesis of ester of kojic acid, the main reaction is the substitution of oleic acid to hydroxyl group at position 7 of kojic acid. It was not only a simple one step procedure but also gave more than 80% product yield. This procedure appears to be superior to the method synthesized 7-*O*-lauroyl kojic acid reported by Kobayashi et al. (2001), since only a yield of 53% was obtained. Kojic acid was a solid powder and oleic acid was liquid at room temperature (28 ± 2 °C), whereas kojic oleate was semisolid substance with the melting point at 34.6 °C and insoluble in water but freely soluble in chloroform.

Span 60 and Tween 61 were chosen to prepare the vesicles because both non-ionic surfactants gave high entrapment efficiency against calcein (Manosroi et al., 2003). DPPC was selected as a representation of phospholipid based vesicles. The 20 mM of kojic acid aqueous solution were used to hydrate the amphiphilic film containing kojic oleate in order to investigate the vesicle formation and the entrapment ability of these vesicles. Kojic acid, which is a hydrophilic substance, was expected to be encapsulated in the inner water phase of the vesicles. The vesicles containing kojic

Table 2
The entrapment efficiencies of kojic oleate in vesicles prepared from various compositions

Compositions[a] (9.5:9.5:1.0 molar ratio)	Kojic oleate concentration (mM)	Entrapment efficiency of kojic oleate	
		Percent kojic oleate ± S.D.[b]	Mol per mol of total vesicular concentration[a]
Span 60/cholesterol/dicetyl phosphate	5	95.29 ± 13.08	0.24
	7	49.86 ± 2.78	0.17
	10	46.88 ± 4.51	0.23
Tween 61/cholesterol/dicetyl phosphate	5	93.65 ± 7.03	0.23
	7	89.13 ± 1.63	0.31
	10	70.41 ± 6.99	0.35
DCCP/cholesterol/dicetyl phosphate	5	84.45 ± 2.90	0.21
	7	98.24 ± 2.16	0.34
	10	71.44 ± 3.72	0.36

[a] Total vesicular concentration = 20 mM.
[b] Experiment data represent the mean ± S.D. of three determinations.

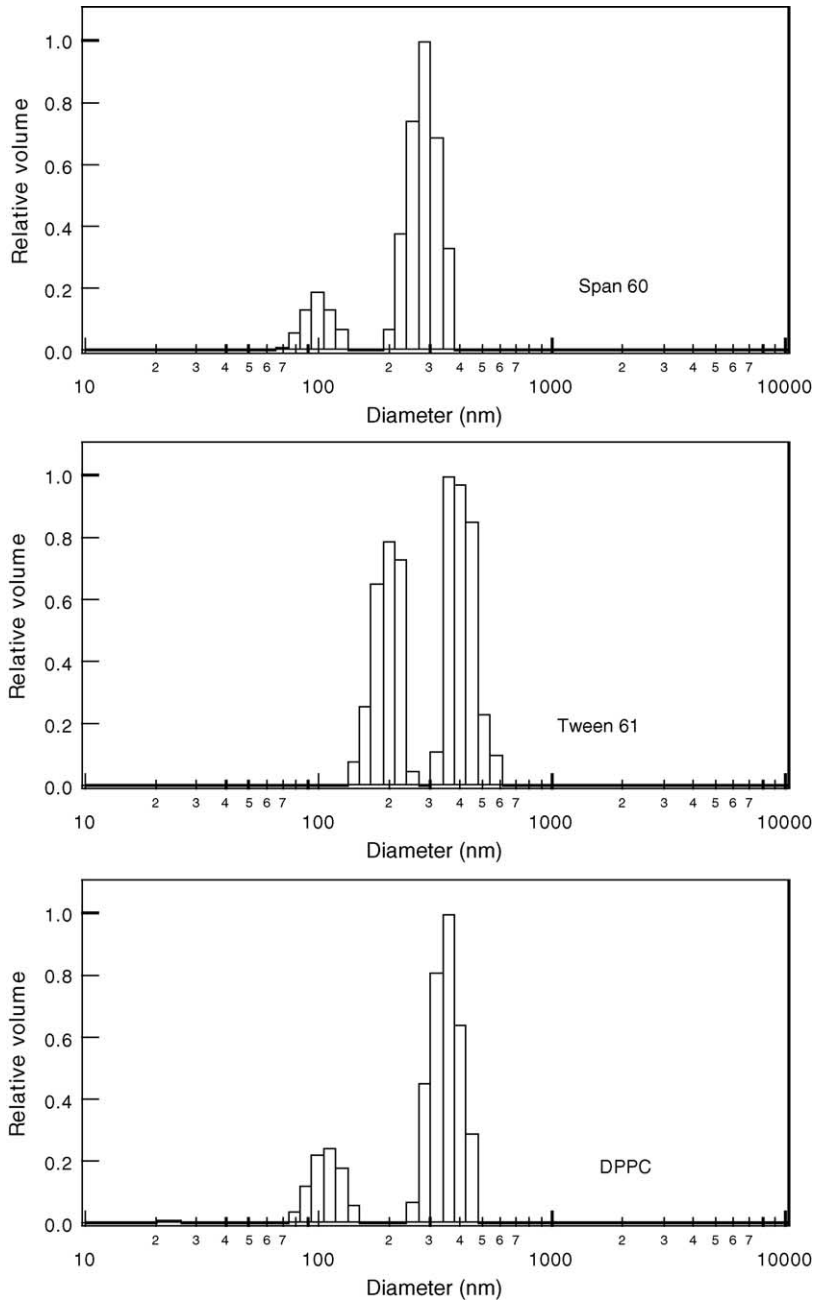

Fig. 4. Particle size measured by zeta potential/particle sizer of the vesicles containing kojic oleate (7 mM) prepared by Bangham method with sonication.

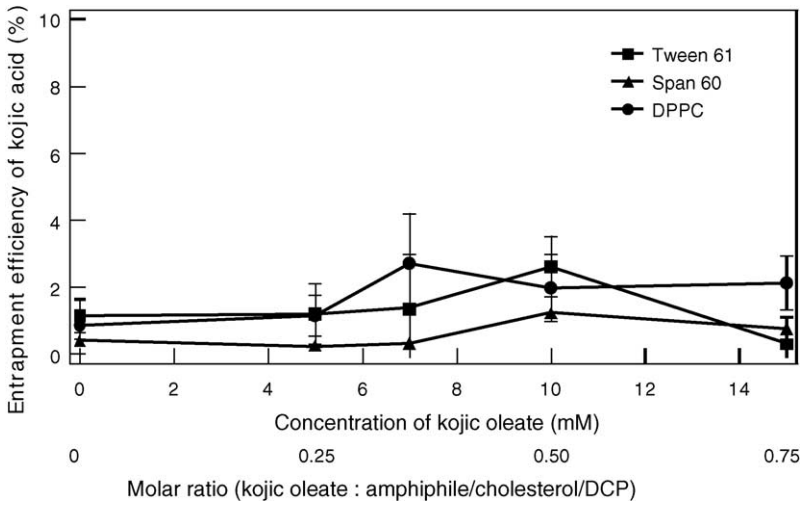

Fig. 5. The entrapment efficiencies of kojic acid in the bilayer vesicles containing various concentrations of kojic oleate. Experimental data represent the mean ± S.D. of three determinations.

oleate in bilayer membrane could also encapsulate kojic acid although entrapment efficiencies were low. This, however, indicated the vesicle formation in the presence of kojic oleate molecule in the vesicular membrane. The entrapment efficiencies of kojic acid in the vesicles were likely independent of kojic oleate addition.

A water dispersion of kojic oleate (5 mM) was a clear solution with floating insoluble particles. Other structures such as micelles or vesicles of kojic oleate in water were not possible because of its high hydrophobicity with high critical packing parameter (CPP). The head group of kojic oleate is a kojic acid molecule, which is very small as compared to its C_{18} unsaturated hydrocarbon chain (oleate). This leads to a small a value, which is surface area of hydrophilic head group of amphiphile, thereby a huge CPP (v_0/al_0). Therefore, kojic oleate as an amphiphilic substance, together with cholesterol could not form the bilayer vesicles, since both substances have higher CPP value than that for vesicle formation ($1/2 < \text{CPP} < 1$) (Israelachvili et al., 1977; Nagarajan, 2002).

Although the concentrations of the amphiphiles forming the vesicles were much above their critical micellar concentrations (CMC), the solubilization of kojic oleate in the micelles of the amphiphilic substances was not prominently expected in the system containing cholesterol, since the bilayer vesicles are the favorable assemblies regarding to the packing constraints. The amounts of kojic acid entrapped in the vesicles containing kojic oleate were not significantly different from the corresponding vesicles without kojic oleate revealed that other assemblies might not form in the systems, since total trapped volumes of the vesicles were not significantly changed by the incorporation of kojic oleate. According to the π–A isotherm data, the oleate chain of kojic oleate was expected to intercalate in the hydrophobic domains between the alkyl chains of the amphiphilic molecules forming the vesicles, since the π–A isotherms of the binary mixtures exhibited a significant shoulder indicating an orientation of both molecules on air/water interface. This implied that kojic oleate was mainly intercalated between the alkyl chains of the amphiphilic molecules forming the vesicles. It also appeared that the amount of kojic oleate entrapped in the vesicles was limited and dependent on the amphiphile and cholesterol concentrations, since the insoluble particles of kojic oleate were observed in the suspensions when the amount of kojic oleate beyond 0.20–0.30 mol/mol of the total concentration of the mixed amphiphile/cholesterol/DCP.

The entrapments of kojic oleate in the vesicles prepared with Tween 61, Span 60 and DPPC were broadly different which could be explained from the

π–A isotherm study. Pure DPPC and Span 60 showed stiffer monolayer at the air/water interface than pure Tween 61. The mean effective area per molecule of pure DPPC (51.7 ± 2.9 Å^2) was larger than that of pure Span 60 (28.0 ± 1.0 Å^2), since DPPC contains double alkyl tails (C_{16}). Span 60 and Tween 61 possess the same alkyl chain length (C_{18}), but the head group of Tween 61 is much larger than Span 60. Therefore, the head group repulsion of Tween 61 was too high for condensed monolayer to be formed so the mean effective area per molecule of pure Tween 61 was larger than that of pure Span 60 and the π–A isotherm of pure Tween 61 showed an isotherm characteristic of a fluid state. Kojic oleate also exhibited an isotherm characteristic of liquid-expanded state at all surface pressure. The π–A isotherms of the mixed substances revealed the significant extension of the liquid-expanded state of the mixed monolayers except for the π–A isotherm of Tween 61–kojic oleate. Moreover, the area per molecule of Tween 61–kojic oleate was larger (46.5 ± 1.5 Å^2) than Span 60–kojic oleate (15 ± 1.3 Å^2). Since Tween 61 head group repulsion was very large, the incorporation of a less bulky head group of kojic oleate allowed for relief of head group repulsion, leading to an increase of monolayer rigidity, whereas the tail groups of both Tween 61 and kojic oleate would exhibit attractive forces. Meanwhile, the head group of kojic oleate was as small as that of Span 60, the mixture of both substances provided very tightly packed monolayer with the lowest area per molecule. This implied that the packing numbers of Span 60 molecules in the vesicular membrane were considerably high, leading to lower amount of kojic oleate allowed to be embedded in vesicular membrane of Span 60 than Tween 61. Thus, entrapment efficiency of kojic oleate in Span 60 vesicles was lower than Tween 61 vesicles.

The π–A isotherm of pure DPPC showed a solid-condensed phase at pressure above 20 mN/m and the LE-to-LC phase transition at $\pi \sim 4$–5 mN/m. The π–A isotherm of DPPC and kojic oleate indicated mixed monolayer on air/water interface. The incorporation of kojic oleate in DPPC extended the liquid-expanded state of DPPC monolayer, while the effective area per molecule at solid-condensed state was decreased, since the intercalation of single alkyl chain of kojic oleate. This clearly revealed the mixing of kojic oleate and DPPC molecules on air/water interface and the intercalation of kojic oleate in the DPPC vesicular membrane could be anticipated.

The release of kojic acid from the multi-lamellar vesicles through the dialysis tube was slightly slower than kojic acid solution revealing that kojic acid almost completely released from the vesicles within 3 h. This indicated not only the entrapment of kojic acid in the vesicles was low but also the intercalation of kojic oleate into the vesicular membrane did not significantly affect a permeability of kojic acid through the vesicles as well. However, the difference in the release profiles of kojic acid between the vesicles with and without kojic oleate could be noticed for Tween 61 and DPPC vesicles but no difference for Span 60 vesicles. This might be attributed to the lower entrapment of kojic oleate into the vesicular membrane of Span 60.

In conclusion, kojic oleate could be intercalated in the bilayer structure of the vesicles composed of amphiphile (Span 60, Tween 61 or DPPC)/cholesterol/dicetyl phosphate at molar ratio of 9.5:9.5:1.0. The best formulations were concluded to be Tween 61 and DPPC vesicles, which exhibited relatively high percentage entrapment of both kojic oleate and kojic acid. The intercalation of kojic oleate in the vesicular membrane did not significantly influence the trapping volume of the vesicles and permeability of the vesicular membrane. These formulations will be further evaluated for irritation and transdermal absorption.

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