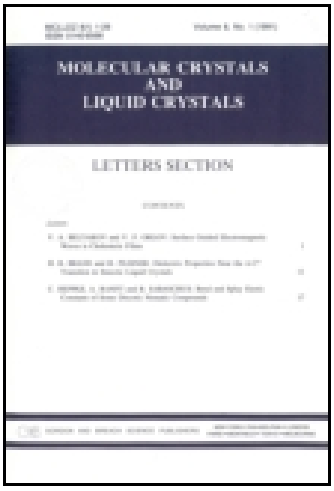

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A Novel Method for the Improved Skin Whitening Effect Based on Nanostructured Lipid Carrier

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A Novel Method for the Improved Skin Whitening Effect Based on Nanostructured Lipid Carrier

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The nanostructured lipid carrier (NLC) encapsulating two potent skin whitening ingredients was developed by our group for cosmetic and dermatological treatment. The particle showed spherical shape morphology under atomic force microscope, and the value of the zeta potential of the NLC was − 65.4 ± 4.3 mV. Good physical stability was observed by dynamic light scattering during 45 days' storage, respectively. The skin whitening effect was improved when applying the NLC. Therefore, the NLC might be a potential delivery vehicle in dermal products.

Keywords Crystal; nanostructured lipid carrier; skin whitening; stability

Introduction

Skin whitening is a very popular treatment in Asian populations. However, consumers in western countries now realize that skin whitening is very desirable for treating pigment spots [1]. Phenylethyl resorcinol (4-(1-phenylethyl)1,3-benzenediol) is a new whitening agent that has been validated to inhibit tyrosinase activity by Schmaus [2]. Many investigations have demonstrated that phenylethyl resorcinol is one of the most potent whitening agents ever reported and is not due to cytotoxicity [2, 3]. Tetrahydrocurcumin (THC), one of the major metabolites of curcumin, has been recently used as an additive in cosmetic formulations, because it has skin whitening and antioxidant properties [4]. Nevertheless, the absorptions of phenylethyl resorcinol and THC are limited due to their low aqueous solubility.

The nanostructured lipid carrier (NLC) is considered as the second generation of the lipid nanoparticles following the solid lipid nanoparticle (SLN) [5 – 7]. Potential problems are associated with SLN, such as limited drug loading capacity and potential drug expulsion caused by an ongoing crystallization process toward a perfect crystal during storage [6]. The NLC is produced by mixing solid lipids with liquid lipids leading to special structures

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of the lipid matrix to avoid or minimize these problems. What is more, enhancement of chemical stability of actives, controlled occlusion, increase in skin hydration, enhanced skin bioavailability of actives, and skin targeting are excellent benefits of NLC [8]. It has been discovered that delivering skin whitening ingredients to the skin in the form of association structures improves efficacy.

The aim of this study was to encapsulate phenylethyl resorcinol and THC in NLC to enhance the skin whitening activity and aqueous solubility. Consequently, the study described the screening for an optimal lipid matrix for the skin whitening ingredients' incorporation. The particle size, zeta potential (ZP), and morphology were characterized. Furthermore, the skin whitening efficacy was evaluated in B16F0 melanoma cells.

Experimental

Materials

Phenylethyl resorcinol and THC were provided by Kans Cosmetic Co., Ltd. (Shanghai, China); glycerin monostearate (GMS) and diglycerides (ACETEM), octyl and decyl glycerate (ODO) were purchased from Zhengtong Chemical Co., Ltd. (Henan, China); Lubrajel oil was purchased from Boyi Co., Ltd. (Guangzhou, China); Olivem® 1000 (Trade Name) was purchased from B&T Chemical Co., Ltd. (Arcore, Italy); PEG-40 stearate was purchased from Will Chemical Co., Ltd. (Nanjing, China); B16F0 melanoma cells (CRL-6322) were obtained from China Center for Type Culture Collection (Hubei, China); fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), trypsin, and penicillin/streptomycin were purchased from HyClone Lab, Inc. (Utah, USA); 3,4-dihydroxy-L-phenylalanine (L-DOPA) was purchased from Aladdin Industrial, Inc. (Shanghai, China); phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), and BCA Protein Assay Kit were purchased from Beyotime Institute of Biotechnology (Beijing, China); Ultrapure water with conductivity of 18.2 MΩ cm was used in all the experiments. All other chemicals used were of analytic grade and commercially available products.

Lipid Screening

The solubility of the phenylethyl resorcinol and THC was determined in different solid lipids and liquid lipids as described elsewhere [9]. For the solubility study in the solid lipid, each dose of the solid lipid was previously melted at 10°C above the respective melting point. The melting points of GMS and ACETEM were about 55°C and 40°C. Small amounts of phenylethyl resorcinol and THC were then added until the saturation of the lipid was achieved. For the liquid lipid, small amounts of phenylethyl resorcinol and THC were dispersed in the liquid lipid and stirred for 1 hr. The solubility was determined visually and microscopically (under polarized light). To determine the presence/absence of skin whitening ingredient crystals in the solidified lipid melts, the samples were investigated using a Microscope (BME Monocular, LEICA, Germany).

Preparation of NLC

The skin whitening mixture-loaded NLC was prepared by the hot high-pressure homogenization method as described elsewhere [10 , 11]. Briefly, solid lipids (GMS and ACETEM) were heated up to 65°C with liquid lipids (ODO and Lubrajel oil). Then, phenylethyl

resorcinol and THC were dissolved in the lipid melt to form a uniform and clear oil phase. Meanwhile, surfactants (Olivem® 1000 and PEG-40 stearate) were dissolved in ultrapure water and heated at 65°C. Then this hot aqueous surfactant solution (aqueous phase) was poured in the oil phase and emulsified by stirring at 600 rpm for 5 min. The pre-emulsion was homogenized by high-pressure homogenizer (AH100D, ATS Engineering, Canada) using two homogenization cycles at 500 bar and 65°C. Finally, the resulting dispersion was cooled at ambient conditions to room temperature to obtain the NLC formulation.

Particle Size and Zeta Potential Analysis

The particle size and polydispersity index (PDI) for all samples were measured by dynamic light scattering (DLS) using a Malvern Zetasize (ZS90, Malvern Instruments, UK). The mean particle size (z-ave) and PDI values of the investigated samples were obtained by calculating the average of three measurements at 25°C with a scattering angle of 90°. The ZP was determined by the measurement of the electrophoretic mobility using a Malvern Zetasizer (ZS90, Malvern Instruments, UK). The ZP was calculated using the Helmholtz–Smoluchowsky equation [12] at 25°C.

Atomic Force Microscope (AFM) Analysis

The morphology of NLC formulation was observed by atomic force microscope (AFM) (Dimension 3100, VEECO, USA). Fresh prepared NLC was diluted with ultrapure water and dropped on a freshly cleaved mica slide, and air-dried for 30 min at room temperature [12]. The air-dried samples were then examined under the AFM. Sample was measured in tapping mode, using pyramidal cantilevers with Pt probes at a scanning frequency of 2 Hz.

Cell Culture

B16F0 melanoma cells were cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin in 5% CO_2 at 37°C. Cells were passaged using 0.25% (w/v) trypsin every third day. B16F0 melanoma cells were seeded at a density of 2×10^4 cells mL^{-1} in 12-well plates overnight. Each dose of sample was added to a well and incubated for another 72 hr. Culture solutions were added as a control. After incubation, cells were washed twice with 1 mL of cold PBS and then harvested with 300 μL of 0.25% (w/v) trypsin for 3 min. Then, 1 mL of fresh PBS (5 mM Mg^{2+}) was added to inactivate the trypsin. After centrifugation (1000 *g* for 3 min), the supernatant was decanted and 200 μL of 0.1% (w/v) Triton X-100 (pH = 6.8) was added to each well. Cells were frozen at −20°C for 30 min and lysed at 4°C for 30 min. After further centrifugation (12,000 *g* for 10 min), the amounts of protein in the supernatant were determined with a BCA Protein Assay Kit. The final protein concentration of each sample was then adjusted to 200 μg mL^{-1}. The precipitate was used for the extraction of melanin.

Melanin Measurement

Melanin content was measured using a modification of a previously reported method [13]. The precipitate was solubilized by treatment with 100 μL of DMSO (1 mol NaOH,

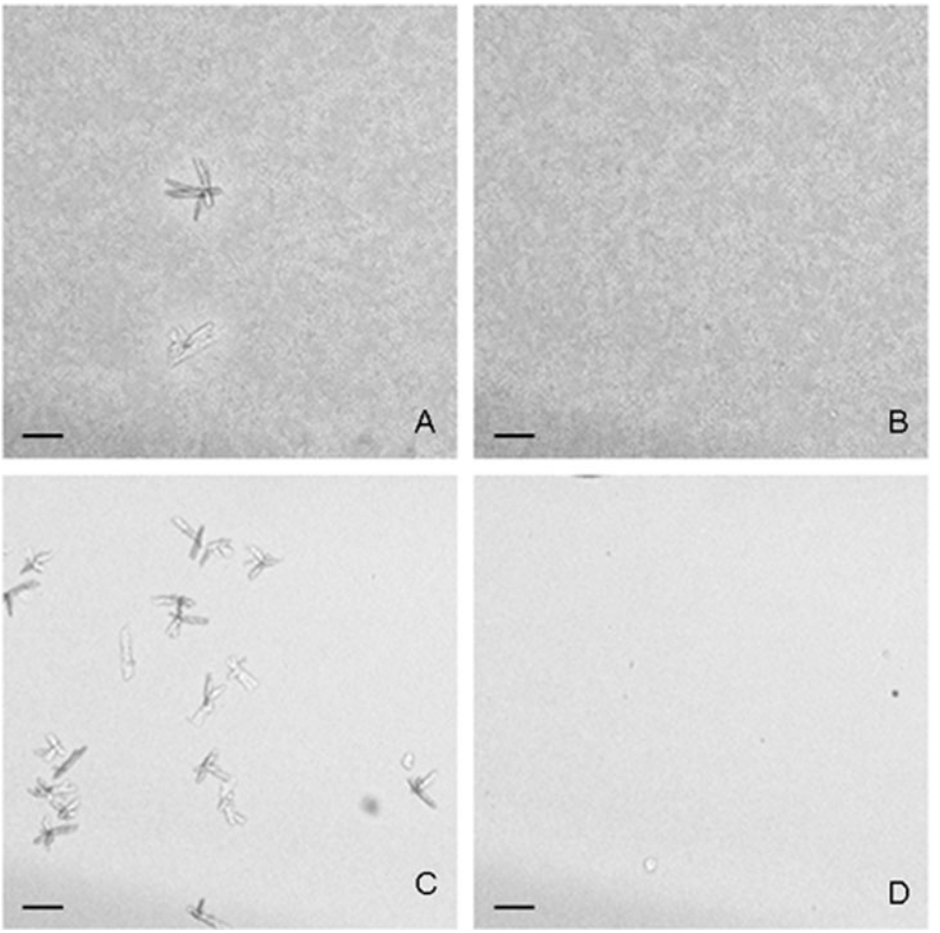

Figure 1. Light microscope pictures of lipid films with phenylethyl resorcinol and THC for GMS (A), GMS/ODO (B), ACETEM (C), and ACETEM/Lubrajel oil (D). The black scale bars on the left bottom of each picture represent 1 μm.

10% DMSO) at 90°C for 2 hr. After centrifugation (12,000 *g* for 10 min), aliquots of supernatant were transferred to 96-well plates, and the optical density of each culture well was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Model 680, Bio-Rad, USA) at 490 nm. The effect of skin whitening mixture and NLC formulation on the melanogenesis of B16F0 cells was examined.

Tyrosinase Assay

Tyrosinase activity was assessed as described previously with slight modifications [14]. 100 μL of each extract was placed in a 96-well plate, and 100 μL of 0.2% L-DOPA was added. The mixture was incubated at 36°C for 20 min. Then, the optical density at 475 nm was read using an ELISA reader (Model 680, Bio-Rad, USA).

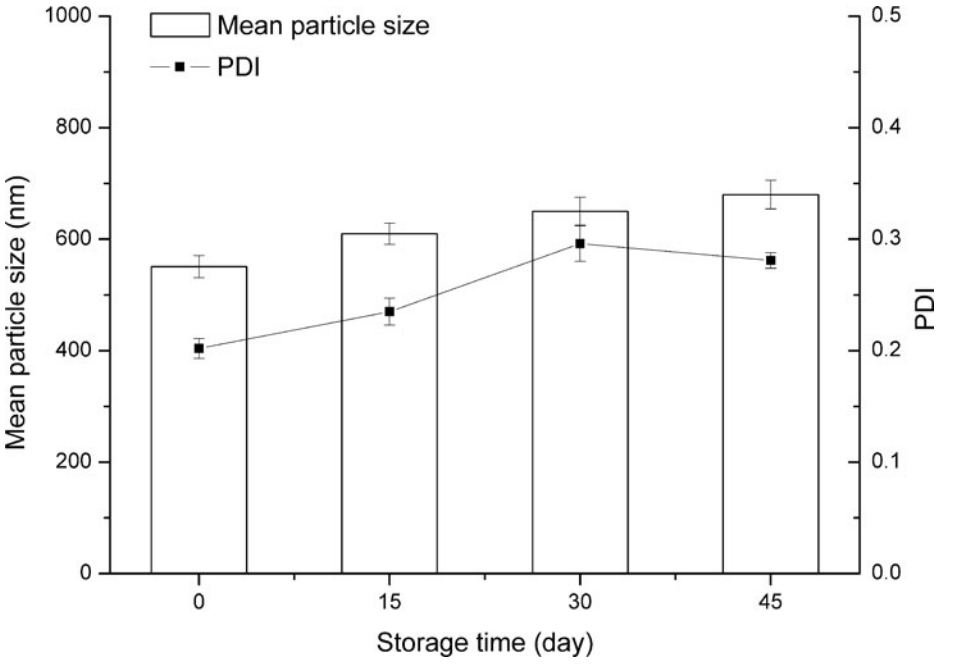

Figure 2. Mean particle size (z-ave) and polydispersity index (PDI) of NLC formulation (mean ± SD, $n = 3$). The lipid mixture was composed of molten GMS, ACETEM, liquid ODO, and Lubrajel oil.

Result and Discussion

Lipid Screening

According to the results (not shown), GMS and ODO allowed maximal solubility for phenylethyl resorcinol, ACETEM and Lubrajel oil were screened to have the best solubility for THC after screening all tested lipids.

The solubility of ingredients is higher in melted lipids compared with solidified lipids. Therefore, the solidification of the liquid mixtures during cooling can cause precipitation of ingredients, leading to the formation of crystals in the formulation. Microscopic investigation was performed to check for crystal formation. The results are shown in Figure 1. Crystals were revealed in solidified GMS (Figure 1(A)) and ACETEM (Figure 1(C)). No crystals were found for the mixtures with GMS and ODO in the ratio 1:1 w/w (Figure 1(B)). Also, no crystals were found for the mixtures with ACETEM and Lubrajel oil in the ratio 1:1 w/w (Figure 1(D)). This could be explained by controlled mixing of solid lipids with spatially incompatible liquid lipids leading to special nanostructures with improved drug incorporation and general imperfections in the crystal [6 , 11]. Therefore, more complex lipids being mixtures of GMS, ACETEM, ODO, and Lubrajel oil showed good drug incorporation capacities for both phenylethyl resorcinol and THC without ingredient crystals. Based on these results, GMS, ACETEM, ODO, and Lubrajel oil were selected to form the lipid mixture.

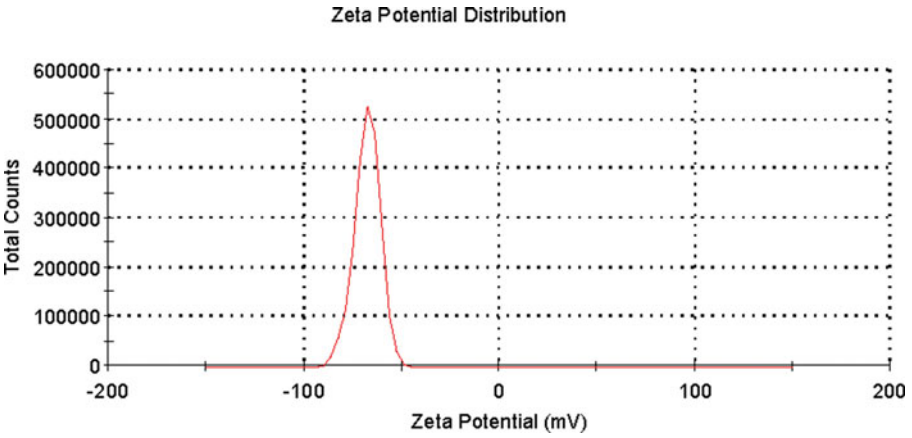

Figure 3. Zeta potential distribution profiles of NLC formulation. The lipid mixture was composed of molten GMS, ACETEM, liquid ODO, and Lubrajel oil. The zeta potential analysis was performed immediately after the preparation.

Particle Size and Zeta Potential Analysis

Particle size analysis is a good indicator of instability and is used to characterize the product. Prior to the measurements, all samples were diluted 100 times by ultrapure water to a weak opalescence. On the other hand, an equal amount of skin whitening mixture was almost

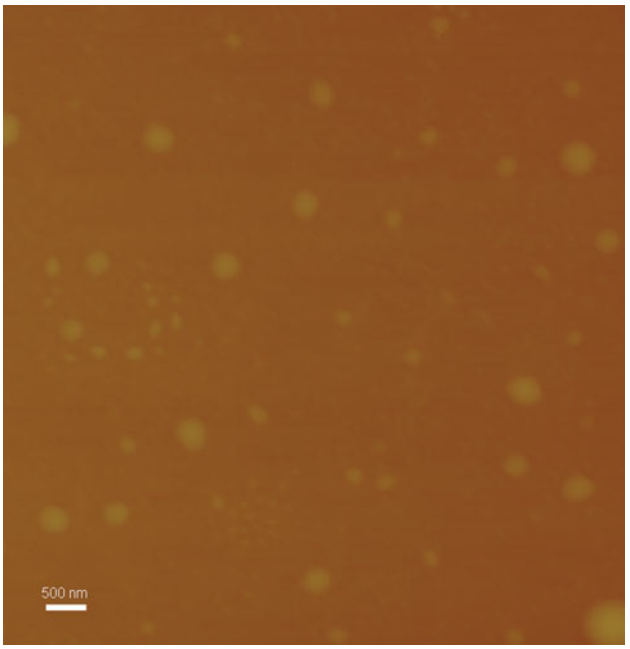

Figure 4. Atomic force microscopy of NLC formulation. The lipid mixture was composed of molten GMS, ACETEM, liquid ODO, and Lubrajel oil. The AFM observation was performed immediately after the preparation.

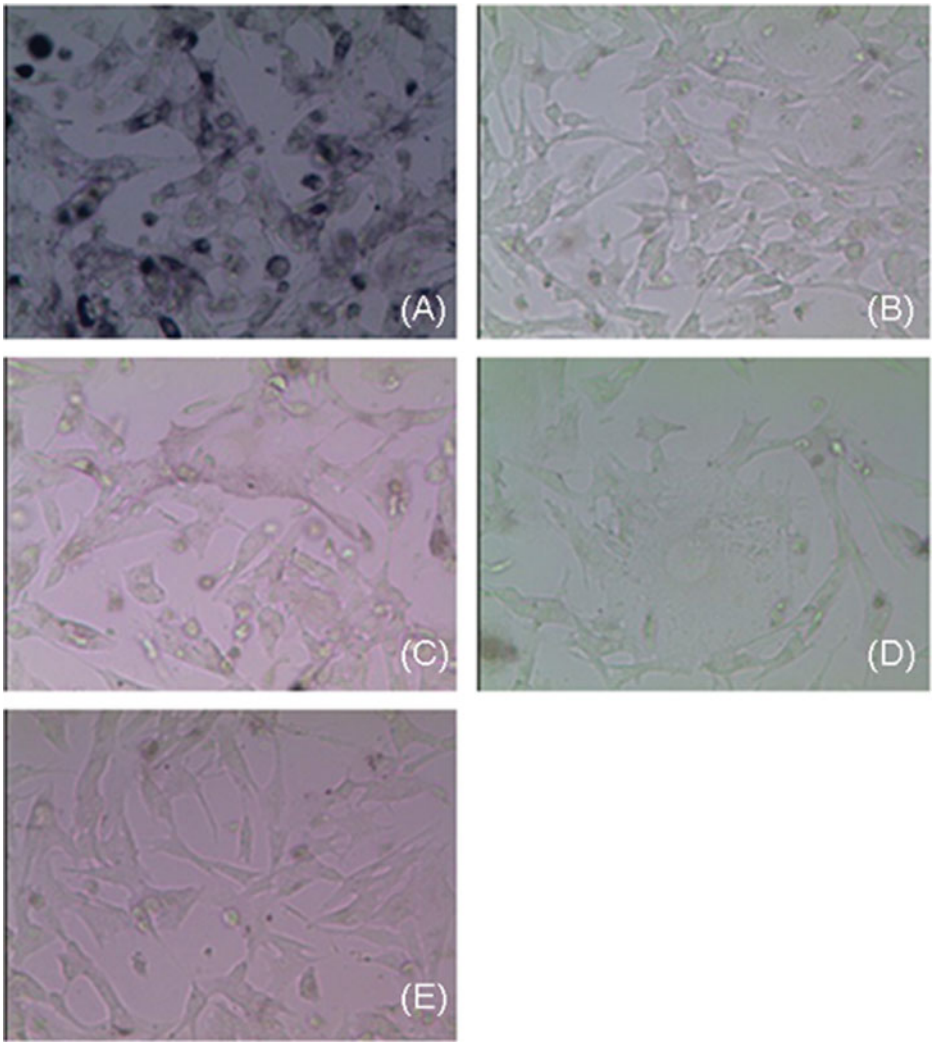

Figure 5. Representative electron microscopic figures of B16F0 melanoma cells after 3 day incubation with 1.2 ng mL^{-1} skin whitening mixture (B), NLC formulation containing 1.2 ng mL^{-1} skin whitening mixture (C), 2.4 ng mL^{-1} skin whitening mixture (D), and NLC formulation containing 2.4 ng mL^{-1} skin whitening mixture (E), compared with untreated control (A).

insoluble in water. It also demonstrated that the NLC possessed properties of hydrophilicity and water solubility. It may be due to the diminutive particle size that granted the NLC to have a favorable surface wettability [15]. Figure 2 shows the mean particle size and PDI of NLC formulation evaluated by DLS after production and during a storage period of up to 45 days at room temperature ($20 \pm 7^{\circ}C$). In 45 days, the mean particles' sizes of all samples were in the range of 550–680 nm. The mean particle size did not change significantly in 45 days' storage. PDI indicates the width of the particle size distribution, which ranges from 0 to 1 while monodisperse populations yield theoretically a PDI of 0. PDI values of samples were lower than 0.3 in all measurements, suggesting that the prepared skin whitening mixture-loaded NLC had a good stability in the long-term storage.

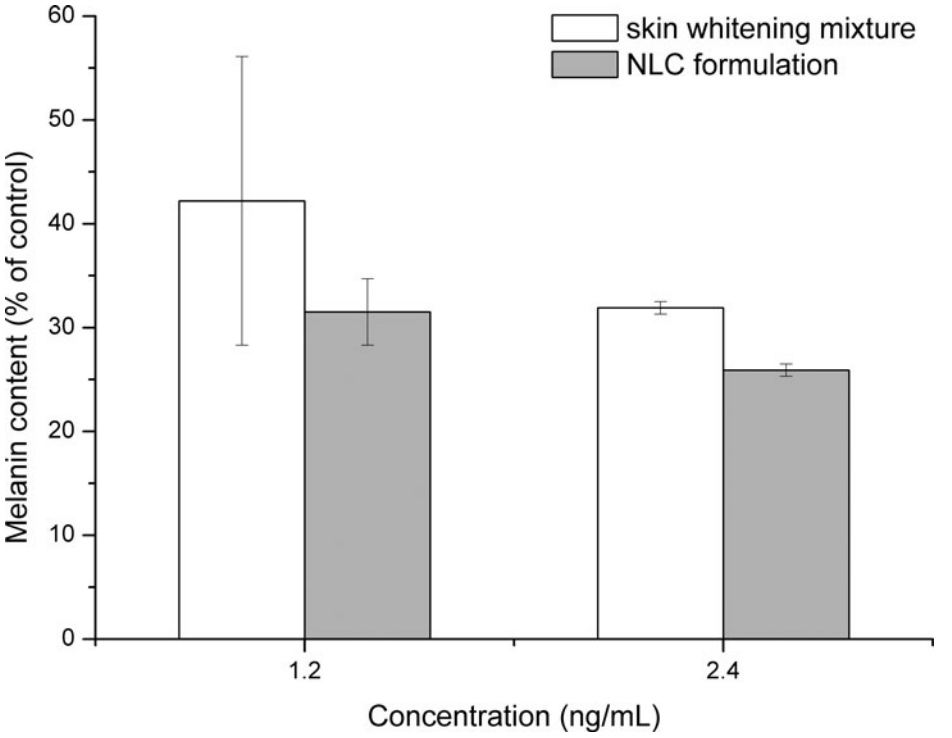

Figure 6. The melanin content of B16F0 melanoma cells after 3 day incubation with skin whitening mixture, and NLC formulation (mean ± SD, $n = 3$).

The ZP gives information about long-term physical stability of colloidal dispersions. In general, particle aggregation is less likely to occur with charged particles (high ZP) due to electrical repulsion [16]. In this research, ZPs of the NLC have been observed immediately after preparation. The ZP was roughly in the range from −61.1 to −69.7 mV for NLC formulation (Figure 3). From the literature, a minimum ZP of higher than −60.0 mV is required for excellent physical stability [16]. The NLC formulation possessed a high ZP and remained unchanged (not shown) during the 45 days of storage suggesting good long-term physical stability.

Atomic Force Microscope (AFM) Analysis

The stability of the NLC formulation could be manifested via particle morphology imaging from another aspect. From the result of AFM measurement (Figure 4), we could see that the particle had a regular spherical shape and did not stick to each other. AFM image of monodisperse NLC formulation corroborated the uniform size distribution with very low PDI. The size of the NLC formulation was slightly larger than that found using DLS. Two reasons [12] may account for the difference: the NLC was not perfectly round but the model for DLS measure was, which may result in bias to the DLS calculating method; it was measured in aqueous circumstance by the DLS, while the sample prepared for AFM was air-dried. The loss of water would be considered as another reason.

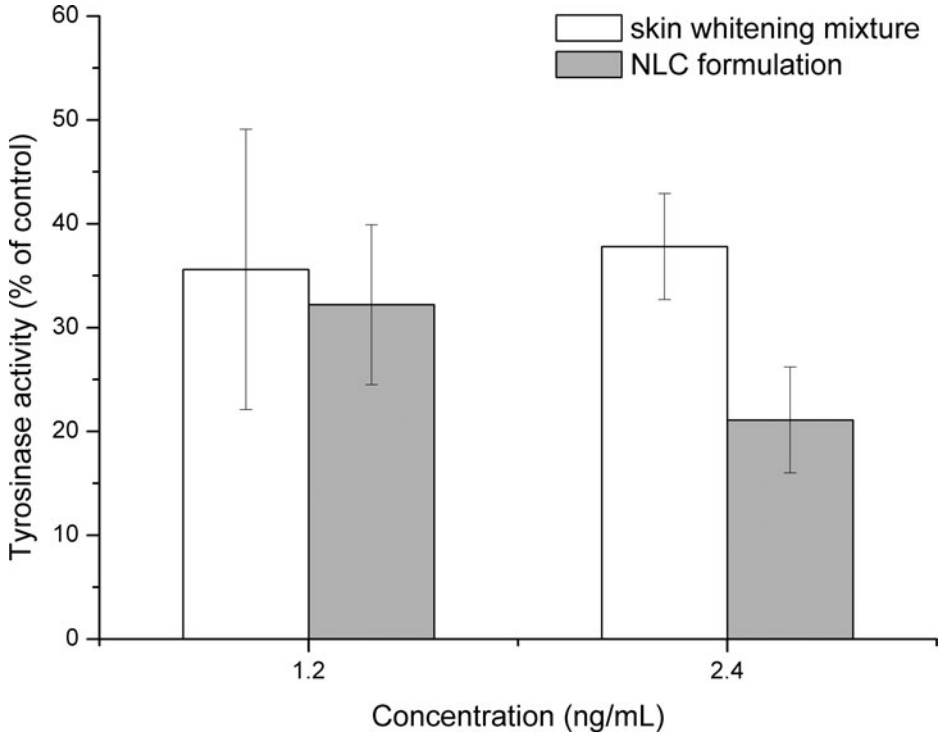

Figure 7. The tyrosinase activity of B16F0 melanoma cells after 3 day incubation with skin whitening mixture, and NLC formulation (mean ± SD, $n = 3$).

Melanin Measurement

Representative electron microscopic figures of B16F0 melanoma cells after 3 day incubation with skin whitening mixture, and NLC formulation, compared with untreated control, are shown in Figure 5. Large numbers of melanosomes were observed in untreated control cells. Cells incubated with skin whitening mixture and NLC formulation had a lower percentage of highly melanized mature melanosomes.

As shown in Figure 6, both skin whitening mixture and NLC formulation inhibited melanogenesis in B16F0 melanoma cells. The samples displayed a stronger melanin synthesis inhibition activity at the higher concentration (2.4 ng mL^{-1}) than the lower treatment concentration (1.2 ng mL^{-1}). Comparing with skin whitening mixture, NLC formulation exhibited higher ability to inhibit the synthesis of melanin. This could be attributed to the higher fusion efficiency of NLC formulation. The NLC formulation might enhance the incorporation of phenylethyl resorcinol and THC into melanoma cells. On the other hand, the NLC formulation was composed of various lipids that also possibly influenced the skin whitening effect.

Tyrosinase Assay

Melanogenesis is known to be controlled by an enzymatic cascade, which is regulated by the level of tyrosinase [14 , 17]. As shown in Figure 7, both skin whitening mixture and NLC formulation inhibited tyrosinase activity. These data suggested that the skin whitening

mixture could initiate the reduction of the deposition of melanin through the inhibition of tyrosinase. The NLC samples displayed a stronger tyrosinase inhibition activity at the higher treatment concentration than the lower concentration. However, the skin whitening mixture samples showed the opposite result. This may be because the quantity of skin whitening mixture was high enough. The extra sample may make the inhibition of tyrosinase activity less effective. The inhibitory effect of NLC formulation on tyrosinase activity was stronger than that of skin whitening mixture. Therefore, the NLC formulation was important for obtaining the enhanced antityrosinase activity of two skin whitening ingredients. The inhibitory activity of samples did not increase even when the treatment concentration was increased up to 12 ng mL^{-1}. This occurred because the inhibition activity of samples at 1.2–2.4 ng mL^{-1} was high enough to inhibit the melanogenesis and tyrosinase activity continuously.

Conclusion

In summary, in this study, the NLC encapsulating phenylethyl resorcinol and THC was developed to increase the skin whitening activity and aqueous solubility of these two ingredients. Satisfactory results were obtained from the study. Relevant data showed that samples were kept stable for at least 45 days. The solubility of two skin whitening ingredients through the preparation of NLC was increased. Improving inhibitory effects on tyrosinase activity and melanin synthesis were obtained, when applying the NLC formulation. Furthermore, this study indicated that NLC is potent and can be used as appropriate carrier for skin whitening agents.

Acknowledgments

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