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# Skin Absorption and Physical Property of Ceramide-added Ethosome

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# 세라마이드 함유 에토좀의 물성과 피부흡수

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**Abstract** : In order to delivery biotin to skin, ethosomes containing both biotin and ceramide were researched by using high pressure homogenizer. Biotin was utilized as a drug and ceramide NP was utilized as a supporter of bilayer. The biotin was entrapped in aqueous core, while ceramide NP was packed in the bilayer of the ethosomes. Looking at the physical properties of vesicles containing ceramide NP, the sized was 80～130 nm, the polydispersity index was 0.09～0.16, and the zeta potential was −40～−49 mV. In vesicles without ceramide NP, the size was 124.80±1.46 nm, and the zeta potential and polydispersity index were −45.48±1.27 mV and 0.088±0.018, respectively. Therefore, the ethosome with ceramide NP has improved physical properties of vesicles compared to the ethosome without ceramide NP. Skin absorption rates of ethosomes with ceramide NP were 6.13～14.98%, while skin absorption rate of ethosome without ceramide NP was 7.08% at 12 h. In conclusion, ethosomes containing ceramide NP not only improved the skin absorption efficiency, but had also a positive effect on the stability of vesicles.

*Keywords : Ethosome, Ceramide NP, High pressure homogenizer, Zeta potential, Skin absorption rate*

**요 약** : 바이오틴을 피부에 전달하기 위해 고압균질기를 사용하여 바이오틴과 세라마이드를 모두 함유하는 에토좀에 대하여 연구하였다. 바이오틴이 주된 성분으로 사용되었으며, 세라마이드 NP는 인지질 이중층의 지지체로 활용되었다. 바이오틴은 수용성 내부에 포획되었고, 세라마이드 NP는 에토좀의 이중층에 흡착되었다. 세라마이드 NP를 함유한 에토좀의 물성을 살펴보면 소포체의 크기는 80～130 nm,

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다분산지수는 0.09～0.16, 제타 전위는 -40～-49 mV로 측정되었다. 세라마이드 NP가 없는 소포체의 크기는 124.80±1.46 nm, 다분산지수와 제타전위는 각각 0.088±0.018과 −45.48±1.27 mV이었다. 따라서 세라마이드 NP가 함유된 에토좀은 세라마이드 NP가 없는 에토좀에 비해 소포체의 물리적 특성이 개선됨을 알 수 있었다. 세라마이드 NP를 함유한 에토좀의 피부흡수율은 12시간 후 6.13～14.98%이었으며, 반면에 세라마이드 NP가 없는 에토좀의 피부흡수율은 7.08%이었다. 결론적으로 세라마이드 NP를 함유한 에토좀은 피부흡수 효율을 향상시킬뿐만 아니라 소포체의 안정성에도 긍정적인 영향을 미쳤다.

*주제어 : 에토좀, 세라마이드 NP, 고압균질기, 제타전위, 피부흡수율*

## 1. Introduction

A variety of cosmetic goods are supplied in the cosmetic market. Creams, ampoules, lotions, serums, etc. are used to deliver the active agent to the deep skin and protect against external irritation. However, these cosmetic formulations have limitations in epidermal penetration because the particle size of each emulsion is too large to do this, and the membrane's flexibility is poor. Also, active agents can be vulnerable to react with external stimuli and then to convert to other materials. Keratinocytes play similar roles in impeding absorption as they protect the skin from external stimuli as skin barriers[1]. Nano-structure can delivery specific drugs to deep dermal skin as their particle sizes are smaller than cell composed of skin. These formulations can especially be absorbed deeply into the skin[2]. Thus, skin absorption of drug could be improved by creating the particle size smaller[3] or increasing of the softness of the particle's membrane[4].

To begin with, one of applicable alternatives of cosmetic formulation is liposome which is vesicle to possibly pass through the epidermal layers. Liposome has characteristics of be biocompatible and biodegradable in human skin due to composition of lecithin and cholesterol. In addition, the liposome makes it possible to encapsulate various drugs. The liposome is composed of aqueous parts and lipophilic bilayer. Thus, hydrophillic groups can be entrapped in the core and lipophilic groups are entrapped in the bilayer of liposome. However, if the particle size is small, the bilayer of liposome is more rigid because the smaller the size of the liposome, the narrower the distance between the phospholipids in the liposome. The following feature has a negative effect on penetration in dermal environment[5,6]. However, ethosome, an ehanced model of liposome by addition of ethanol, has more flexible bilayer than the liposome[7].

Ceramides, known as sphingolipids, are built by amide bond with link between a sphingoid base and a fatty acid chain. As the two chains both have diverse lengths and extra functional groups, there are diverse subtypes of ceramides. Ceramides are most plentiful extracellular lipid component, accounting for 40～50% (w/w) of the total extracelluar lipids [8]. Ceramide account for about 50% of the intercellular lipid mass and are known to play an important role in damaged skin[9]. In skin irritation studies, a significant reduction of ceramides was observed in lesions as well as in normal skin, affected by skin diseases such as atopy, psoriasis, and LPS due to inflammation caused by chemicals. Transepidermal water loss by the decrease of exogeneous ceramides has been reported[10].

Biotin, known as vitamin H or vitamin B7, is a water-soluble vitamin of B-complex. It is composed of an ureido (tetrahydroimidazolone) ring fused with a tetrahydrothiophene ring.

Biotin is often recommended as a dietary supplement for strengthening hair and nails [11]. It is popular with consumers who like healthy hair and nails, and it is also relatively inexpensive[12].

In this paper, the purpose of the research was to study more desirable skin absorption and stable cosmetic formulations using ethosome containing ceramide NP. It has been used to make more stable ethosome by utilizing the cholesterol[13,14]. Also, when preparing ethosome, both addition of ceramide and the high pressure homogenization[15-17] helped to generate nano-sized and homogeneous ethosome formulation[18]. In this research, drug was biotin that was dissolved in aqueous part and arginine was used in order to facilitate solubility of the biotin in deionized water. Thus, by utilizing those chemical compounds and process, the aim of this study researched the ethosome containing ceramide which was stable and high skin absorption efficiency.

## 2. Materials and methods

### 2.1. Materials

Hydrogenated lecithin (Lipoid S75-3, Lipoid GmbH, Germany), used as a surfactant, was purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (Cholesterol JP, Nippon Fine Chemical Co. LTD, Japan), ceramide NP (DS-Ceramide Y30, Doosan Solus Advanced Materials, Korea), anhydrous ethanol (95~96%, Daejung, Korea), biotin (HPLC grade, Sigma Aldrich, USA), and arginine (L-arginine, Ajinomoto, Japan), ethylhexylglycerin (Saskine 50, Sachem Corporate, USA) were used. Deionized water (< 0.1 $\mu$S/cm) was manufactured by instrument (EXL1 Analysis, Vivagen, Korea). All chemicals not mentioned used an analytical grade.

### 2.2. Preparation of Primary Ethosome

For preparing primary ethosome, water part and oil part were prepared. First, hydrogenated lecithin, cholesterol, ceramide NP, and ethanol were weighed as shown in Table 1 for oil part. In order to prepare a water part, biotin, arginine, ethylhexylglycerin, and water were also weighed as shown in Table 2.

Weighed oil part and water part were heated at 70~75℃. The oil part was added into the water part slowly and the mixed material emulsified at 2,500~3,000 rpm for 5 min by using a homomixer (Homogenizing mixer Mark Ⅱ Model 2.5, PRIMIX, Japan). After 5 min, the primary ethosome was cooled at 40~45℃ and air bubble in the primary ethosome was eliminated by utilizing an instrument of defoaming (Eyela A-1000S, Eyela, Japan).

### 2.3. Preparation of nano-sized ethosome

In order to prepare nano-size ethosome, the primary-ethosomes in Chapter 2.2. were passed 2 times at 800 bar in high-pressure nanodisperser (NLM 100, Ilshin Autoclave, Korea). When the vesicles passed nanodisperser, particles were shrunk and converted heterogeneous dispersity to homogeneous dispersity due to impact, shear force and cavitation.

### 2.4. Measurement of particle size and polydispersity index

The particle size and polydispersity index (PDI) of ethosome were evaluated by dynamic light scattering[19,20]. The ethosome was diluted to 10% by water in vial. The sample was mixed for 30 s by vortex instrument (Vortex Genie 2, Scientific Industries, Bohemia). Cells of the zetasizer were filled with ethosome suspension. The particle size and PDI of samples were measured in the nano-zetasizer (Nano-ZS, Malvern Panalytical, England) at 25℃. Its software used Zetasizer software and the measurement of both particle size and PDI used Stokes-Einstein equation.

Table 1. The weight percent of oil and water part in the ethosome

| | Materials | #1-1 | #1-2 | #1-3 | #1-4 | #1-5 | #1-6 | Control |
|---|---|---|---|---|---|---|---|---|
| Oil part | Hydrogenated lecithin | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | - |
| | Cholesterol | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | - |
| | Ceramide NP | - | - | 0.15 | 0.3 | 0.45 | 0.6 | - |
| | Ethanol | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 |
| Water part | Biotin | - | 0.1 (Drug) | | | | | |
| | Arginine | 0.06 | | | | | | |
| | Ethylhexylglycerin | 0.1 | | | | | | |
| | Water | to 100 | | | | | | |

Table 2. The weight percent of oil and water part in the biotin-free ethosome

| | Materials | #2-1 | #2-2 | #2-3 | #2-4 | #2-5 | #2-6 |
|---|---|---|---|---|---|---|---|
| Oil part | Hydrogenated lecithin | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| | Cholesterol | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | - |
| | Ceramide NP | - | 0.15 | 0.3 | 0.45 | 0.6 | 0.6 |
| | Ethanol | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 |
| Water part | Arginine | 0.06 | | | | | |
| | Ethylhexylglycerin | 0.1 | | | | | |
| | Water | to 100 | | | | | |

### 2.5. Measurement of zeta potential

The zeta potential was measured in the nano-zetasizer (Nano-ZS, Malvern Panalytical, England) at 25℃. Each 10% diluted samples was put into the zetasizer cell. The software of the instrument was Zetasizer software and the data processing was conducted by principle of Smoluchowski's equation.

### 2.6. Measurement of pH

The pH of ethosome was confirmed at 25℃ by pH meter (Orion Star A111, Thermo Scientific, USA). The ethosome was diluted by water and the ratio was sample of ethosome : water = 2 : 30 (w/w) in conical tube. The sample was mixed for 30 s by vortexing (Vortex Genie 2, Scientific Industries, Bohemia).

### 2.7. Image of nano-sized ethosome

All image of sample was pictured in tabletop darkroom by Smart Phone (iPhone SE2, Apple, USA). The photo was taken a day, a week, two weeks, three weeks, and four weeks after preparation of ethosome at 25℃. The image of ethosome samples after 4 weeks was only showed in Fig. 1 and 2. Pictures of other samples were not shown in this paper.

### 2.8. Franz diffusion cell experiment of nano-sized ethosome

The skin absorption efficiency of biotin was processed by Franz diffusion cell experiment. The Franz cell diffusion is processed by DHC-6TD (Logan Instruments, USA). First, receptor part was 50% ethanol (ethanol : water = 1:1 (v/v)). It was filled into receptor part in Franz diffusion cell instrument. Artificial skin membranes (Strat-M membrane 25 mm discs, Merck Millipore, USA) were mounted onto Franz diffusion cells and the donor parts cap also were put above membrane. The membranes were stabilized for 20 min by stirring the magnetic bar in the receptor part at 37±1℃. Second, 400 μL of

ethosome samples were applied on the skin membranes and allowed to spread over the membranes. The solution in the receptor parts was taken after 4 h, 8 h, 12 h, and 24 h, respectively[21]. The donor parts were withdrawn and diluted with 0.05% phosphoric acid (v/v) (solvent : methanol). Each skin membrane was cut into 8 pieces and was put into conical tube with 0.05% phosphoric acid (v/v) (solvent : methanol). The remaining donor and receptor samples were sonicated for 1 h (Ultrasonic cleaner JAC-3010, Kodo, Japan) to destructure the bilayer of ethosome. All samples were analyzed by HPLC (2695, Waters, USA) equipped with PDA detector (2998, Waters, USA). Method of HPLC was illustrated in the Table 3. Skin absorption of #1-1 sample was not conducted because of biotin-free ethosome.

### 2.9. Encapsulation efficiency of nano-sized ethosome

The method of encapsulation efficiency is referred from H. J. Gwak et al.[22], and C. K. Kim[23]. The ethosome samples were centrifuged (21,000 ×g, 4℃, 3 h) by ultracentrifuge instrument (Varispin 15R multi centrifuge, Novapro, Korea) to evaluate encapsulation efficiency of biotin in ethosome aqueous core. After separating the ethosomes into supernatant and lower layers, each of 100 μL supernatant was sampled. Each supernatant was diluted with 10 mL of 0.05% methanol and sonicated for 3 min. These were analyzed by HPLC and the method was shown in the Table 3. The formula for calculating the encapsulation efficiency is to divide the difference between the amount of biotin in the total ethosome sample and the amount of biotin in the supernatant by the amount of biotin in the total ethosome sample. Encapsulation efficiency experiment of #1-1 sample was not conducted because of biotin-free ethosome.

$$EE\ (\%) = \frac{C_t - C_s}{C_t} \times 100\ (\%)$$

$C_s$ = Amount of biotin in supernatant (μg)
$C_t$ = Amount of biotin in total liposome sample (μg)

### 2.10. Statistical analysis

Experiments were repeated three times and data were presented as mean±standard deviation (SD). All data of experiment were processed by the one-way ANOVA task in SAS program (version 9.4, SAS Institute, USA) and significance was acknowledged for p values less than 0.05.

Table 3. Method of biotin for HPLC with PDA detector

| System | Condition | |
|---|---|---|
| Column | Kromasil C18 column, 5 μm, 4.6×250 mm (AkzoNobel, Netherlands) | |
| Temperature of column and sample | 40℃ and 25℃ | |
| Mobile phase | A : acetonitrile<br>B : 0.02% phosphoric acid (solvent : water) | Isocratic |
| | A : B = 15 : 85 | |
| Flow rate | 1.0 mL/min | |
| Injection volume | 10 μL | |
| Detector | Photodiode Array | |
| Wavelength | 200 nm | |

## 3. Results and Discussion

### 3.1. Physical characteristics of ethosome

The ethosome samples were stored at room temperature (25℃) for a month. One day after preparing the ethosomes, the physical properties were measured with a nano-zetasizer. #1-1 was biotin-free and ceramide-free ethosome. Its particle size was 112.12±1.40 nm, zeta potential was −68.85±3.84 mV and PDI was 0.272±0.001. #1-2 was added only biotin except for ceramide NP. Its particle size was 124.80±1.46 nm, zeta potential was −45.48±1.27 mV and PDI was 0.088±0.018. #1-3∼6 were added with biotin and ceramide NP. As the number of samples increased, the amount of ceramide were increased respectively. Their particle size, zeta potential, and PDI were clarified in Table 4. The PDI of all was lower than 0.300. It refered that #1 ethosomes were formed in homogeneous vesicles. Absolute values of zeta potential for all samples were higher than −40 mV. The stability of all vesicles was expected to be good.

As the amount of ceramide NP increased, the particle size of ethosome decreased. paritlce size of #1-6 was the smallest among the samples. The appearance of ethosome samples were showed after 4 weeks in Fig. 1. It seems that ceramide NP played a similar role in bilayer of ethosome like cholesterol. The cholesterol has been studied to be trapped in a bilayer composed of phospholipids. Ceramide

Table 4. Appearance, pH, particle size, PDI, and zeta potential of #1 and #2 samples (25℃, 1 day, mean±SD, n=3)

| No. | Feature* | Appearance | pH | Particle size (nm) | Polydispersity Index | Zeta potential (mV) |
|---|---|---|---|---|---|---|
| #1-1 | ceramide, biotin | translucent | 9.29±0.04 | 112.12±1.40 | 0.272±0.001 | −68.85±3.84 |
| #1-2 | ceramide | pinkish opaque | 5.69±0.04 | 124.80±1.46 | 0.088±0.018 | −45.48±1.27 |
| #1-3 | - | pinkish opaque | 5.52±0.03 | 127.18±1.13 | 0.098±0.019 | −46.40±1.50 |
| #1-4 | - | pinkish translucent | 5.47±0.02 | 102.82±1.17 | 0.107±0.015 | −48.65±4.67 |
| #1-5 | - | pinkish translucent | 5.56±0.01 | 88.55±0.82 | 0.157±0.007 | −43.05±1.85 |
| #1-6 | - | pinkish translucent | 5.38±0.01 | 74.61±0.75 | 0.161±0.006 | −49.23±1.52 |
| #2-1 | ceramide, biotin | off-white translucent | 9.39±0.03 | 121.87±0.47 | 0.224±0.010 | −62.83±1.26 |
| #2-2 | biotin | translucent | 9.30±0.02 | 79.67±0.09 | 0.485±0.006 | −65.53±0.85 |
| #2-3 | biotin | translucent | 9.23±0.02 | 57.97±0.54 | 0.436±0.009 | −62.03±0.65 |
| #2-4 | biotin | translucent | 9.22±0.04 | 66.91±0.54 | 0.458±0.006 | −61.17±1.50 |
| #2-5 | biotin | translucent | 9.26±0.01 | 61.60±0.39 | 0.445±0.002 | −60.47±0.93 |
| #2-6 | cholesterol, biotin | translucent | 9.26±0.03 | 83.37±0.53 | 0.224±0.008 | −56.60±0.82 |

*materials not included in #1 and #2 samples

NP were also entrapped between phospholipids not at the tail end of the phospholids in the bilayer[24]. Therefore, it is suggested that ceramide NP is effective in reducing the particle size of vesicles.

Samples #2 were ethosomes which contained ceramide NP without biotin in the aqueous portion. When comparing #2-1 with the other #2-2~6, the particle size of #2-1 without ceramide NP was larger than the other samples. It alluded that prescription of ceramide NP helped particle size of vesicles to reduce. The values of PDI of #2-1 and #2-6 were lower than 0.300. It means that the particle size of the vesicles were homogeneous form. In contrast, the PDI of the other samples was higher than 0.300 and meaning heterogenous form. Absolute value of zeta potential of all samples were higher than -40 mV. #2-6 was ethosome which did not have cholesterol. The data from #2-6 showed that the particle size was less than 100 nm, the value of PDI was 0.224±0.008, and the measured zeta potential was -56.60±0.82 mV. The appearance of #2 samples were showed after 4 weeks in Fig. 2. Ethosome was stablized by containing ceramide NP without cholesterol. It indicates that ceramide NP is utilized as a bilayer supporter instead of cholesterol.

### 3.2. Physical changes of the ethosomes over time

The physical changes of the ethosomes were analyzed with a nano-zetasizer at 25 ℃ for 4 weeks as shown in Fig. 3. The particle size of #1-1 tended to decrease over time. This is because that the biotin and ceramide NP were no addition in #1-1. Ceramide NP is located in the phospholipid bilayer and biotin is located in the aqueous part of the ethosomes. As over time, it indicates that the aqueous part of ethosome was leaking. Thus, the particle size of ethosome was waning at 25℃ in accordance with time. Contrast to #1-1, particle sizes of #1-2~6 samples were swelling over time. Firmness of bilayer structure of vesicles was declining to release the drug. Therefore, this tendency illustrates that the formulation of vesicles was able to apply in drug delivery system.

In the case of the zeta potential, it was found that the stability was maintained as it was greater than the absolute value of 40 even

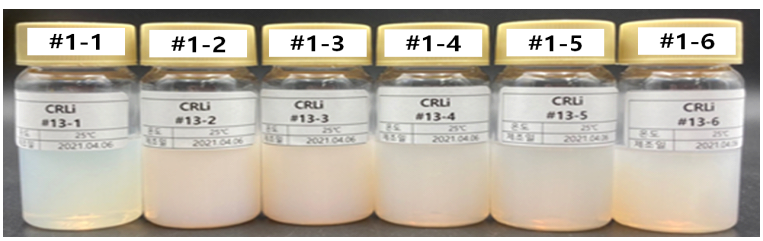


Fig. 1. Appearance of #1-1~6 at 25℃ after 4 weeks.

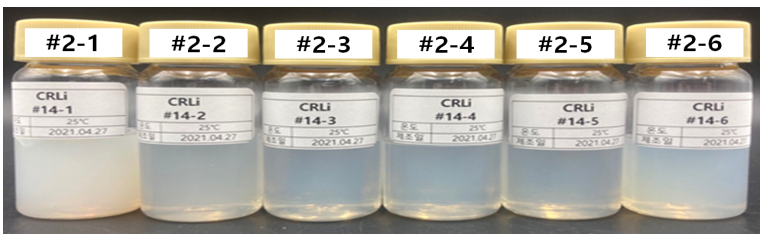


Fig. 2. Appearance of #2-1~6 at 25℃ after 4 weeks.

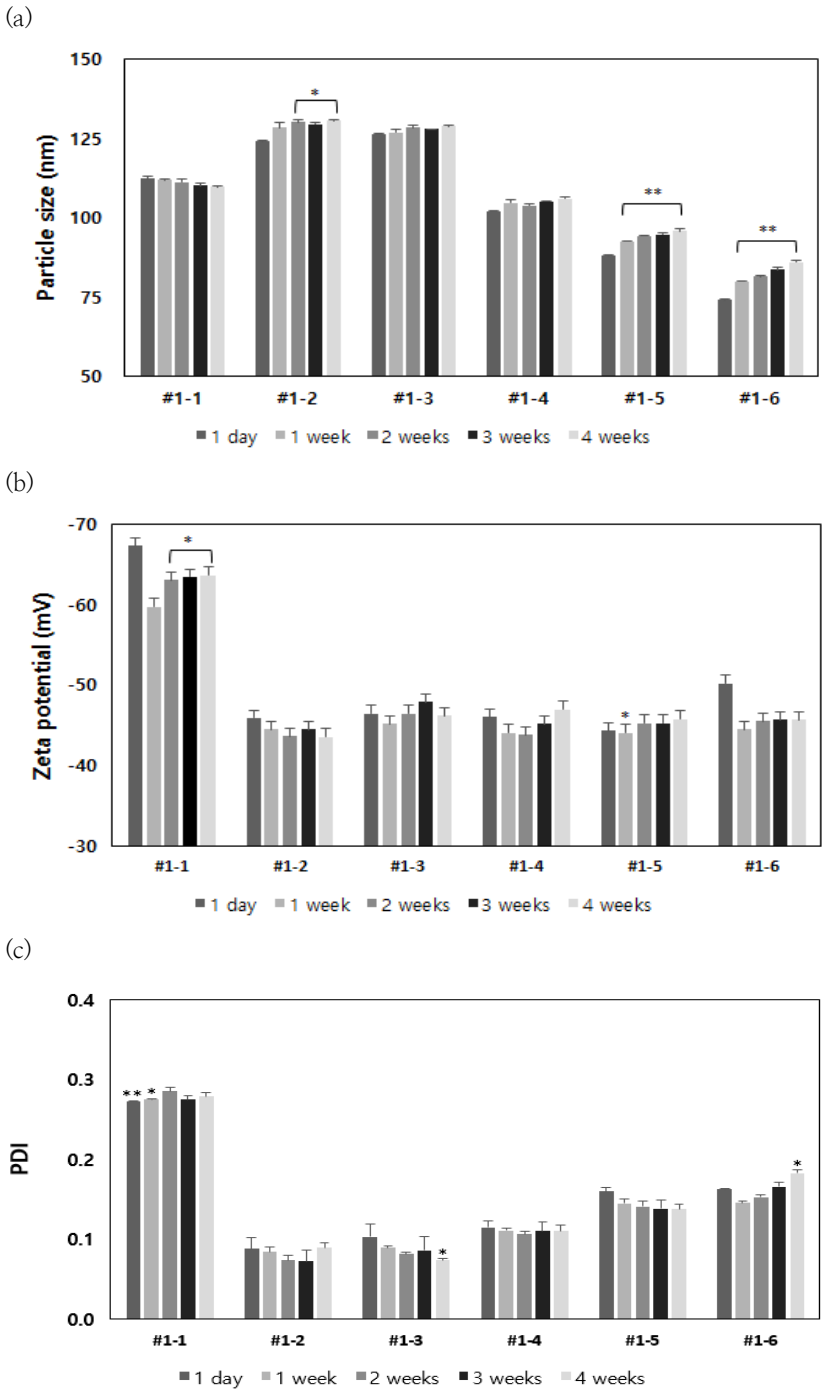


Fig. 3. Physical propertiy changes in #1 over time. The results were expressed as the mean±SD (n=3), $^{*}p < 0.05$, $^{**}p < 0.01$ compared with each 1 day data; (a) particle size, (b) zeta potential, (c) polydispersity index.

after 4 weeks. When measuring the PDI after 4 weeks, all samples were not over 0.300. Hence, it is considered a formulation with a uniform particle size. As a result, the addition of ceramide NP did not significantly affect the zeta potential and PDI, whereas the addition of 0.6% ceramide NP (w/w) significantly reduced the particle size.

### 3.3. Skin absorption efficiency

The Franz diffusion cell experiment was proceeding for 24 h in order to measure skin absorption efficiency of biotin. #1-1 was a biotin-free ethosome, so Franz diffusion cell experiment was only not proceeding.

The skin absorption efficiencies of # 1-2 to 6 were performed for 24 hours by the Franz diffusion cell method as shown in Fig. 4. There was no significant difference in the skin absorption efficiency of each sample for 0-8 hours. As time passed, the skin absorption rate increased. The absorption rate of #1-6 containing 0.6% ceramide NP was 14.99±0.48%, #1-2 without ceramide NP was 7.09±0.17%, and the control group was 5.79±0.15%. Sample #1-6 showed the greatest skin absorption efficiency among them. The particle size of #1-6 was 74.61±0.75 nm, the smallest compared to other samples. These results mean that the skin absorption efficiency is inversely proportional to the particle size.

### 3.4. Encapsulation efficiency

The encapsulation efficiency (EE) was measured by ultracentrifuge method. After ultracentrifuging each sample, the supernatant was analyzed by HPLC. It indicates that amount of biotin in supernatant was not entrapped in the aqueous core of ethosome. The encapsulation efficiency was marked in the Table 5. The range of EE ranges from 14 ~23%, showing that the primary homogenization and high pressure homogenization methods are not higher EE than the traditional liposome method (thin film hydration method or Bangham method). This is because the thin film hydration method involves evaporation of the organic solvent, this step motivated the drug to be encapsulated in the aqueous core of vesicles. However, the thin film hydration method uses organic solvents, therefore there are restrictions in the manufacture of cosmetics.

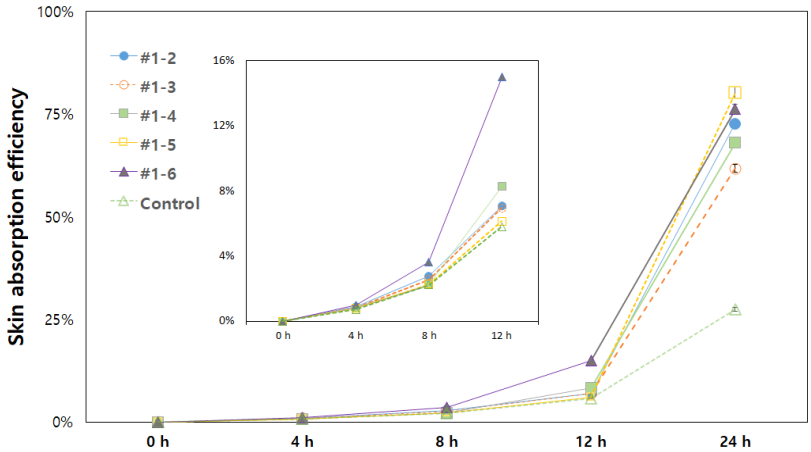


Fig. 4. Data of Franz diffusion cell experiment in accordance with time; the results were expressed as the mean±SD (n=3).

Table 5. Encapsulation efficiency of #1 by ultracentrifuge method (mean±SD, n=3)

| No. | Encapsulation Efficiency (%) |
|---|---|
| #1-2 | 14.29±0.85 |
| #1-3 | 17.58±2.09 |
| #1-4 | 14.85±0.86 |
| #1-5 | 23.59±1.53 |
| #1-6 | 23.53±1.82 |

## 4. Conclusion

The aim of this study was to confirm the stability and skin absorption rate by adding ceramide NP to the ethosomes. As a result of observing the ethosomes containing ceramide NP for 4 weeks, it was determined that the addition of ceramide NP did not adversely affect the stability of ethosomes. It was confirmed that the bilayer structure of ceramide NP as a supporter of the bilayer structure was more rigidly improved than that of ethosomes without ceramide NP, and it was also confirmed that ethosome particles decreased when the amount of ceramide NP was increased from 0 to 0.6%.

The skin absorption efficiency of the ethosome containing 0.6% ceramide NP was 14.98±0.48%, and 7.08±0.17% in the absence of ceramide NP after 24 h. It has been shown that the addition of ceramide NP to the ethosomes improves the skin absorption effect. The encapsulation efficiency of ethosomes was 14~23% by the high pressure homogenization method.

In conclusion, the ethosome containing 0.6% ceramide NP did not change to any other form after 4 weeks. The skin absorption rate of ethosomes containing ceramide NP was higher than those without ceramide NP. This indicates that utilizing the ethosomes with ceramide NP can be expected as a high drug delivery system in the cosmetic market.

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