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"A Breakthrough in Liposomal Technology: Enhanced Stability and Skin Barrier Function of Liposomes Through the Utilization of Acidic phospholipids"

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1. Introduction

The increasing global prevalence of sensitive skin, attributed to environmental factors and lifestyle changes, has driven a significant demand for effective skincare products. These formulations frequently incorporate active ingredients such as vitamin C and retinol. However, the stratum corneum, the layer of the skin, presents a barrier composed of lipids and water, impeding the efficient percutaneous penetration of these beneficial agents and consequently limiting their efficacy.

Liposomes, microvesicles primarily composed of lecithin, have garnered considerable attention as a promising approach to address this challenge. Liposomes offer the advantage of encapsulating a wide range of substances and enhancing their penetration across the skin barrier. Consequently, they have been extensively utilized as delivery systems for active ingredients within the cosmetic industry[1–2)].

Nevertheless, the structural instability of conventional liposomes remains a significant issue. Particularly under conditions of prolonged storage and exposure to external environmental factors such as temperature, liposomes are susceptible to aggregation, fusion, and leakage of their encapsulated contents, potentially compromising product quality and diminishing efficacy. Furthermore, the diversification of products within the skincare market has led to a growing demand for liposomes with enhanced added value, necessitating the development of innovative technologies in this field.

Acidic phospholipids (APs), a class of phospholipids, are essential for maintaining biological activity within the organism. They are known to contribute to the maintenance and expression

of the higher-order structures of various proteins and are recognized as crucial for sustaining biological homeostasis and preserving diverse physiological functions[3].

In this study, we aimed to develop a novel liposomal formulation containing APs at half of the total phospholipid content, with the objective of achieving superior stability and enhanced skin barrier function compared to conventional liposomes. The developed APs-rich liposome, along with conventional phosphatidylcholine-rich (PC-rich) liposome, were comparatively evaluated for their storage stability and contribution to tight junction reinforcement.

2. Materials and Methods

2.1. Preparation of Pre-mixtures and Formulations

Cosmetic-grade phospholipid (PC, phosphatidylcholine), acidic phospholipids (APs; phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid), and pentylene glycol were employed. The specific concentrations of each component in the pre-mixtures and final formulations are detailed in Table 1. To prepare the pre-mixtures, the respective lipids were added to the solution of pentylene glycol and water, followed by heating to 90 °C to ensure complete dissolution. Subsequently, the pre-mixtures, maintained at 80 °C, were diluted with water at 80 °C to achieve a final lipid concentration of 0.8% (w/v), and then cooled to form the formulations. Formulation A and Formulation B were utilized for liposome particle characterization, evaluation of the stability of each formulation, and assessment of their efficacy on a three-dimensional skin model. Formulation C served as a comparative control in the efficacy evaluation.

Reagents	Pre-mixture for Formulation A (%)	Pre-mixture for Formulation B (%)	Pre-mixture for Formulation C (%)
PC	5	10	0
APs	5	0	0
Pentylene glycol	50	50	50
Deionized water	40	40	50

Pre-mixtures were diluted to 8% (w/v) with deionized water and employed as formulations for experiments.

Reagents	Formulation A (%)	Formulation B (%)	Formulation C (%)
PC	0.4	0.8	0.0
APs	0.4	0.0	0.0
Pentylene glycol	4.0	4.0	4.0
Deionized water	95.2	95.2	96.0

Table 1. Composition of pre-mixtures and formulations (w/v)

2.2. Liposome Particle Characterization

Particle size distribution of Formulation A and Formulation B was determined using dynamic light scattering (DLS) (Zetasizer, Malvern Panalytical Ltd.). The formation of Formulation A particle was confirmed by cryogenic transmission electron microscopy (cryo-TEM). Briefly, a small aliquot of the sample suspension was deposited onto a copper TEM grid coated with a holey carbon film, held by self-locking tweezers mounted on a spring-loaded shaft within a cryo-preparation system (LEICA EM CPC, Leica Microsystems). Excess liquid was blotted with filter paper to create a thin film on the grid, which was immediately plunge-frozen in liquid ethane cooled by liquid nitrogen. The grid was then transferred to the tip of a liquid nitrogen-cooled cryospecimen holder (CT-3500, Oxford Instruments). Specimens were maintained below −170 °C and imaged using a transmission electron microscope (H-7650, Hitachi Science Systems, Ltd.) operating at an accelerating voltage of 100 kV with a low electron dose. Furthermore, the zeta potential of each formulation was also analyzed with the zeta potential analyzer (Zetasizer, Malvern Panalytical Ltd.).

2.3. Storage Stability Assessment of Formulations

Each Formulation was stored in a environment chamber at 40 °C. After a period of two months, the particle size distribution was evaluated using DLS, and the particle morphology was examined using optical microscopy.

2.4. Transepidermal Water Loss (TEWL) Measurement Using a Three-Dimensional Reconstructed Human Epidermis Model

A three-dimensional reconstructed human epidermis model, SkinEthic™ RHE model (RHE, EPISKIN Laboratories), was utilized in this study. Each well of a 12-well plate was filled with 2 mL of growth medium (SkinEthic™ Growth Medium) and incubated overnight for acclimatization. Subsequently, 150 µL of each formulation was applied topically to the stratum corneum side of the RHE and cultured at 37 °C under a 5% CO_2 atmosphere. After 24 hours of incubation, each formulation was removed, and the stratum corneum surface was washed with 0.5 mL of phosphate-buffered saline (PBS). The medium was then replaced with Hank's balanced salt solution (HBSS), and the RHE was irradiated with 100 mJ/cm² of UV-B. Immediately following UV-B irradiation, the medium was exchanged back to growth medium, followed by incubation at 37 °C under a 5% CO_2 atmosphere. After a further 24 hours of incubation, the stratum corneum surface was washed with 0.5 mL of PBS. Transepidermal water loss (TEWL) was then measured using a TEWL meter (AS-CT1: Asch Japan Co., Ltd.). TEWL values were compared between UV-irradiated and non-UV-irradiated skin models to evaluate the extent to which the formulations could inhibit water loss after UV irradiation.

2.5. Evaluation of Tight Junctions by Electrical Conductivity Measurement

Normal human epidermal keratinocytes (NHEK cells, Kurabo Industries Ltd.) were seeded at a density of 1 × 10⁵ cells per well in a 24-well plate and cultured in KG2 medium (Kurabo

Industries Ltd.) for 48 hours. Following this initial culture period, the medium was replaced with KG2 medium containing 0.3% of each formulation, and the cells were cultured for an additional 24 hours. Subsequently, the medium was removed and replaced with 30 mM HEPES buffer. The cells were then irradiated with 20 mJ/cm² of UV-B, after which the medium was immediately replaced with KG2 medium. Transepithelial electrical resistance (TEER) values were measured 24 hours later using a Millicell ERS-2 Voltohmmeter (Merck Millipore).

2.6. Quantification of CLDN1 and OCLN by Enzyme-Linked Immunosorbent Assay (ELISA)

RHE models were prepared following the same procedure as described for the TEWL measurement. After the final 24-hour incubation period with each formulation, the cultured tissues were transferred to 1.5 mL microcentrifuge tubes, and 0.5 mL of PBS was added. The cells were lysed using a TissueLyser II (QIAGEN K.K., Japan), and the resulting lysates were centrifuged. The supernatants were collected and appropriately diluted. The expression levels of Claudin-1 (CLDN1) and Occludin (OCLN) proteins were quantified using commercially available ELISA kits (CLDN1: SEC388Hu, OCLN: SEC288Hu, Clone Corp., USA) according to the manufacturer's instructions.

3. Results

3.1. Preparation of Liposome Solutions

Liposome solutions (Formulation A and Formulation B) were prepared using pre-mixtures with the compositions shown in Table 1 (Pre-mixture A and Pre-mixture B). The particle size distribution of each formulation was analyzed using DLS, and the results are presented in Figure 1. Both Formulation A and Formulation B exhibited uniform particle size distributions, with peak tops at 91.3 nm for Formulation A and 122.4 nm for Formulation B. The morphology of Formulation A particles was confirmed using cryo-TEM. As shown in Figure 2, the formation of liposomes with a size of approximately 70 nm was observed in Formulation A. The zeta potential of each formulation was also analyzed (Table 2).

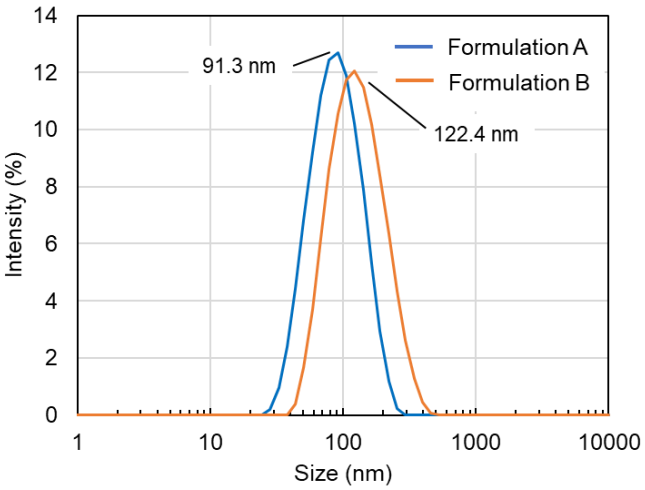

Figure 1. Particle size distribution of each formulation

Formulation A	Formulation B
−44.8 mV	−22.9 mV

Table 2. Zeta potential of each formulation

Formulation A exhibited a lower zeta potential (−44.8 mV) compared to Formulation B (−22.9 mV).

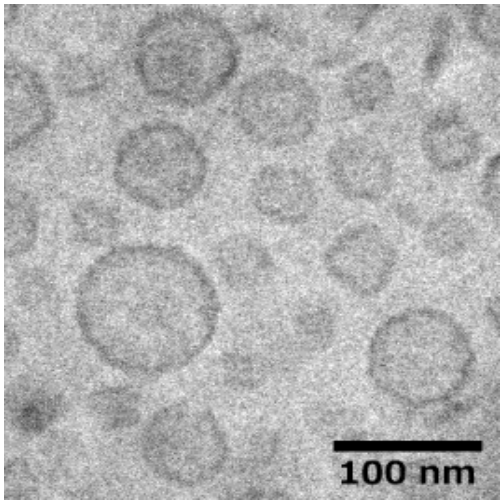

Figure 2. cryo-TEM image of formulation A

3.2. Storage Stability Assessment of Formulations

To compare the storage stability of Formulation A and Formulation B, each sample was stored at 40 °C for 8 weeks, and changes in particle size distribution and particle state were examined (Figure 3). For Formulation B, after storage resulted in the initially uniform particle size distribution becoming bimodal, indicating particle aggrigation. Furthermore, microscopic observation of Formulation B after storage revealed the presence of large particles and aggregated particles. In contrast, Formulation A showed no significant changes in particle size distribution. Microscopic observation also did not reveal any large or aggregated particles in Formulation A. These results suggest that Formulation A possesses higher storage stability than Formulation B.

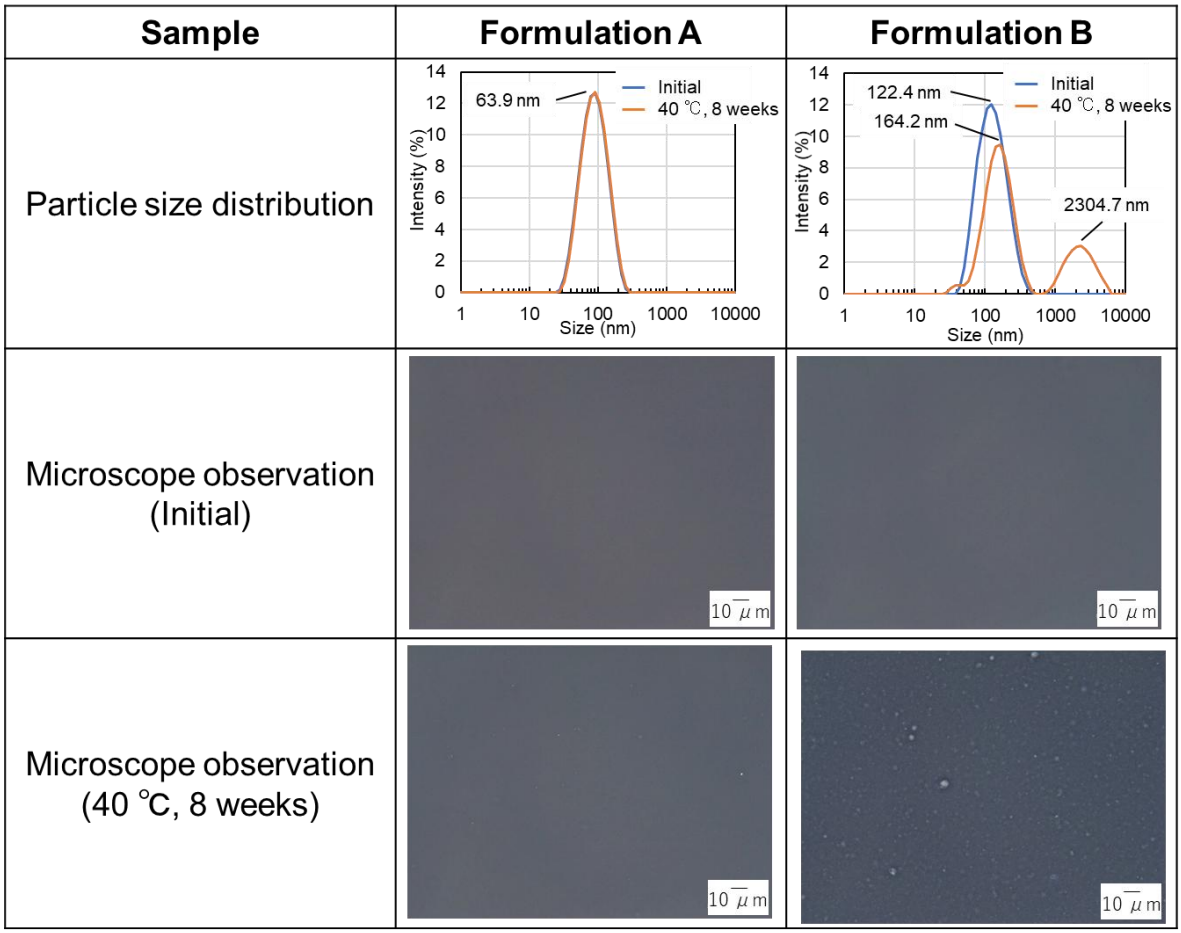

Figure 3. Particle size distributions and microscope observations on storage stability

3.3. Evaluation of the Efficacy of Each Formulation on Skin

To evaluate the skin barrier function exhibited by Formulation A and Formulation B, a three-dimensional skin model was utilized to compare the transepidermal water loss (TEWL) after application of each formulation (Figure 4). In the three-dimensional cultured skin model treated with Formulation C, which does not contain lipids, the TEWL rate after UV irradiation was 1.80. In contrast, Formulation B, containing only PC, showed a reduced TEWL rate of 1.38 compared to Formulation C. Formulation A, containing AP and PC at a 1:1 ratio, exhibited the most significant reduction in TEWL rate, with a value of 0.92.

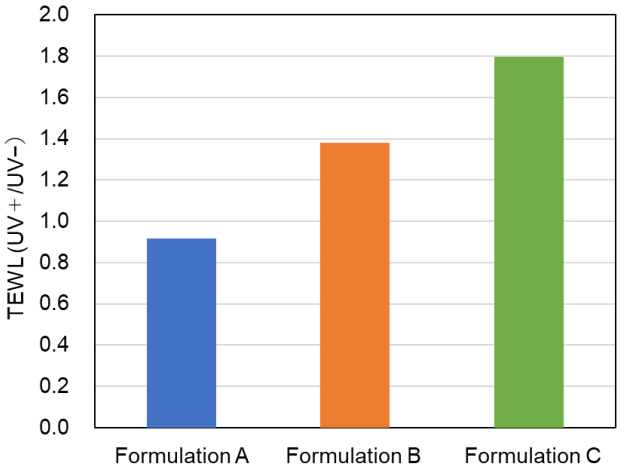

Figure 4. TEWL after application of each formulation

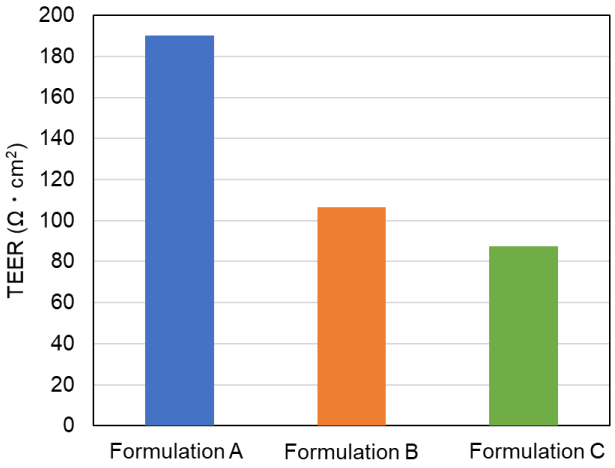

Figure 5. TEER after application of each formulation

Furthermore, to conduct a more in-depth evaluation of skin barrier function, the transepithelial electrical resistance (TEER) values were compared in an NHEK cell model after application of each formulation. TEER values are known to correlate with the barrier strength of tight junctions in the epithelium, with higher TEER values suggesting the formation of more robust tight junctions [4]. The results of TEER measurements 24 hours after application of each formulation are shown in Figure 5. Application of Formulation A significantly increased TEER values, indicating a significant enhancement of tight junctions in the NHEK cell model.

To elucidate the mechanism by which Formulation A enhances tight junctions, the expression levels of proteins forming tight junctions were evaluated using a three-dimensional cultured skin model. Tight junctions are cell adhesion complexes that fill the intercellular space in the epidermis, the outermost layer of the skin, and play a crucial role in preventing water loss. Their main structural components are Claudin-1 (CLDN1) and Occludin (OCLN) [5-6]. Therefore, the expression levels of CLDN1 and OCLN in the three-dimensional cultured skin model after UV irradiation were compared using ELISA (Figure 6). Regarding CLDN1 expression, a significant increase in expression was observed only in Formulation A after UV

irradiation. In contrast, no significant difference in OCLN expression was observed. These findings suggest that Formulation A, which contains a high concentration of APs, promotes CLDN1 expression in the epidermal cell layer, thereby enhancing tight junction function.

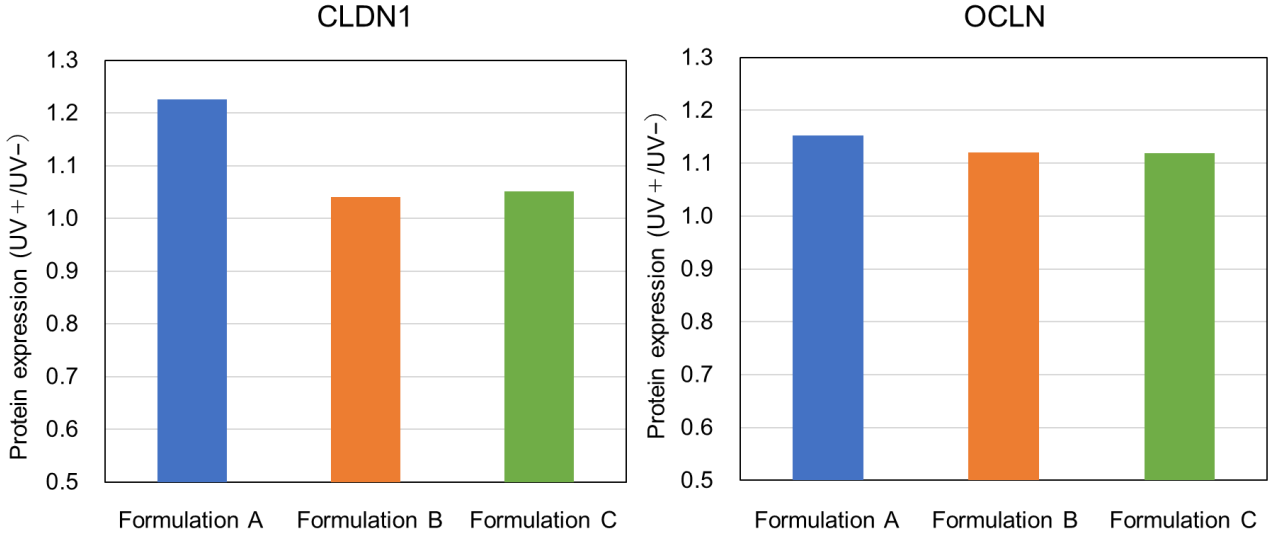

Figure 6. Protein expression after application of each formulation

4. Discussion

The results of the storage stability tests for each formulation demonstrated that Formulation A possesses high stability, capable of withstanding storage at 40 °C for 8 weeks. Formulation A contains negatively charged APs, leading to a more negative surface charge compared to Formulation B (Table 2). It is hypothesized that this negative surface charge results in electrostatic repulsion, contributing to its high dispersion stability. Furthermore, the efficacy test results revealed that Formulation A, which contains a higher concentration of APs, increased the expression level of CLDN1, a key component of tight junctions, in epidermal cells. This increase in CLDN1 expression was associated with enhanced tight junction integrity. While APs are essential for maintaining various physiological activities and are known to contribute to the maintenance and expression of higher-order structures of various proteins, their impact on tight junctions has not been previously established. Further research is warranted to determine whether the efficacy observed with Formulation A is attributed to the effects of APs themselves or the liposomal formulation.

5. Conclusion

We successfully utilized APs in liposomes, resulting in a novel liposomal formulation with significantly higher physical stability and remarkable tight junction formation-promoting activity compared to conventional phospholipid formulations. Notably, the CLDN1 expression-promoting effect of this formulation is considered crucial for repairing damaged skin barriers

and protecting against external stimulation. This innovative technology offers a promising new option for gentle and effective care for the skin of modern individuals, which tends to be sensitized by environmental changes and stress. Future prospects include combination with active ingredients and application to various formulation formats, with the expectation of benefiting a broader consumer base.

6. Acknowledgment

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7. References

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