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Ceramide 3 Effect on the Physical Properties of Ambora Extract and Chromabright-Loaded Transethosomes

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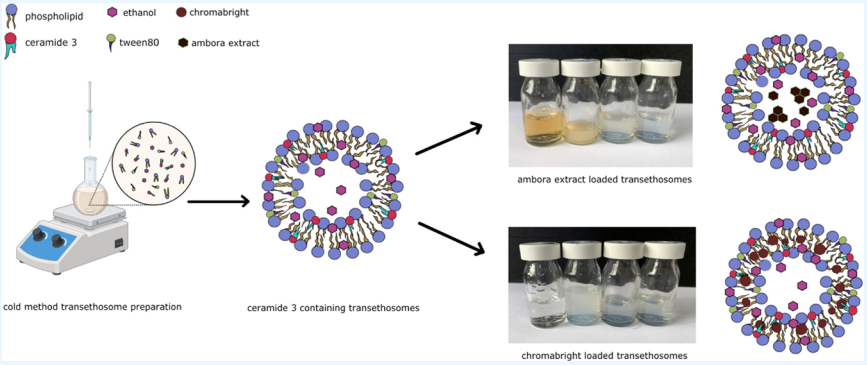

ABSTRACT: Spontaneous self-assembly of phospholipids into lipid vesicles in aqueous media is called liposomes, and these structures are widely used as nanocarriers in the cosmeceutical industry. Transethosomes are ethanol and edge activator-containing liposomes that are proven to be very effective in topical applications for penetrating the skin barrier. Many cosmeceutical products contain formulations with ceramides to restore the skin barrier and treat eczema. However, due to the low solubility and penetration ability of the ceramides, the effectiveness of these products is limited. In this study, a transethosome formulation containing ceramide 3 (Cer 3) was achieved by introducing varying concentrations of cholesterol and an edge activator (Tween 80) to improve the effect of the skin products used to treat eczema. The obtained transethosomes were examined in terms of size, homogeneity, zeta potential, morphology, and one-month stability. Loading capability experiments were carried out with lipophilic Chromabright and hydrophilic Ambora extract. The effect of Cer 3 on the loading of the selected payloads was evaluated. Data were analyzed statistically with linear regression analysis and two-way analysis of variance. The results showed that the inclusion of Cer 3 had almost no effect on the physical properties of the loaded or empty transethosomes. Independently of the presence of Cer 3, loading of the lipophilic compound was more efficient than that of the hydrophilic one.

1. INTRODUCTION

Human skin is the vital organ that acts as the main barrier for protection against outer agents because of its three-layered structure: epidermis, dermis, and hypodermis. The outermost protective barrier in the epidermis is known as stratum corneum, SC. The function of the skin barrier is basically characterized by SC lipids such as ceramides, cholesterol, and fatty acids, and the ratio between these lipids determines the skin integrity and health. It has been reported that disorganization in ceramides, fatty acids, and cholesterol ratio causes skin impairments such as eczema. Cer 3, or N-acylated phytosphingosine, is the main ceramide subtype found in healthy skin, and decreased levels of this molecule cause eczema symptoms.[1−3] Cer 3 is used in a broad variety of cosmeceutical products because of its known efficiency in improving skin barrier function and reducing the risk of transepidermal water loss as well as being a restoring lipid in eczema treatment.[4,5] In the cosmeceutical industry, many brands like Triple Lipid Restore, SkinCeuticals, Avène, and Dr. Jart+ develop formulations containing ceramides with fatty acids and cholesterols in order to replace the skin barrier components. However, conventional formulations may have limited penetration abilities through the main protective layer of the skin, which is SC, and incorporation of ceramides into liposomal carriers has been studied recently.[6−9]

Applying cosmeceuticals or medical products directly to the skin is a useful method for delivery of the necessary compounds to the targeted areas by favoring their penetration throughout the skin bilayers.[10] Topical agents that have similar structure to the skin's outer layer can mimic the biological skin

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membrane and penetrate better into the skin.[11] Recently, nanoscale topical delivery agents like liposomes have been introduced to skin applications in order to make an impact with their physicochemical properties on the different components of skin layers.[12,13] When amphiphilic phospholipid molecules self-assemble in aqueous media, they construct nanosized spherical bilayer vesicles, which are known as liposomes. These structures have an outer spherical lipid bilayer and an aqueous inner space that make them available for both hydrophilic and hydrophobic active agent loading. One of the industries where liposomes are used commonly as topical delivery agents is cosmeceuticals. The bilayer lipid structure of liposomes makes them similar to biological skin lipid membranes, and this provides a unique penetration property for the delivery of topical cosmeceutical agents.[14] Transethosomes (TEs) are liposomes that are prepared with ethanol and contain an edge activator as an extra ingredient. Ethanol serves as a penetration enhancer through skin layers, whereas the edge activator gives liposomes deformability during penetration by improving their fluidity. Therefore, TE usage has become very important in the cosmeceutical industry lately due to their unique characteristics.[15−19]

In this study, a TE formulation containing soy phospholipid, cholesterol, Tween 80, and Cer 3 was developed for optimum skin conformance for eczema patients. Cer 3 inclusion was aimed to improve the efficiency of the products used to treat eczema. The effect of Cer 3 addition on the physiological properties of the TEs in terms of size, morphology, size distribution, and stability was observed and discussed. The high hydrophobicity of Cer 3 was overcome by using ethanol and Tween 80 in the formulation, and Cer 3 inclusion within lipid bilayers was optimized. To examine the loading yield of the obtained TEs, two different payloads were chosen. Ambora extract was preferred as a hydrophilic cargo because of its therapeutic effects on rosacea and eczema skin diseases. It is rich in flavonoids that have potential usage as anti-inflammatory, anti-irritant, and antioxidant agents in cosmeceutical formulations.[20] Dimethylmethoxy chromanyl palmitate (Chromabright), which is a cutting-edge molecule for a radiant skin tone, was preferred as a hydrophobic cargo because of its inhibitory effect on photoaging. In vitro studies have shown that it regulates the process of skin pigmentation by inhibiting melanin synthesis, which is responsible for skin color.[21] The obtained TEs were analyzed via dynamic light scattering (DLS), scanning transmission electron microscopy (STEM), and ultraviolet−visible (UV/vis) spectroscopy.

2. MATERIALS AND METHODS

2.1. Materials. Soybean phosphatidylcholine (Soy PC) with 94% PC content, in light orange-colored grease form, was kindly provided by Lipoid GmBH (Ludwigshafen, Germany) under the brand name Lipoid S 100. Tween 80, also known as polysorbate 80, was purchased from Merck in the form of a yellow liquid and was used as an edge activator. Cholesterol was purchased from Sigma-Aldrich in white powder form with >99% purity. Cer 3, Ambora extract, and Chromabright were all kindly provided by Novodist Turkey. The chemical structure and functional groups of Cer 3 are provided in Figure 1. Ethanol with >99% purity was purchased from Merck. All of the experiments were performed using ultrapure Milli-Q water (Type 1) from Millipore Instrument.

2.2. Preparation of TEs. TEs were prepared using the cold method.[22,23] Briefly, all of the lipids and hydrophobic contents

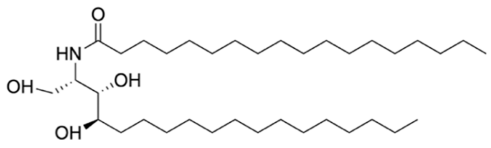

Figure 1. Structure of Cer 3.

were dissolved fully in ethanol in a 25 mL round-bottom flask. The temperature of the medium was kept at 35 °C with magnetic stirring at 750 rpm. For Ambora extract, the extract was dissolved in aqueous phase containing ultrapure water and heated separately and maintained at 35 °C. The aqueous phase was added to the alcoholic solution drop by drop using a 1 mL syringe with a speed of 250 μL/min. In all of the formulations, the total lipid concentration in the final vesicular dispersion was kept constant as 10 mg of lipid per mL of total dispersion, and the ethanol:water volume ratio was fixed as 3:7. All of the loaded vesicles contained a 1:10 mass ratio of loading material (both hydrophobic and hydrophilic) to total lipid mass. TEs were downsized using an Avanti Polar Lipids mini extruder equipped with 0.1 μm pore size polycarbonate membranes and 1000 μL syringes. Each sample was subjected to 11 consecutive filtrations through polycarbonate filters to ensure uniformity. The final solutions were wrapped in aluminum foil and placed in the refrigerator at 4 °C. The variable lipid mass ratios used in different TE formulations can be found in Table 1, presented as Soy PC/Tween 80/cholesterol/Cer 3 mass ratios.

Table 1. Mass Ratios of Soy PC, Tween 80, Cholesterol, and Cer 3 in Different TE Formulations

sample no.	Soy PC	Tween 80	cholesterol	Cer 3
TE1	15	0.5		
TE2	15	1.0		
TE3	15	1.5		
TE4	15	1.5	1.0	
TE5	15	1.5	1.5	
TE6	15	1.5	2.0	
TE7	15	1.5	1.5	0.9
TE8	15	1.5	1.5	1.5
TE9	15	1.5	1.5	1.9
TE10	15	1.5	1.5	2.8

2.3. Characterization of TEs. The Brookhaven Instruments 90 Plus Particle Size Analyzer was utilized to conduct DLS measurements to determine the size, size distribution, and zeta potential of the samples. The measurements were performed after each final TE dispersion was diluted with Type 1 water (1:10). TEs were then compared in terms of zeta potential, size, and polydispersity index (PDI), which gives the overall quality of the TE solution with respect to homogeneity. As well as the size measurement, stability measurements were also followed by the zeta potential data of the colloidal systems. The magnitude of the zeta potential provides crucial information about the particle stability. A high zeta potential will confer stability; the solution or dispersion will resist aggregation. If the potential is small, then attractive forces may exceed this repulsion. So, colloids with high zeta potentials (negative or positive) are electrically stabilized, while colloids with low zeta potentials tend to aggregate.[24] Generally, the zeta potential of nanosized vesicles falls within the range of ±10 to ±70 mV depending on the composition.[25] The optimum

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vesicle size was expected to be below 200 nm for a good skin penetration and PDI of <0.2 indicated a monodisperse, acceptable size distribution for homogeneous vesicle systems.[26] The shapes, sizes, and surface morphologies of the TEs were examined by the STEM technique using Thermoscientific QUATTRO S SEM equipment centered at the Boaziçi University Center for Life Sciences and Technologies. The measurements were performed after diluting each final TE dispersion with 10-fold Type 1 water. Dispersions were dropped onto copper STEM grids and air-dried for 3 h prior to examination. Dried film specimens were imaged at 8−12 kV with a magnification range changing from 50.000 to 300.000.

2.4. Calculation of Loading Efficiencies. The ultracentrifugation technique was employed as a simple and efficient method for separating loaded vesicles from the bulk solution. Loaded vesicle suspensions were filtered using the combination of a Rotafix 32 Hettich centrifuge and a Vivascience Vivaspin 2 mL concentrator. Each sample was centrifuged at 6000 rpm and at room temperature. Solutions at the bottom part of the filtration tube were examined periodically with a UV/vis spectrophotometer, and elimination of the excess payload was followed. The maximum absorbance wavelength for Ambora extract was detected as 286 nm, whereas it was 274 nm for Chromabright. The other lipid ingredients did not show any interference with the loaded ingredients. Once the loaded TEs were all separated from their exterior medium, they were broken down and the released payload was then quantified with a UV/vis spectrophotometer. Breakdown of the TEs was achieved upon dilution of each sample with 1:10 (v/v) ethanol and 30 min vigorous shaking with a vortex to be able to analyze their loading efficiencies. The broken TEs did not show any meaningful peaks upon DLS measurements, proving the disruption of the lipid bilayer, and also the unique bluish color of the vesicles disappeared upon the breakage process. The loading efficiencies were calculated by observing the ratio of the amount of loaded payload to that of the initially added payload amount. All of the samples were kept in a refrigerator at 4 °C to follow their physical stabilities upon one month of storage and analyzed in terms of their particle size, homogeneity, and percent loading efficiency values.

2.5. Statistical Analysis. The results of each experiment were run in at least triplicate, and the data were then presented as the average ± standard deviation. Linear regression analysis was done to analyze the effect of increasing the amount of Cer 3 content on the size and homogeneity of the TEs. Two-way analysis of variance (ANOVA) was used for statistical analysis to compare the effect of Cer 3 addition on loading hydrophilic and lipophilic payloads. A difference was deemed statistically significant if the p-value was less than 0.05.

3. RESULTS AND DISCUSSION

3.1. Preparation and Characterization of Cer 3-Containing TEs. To be able to formulate a TE, first, Tween 80 was added as an edge activator with Soy PC:Tween 80 mass ratios of 15:0.5, 15:1, and 15:1.5 and the effects of the mass ratio of Soy PC to Tween 80 on the vesicle size, homogeneity, and morphology were investigated. Size and size distributions were measured via DLS; the obtained vesicular dispersions were visualized with STEM. The zeta potential of the final selected formulation was measured with the zeta-sizer module of the particle-size analyzer.

TEs prepared with all mass ratios of Tween 80 had significantly low PDI values that showed great homogeneity, and they were in the acceptable size range for skin penetration applications. As seen in Table 2, upon increasing the Tween 80 content, the size decreased without affecting the homogeneity. Increasing the mass ratio of Tween 80 can balance the surface tension of the vesicle membranes, causing an increase in the stability and decrease in the payload leakage. However, when the amount of Tween 80 added exceeds a certain amount, it may disturb the ordered phospholipid arrangement and destroy the liposome structure, causing transformation into spherical micelles instead of bilayers.[27−29] So, adding a suitable amount of Tween 80 into the TE formulation may contribute to a decrease in vesicle size while causing an increase in stability and the loading efficiency of the TE. Thus, 15:1.5 Soy PC:Tween 80 containing TE3 formulations was chosen for the following experiments. In Figure 2, the STEM image of homogeneously dispersed TEs containing 15:1.5 Tween 80 with around 100−150 nm size range could be observed with and without size tags. The DLS results of this formulation resulted in 62 nm average size, while in this figure the vesicles seemed bigger. The reason behind this fact could be that DLS analysis shows the average size of the entire system, whereas STEM can focus only on a small area of the sample and gives information about morphological analysis mostly. So the shapes of the particles could be seen, and the images could be used to judge whether good dispersions were achieved or agglomerations were present in the system. In combination with DLS, STEM becomes a valuable aid in the characterization of the vesicles. So, the observed area of the TE3 sample showed homogeneously dispersed vesicles with round shapes and no aggregations, which was consistent with the DLS results.

Table 2. Effect of Soy PC:Tween 80 Mass Ratio on the Size and Homogeneity of TEs

sample no	Soy PC	Tween 80	size (nm)	PDI
TE1	15	0.5	71.40 ± 2.96	0.09 ± 0.01
TE2	15	1.0	64.77 ± 4.97	0.09 ± 0.01
TE3	15	1.5	62.60 ± 4.25	0.11 ± 0.06

Addition of cholesterol in lipid composition stabilizes the bilayers by providing membrane fluidity, elasticity and permeability as well. A rigid and more saturated membrane with higher ratio of cholesterol increases the stability and reduces the leakage problems.[30−32] The major lipids found in human skin are ceramides, cholesterol, and free fatty acids, which make up approximately 50, 25, and 15% by mass of the SC lipid matrix, respectively.[33] Hence, addition of cholesterol up to a maximum of 25 mass percent in our vesicular system can be a wise move to obtain more stable vesicles with better skin penetration properties.

To achieve this, three different formulations containing Soy PC:Tween 80:cholesterol with mass ratios of 15:1.5:1,15:1.5:1.5, and 15:1.5:2.0, respectively, were prepared. In Table 3, a comparison of TEs with different cholesterol contents can be seen. Immersion of 15:1:1 cholesterol first decreased the vesicle size but then it started to increase on raising the cholesterol amount. The desired size range was achieved with all TE formulations, but the monodispersity of the colloidal solutions started to disrupt upon addition of more than 15:1.5:1.5 cholesterol. The results obtained were

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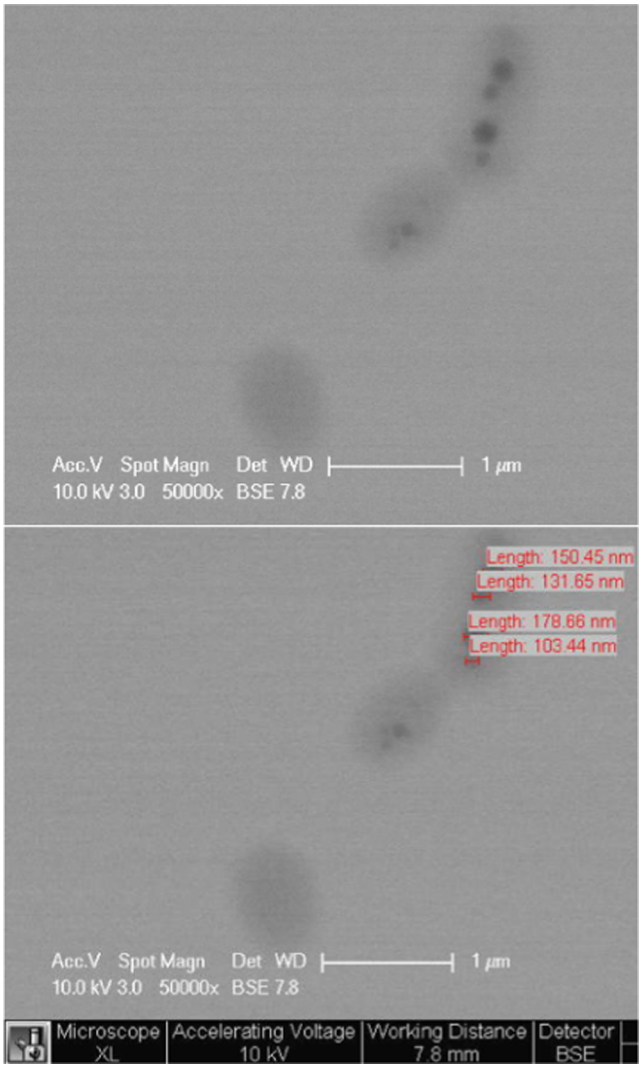

Figure 2. STEM images showing TE3 samples containing 15:1.5 mass ratio of Soy PC:Tween 80 with and without size tags.

consistent with the literature data given by S.C. Lee et al., who concluded that the average particle size increased as the cholesterol content increased.[34] All of the vesicle sizes and PDIs were below the maximum size and PDI limits to be further used in transdermal delivery studies (smaller than 200 nm and PDI lower than 0.2), but increasing the cholesterol ratio more than 15:1.5:1.5 would decrease the loading efficiency; cholesterol would penetrate through the bilayer system by resulting in a competition with the loaded materials.[35] Therefore, the TE5 formulation containing Soy PC:Tween 80:cholesterol with the ratio of 15:1.5:1.5 was chosen for further experiments due to its being a more homogeneous system with a lower PDI value and not decreasing the loading efficiency in the following encapsulation studies. The zeta potential of this formulation was measured as −22.22 ± 0.47 mV. The STEM data shown in Figure 3 also

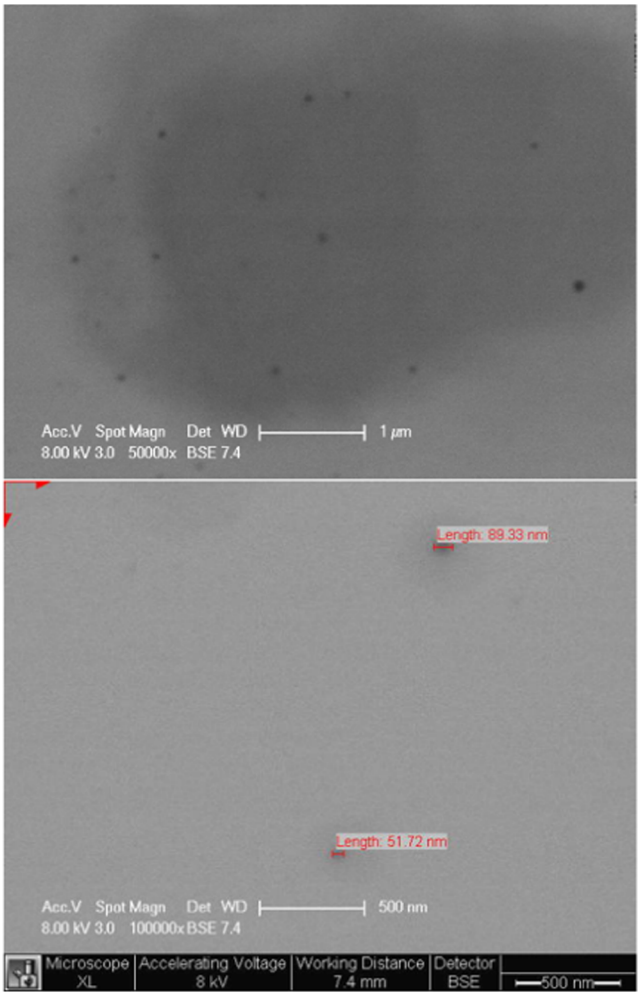

Figure 3. STEM images showing TE5 samples containing 15:1.5:1.5 mass ratio of Soy PC:Tween 80:cholesterol with and without size tags.

verified the size and homogeneity results obtained using a particle-size analyzer, showing a TE medium containing nearly 100 nm sized, homogeneously dispersed, round-shaped particles.

The regulating skin barrier is managed by intercellular SC lipids and among them, ceramides play a key role in keeping this barrier healthy. Eczema patients suffer from genetic disorders that cause a decrease in Cer 3 levels and alteration in ceramide contents. It has been reported that total ceramide:cholesterol ratios were significantly lower in eczema patients with respect to normal skin. Healthy skins contained roughly 30% higher total ceramide amounts than eczema-

Table 3. Mass ratios of Soy PC, Tween 80 and cholesterol containing formulations and comparison with respect to mean size and PDI values

sample no	Soy PC	Tween 80	cholesterol	size (nm)	PDI
TE3	15	1.5		62.60 ± 4.25	0.11 ± 0.06
TE4	15	1.5	1.0	53.70 ± 4.12	0.09 ± 0.01
TE5	15	1.5	1.5	75.28 ± 15.17	0.07 ± 0.03
TE6	15	1.5	2.0	76.90 ± 3.76	0.16 ± 0.08

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Table 4. Mass Ratios of Soy PC-, Tween 80-, cholesterol-, and Cer 3-Containing Formulations and Comparison with Respect to Mean Size and PDI Values

sample no.	Soy PC	Tween 80	cholesterol	Cer 3	size (nm)	PDI
TE5	15	1.5	1.5		75.28 ± 15.17	0.07 ± 0.03
TE7	15	1.5	1.5	0.9	83.30 ± 8.09	0.07 ± 0.01
TE8	15	1.5	1.5	1.5	80.17 ± 8.60	0.06 ± 0.03
TE9	15	1.5	1.5	1.9	88.04 ± 5.32	0.07 ± 0.04
TE10	15	1.5	1.5	2.8	110.90 ± 51.71	0.14 ± 0.10

suffering skins; in healthy skins, the total ceramide:cholesterol ratio was found to be 2.24, whereas in eczema-suffering skins it was 1.53. Also, the importance of Cer 3 through all ceramide subgroups was found to be strongly related to the trans-epidermal water loss, which is an active sign of eczema.[36−39] Hence, transdermal delivery of this lipid may provide opportunities for diminishing ceramide deficiency and improving skin condition. This experimental design was conducted to supply Cer 3 through transdermal delivery using TEs for eczema patients with 15:1.5:1.5:0.9, 15:1.5:1.5:1.5,15:1.5:1.5:1.9, and 15:1.5:1.5:2.8 Soy PC:Tween 80:cholesterol:Cer 3 mass ratio-containing compositions as seen in Table 4.

According to the results presented in Table 4, addition of Cer 3 caused a small increase in size without affecting the homogeneity much, which was the desired result. These results are also consistent with the previous research reported by Mir-Palomo that adding a lipophilic molecule to the TE systems, such as ceramides, causes an increase in the mean diameters of the obtained vesicles.[40] The impact of Cer 3 concentration on size and PDI was also investigated using a simple linear regression model, as represented in Figure 4 and 5, with 21

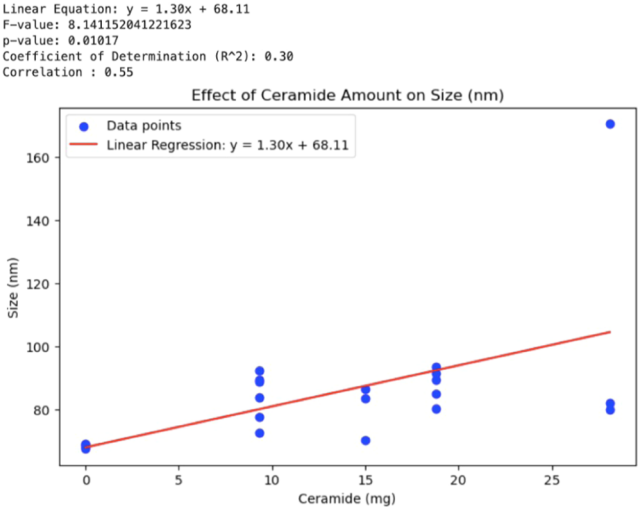

Figure 4. Statistical regression analysis showing a positive relationship between Cer 3 increase and TE mean diameter results with a *p*-value of 0.0102.

observations. The model was statistically significant for Cer 3's association with size with a p-value of 0.0102, confirming a positive and meaningful relationship between Cer 3 amount and size. The coefficient for the Cer 3 effect on PDI values was 0.0014, with a p-value of 0.309, indicating that changes in Cer 3 amount were not significantly associated with changes in homogeneity of the TEs within the confidence levels considered. From these figures, separate results of the

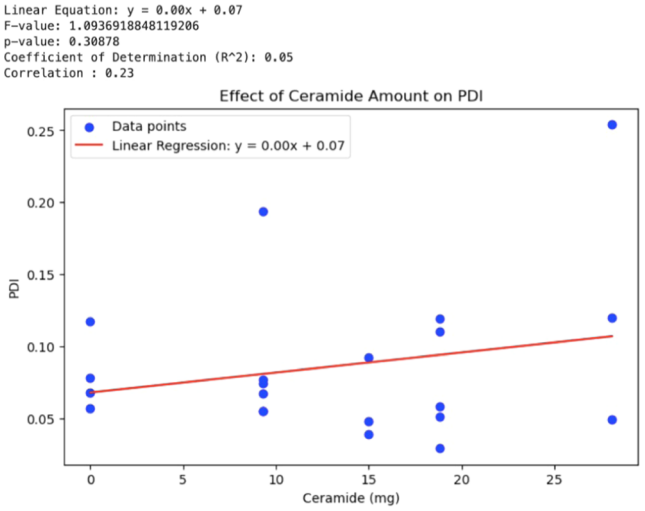

Figure 5. Statistical regression analysis showing no significant association between Cer 3 increase and homogeneity results with a *p*-value of 0.309.

measurements belonging to the same formulations (i.e., TE5, TE7, TE8, and so on) could be seen individually. As seen, the last points of the graphs, around 28 mg of Cer 3, showed three values of a triplicate measurement that were very far from being precise, which were consistent with the DLS results presented in Table 4. TE10 formulations had the biggest standard deviation among all of the formulations.

However, there was a dramatic increase in PDI after 15:1.5:1.5:1.9 Cer 3 introduction, which could be explained by an initiation of heterogeneity within the colloidal system, but this homogeneity value was also within the accepted range for monodispersion, which is under 0.2.[26]

Even though the TE9 formulation had a mean size and PDI range within the acceptable boundaries, TE9 and TE10 formulations started to precipitate after 2 days of storage, and white, jelly aggregates were observed. Due to the instability issues of these formulations, all further experiments from then on contained a 15:1.5:1.5:1.5 mass ratio of Soy PC:Tween 80:cholesterol:Cer 3, and had a mean size of 80.17 ± 8.60 nm with a 0.06 ± 0.03 PDI value. The zeta potential of this formulation was measured as −23.37 ± 0.35 mV. The STEM results of this formulation, TE8, were in agreement with the DLS results and can be seen in Figure 6. In these images, TEs having approximately 90 nm size within small groups could be observed. Vesicles were seen within agglomerates in these images, but distinct round shapes could clearly be observed. Those aggregations could have resulted from the drying process in STEM imaging. Vesicles tend to come together while they are drying. If there were aggregates within the dispersions, DLS results would give higher PDI results.

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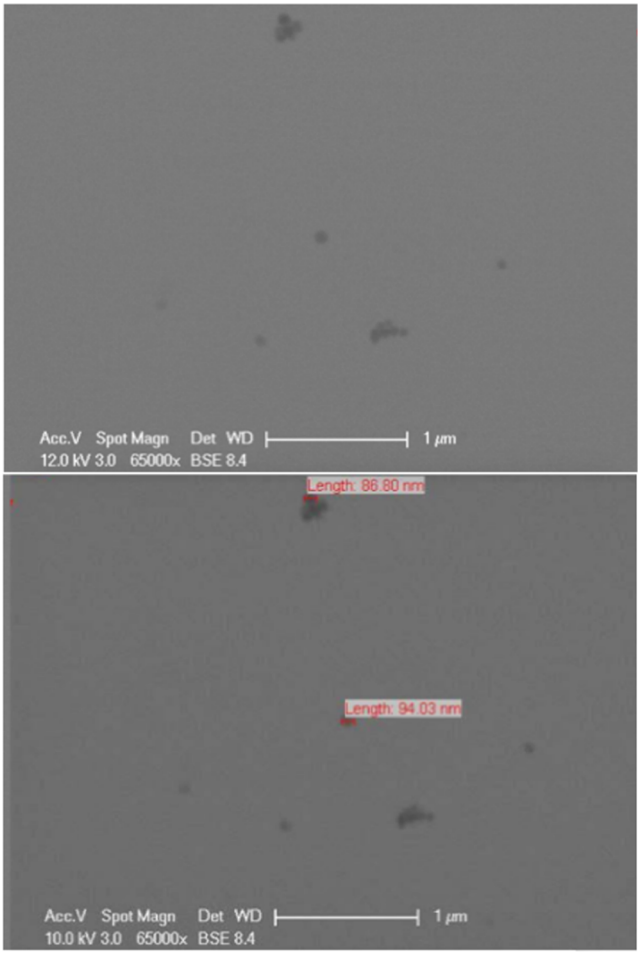

Figure 6. STEM images of TE8 formulations with and without size tags showing the obtained mean diameter around 90 nm.

With this experiment, a TE formulation containing Cer 3, cholesterol, and Tween 80 was formulated successfully.

The stability of vesicular systems is a big concern for their further usage in cosmeceutical formulations. All cosmeceutical products have a certain shelf life, and these vesicles should carry the loaded materials up to the end of this time period. To examine the stability, the mean size and homogeneity values of the TE8 formulation were analyzed and compared with the TE5 formulation that did not contain Cer 3 after 30 days prior to the preparation as seen in Table 5. The results given in

Table 5. One-Month Stability of TE5 (No Ceramide) and TE8 (Cer 3-Containing) Formulations with Respect to Mean Size and PDI Values

	size (nm)	one-month size (nm)	PDI	one-month PDI
TE5	75.28 ± 15.17	80.46 ± 18.66	0.07 ± 0.03	0.06 ± 0.04
TE8	80.17 ± 8.60	85.12 ± 6.51	0.06 ± 0.03	0.09 ± 0.03

Table 5 showed that TE8 formulation was stable even after 30 days, very similar to the TE5 formulation; neither the mean size nor the homogeneity of the samples was disturbed. The size increase upon one-month storage was almost the same for both formulations; no significant difference occurred in their PDI values. Also, TE5 had −22.22 ± 0.47 mV zeta potential while TE8 had −23.37 ± 0.35 mV zeta potential. The closeness of the two data sets also indicated that there would be no big difference in the colloidal stability of these formulations.

3.2. Effect of Cer 3 on the Physical Properties of Ambora Extract and Chromabright-Loaded TEs. As mentioned earlier, one hydrophilic and one lipophilic compound were chosen to observe the effect of Cer 3 on the mean size, homogeneity, and loading efficiencies of the TEs. A series of experiments were carried out; TE11 and TE12 did not contain any loaded material; TE13 and TE14 contained 10% of hydrophobic Chromabright by total lipid mass; TE15 and TE16 contained 10% of hydrophilic Ambora extract by total lipid mass as summarized in Table 6.

The absorbance peaks of the components of the TE (i.e., Soy PC, Tween 80, cholesterol, and Cer 3) are shown in Figure 7, along with the absorbance line of the empty T11, shown in bold. As seen in this figure, once the TE was formed, its peak did not coincide with the peaks of any of the other ingredients. Also, no overlap occurred at 274 and 286 nm wavelengths, which enabled us to follow the peak for loading efficiencies.

The idea behind using the maximum absorbance wavelengths to calculate the loading yields relies on the following method: (a) after preparing the loaded TEs, they were subjected to ultracentrifugation under 6000 rpm to get rid of all of the unloaded free materials; (b) the filtrate was examined with a UV/vis spectrophotometer at the maximum absorbance wavelengths; (c) centrifugation was continued until all of the filtrate was cleaned from free Ambora extract or Chromabright. The vesicles were then broken down, as explained in Section 2.4, and the absorbance (i.e., the amount) of the released content was calculated.

As seen in Table 6, a comparison of Cer 3-containing TEs TE 11, 13, and 15 shows that loading of a lipophilic cargo had almost no change in size and homogeneity, whereas introduction of a hydrophilic cargo yielded a 45% increase in size and almost 100% decrease in homogeneity. A plausible explanation could be that Chromabright, as a lipophilic compound, was incorporated within the bilayer structure and behaved as a constitutional lipid and strengthened the vesicle lipid wall. Lipophilic cargo did not seem to cause a significant chance in the size and homogeneity of the bilayer system upon one-month storage. Also, loading studies strengthened this hypothesis since almost all of the Chromabright molecule got inside both TE structures produced with and without Cer 3, and remained stable up to one month. On the other hand, the huge increase in size and PDI values of the TEs containing Ambora extract could be explained by encapsulation within the aqueous core of the TEs. The slight increase in size after one-month storage could arise from the swelling of the bilayer structure, which was also compatible with the loading data. Upon one-month storage, the loading efficiency of TE15 was decreased from 74% to 60%.

Again, as seen in Table 6, a comparison between TEs not containing Cer 3, TE 12, 14, and 16 was also made. It was observed that the size increase upon Chromabright addition was more distinct between TE12 and TE14 than between TE11 and TE13. This result supported the idea that Chromabright participated within the bilayer structure since without Cer 3, Chromabright could position itself in the bilayer more effectively. Again, the hydrophilic material acted as a cargo that went to the aqueous core of the TE and caused an increase in size and decrease in homogeneity. Ambora

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Table 6. Effect of Cer 3 Existence on the Physical Properties of Ambora Extract (A.Extract) or Chromabright-Loaded TEs in Terms of Mean Size, PDI, and Loading Efficiency (%LE) and Their One-Month Stabilities

sample no.	Soy PC: Cer 3	cargo	size (nm)	size (nm) (one-month)	PDI	PDI (one-month)	%LE	%LE (one-month)
TE11	15:1.5		80.17 ± 8.60	85.20 ± 6.51	0.06 ± 0.03	0.09 ± 0.03		
TE12			75.28 ± 15.17	80.47 ± 18.66	0.07 ± 0.03	0.06 ± 0.04		
TE13	15:1.5	chromabright	84.68 ± 4.45	85.25 ± 5.46	0.08 ± 0.01	0.11 ± 0.03	98.85 ± 0.94	98.39 ± 1.97
TE14		chromabright	85.62 ± 11.52	86.50 ± 17.38	0.06 ± 0.01	0.05 ± 0.01	98.36 ± 1.35	98.38 ± 0.25
TE15	15:1.5	A. extract	116.20 ± 10.83	117.97 ± 8.56	0.12 ± 0.02	0.13 ± 0.03	74.41 ± 3.13	60.40 ± 17.14
TE16		A. extract	127.40 ± 11.74	134.20 ± 6.50	0.13 ± 0.02	0.10 ± 0.04	73.40 ± 5.97	67.50 ± 10.64

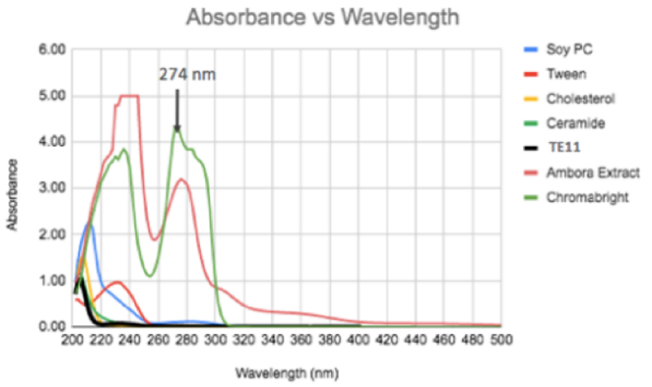

Figure 7. Maximum absorbance wavelengths of the lipids used in TE formation, TE11, and the payloads; Ambora extract and Chromabright.

extract loading efficiencies were slightly less, but loading stability after one-month storage was better in TEs without Cer 3. The size increase after one month was larger in TE16 than in TE15. Cer 3 could strengthen the bilayer wall and avoid swelling. Hydrophilic cargo also seemed to cause damage to the uniformity of the colloidal system. The PDI values of TE15 and TE16 were much higher compared to the empty and lipophilic material-containing vesicles. This could have arisen from the fact that Ambora extract was a mixture of chemicals and had a complex structure.

The obtained data were analyzed statistically with a two-way ANOVA test as shown in Figures 8 and 9. By using a two-way ANOVA, the variance in size and homogeneity across different materials was assessed. This statistical approach enabled us to isolate the effect of material choice on the size and PDI measurements. The ANOVA analysis revealed a statistically significant effect of material type on size ($F(2, 17) = 10.441723$, $p = 0.001102$) as stated in Figure 8. This significant finding indicates that the variation in size among the tested materials was not random but influenced markedly by the choice of material.The p-value for Cer 3 was greater than the significance level, suggesting that Cer 3 was not statistically significant in relation to size.The p-value for the interaction between payload and Cer 3 was also greater than the significance level, indicating that this interaction was not statistically significant with respect to size. Therefore, in summary, the nature of the chosen material had a statistically significant effect on size, while Cer 3 and the interaction between the payload and Cer 3 did not. The variation in size, attributed to material choice, pointed to inherent differences in the physicochemical properties of the materials, which in turn

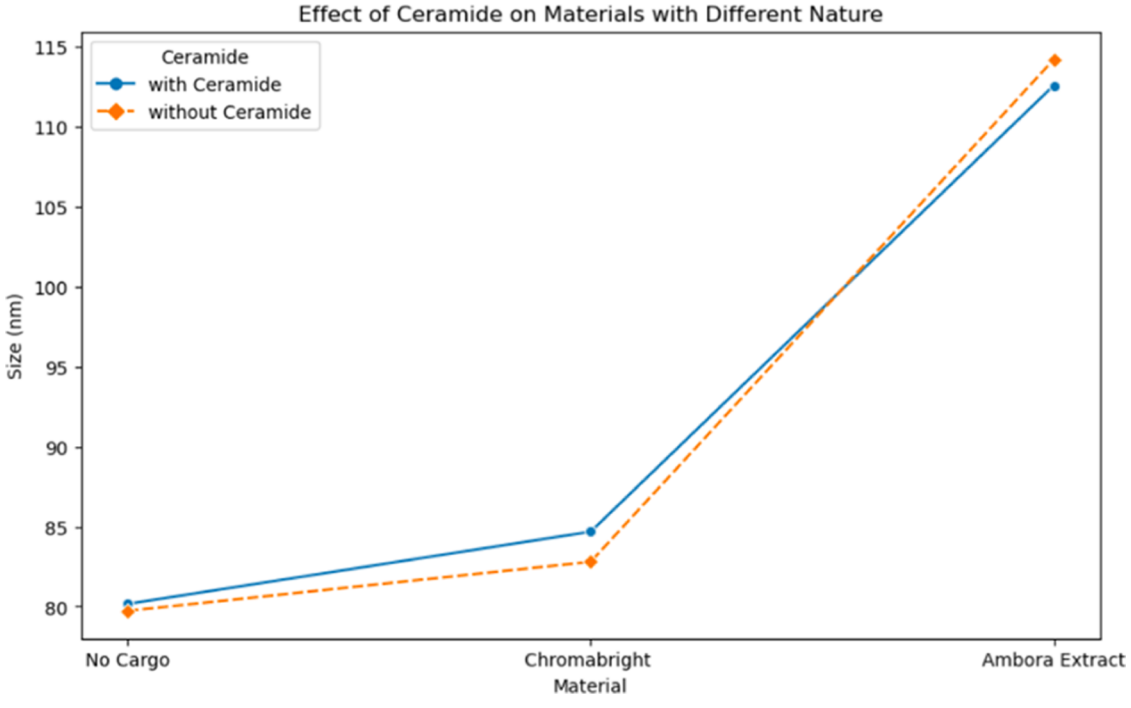

Figure 8. Statistical analysis on the effect of Cer 3 existence on hydrophilic and hydrophobic payloads containing TEs in terms of mean size.

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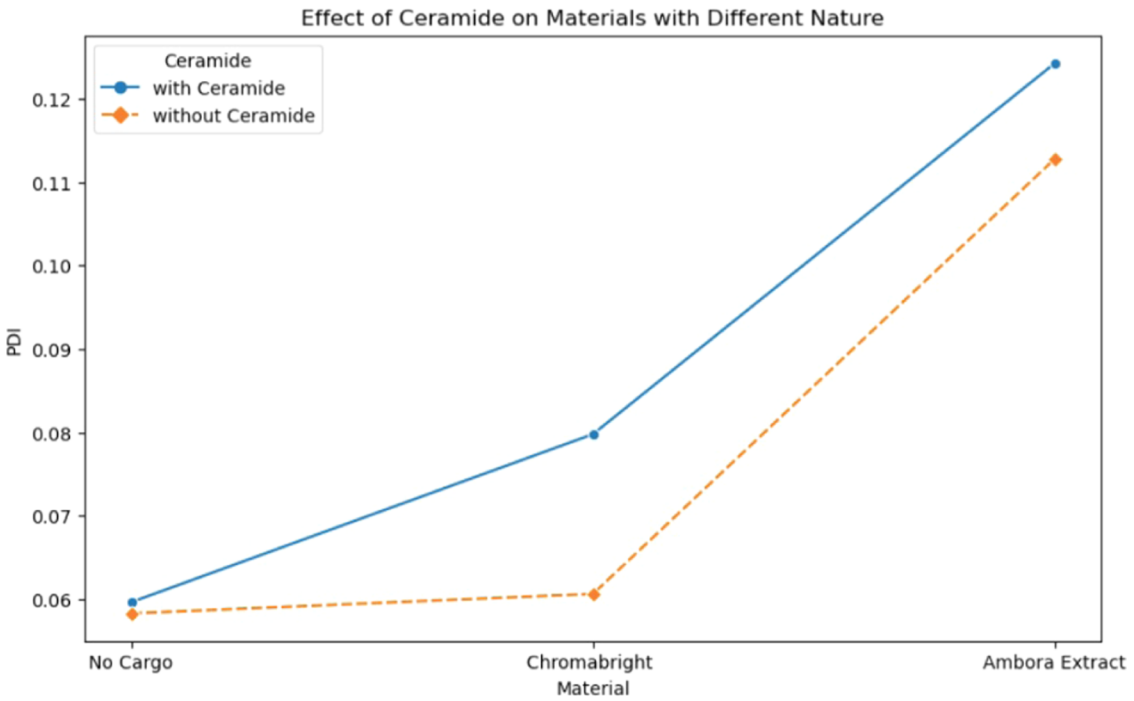

Figure 9. Statistical analysis on the effect of Cer 3 existence on hydrophilic and hydrophobic payloads containing TEs in terms of homogeneity.

affected their dispersion characteristics and stability in a given medium.

Based on the two-way ANOVA analysis on PDI results as shown in Figure 9, the *p*-value for material type was less than the significance level (0.05), indicating that material choice was statistically significant for homogeneity. The p-value for Cer 3 existence was greater than the significance level, suggesting that the presence of Cer 3 was not statistically significant in terms of homogeneity. The p-value for the interaction between the payload and Cer 3 was also greater than the significance level, indicating that this interaction of Cer 3 and material nature was not statistically significant. Further investigations could expand on this foundation by exploring the specific material properties influencing size and homogeneity and by including a broader range of materials to encompass a wider spectrum of applications.

Figures 10 and 11 showed the difference in physical properties of the loaded and empty TEs observed by the naked eye. The distinct orange color of the dissolved Ambora extract seen in Figure 10(a) became cloudy after the loading process (b) and further turned bluish upon extrusion (c). Vesicular suspensions like TEs have a certain bluish color because of the scattering of light by the vesicles.[41,42] Difference between (c) and (d) stated a successful loading of the hydrophilic cargo, whereas there was no trace of orange color in (d) and a bluish-orange color in (c) could be clearly seen, demonstrating the presence of the orange cargo within the vesicles.

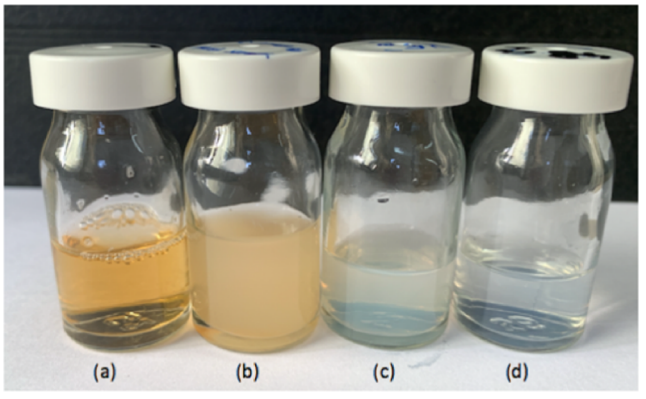

Figure 10. (a) Ambora extract dissolved in water (b) Ambora-extract-containing TEs prior to extrusion (c) Ambora-extract-containing TEs after extrusion (d) Empty TEs after extrusion.

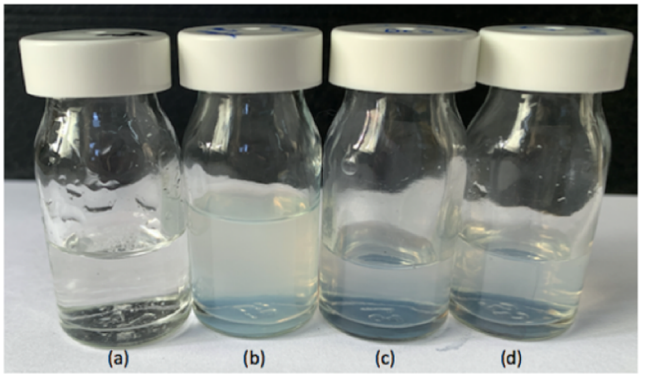

Figure 11. (a) Chromabright dissolved in ethanol. (b) Chromabright-containing TEs prior to extrusion. (c) Chromabright-containing TEs after extrusion. (d) Empty TEs after extrusion.

Also, the pH values of these samples were measured and obtained as 3.83, 5.38, 6.20, and 6.81, respectively. The low pH value of the Ambora extract diminished after being loaded within vesicles and further increased upon extrusion, which could be interpreted as getting rid of excess Ambora extract from the outer medium. Vesicles without any loaded material had the highest pH among them, which was 6.81.

Similarly, the colorless Chromabright solution in Figure 11(a) turned bluish upon loading (b) and further became

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more bluish after the extrusion process (c). There was also an obvious color difference between the empty TE sample (d) and the Chromabright-containing TE sample (c). The obtained pH values were 5.07, 7.34, 7.55, and 6.81, respectively. Again, the low pH value of Chromabright diminished upon being loaded and further increased after the extrusion process, which eliminated excess unloaded material from the bulk medium.

4. CONCLUSIONS

Although there are numerous studies reporting the potential use of liposomes as transdermal delivery systems, there seems to be a lack of understanding regarding the formulation of a vesicular system with optimum stability, homogeneity, and loading efficiency. Therefore, the present study was focused mainly on designing a TE system by utilizing ethanol and edge activators in vesicular structures and having a better alternative to liposomes as skin delivery agents. The effects of edge activator and cholesterol concentrations and ceramide usage on the physical properties, stability, homogeneity, and loading efficiencies were investigated. A successful TE composition with very narrow size distribution and optimum size for skin applications was achieved by introducing ethanol, cholesterol, Tween 80, and Cer 3 into the vesicular system. The loading efficiencies and one-month stabilities of the obtained TEs were examined. The results showed that increasing Tween 80 concentrations caused a decrease in size while not affecting the homogeneity. Addition of cholesterol into the formulation caused a growth in size, but after 15:1.5:1.5 mass ratio of Soy PC:Tween 80:cholesterol, a dramatic decline in size distribution occurred. TE size increased with increasing Cer 3 content and optimum size and homogeneity was achieved using a 15:1.5:1.5:1.5 mass ratio of Soy PC:Tween 80:cholesterol:Cer 3. This TE preserved its size and homogeneity stability upon one month of storage. By using the optimized TE, loading efficiency experiments were carried out with Chromabright and Ambora extracts that have different water solubility natures. The effects of Cer 3 and the nature of the chosen material were studied, and the results were analyzed statistically. According to the results, loading yield and loading stability were more successful with the lipophilic cargo irrespective of the presence of the ceramide. Hence, Cer 3 had almost no effect on the loading efficiency of the TEs.

Our study provides a valuable contribution to the field of liposome research highlighting the impact of ceramides on the physical characteristics, stability, homogeneity, and loading properties of the vesicular systems. These results suggest that Cer 3 plays an important role in vesicle characteristics, and the findings can be used in potential applications in eczema curing studies. Future work can be done by examining the skin penetration characteristics and cytotoxicity of the obtained TEs.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c04992.

DLS data for all samples and zeta potential results for TE3, TE5 and TE8 (PDF)

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Notes

The authors declare no competing financial interest.

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