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Effect of Freeze-Thaw Cycles Method to Transfersome Characteristics for Growth Protein Encapsulation

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Abstract. Transfersome, a lipid-based nanovesicle, can be a suitable tool to improve the delivery of valuable growth factors. Through transfersome technology, growth factors and active compounds can be transferred transdermally without the need for invasive delivery procedures. In this study, we evaluated the impact of freeze-thaw cycles on transfersome characteristics, particularly particle size, polydispersity, and encapsulation efficiency. Transfersome particles were prepared from dipalmitoylphosphatidylcholine (DPPC) and Tween 80 with a 97.5:2.5 w/w% using thin film hydration at a temperature of 45° – 50°C. Then, the transfersome suspension was subjected to repeated freeze-thaw for 1 minute of freezing and 3 minutes of thawing. The protein release from all transfersome samples were evaluated using Bradford assay, while the particle size and polydispersity were determined with a dynamic scattering analyzer. It was found that freeze-thaw increased encapsulation efficiency, particle size, and polydispersity of transfersomes up to 81.63±0.00%, 180.70±0.87 nm, and 0.369±0.02, respectively, from those without freeze-thaw steps (73.35±0.03%, 144.93±0.21 nm and 0.202±0.02). Moreover, freeze-thawed transfersomes exhibited a release of up to 52.80% of loaded protein within 78 hours, in contrast to 37.48% in non-freeze-thawed transfersomes. This study shows that an additional freeze-thaw step is a promising method to improve the properties of transfersome particles, especially encapsulation efficiency and sustained protein release.

Keywords: Dipalmitoylphosphatidylcholine; Encapsulation; Freeze-thaw; Growth protein; Transfersome

1. Introduction

Degenerative disease, or disease caused by the functional and structural decline of tissues or organs, becomes a common disease among the elderly or even young people. The factors affecting degenerative disease are age, lifestyle, genetics, or the impact of an accident. In therapeutic approaches, there are various methods for treating degenerative

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diseases, including the utilization of stem cells in cell-based therapy. Stem cell therapy can be conducted by transplanting healthy cells to recipients (Mahla, 2016). However, cell-based therapy is a complicated process and may pose risks of immune rejection, tumorigenesis, and disease transmission (Rhatomy *et al.*, 2020).

The secretome is a stem cell culture medium containing protein, hormone, cytokine, chemokine, an angiogenic factor, and growth factor that is secreted by stem cells (Umar, 2023; Nurhayati *et al.*, 2021). Secretomes can be an alternative to stem cell therapy due to their ability to be immunomodulatory, anti-inflammation, homeostasis, angiogenesis, and regenerative capacity. However, some bioavailability challenges were caused by the first-pass effect and secretome distribution into the unintended target tissue (Umar, 2023). To overcome bioavailability problems, valuable molecules, including secretome, can be encapsulated within nanovesicles composed of proteins, peptides, phospholipids, or synthetic polymers (Aguilar-Toalá *et al.* 2022; Kusrini *et al.*, 2020; Sahlan *et al.*, 2019).

Transfersomes, lipid-based nanovesicles, are also known as elastic or deformable liposomes due to the presence of surfactants contained in the bilayers, which make them more flexible and less stiff to pass through holes and provide smaller vesicles than the conventional liposome (Apostolou *et al.* 2021). The unique characteristic of transfersome is ultra-flexibility, able to shrink its size 5 to 10 times smaller than its normal size (Opatha Titapiwatanakun, and Chutoprapat, 2020), which is needed for transdermal route administration of secretome. Transfersomes have the ability to encapsulate molecules with a molecular weight ranging from 200 to 10^6 Da, making them intriguing candidates for utilization in secretome-based delivery methods (Das, Nayak, and Mallick, 2022).

The encapsulation efficiency of transfersome may vary depending on materials and fabrication methods, ranging from 55 to 78% (Nojoki *et al.*, 2022; Vasileva *et al.*, 2022; Luiz *et al.*, 2021). In this study, we employed the freeze-thaw method to promote the encapsulation efficiency of transfersome. The lipid vesicle obtained from the thin film hydration method is an inhomogeneous multilamellar vesicle (MLV), with the freeze-thaw method can be used to homogenize the lamellarity and increase encapsulation at once (Costa, Xu, and Burgess, 2014; Sriwongsitanont and Ueno, 2011; Colletier *et al.*, 2002). Freezing step expands the vesicle size due to ice crystal formation and dehydration of lipid groups (Sriwongsitanont and Ueno, 2011). This process makes the membrane structure more brittle and susceptible to disruption. Additionally, during the thawing process, membrane fusion occurs, giving rise to new vesicles with a reduced lamellar number (Sriwongsitanont and Ueno, 2011).

Bovine serum albumin (BSA), a common protein source for human cell culture, was used in this study as a protein model loaded onto transfersomes. It consists of 583 amino acids (Topală *et al.*, 2014), which can represent various proteins in the secretome. Furthermore, BSA has a size of 66.4 kDa, which is close to secretome-derived protein, sizing 20 – 60 kDa (Weigent, 2011). In this study, we evaluated the impact of freeze-thaw cycles on transfersome characteristics, particularly particle size, polydispersity, and encapsulation efficiency, which could be further utilized to produce transfersome particles with desirable characteristics.

2. Methods

2.1. Materials

All chemicals used in this study were analytical grade (purity >95%) and purchased from Merck (Germany), otherwise specifically mentioned.

2.2. Protein Quantification

Protein quantification was measured with a Bradford method, as described elsewhere. Bradford reagent (1 mL) was mixed with 100 µL standard solution or protein sample. The mixture was incubated for 5 minutes at room temperature and then measured by UV-Vis spectrophotometer (Shimadzu, Japan) at 590 and 450 nm (Ernst and Zor, 2010).

2.3. *Transfersome Preparation*

Transfersome particles were prepared using a thin film hydration method (Nojoki *et al.*, 2022; Luiz *et al.*, 2021; Opatha, Titapiwatanakun, and Chutoprapat, 2020). Briefly, Tween 80 (Vivantis, Malaysia) was dissolved in ethanol and then mixed with DPPC (Avanti Polar Lipids, Inc., USA) with a ratio of 97.5:2.5 %w/w using 100 mL round bottom flasks. The mixture of DPPC and Tween 80 was dissolved with chloroform and methanol mixture with a 2:1 v/v ratio. The organic solvent in the mixture was removed by vacuum rotary evaporation at 50°C and 90 rpm for at least 10 minutes until a thin film formed. The flask containing the lipid thin film was stored in a vacuum desiccator overnight to remove the remaining organic solvent. The amount of BSA used was a quarter of phospholipid mass. The completely hydrated lipid film was rehydrated by BSA (ratio 1: 3) in PBS solution and by rotation at 90 rpm and 45°C for 5 minutes. After rehydration, transfersome suspension was vortexed for 1 minute. The heating in the 45°C water bath process and vortex step were done with 3 times repetition for 1 minute in every procedure.

After the rehydration process, transfersome suspension inside the egg flask was immersed in liquid nitrogen (-196°C) for 1 minute, then thawed in a 45°C water bath while shaking the flask by hand for 3 minutes (Castile and Taylor, 1999). The freeze-thaw steps were 5 cycles (S1), 10 cycles (S2) or none (S0). The freeze-thawed suspension was subsequently extruded 10 times by a mini extruder (Avanti Polar Lipid, Inc., Alabaster, AL, USA) at 45°C. The extruded transfersome particles were purified by mini-column centrifugation. The sample was transferred into the top part of an ultra-centrifugal filter (Amicon tube, 100,000 MWCO) (Merck, Darmstadt, Germany). Then, the tube was centrifuged for 15 minutes at 5000 rpm and 4°C temperature. The centrifugation step was repeated four times under the same operating conditions, with the addition of 500 µL fresh PBS to the top part of the Amicon tube at each repetition interval. The settled particle and filtrate were separated for further analysis.

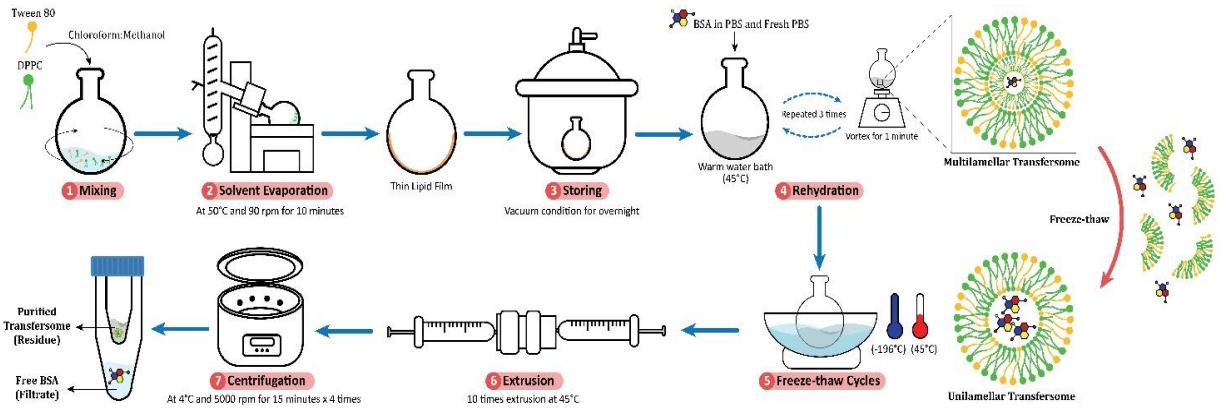

Figure 1 Schematic drawing of preparation technique for protein encapsulation with transfersome

2.4. Determination of Physicochemical Properties

The residue obtained from the purification process contains transfersome particles used for physicochemical analysis, namely particle size (diameter), polydispersity index

(PDI), and zeta potential. A total of 500 µL residue sample was dispersed into 5 mL bidistilled water. The sample was examined using the dynamic light scattering (DLS) method with a particle size analyzer (SZ 100z, Horiba, Japan).

2.5. Determination of Encapsulation Efficiency (%EE)

The filtrate obtained from the purification process contains free BSA protein or non-encapsulated BSA. The filtrate sample was tested for protein content using the Bradford Assay procedure as described previously. Encapsulation efficiency can be calculated using Equation 1:

$$\%EE = \frac{M_i - M_f}{M_i} \quad (1)$$

where M_i is the total amount of initial protein added, and M_f is the total amount of free or unencapsulated protein.

2.6. In vitro Protein Release Assay

The procedure of this assay was carried out using a modified method (Pisani *et al.*, 2022; D'Souza, 2014; Li *et al.*, 2001). The other residue sample obtained from the purification process was transferred into the top part of an ultra-centrifugal filter (Amicon tube, 100,000 MWCO) with a volume (ranging from 600 – 700 µL, depending on sample encapsulation efficiency) that is equivalent to 1 mg of BSA. After that, 500 µL fresh PBS (pH 7.4) solution was added to create a gradient concentration of protein. The sample inside the tube was incubated in a shaker incubator with 37°C set temperature and 100 rpm. The released BSA was measured with Bradford assay. Hereinafter, the protein release profile data were fitted into several drug release kinetic models, namely zero-order, first-order, Higuchi, and Korsmeyer-Peppas. The data fitting model was analyzed by Microsoft Excel with DDSolver add-ins (Mazhar *et al.*, 2023; Khan *et al.*, 2022; Zhang *et al.*, 2010), and the coefficient of determination (R^2) was used as a goodness-of-fit indicator (Costa and Lobo, 2001).

2.7. Statistical Analysis

Data were collected from triplicate experiments and presented as mean ± standard deviation. Mean comparisons were carried out using One-way ANOVA, followed by the Least Significant Difference (LSD) multiple comparisons test. The *p*-value less than 0.05 was considered statistically significant. For the BSA release profile, the difference factor (f_1) was used to compare the profile release curve between samples (Zhang *et al.*, 2010; Costa and Lobo, 2001). Furthermore, the difference factor (f_1) can be calculated using Equation 2 in Microsoft Excel with the DDSolver add-ins.

$$f_1 = \left[\frac{\sum_{t=1}^{n} |R_t - T_t|}{\sum_{t=1}^{n} R_t} \right] \quad (2)$$

where R_t and T_t are cumulative amounts of released BSA from the reference data group and test data group, respectively. t is a time-t point, and n is the number of test repetitions. The BSA release profile between the two samples was considered different if $f_1 > 15$ and vice versa.

3. Results and Discussion

3.1. Encapsulation Efficiency (%EE)

In this study, all samples were prepared with a thin film hydration method; therefore, protein encapsulation or any active compound is affected by the thin film rehydration

process. This is due to the thin film swelling and increased fluidity and permeability of the formed lipid bilayer (Lombardo and Kiselev, 2022). Two different freeze-thaw cycles (5 and 10 cycles) were evaluated in terms of encapsulation efficiency (Figure 2).

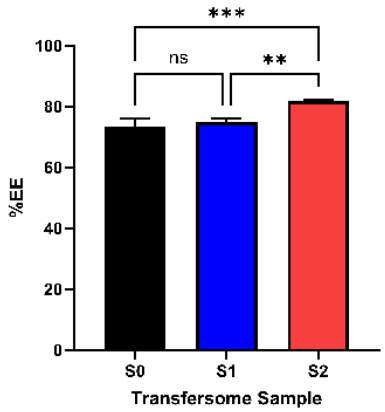

Figure 2 Encapsulation efficiencies of transfersomes prepared with or without freeze-thaw (n=3). S0, S1, and S2 indicated transfersomes prepared without freeze-thaw, with 5 cycles of freeze-thaw and 10 cycles of freeze-thaw, respectively. (**) $p < 0.01$; (***) $p < 0.001$; (*ns*) no significance

Thin film hydration creates multilamellar vesicle (MLV) transfersome (Lombardo and Kiselev, 2022; Khayrani *et al.*, 2019). Structural changes will increase the encapsulated protein amount within transfersome particles due to disruption and membrane fusion during the freeze-thaw process (Sriwongsitanont and Ueno, 2011). The formed multilamellar becomes fragile and disrupted into bilayer fragments during the freezing process because of ice crystal formation on the polar part membrane of the vesicle. Furthermore, during the thawing process (45°C) above the transition lipid temperature (DPPC = 41.3°C), the bilayer fragments will be re-assembled by membrane fusion to form new vesicles with less lamellar number, which subsequently makes them able to encapsulate free proteins. Besides that, a decrease in the lamellar number on the membrane obtains a bigger internal volume within the particle, so it will increase the encapsulation efficiency (Sriwongsitanont and Ueno, 2011).

The encapsulation efficiency of transfersome with 5 cycles of freeze-thaw had no significant differences ($p > 0.05$) as compared to those without freeze-thaw. The possible explanation for this result is a shorter freezing process compared to 10 cycles of freeze-thaw, leading to suboptimal vesicle disruption. In other words, each bilayer layer in the formed multilamellar vesicle was not completely disrupted with 5 cycles of freeze-thaw, resulting in suboptimal encapsulation of free BSA by the new vesicle.

3.2. Physicochemical Properties

The physicochemical characteristics such as size (diameter), polydispersity index (PDI), and zeta potential can give information about particle ability in drug delivery, particle homogeneity, and particle stabilization. Based on Figure 3, transfersome particles with a diameter lower than 300 nm were obtained. That particle size indicates that transfersome particles can penetrate the skin epidermis (Das, Nayak, and Mallick, 2022; Wang *et al.*, 2020). In other words, the obtained transfersome particle is able to deliver protein transdermally. Moreover, based on the polydispersity index value, the obtained particle has a uniform size or homogenous size distribution because the PDI value is lower than 0.5 (Danaei *et al.*, 2018). Because of that PDI value, the obtained transfersome particles could efficiently deliver valuable molecules to a precise target (Danaei *et al.*, 2018). Nevertheless, the three samples have low particle stability because the zeta potential value

is between -10 mV and 10 mV (Wang *et al.*, 2020). The low zeta potential value indicates that the obtained transfersome particle is prone to agglomeration, coagulation, and flocculation during storage in the colloidal system (Manaia *et al.*, 2017). The low transfersome particle stability in the colloidal system can lead to inadequate transdermal delivery and suboptimal release of protein or drugs.

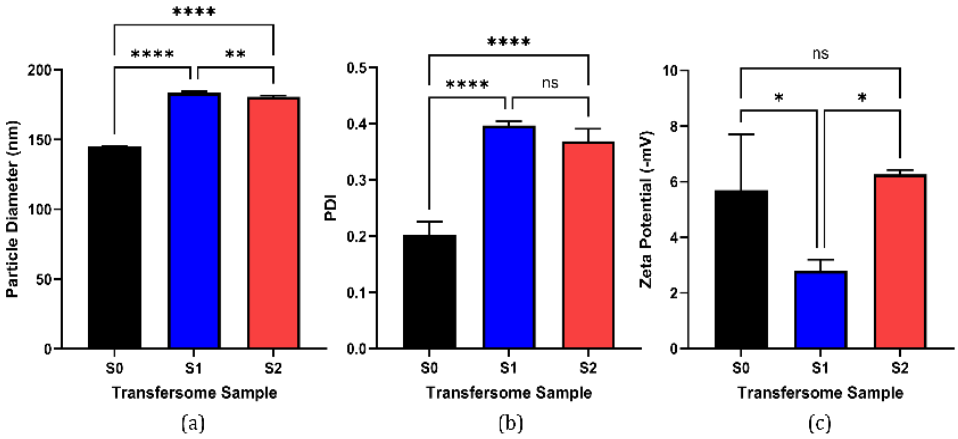

Figure 3 Physicochemical characteristics of transfersome (n = 3). (a) Particle diameter (b) PDI, (c) Zeta potential. S0, S1, and S2 indicated transfersomes prepared without freeze-thaw, with 5 cycles of freeze-thaw and 10 cycles of freeze-thaw, respectively. (**) $p < 0.01$; (***) $p < 0.001$; (*ns*) no significance

The results of particle diameter and polydispersity index measurement showed that the diameter and polydispersity increased ($p < 0.0001$) due to the freeze-thaw cycle process. The increase in diameter and PDI can be caused by the formation of new particles and aggregate during the thawing process, which can increase both the particle size and size distribution. This result is consistent with Castile and Taylor (1999) study, reporting an increase in diameter and size distribution caused by freeze-thaw. In addition, the freezing step can decrease the repulsive force and distance between membranes because of particle dehydration caused by ice crystal formation (Bernal-Chávez *et al.*, 2023; Boafo *et al.*, 2022).

Based on the diameter and polydispersity measurement, transfersome particles produced in this study were quite suitable for transdermal protein delivery. However, the obtained particle diameter and polydispersity index can still be reduced by cryoprotectant addition. The presence of cryoprotectants, such as cellobiose, sucrose, lactose, and glycerol on transfersome formulation can inhibit ice crystal formation, which can prevent nanovesicle damage that impacts particle size distribution (Bernal-Chávez *et al.*, 2023; Boafo *et al.*, 2022). Based on that statement, inhibition of ice crystal formation is slightly contradicted by the freeze-thaw cycle, which is intended to reduce lamella numbers and increase encapsulation efficiency. However, the report by Susa *et al.* (2021) showed that particle sizes could be reduced with cryoprotectant due to the freezing process, which further disrupts membranes into bilayer fragments. Then, the fragments are re-assembled to form new vesicles with smaller sizes in the thawing process. Therefore, the addition of cryoprotectant into transfersome that contain surfactant can prevent particle aggregation during the freezing process. Further research is needed to study the influence of cryoprotectants in lamellarity and encapsulation efficiency of freeze-thawed transfersome.

The zeta potential measurement showed zeta potential changes on S1 transfersome particles, which indicated more neutral potential than the control sample (S0) and sample S2. These changes can be affected by constituent material and/or hydration medium (Khan

et al., 2021; Heurtault *et al.*, 2003). If the constituent material affects the particle zeta potential, particle structure changes due to freeze-thaw can lead to a zeta potential shift (Bernal-Chávez *et al.*, 2023; Sungpud *et al.*, 2020; Costa, Xu, and Burgess, 2014). Nevertheless, in this study, zeta potential changes on sample S1 were not affected by freeze-thaw cycles. It happens because the DPPC used is neutral in a PBS medium with pH 7.4, and Tween 80 is categorized as a non-ionic surfactant. Then, the structural changes due to freeze-thaw cannot alter the zeta potential of samples S1 and S2. It indicates that the zeta potential shift is due to pH changes in the medium. In the S1 sample preparation, there may be impurities that lower the pH of the medium, thereby reducing the negative charge on the S1 sample transfersome (Heurtault *et al.*, 2003).

The zeta potential is one of the important parameters to measure particle stability. Generally, transfersome is used for topical administration of protein or drugs through the skin in the colloidal form like ointment, so it must have high particle stability to prevent particle aggregation during storage. The transfersome particles are considered stable if the zeta potential is more than 30 mV and less than -30 mV (Manaia *et al.*, 2017). However, every transfersome particle obtained in this study, mainly with the freeze-thaw cycles, has low stability in the colloidal system. Consequently, these particles are prone to aggregation and an increase in diameter. To enhance transfersome particle stability in the colloidal system, cholesterol addition to the transfersome formulation can be employed (Maritim, Boulas, and Lin, 2021). The increased particle stability is obtained from the increased rigidity of the particle membrane due to cholesterol presence, which can maintain particle fluidity by lowering the tilt angle of the phospholipid component (Yeo, Yoon, and Lee, 2022; Khan *et al.*, 2021). On the other hand, cholesterol addition also reduces leakage of the encapsulated compound so it can maintain or even increase the encapsulation efficiency (Hsieh *et al.*, 2021; Khan *et al.*, 2021; Maritim, Boulas, and Lin, 2021; Lu *et al.*, 2014). Despite this, cholesterol addition into transfersome formulation also contradicts the ultra-flexibility properties of transfersome because the addition of cholesterol can reduce deformability and transfersome permeability into the skin (Duangjit *et al.*, 2013). The cholesterol addition into transfersome formulation needs further research to obtain optimal cholesterol amounts so it can form a stable transfersome but still maintain its flexibility.

3.3. In vitro Protein Release Studies

In vitro BSA release from transfersome was evaluated for 78 hours using an incubation temperature of 37°C and phosphate-buffered saline (PBS) with pH 7.4 as the release medium in order to mimic human physiology conditions (Yilmaz *et al.*, 2020; D'Souza, 2014). Figure 4 shows protein released from various transfersomes. In 78 hours, the BSA release for samples S0, S1, and S2 were 37.48%, 40.39%, and 52.80%, respectively. The BSA release profile showed a biphasic release pattern, which was indicated by initial burst release and sustained release.

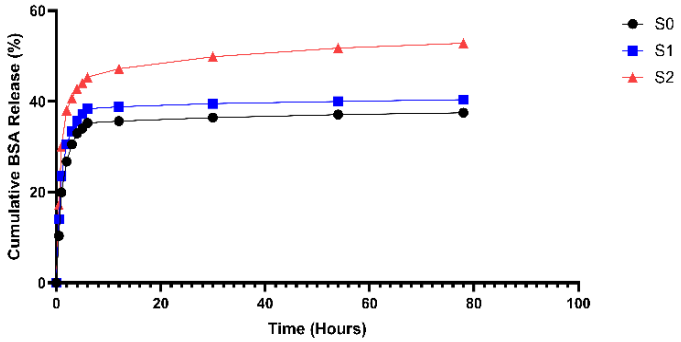

Figure 4 Protein release profile of encapsulated BSA

Based on different factors (f_1), results show that between S0 and S1 have similarities (f_1 < 15), whereas between S2 with two other samples have differences (f_1 > 15). Furthermore, the BSA release profile graph shows that for 78 hours, a transfersome sample with 10 cycles of freeze-thaw tends to release BSA more than the two other samples. The difference in the amount of BSA released by each transfersome sample is closely related to the number of lamellar on the transfersome membrane. Transfersome sample S2 is suspected of having a lower lamellar number or even an unilamellar membrane that makes BSA diffuse out of particles easily. On the other hand, S0 and S1 transfersome samples have less released BSA compared to the S2 sample. The slower release observed in S0 and S1 samples can be attributed to a higher number of lamellar structures compared to S2 samples. Due to the presence of multilamellar on particle membranes, the particle has thicker boundaries, so BSA takes more time to diffuse out (Khan *et al.*, 2021; Sungpud *et al.*, 2020). It is also consistent with the research results of Matsuura-Sawada *et al.* (2023), which show that paclitaxel (PTX) release is slower in multilamellar liposomes.

In the sustained release phase in vitro, there will be a slow decline in vivo due to drug distribution, metabolism, or drug excretion in drug pharmacokinetics (Adepu and Ramakrishna, 2021; Yoo and Won, 2020; Rahul *et al.*, 2015). On the other hand, the BSA release profile in this study shows the sustained release of the amount of BSA released less than 60%, which suggests the transfersome particle can increase protein bioavailability. The biphasic release pattern can reduce dosage frequency and improve patient compliance (Rahul *et al.*, 2015). However, the drawbacks of this pattern are the possibility of drug excess to the toxicity threshold in blood circulation due to initial burst release. Thus, it would give drug side effects (Yoo and Won, 2020).

The mathematical modeling of release kinetic was performed using Microsoft Excel add-ins, namely DDSolver (Mazhar *et al.*, 2023; Khan *et al.*, 2022; Zhang *et al.*, 2010). The model fitting results for BSA release kinetic were summarized in Table 1.

Table 1 Release Kinetic Model-fitting Results

Kinetic Model	Parameters	Sample Code		
		S0	S1	S2
Zero Order	k_0	0.703	0.761	0.974
	R^2	-2.79	-3.26	-2.97
First Order	k_1	0.013	0.015	0.020
	R^2	-2.41	-2.76	-2.19
Higuchi	k_H	6.510	7.082	8.925
	R^2	-0.72	-0.98	-0.76
Korsmeyer-Peppas	K	20.42	24.26	29.50
	n	0.191	0.160	0.169
	R^2	0.77	0.82	0.87

The BSA release kinetic model fitting shows a negative coefficient of determination (R^2) for all samples in zero order, first order, and Higuchi models. A negative coefficient of determination indicates that release profile data has a worse fitting with those three models. When compared with all release kinetic models, Korsmeyer-Peppas has the highest R^2 value in all samples. This indicates that S0, S1, and S2 have the Korsmeyer-Peppas release model. Based on the Korsmeyer-Peppas model, transfersome particles in every sample have a Fickian diffusion mechanism with n value less than 0.43 (Bayer, 2023). That parameter shows that the BSA release mechanism from a transfersome particle is diffusion caused by the concentration gradient between the vesicle core and the outer side of the particle. Such diffusion mechanisms can be triggered by the hydrophilic properties of BSA

and the presence of pores in the transfersome membrane due to the surfactant content as an edge activator, so the BSA can diffuse easily (Opatha, Titapiwatanakun, and Chutoprapat, 2020). In addition, Korsmeyer-Peppas release kinetic model in every transfersome sample also indicates that protein release occurs over a long period of time due to slow release on the release profile (Mazhar *et al.*, 2023), which may benefit in less frequency for repeated protein injection for a therapy (Khan *et al.*, 2022).

4. Conclusions

This study evaluated the influence of repeated freeze-thaw process for transfersome characteristics in terms of encapsulation efficiency, particle size, and polydispersity. It was found that 10 cycles freeze-thaw had more significant effects than 5 cycles freeze-thaw. The encapsulation efficiency of transfersome was improved up to 81.63±0.00% (10 cycles freeze-thaw) from 73.35±0.025% (without freeze-thaw). However, the particle sizes of transfersome fabricated with freeze-thaw were enlarged from 144.93±0.21 to 180.70±0.87 nm, and polydispersity was increased from 0.202±0.02 to 0.369±0.02 in the freeze-thawed samples. The proteins released from the transfersome were then evaluated in the in vitro system. Out of the four kinetic models, it was observed that the protein release from transfersomes exhibited the best fit with the Korsmeyer-Peppas model. After 78 h incubation, transfersomes prepared from freeze-thaw had better protein release (52.80%) than those without freeze-thaw (37.48%). In conclusion, freeze-thaw is a suitable method to improve the characteristics of DPPC-based transfersome, and further study is needed to understand the impact of cryoprotectant addition to freeze-thawed transfersome.

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