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The Influence of Hydration Temperature and Lipid Weight in *C*-4-hydroxy-phenylcalix[4]pyrogallolarene Liposome Preparation as Skin Brightening

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ABSTRACT

The *C*-4-hydroxy-phenylcalix[4]pyrogallolarene is a compound proven to inhibit tyrosinase enzyme activity. This activity makes *C*-4-hydroxy-phenylcalix[4]pyrogallolarene as a potential skin-brightening agent. However, the poor water solubility of this compound limits its application as a skin brightener. Therefore, this study formulated *C*-4-hydroxy-phenylcalix[4]pyrogallolarene liposomes to address this issue. The liposomes were prepared using the thin-layer lipid film hydration method with L-α-phosphatidylcholine as the lipid base. The resulting C-4-hydroxy-phenylcalix[4]pyrogallolarene liposomes were found in spherical forms, and they exhibited multilamellar vesicle characteristics. This study also examined the influence of hydration temperature and L-α-phosphatidylcholine concentration on liposome size and polydispersity index (PDI). ANOVA analysis using Design Expert-13 indicated that the factors of hydration temperature, L-α-phosphatidylcholine weight, and their interaction all influenced the size and PDI of the resulting *C*-4-hydroxy-phenylcalix[4] pyrogallolarene liposomes.

1. Introduction

Melanin production is a complex process involving the crucial role of tyrosinase.[1] The overactivity of tyrosinase is often implicated in the overproduction of melanin, resulting in hyperpigmentation[2]. Hyperpigmentation is characterized by dark spots and uneven skin tone.[3] This condition can affect a person's appearance. A strategy to address hyperpigmentation is to inhibit tyrosinase activity. Various natural and synthetic inhibitors, such as kojic acid, arbutin, and hydroquinone, have been extensively studied for their ability to reduce melanin production by directly inhibiting tyrosinase activity.[4] Furthermore, the quest for new and more effective tyrosinase inhibitors continues, with ongoing research focusing on both synthetic derivatives and natural compounds that can provide safe and effective solutions for hyperpigmentation.[5,6]

The synthetic compound of calixpyrogallolarene derivative, specifically *C*-4-hydroxy-phenylcalix[4] pyrogallolarene, as shown in Figure 1, has garnered significant attention in the field of medicinal chemistry, particularly for its role as a tyrosinase inhibitor.[7] The mechanism by which *C*-4-hydroxy-phenylcalix[4]pyrogallolarene acts as a tyrosinase inhibitor can be attributed to its phenolic structure. The presence of hydroxyl groups in calixpyrogallolarene structure enhances its ability to form hydrogen bonds with tyrosinase, thereby inhibiting its activity. This interaction is crucial as it can lead to a decrease in melanin production, making it as a potential candidate for cosmetic applications aimed at skin brightening.[8] Moreover, the antioxidant properties of *C*-4-hydroxy-phenylcalix[4]pyrogallolarene further support its role in inhibiting tyrosinase, as oxidative stress has been linked to increased tyrosinase activity and subsequent melanin production.[7] These properties make *C*-4-hydroxy-phenylcalix[4]pyrogallolarene more effective as a tyrosinase inhibitor compared to kojic acid, which is already used in cosmetic formulations.[9] While *C*-4-hydroxy-phenylcalix[4]pyrogallolarene presents intriguing properties as a tyrosinase inhibitor, its limitation in solubility restricts its practical applications in cosmetics. Addressing these challenges, we formulated liposomes with *C*-4-hydroxy-phenylcalix[4]pyrogallolarene in the present work.

Liposomes are lipid-based vesicles consisting of phospholipid bilayers that encapsulate an aqueous core, allowing for the simultaneous delivery of both hydrophilic and lipophilic drugs.[10-11] The structural versatility of liposomes is one of their most significant advantages. They can be engineered to vary in size, lamellar structure, and lipid composition, which influences their pharmacokinetic properties and drug release profiles.[12-14] This adaptability allows for the optimization of liposomal formulations to achieve desired therapeutic outcomes.[11,15] This is particularly relevant for calixpyrogallolarene, which may face solubility challenges in conventional formulations. By utilizing liposomes as a delivery vehicle, the bioavailability of *C*-4-hydroxy-phenylcalix[4]pyrogallolarene can be significantly enhanced. The ability to tailor the liposomal formulation to the specific physicochemical properties of *C*-4-hydroxy-phenylcalix[4]pyrogallolarene can lead to improved therapeutic outcomes as a tyrosinase inhibitor.

In this research, we employed L-α-phosphatidylcholine as lipid-based. This lipid-based agent serves as a highly effective liposome carrier due to its unique physicochemical properties and biocompatibility, and it can be tailored for specific applications. It can enhance the stability and integrity of the liposomal bilayer, maximizing therapeutic efficacy and minimizing the risk of adverse reactions when used in drug delivery applications.[16-18] These advantages are important for maintaining the therapeutic payload during storage and transit through the biological environment. The application of liposomes formulated with L-α-phosphatidylcholine has been successfully used to improve the topical delivery of ascorbic acid, enhancing its penetration into the skin and thereby maximizing its antioxidant effects.[19] The spherical *C*-4-hydroxy-phenylcalix[4] pyrogallolarene liposomes are observed, and they exhibit multilamellar vesicle (MLV) characteristics. In this study, the effects of hydration temperature and L-α-phosphatidylcholine weight on the size and polydispersity index (PDI) were examined using Design Expert-13 with ANOVA statistical methods. The influence of each factor and their interaction on the

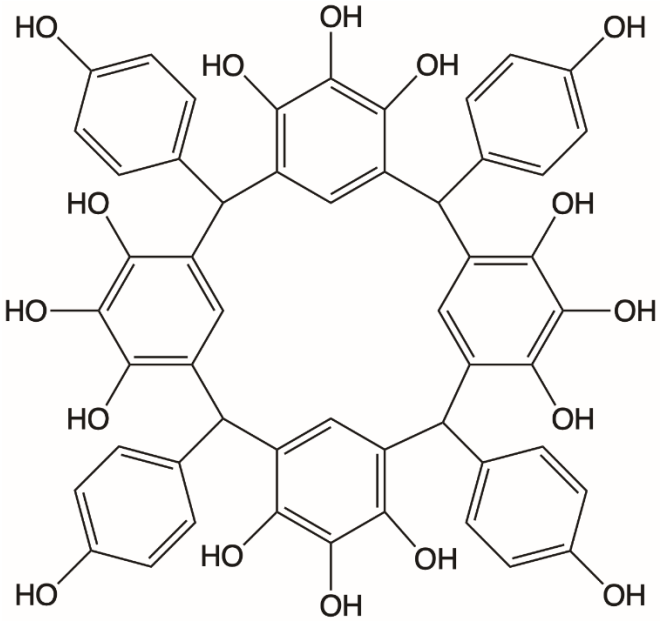

Figure 1. *Structure of C-4-hydroxy-phenylcalix[4]pyrogallolarene*

size and PDI of the *C*-4-hydroxy-phenylcalix[4]pyrogallolarene liposomes was discussed.

2. Material and Methods

2.1. Material

The L-α-phosphatidylcholine (soy lecithin) (Merck), chloroform (Merck), *C*-4-hydroxy-fenilcalix[4]pyrogallolarene (provided by Department of Chemistry, Universitas Gadjah Mada), and deionized water generated by a Thermo reverse osmosis water purification system with a resistance of 18.2 MΩ.cm were employed in this experiment.

2.2. Methods

2.2.1. Liposome Preparation Procedure

Liposome preparation was conducted using the thin layer lipid film hydration method.[20] A two-factor, two-level factorial design was employed to investigate the influence of L-α-phosphatidylcholine weight (0.5 and 1.5 g) and hydration temperature (60 and 80°C) on liposome characteristics. The experimental design was generated using Design Expert-13 software trial version (Stat-Ease, MN, USA), resulting in four experimental runs.

Liposomes were prepared by dissolving L-α-phosphatidylcholine in chloroform in a round-bottom flask, with concentrations of either 0.5 or 1.5 g/50 mL as per the experimental design. The organic solvents were removed using a rotary evaporator (Buchi Rotavapor R-300, Flawil, Switzerland) to form a thin lipid film, which was then dried in a desiccator overnight to remove any residual solvent. The lipid film was hydrated with deionized water, which contains 0.01 g *C*-4-hydroxy-phenylcalix[4]pyrogallolarene at either 60 or 80 °C according to the experimental design. The mixture was then stirred

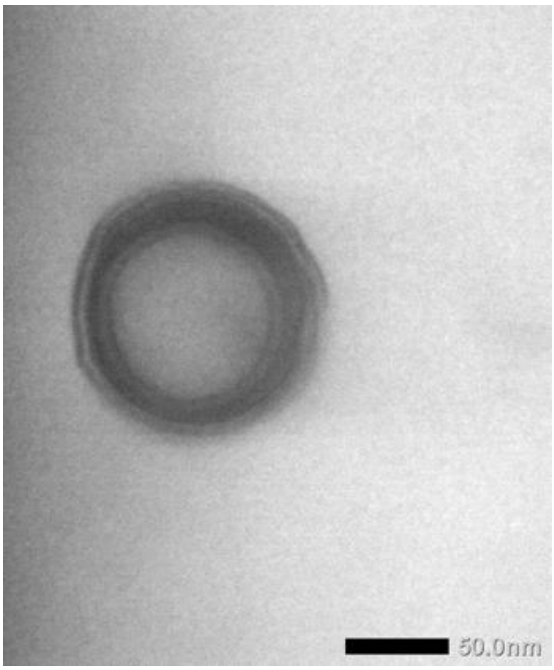

Figure 2. *TEM micrograph of the C-4-hydroxy-phenylcalix[4]pyrogallolarene liposomes*

for 3 hours. The resulting dispersion was refrigerated for 24 hours. Morphological liposomes were analyzed using transmission electron microscopy (TEM). Characterization of the liposomes focused on size and polydispersity index (PDI) measurements, which were performed using scattering light intensity (Horiba Scientific SZ-100, Japan). The measurement was conducted at 25 °C, with a scattering angle of 90°. Data analysis was performed using Design Expert-13 software. The effects of soy lecithin concentration and hydration temperature on liposome size and PDI were evaluated using analysis of variance (ANOVA). Main influences and interaction plots were generated to visualize the impact of each factor on the responses. The statistical significance of the factors and their interaction was determined at a 95% confidence level ($p < 0.05$).

2.2.2. TEM Analysis

The morphological features of the liposomes were observed using TEM. For TEM sample preparation, a drop of the liposome suspension was placed on a carbon-coated copper grid and allowed to adsorb for 5 minutes. Excess liquid was removed using filter paper. The sample was then negatively stained with 2% (w/v) uranyl acetate solution for 2 minutes. The excess stain was removed, and the grid was air-dried at room hydration temperature. TEM images were captured using a JEOL JEM-1400 operating at an acceleration voltage of 100 kV. Multiple fields were viewed, and representative images were captured to analyze the shape, lamellarity, and overall structure of the liposomes.

1.1.3. Particle Size Analysis

Liposome physical properties were characterized using a Horiba Scientific SZ-100, Japan. The PDI, zeta potential, and Z-average (intensity-weighted mean hydrodynamic diameter) were measured for each liposome formulation. Samples were diluted with filtered deionized water to achieve an optimal concentration for analysis. Measurements were performed

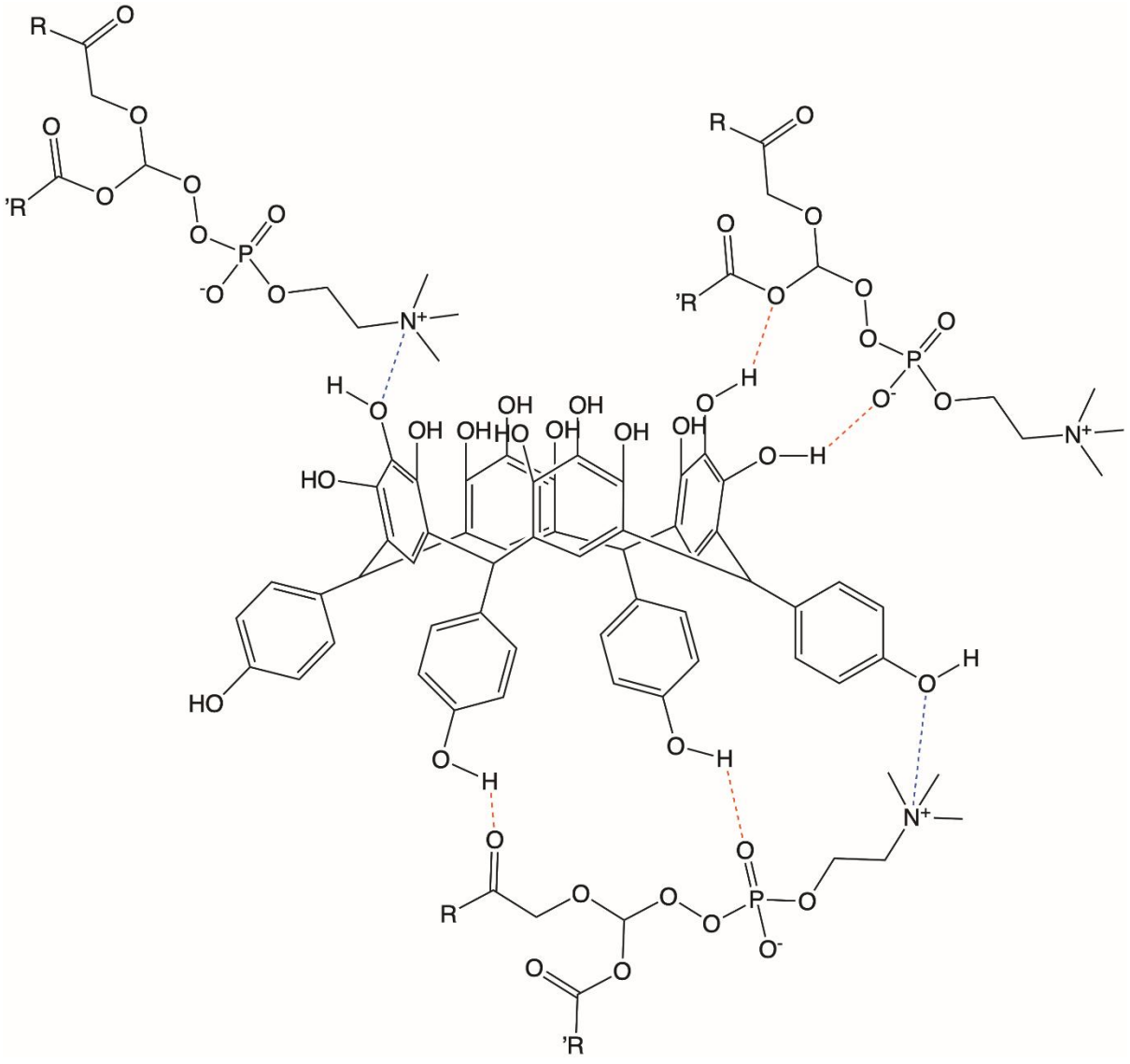

Figure 3. *Hydrogen bonds and electrostatic interactions between C-4-hydroxyphenylcalix[4]pyrogallolarene and L-α-phosphatidylcholine. Hydrogen bond and electrostatic interaction are shown as red and blue dotted lines, respectively.*

at 25 °C after a 2-minute equilibration period. The PDI, which indicates the width of the particle size distribution, was obtained through cumulant analysis of the dynamic light scattering data. Zeta potential was determined using laser Doppler micro-electrophoresis, providing information on the surface charge and stability of the liposomes. The Z-average size was calculated from the intensity of scattered light, offering a measure of the overall mean diameter of the liposome population. All measurements

Table 1. ANOVA results for liposome size factorial model

Source	Sum of Squares	df	Mean Square
Model	14209.63	3	4736.54
A-Lipid Weight	1668.72	1	1668.72
B- Hydration Temperature	7577.70	1	7577.70
AB	4963.20	1	4963.20
Pure Error	0.0000	0	
Cor Total	14209.63	3	

were repeated on three independently prepared batches to ensure reproducibility.

3. Results and Discussion

The characterization of the *C*-4-hydroxy-phenylcalix[4]pyrogallolarene liposomes was performed using TEM, as shown in Figure 2. The TEM analysis results indicated that the liposomes were spherical in shape and exhibited multilamellar vesicle (MLV) characteristics. Spherical liposomes demonstrated the lowest energy configuration, rendering them as the most stable form. This stability was particularly advantageous in the encapsulation of drugs, where achieving a minimum energy state was crucial for preserving liposome integrity during drug delivery.[21] Moreover, the spherical geometry promotes uniform stress distribution across the liposome surface, minimizing the risk of rupture and ensuring controlled and consistent drug release.[22] From the chemistry point of view, the spherical morphology of liposome could be due to hydrogen bonds and electrostatic interactions between *C*-4-hydroxy-phenylcalix[4]pyrogallolarene and L-α-phosphatidylcholine, as proposed in Figure 3.

MLVs offer several advantages that make them highly suitable for drug delivery applications. Their increased hydrophobic volume compared to unilamellar vesicles (ULVs) allows for the efficient encapsulation of hydrophobic drugs, potentially enhancing both delivery and therapeutic efficacy.[23] Additionally, the multilamellar structure imparts greater stiffness, which can improve cellular uptake by enhancing the interaction between MLVs and cellular membranes. The stiffness increases with each additional lipid bilayer, further optimizing this interaction.[23] Moreover, MLVs demonstrated high encapsulation efficiency for a wide range of drug molecules, including small molecules, peptides, and proteins, within their internal aqueous compartments, facilitating sustained drug release, particularly in non-vascular administration routes.[24]

3.1. Liposome Size

We used a two-level factorial design to determine the influence of hydration temperature and lipid weight on the size and PDI of the *C*-4-hydroxy-phenylcalix[4]pyrogallolarene liposomes. The ANOVA results indicate that neither the model nor the interaction between lipid weight and hydration temperature significantly influenced the size of the produced liposomes ($p > 0.05$), as shown in Table 1. Despite the lack of statistical significance, the derived regression equation $Y = 800.28 + 20.42X_1 - 43.53X_2 + 35.23X_1X_2$ provides insight into potential trends affecting liposome size. In this equation, Y represents liposome size, X_1 denotes lipid weight, and X_2 signifies hydration temperature, while the term X_1X_2 captures the interaction between lipid weight and hydration temperature.

The equation suggests that lipid weight has a more substantial impact on increasing liposome size compared to hydration temperature. Specifically, lipid weight exhibits a positive correlation with liposome size, indicating that an increase in lipid weight tends

Table 2. ANOVA results for liposome PDI factorial model.

Source	Sum of Squares	df	Mean Square
Model	0.0144	3	0.0048
A-Lipid Weight	0.0006	1	0.0006
B-Hydration Temperature	0.0005	1	0.0005
AB	0.0132	1	0.0132
Pure Error	0.0000	0	
Cor Total	0.0144	3	

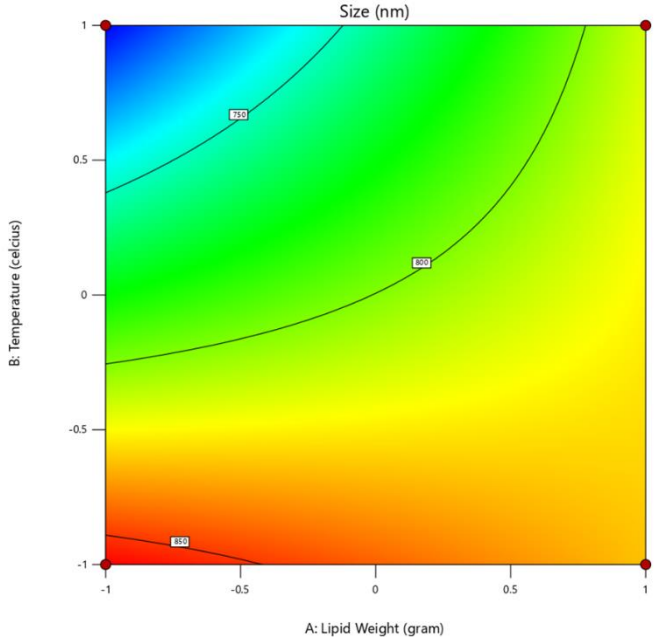

Figure 4. *Contour plot of lipid weight and hydration temperature Influence on liposome size*

to result in larger liposomes. Conversely, hydration temperature shows an inverse relationship, where higher hydration temperatures are associated with smaller liposome sizes. Additionally, the interaction term implies that the effect of hydration temperature on liposome size varies depending on the lipid weight. At lower lipid weights, an increase in hydration temperature leads to a reduction in liposome size. In contrast, at higher lipid weights, an increase in hydration temperature contributes to an increase in liposome size.

Figure 4 clearly demonstrates the influence of lipid weight and hydration temperature on the sizes of liposomes. With increasing lipid weight, the size of the liposome increases due to the larger sizes (red and orange regions) with higher lipid weights. An increase in hydration temperature results in a decrease in the size of the liposomes, as observed from the change in color from warm to cooler colors in the plot. This inverse relationship would imply that the lipid weight is conditioning liposome growth in a hydration temperature-dependent manner, which

is bound to reduce size, likely because of its effect on the formation of the lipid bilayer. In non-linear statistics, two variables interact non-additively if their combined action on the dependent variable is something more than an additive relationship. For instance, the size reduction is greatly enhanced by hydration temperature at lower lipid weights, while at higher lipid weights, the increase in hydration temperature has less effect on the resulting size. This would imply that there is a need to carefully optimize both lipid weight and hydration temperature for size control of liposomes in formulations.

Our findings showed a difference in liposome size between the light scattering assay (particle size analyzer) and TEM results. This is consistent with research conducted by Filipov et al., 2023,[25] which found that DLS typically reports larger sizes than TEM due to the inclusion of the hydration shell and potential particle agglomeration in solution. In contrast, TEM requires drying the sample, which can cause shrinkage or morphological changes, potentially leading to smaller size estimations.

1.2. **PDI Results of Liposome**

The analysis suggests that lipid weight and hydration temperature may have an influence on the PDI of the liposomes, as shown in Table 2. However, ANOVA results indicate that the effects of both variables, as well as their interaction, do not reach statistical significance ($p > 0.05$). Despite this, the fitted model, described by the equation $PDI = 0.4055 - 0.0125X_1 - 0.0115X_2 - 0.0575X_1X_2$, offers some insight into potential patterns. In this model, PDI represents the polydispersity index, X_1 corresponds to lipid weight, and X_2 represents hydration temperature, with X_1X_2 representing the interaction between these factors.

According to the model, lipid weight has a notable negative effect on PDI, suggesting that increasing lipid weight results in a reduction in PDI, which implies improving uniformity in liposome size distribution. Hydration temperature, in contrast, has a more modest impact, with higher hydration temperatures slightly decreasing PDI, pointing to a minor improvement in size distribution. The interaction between lipid weight and hydration temperature suggests that the combined increase of these two variables could further decrease PDI. Although the ANOVA results did not reach statistical significance, the model hints at a potential relationship between lipid weight, hydration temperature, size, and PDI.

The contour plot of the level of lipid weight (A) and hydration temperature (B) on the PDI of liposomes reflects the size distribution of liposomes, as shown in Figure 5. PDI values between 0.34 and 0.44 are obtained, through which the color gradient and contour lines represent an interaction of both factors in the size distribution of liposomes. The lipid weight drastically affects PDI, which generally goes up along with relatively higher lipid weights, corresponding to a broader size distribution. This is mainly observed in the red and orange areas, where lipid weight is highest, corresponding to approximately 1 g. On the other hand, hydration temperature has a subtle effect: lower hydration temperatures develop liposomes with higher PDI values. At the same time, PDI decreases as the hydration temperatures rise, which results in a more homogeneous size distribution of liposomes. The relationship between lipid weight and hydration temperature is non-linear; the lowest values for PDI are achieved for low weight of lipid and high hydration temperature, which means that this combination provides a better homogenizing effect on size. However, the high lipid weight and low hydration temperature cause high PDI values, which means the size of the liposome formed was found to be highly heterogeneous in these conditions. In other words, both lipid weight and hydration temperature were significant in their interaction effects on PDI, which defines the homogeneity of liposomal size.

3. **Conclusions**

This study emphasizes the influence of lipid weight and hydration temperature for stable *C*-4-hydroxy-phenylcalix[4]pyrogallolarene liposome size and PDI. Even though the effects were not statistically significant, lipid weight increased liposome size, and higher hydration temperatures reduced it. The interaction between these factors influenced both

size and PDI, with lower lipid weight and higher hydration temperatures resulting in more uniform liposomes. These findings offer insights for improving liposomal formulations, especially for compounds with solubility challenges, and suggest further investigation into other formulation factors.

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