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Freeze-Dried Liposome Formulation for Small Molecules, Nucleic Acid, and Protein Delivery

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ABSTRACT

Liposomes are one of the means in the drug delivery system that is quite promising because of its ability to carry active ingredients, both hydrophilic and lipophilic. In addition, the structure of lipid bilayers that resemble cell bilayer membranes is useful in optimizing liposomes' ability through increased internalization of active ingredients into cells. However, liposomes themselves are physically and chemically vulnerable systems, so increasing liposome stability is one of the primary considerations in liposome formulations. Lyophilization or freeze-drying can increase the shelf-life of liposomes, but the process itself is very extreme and prone to triggering damage to vesicle structures. Lyoprotectans from sugars and polyols are usually employed to improve the formulations' stability during the drying process. Physical characterization of the liposome end product is done by looking at the system's morphological appearance, crystallinity profile, thermal analysis, and functional group analysis.

Keywords: Liposomes, stability, lyophilization, freeze-drying, lyoprotectants

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INTRODUCTION

Liposomes are defined as microscopic spherical vesicles with bilayer membranes formed when phospholipids are hydrated or exposed to a water-rich environment. Liposomes have been widely used in drug formulations because of its advantages, among others: can reduce toxicity by ensuring the specificity of target organs and reducing the risk of other organs that can experience adverse effects, the half-life of drugs in the body will increase because the drug is maintained in stable conditions, delivery with liposomes can reduce the effective dose of the drug, and liposomes are suitable carriers for drugs that are hydrophilic or lipophilic[1]. Not only in drug delivery, but liposomes also play an essential role in enhancing the immune system in the vaccine delivery system. Liposome composition, such as the structure of phospholipids, membrane fluidity, and liposome loading plays a vital role in terms of vaccine retention[2].

Despite promising great potential as a carrier in drug delivery systems, the physical and chemical instability of liposomes is limited in the application of liposomes. This instability causes liposomes to experience increased bilayer permeability, leakage of drug ingredients, as well as aggregation of vesicles and precipitation. Instabilities occur due to chemical degradation, namely oxidation of phospholipid fatty acid chains, which results in changes in the permeability of the bilayer membrane. Besides, physical factors such as heating and freezing can also affect the stability of the bilayer membrane[3].

One of the most efficient ways to overcome this problem of instability is to make liposomal dry products by lyophilizing or freeze-drying. Freeze-drying is the leading way to extend the shelf-life of drugs, especially in thermolabile medicinal substances such as active biopharmaceutical ingredients. Removal of water from the liposome system prevents phospholipid hydrolysis. Another advantage is that the final product in the form of a solid phase will reduce the movement of molecules in the system so that it is generally able to reduce chemical and physical instability[3].

However, this process also creates its problems because the freeze-drying procedure can affect the structure of the liposome. Bilayer membranes may shrink when frozen, and the following reconstitution of the products will promote vesicle aggregation/fusion and phase transitions [4]. Therefore, lyoprotectants that function as a stabilizer in the drying process are employes; the commonly used lyoprotectans are compounds with many hydroxy (OH) groups (polyhydroxy compounds) as sugar or saccharides. The mechanism of action of this lyoprotectant includes replacing the hydrogen bonds that were initially occupied by water molecules and preventing hydrolysis. Secondly, the mechanism of stabilization is achieved by creating a viscous glass matrix, thereby reducing the molecular mobility of liposomes during the process and forming barriers between adjacent bilayers [5,6]. For sufficient stabilization power, it is necessary to optimize the lyoprotectant concentration in the system because the phospholipid-lyoprotectant ratio affects the system's overall stability [3].

LIPID BILAYER COMPOSITION IN LIPOSOME FORMULATIONS

The composition of lipid bilayers largely determines the physicochemical characteristics of the liposome system, including the charge and fluidity of the bilayer membrane and the permeability of the membrane to molecules inside

and outside the system. The fluidity of a bilayer membrane can be characterized by the phase transition temperature (Tm) of a bilayer membrane. The Tm value can be adjusted based on the composition of the phospholipids in the liposome system [7]. For example, cationic lipid DDA (dimethyl-dioctadecyl-ammonium) is one of the main components in the vaccine adjuvant system and gene delivery. However, DDA has low physical stability because it easily aggregates in salt-containing solutions, even at a small concentration [8].

For this reason, DDA is combined as a minor lipid component with major lipid components such as soy phosphatidylcholine [9].

The addition of cholesterol to the composition of liposomes can increase lipid packing so that it decreases or negates the phase transition temperature in the liposomal system with the lipid component DDA: Chol: TDB (trehalose-6,6-dibehenate). DDA: TDB has a phase transition temperature above 37 ° C, so this system is like a regular dense membrane at body temperature. The reduced temperature of the gel-liquid phase transition results in increased fluidity of the bilayer membrane and decreases the retention of active ingredients in vitro. However, the addition of high cholesterol in the lipid mixture triggers the formation of crystal habits. Cholesterol is a membrane stabilizer which optimally used in 20-50 of molar percentage in the lipid phase [10]. Cholesterol levels can also affect the pattern of release of active ingredients that are absorbed in the membrane. Increased cholesterol levels slow the release of doxorubicin from the DPPC / DSPC liposome system (75:25) [11].

THE PRINCIPLE OF DRYING USING LYOPHILIZATION TECHNIQUES

Lyophilization is the main technique used in drying pharmaceutical products from biological materials. In contrast to drying with an oven, which involves changing the liquid phase to gas, removal of water in lyophilization utilizes the solid phase's change directly into gas or sublimation. This process involves three main stages: freezing of a system that is rich in water contents, removal of water due to sublimation in primary drying, and finally removal of water that does not freeze with secondary drying[12].

Freezing is the first step in lyophilization and removal of water content from drug ingredients and dissolved excipients because most of the water will be converted into solid form, i.e., ice crystals. Solut in the system will increase in concentration as a result of reduced water (liquid) solvents. This increase in concentration is commonly referred to as freeze concentrate. At this stage, the system is separated into several phases; for instance, the water phase and drug phase interfaces are formed. The freezing stage may cause the destabilization of preparations called freezing damage because of the ingredients' increased concentration level [13,14]. During freezing, the degree of supercooling of the samples affects the ice crystal habit, i.e., size, number, and morphology. This stage impacts the following drying process, for example, the resistance to water vapor flow. Therefore, various degrees of supercooling will eventually lead to heterogeneous drying behavior [15].

Primary drying, which is the phase of removing ice through sublimation, is the most extended process and requires much energy. Optimization at this stage will have a significant effect on production efficiency; thus, research is conducted to shorten this stage's duration. The duration of the step is the most essential to speed up process development and product manufacturing [16,17]. In contrast, secondary drying usually only takes a few hours, so the optimization process does not play a significant role. During the primary drying, sublimation occurs when the pressure in the chamber is lowered to below the ice vapor pressure, and the temperature is raised to supply heat to the product. Secondary drying removes residual water from the product (desorption), generally at low pressure, with an increase in temperature [18].

Primary drying is carried out at a temperature of 5-10 ° C below the temperature of the lyoprotectant or the crystallized material after freezing to obtain a porous solid product. Determination of the glass transition temperature (Tg') during the frozen state is essential because the dried cake resistance sharply decreases above the temperature [19]. Thus, maintaining the process above the Tg' will avoid collapse and successfully form an elegant cake [20]. If the drying temperature is maintained around Tg', vesicle aggregation or fusion occurs, which is thought to be caused by the inability to form a glass matrix. The drying temperature is the factor that has the most considerable influence on the rate of sublimation. The higher the temperature, the greater the sublimation rate and the shorter drying time. In addition to the temperature factor, drying time is also greatly influenced by the height of the sample filling in vials and sample volume. High sample filling and large volumes will result in longer drying times. Secondary drying cannot be easily quantified because the drying rate is affected by the diffusion of water from the product filament and is followed by the desorption process. This process can be accelerated by raising the temperature, but care must be taken to ensure the stability of the active ingredients in the product[4].

EFFECT OF LYOPHILIZATION ON THE STABILITY OF THE BILAYER MEMBRANE

Although lyophilization can help improve the stability of liposome products, the stress that occurs during the process can damage the liposome structure. The freezing process can induce liposomal instability due to increased liposomal concentrations in water media resulting in aggregation or fusion of liposomes, damage to the bilayer structure due to the formation of interfaces of solids and liquids (ice and residual water), and phase separation that can end in segregation liposomes with stabilizers. Process optimization at this stage includes freezing rate, freezing temperature, and duration of the process. Fast freezing (fast freezing rate) generally results in fine ice crystals and a more homogeneous distribution of lyoprotectants [6]. Optionally, the freezing step can be continued with annealing, which aims to allow the crystalline component to be recrystallized, for example, in mannitol and glycine. If the crystalline material fails to crystallize, there will be a

decrease in Tg' and may even cause recrystallization during storage. Also, if crystalline material is present in large quantities and undergoes recrystallization during primary drying, it can cause vial rupture[18].

It was reported lyophilized the positively charged liposome formulation containing the DOTAP lipid component (1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP) chloride) and the active ingredient model of the linear hydrophilic decapeptide without the addition of lyoprotectant. Lyophilization is carried out with three different protocols in several parameters: primary drying temperature, freezing rate, and secondary drying. Protocols 1 and 2 involve annealing and a faster freezing rate. Whereas in protocol number 3, the annealing procedure is eliminated, and the freezing rate is slower than the previous. Primary and secondary drying process parameters in the three protocols differ in terms of the plate temperature used, chamber pressure, and duration. There was a significant decrease in vesicle size of the liposome product after drying compared to before the lyophilization process in all protocols, indicate the occurrence of shrinkage caused by the lyophilization process. There was a slight increase in the Poly Dispersity Index (PDI) after lyophilization[21].

MECHANISM OF LYOPROTECTANT IN PROTECTING LIPOSOME STRUCTURE

The mechanism of monosaccharide compounds and disaccharides as lyoprotectants, among others, by replacing hydrogen bonds that have initially been occupied by water molecules, also prevents hydrolysis. Another mechanism is stabilization with a viscous glass matrix, reducing the molecular mobility of liposomes during the process and forming barriers between adjacent bilayers. The ability of sugar as a lyoprotectant is affected by the glass transition temperature (Tg) of the sugar. A matrix is formed at temperatures less than Tg, but at temperatures more than Tg, there will be a decrease in viscosity so that molecular mobility increases[6].

Effect of Lyoprotectant Type and Concentration

Effective lyoprotectants must have several properties: (1) compatibility with other ingredients or excipients in the formula; (2) have sufficient water solubility and miscible with other ingredients; (3) no phase separation occurs during the freeze-drying process; (4) no adverse chemical reactions occur or reduce the ability of active ingredients; for example, the peptide glycation reaction has a glass transition temperature suitable for dry storage, which can undergo in vivo clearance[5].

Drug retention in liposomes is related to phase transitions and the occurrence of aggregation and fusion of vesicles. Bilayer membranes will easily leak when the temperature reaches Tm; this can cause the failure of liposomes in the retention of drug ingredients. Therefore, phase transitions must be avoided during the drying process to increase the drug, which is retained in the liposome [4]. The interaction between sugar and lipids in the liposomal bilayer membrane is responsible for suppressing the transition temperature of the gel to liquid crystalline (Tm) in dry bilayer phospholipids, to avoid the occurrence of the transition phase and avoid the loss of encapsulated drug ingredients. The sugar: lipid ratio is known to affect the ability of lyoprotection. Low sucrose levels can only suppress the phase transition but do not protect liposomes[22].

Different types of encapsulated materials in liposomes or even blank liposomes may have different lyoprotectants to protect them from degradation during the freeze-drying process. Phosphatidylcholine (PC) and its derivatives often use trehalose since it causes stronger interactions with the membrane bilayer and able to form highly ordered clusters around the lipids [23–26]. On the other hand, sucrose is also interesting since it provides an excellent ability to protect neutral and charged liposomes. The sugars play an essential role in stabilizing the liposome formulations, as the lipid head group will interact with each other in close proximity. These interactions result in the increase of van der Waals forces between the lipids resulting in increases in Tg'. Stability can be improved by adding lyoprotectants such as sucrose, which reduces the Tg'values [20].

Another type of lyoprotectants was oligosaccharides and compounds abundant in a hydroxyl group, such as mannitol and glycerol. Oligosaccharides like maltodextrins and beta-cyclodextrin were reported to give proper protection for encapsulated substance in liposomes [26,27]. As it has amorphous nature, maltodextrin has a vitrification effect of maintaining the integrity of the liposomes. Mannitol is suitable for protein and inhalable products. Nonetheless, it quickly recrystallizes during drying. Mannitol can be employed in combinations with the other lyoprotectants to solve these problems since mannitol recrystallization during the freeze-drying process and might be causing drug leakage in the liposome [28–30]. The summary of the different formulations reported in the literature is presented in **Table 1.**

Non-Carbohydrate Excipients in the Dry Liposome Formula

The use of carbohydrate lyoprotectant is the most common approach to maintain liposome stability in the lyophilization process. However, some researchers also conduct original research using other excipients to reduce the level of damage caused by ice crystals or prevent the fusion and aggregation of vesicles. Until now, ingredients other than carbohydrates that are often used as excipients to increase the stability of the liposome system are amino acids, polyalcohols, and several other additives[4].

Guan et al. (2015) used gelatin to maintain the structure of liposomes during freeze-drying because gelatin was mixed in a hot state at temperatures above the gel temperature (Tgel) so that when the system temperature was lowered a transition from sol to gel was formed. The result is liposomes with gelliposome internal structures and is assumed to protect liposome structures better than conventional liposomes. Product quality testing includes adsorption efficiency, particle size before and after reconstitution, reconstitution time, and morphology of liposomes, and it is known that gelliposomes can increase physical stability better than conventional liposomes. In conventional liposomes, structural deformation and recrystallization of the active ingredient of paclitaxel are observed[31].

Table 1: Summary of different formulations of freeze-dried liposomes

Category	Lit.	Encapsulated Substance	Lipid Components	Lyoprotectants	Preparation Methods	Main Findings
Blank Liposome	S. Franzè et al., 2020 [24]	N/A	1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol (CHOL) (70:30, mol:mol ratio)	sucrose, trehalose and/or poly(vinyl pyrrolidone) (PVP),	ethanol injection	Trehalose provided stronger lyoprotectant effect compared with sucrose
	D. Marín et al., 2018 [26]	N/A	Partially purified phosphatidylcholine (PC)	Trehalose	Phosphatidyl choline was mixed with each bioactive solution, and it was kept in a water bath with gentle stirring.	significant increase in particle size and PDI (Polydispersity index) after freeze-drying, no significant change of Zeta potential indicating stable liposomes
	R.W. Nugraheni, et al., 2017 [38]	N/A	SPC, DDA, and cholesterol in the molar ratio of SPC: DDA: Cholesterol = 9:3:1.	maltodextrin and mannitol,	thin film hydration methods	Formulations containing maltodextrin were relatively homogenous compared to the formulations using mannitol, which showed phase separation.
Small Molecules	F. Zhou et al., 2018 [39]	Acteoside	Soybean phosphatidylcholine, cholesterol, and acetonide (in a weight ratio of 10:1:1)	Mannitol and coated with Chitosan	ethanol injection method	Interactions between Chitosan and phospholipid stabilized the thermal property of the API and liposomes
	S. Yu et al., 2020 [30]	Ciprofloxacin and colistin	HSPC, DSPG, PEG, and Chol in a 3:2:0.5:1.7 mass ratio	Different concentrations of sucrose or mannitol were listed as "internal lyoprotectant." The External lyoprotectants were added before USFD using mannitol, sucrose, and leucine alone or in combinations.	thin film evaporation	The Entrapment Efficiency (EE) of Ciprofloxacin increased after the addition of sucrose as an external lyoprotectant.
	T. Ye, et al., 2019 [40]	Clarithromycin	SPC/Chol/CLA in ratio 4:1:2 by mass	15% mannitol and 5% sucrose (W: V) as combination lyoprotectants	thin lipid film hydration	The morphological appearance of the formulations had a porous structure with uniform drug content and recovery.

	M.A. Vélez, et al., 2019 [41]	Conjugated linoleic acid (CLA)	soy phosphatidylcholine and CLA (conjugated linoleic acid isomers 9c, 11t 127 and 10t, 12c) were mixed at 2:1 molar proportion	It is worthy of highlighting that no lyoprotectants were utilized in the formulations.	ethanol injection 33 method ethanolic injection technique	No lyoprotectants were included in the formulations; however, CLA loaded liposomes were more stable than the blank counterparts.
	Q. Wang, et al., 2019 [42]	Daidzein	55:40 for the molar ratio of soybean phosphatidylcholine: cholesterol and 1:10 for the mass ratio of daidzein to total lipid.	3% sucrose (used as a lyoprotectant)	lipid film-hydration method.	Stable liposomes with high drug loading
	CF de Freitas, et al, 2019 [43]	Erythrosine-decyl ester (ERYDEC) and biotinylated-F127 (f127eb)	DPPC (1.5×10−3 mol L−1) and copolymer (F127 or F127eB) 0.02% or 0.20% w/V were co-solubilized in a mixture of CHCl3:CH3OH (4:1, V/ V)	trehalose (3.0×10−4 mol L−1)	thin film hydration	High encapsulation efficiency and slow-release kinetics
	T. Toniazzo, et al., 2017 [44]	Quercetin	Liposomes containing 12, 16, and 20 mM of phospholipid incorporated with 0.1, 0.2, 0.3, 0.4, 0.5% of quercetin,	Sucrose was used as lyoprotectant at 2:1, 3:1, and 4:1 ratio (sucrose: phospholipid)	ethanol injection	the encapsulated quercetin had not degraded after 100 days of storage. In addition, the lyophilized liposomes presented a low propensity for water adsorption and low hygroscopicity.
	M. Wang et al., 2016 [45]	Ursolic acid (UA)	SPC, CHOL, and UA (in a weight ratio of 50: 6: 5)	Mannitol and sucrose	ethanol injection method	Chitosan was successfully modified on the liposomes containing Ursolic acid. Chitosan gives more positive charges and improves the stability of the vesicles.
Essential Oil	Y. Zhu et al., 2020 [46]	Nutmeg (Myristica fragrans Houtt) essential oil (NEO)	NEO (5 mg/ mL), soybean lecithin (20 mg/mL), cholesterol (4 mg/mL)	β-cyclodextrin (β-cyclodextrin:soy bean lecithin at 6:1 ratio w/w	thin film hydration	The formulations are able to maintain their functional properties. i.e., provide protections for the meat batters, even after the freeze-drying process.
Nucleic Acid	M. Rasoulianboroujeni, et al., 2017 [47]	DNA (LacZ)	DOTAP/DOPE/cholesterol with molar ratio of 1:1:2 were dissolved in chloroform (0.5 ml, total	sucrose	lipid film hydration method	There was no significant change in particle size and Polydispersity index after the freeze-drying process.

			concentration of 5 mg/ml).			
	H. Lujan, et al., 2019 [48]	siRNA or synthetic microRNA	Didodecyldimethylammonium bromide), ovine cholesterol, and tocopherol PEG 1000 succinate (TPGS) with molar ratios of 12:7:1 M	no protectants included in the formulations	Ethanol injection methods	Liposomes were able to maintain their homogeneous average sizes and spheroidal shape without notable degradations or artifacts. There was also evidence of the ability to extend the shelf-life based on encapsulation efficiency determination.
Protein	H. Yusuf, et al., 2019 [27]	Ovalbumin (OVA)	Soy phosphatidylcholine (SPC), DDA, and cholesterol were dissolved in methanol in the molar ratio of SPC:DDA: cholesterol (9:3:1)	Maltodextrin	thin film hydration methods	Oligosaccharides and cellulose matrix miscible with cationic liposomes constituents in the amorphous solid state while maintaining the spherical shape of the liposomes.
	M.T. Hussain, et al., 2019 [20]	Ovalbumin (OVA),	Lipids and cholesterol were dissolved in methanol at varying concentrations (maintaining a 2:1 wt/wt ratio)	Sucrose	microfluidics	The sucrose as lyoprotectants dominating the Tg' of the mixture and has a vital role in stabilizing liposomes formulations. Reduces in Tg' value improves the stability of the liposomes.

Amino acids are used as substitutes for sugar excipients in dry liposome formulas because amino acids can form hydrogen bonds with phospholipids and carbohydrates, in addition to the damage due to ice on the surface of liposomes can be prevented through the interaction of phospholipids and amino acids electrostatically[4]. Lysine is reported to have a protective ability comparable to trehalose for EPC liposomes with the active ingredient ibuprofen. Freeze drying protocols used are freezing temperatures of -50 ° C, primary drying of -40 ° C for 6 hours, and secondary drying at -25 ° C for 24 hours[32].

CHARACTERIZATION OF FREEZE-DRIED LIPOSOME PRODUCTS

Powder X-Ray Diffraction (PXRD)

Characterization by X-ray diffraction method was carried out to determine the crystallinity of a compound. This method can be used for quantitative identification and determination of a crystal phase in solid or powder samples. The diffractogram results can provide information in the form of peak position, peak height (crystal intensity), and the presence/absence of amorphous formation. X-rays have wavelengths that are proportional to the distance between atoms in the crescent grille. Each crystal form of a compound can produce a characteristic x-ray diffraction pattern. This pattern is commonly called X-ray diffractograms.

Scanning Electron Microscope (SEM)

It is a microscopy technique with a higher resolution than optical microscopy and can be used to determine the surface characteristics of a solid. Inspection with an electron microscope is intended to see the outer surface or morphology of an object in a vacuum. In this case, we aim to determine knowing information about vesicle shape and surface morphology of liposomal vesicles. Also, SEM observations can be used to determine the matrix structure of the trapped liposome that has been formed. The optimal form of liposome vesicles in a spherical shape and trapped in the matrix as results conducted by Chandran and Pichandy (2015) demonstrating spherical liposome shape at magnification 10,000 times and 5,500 times [33].

Differential Scanning Calorimetry (DSC)

The DSC method is used to observe changes in energy in the form of heat during heating the sample. Thermal events that involve absorption of energy are called endothermic. Meanwhile, thermal events that involve the release of energy are called exothermic. The amount of energy released or absorbed will be directly proportional to the area below the peak. DSC method is used to provide information about the presence or absence interaction between drug ingredients and phospholipid lipid bilayer and amorphicity of drug ingredients after formulation

[34]. The amorphous nature of the material is determined by glass transition temperature (Tg); therefore, it is crucial for the formulation and process development of lyophilized products, and even to determine the storage condition based on their thermal stability. The graph is plotted as heat flow at the y-axis with the temperature at the x-axis [35].

Thermogravimetry Analysis (TGA)

Thermogravimetric analysis is a technique for measuring the weight of a compound as a function of temperature or time. The result is usually a continuous diagram recording. The sample used, weighing several milligrams, is heated at a constant rate, ranging from 1 - 20^{0}C / min, maintaining its initial weight, Wi, until it begins to decompose at Ti temperature. Under dynamic heating conditions, decomposition usually occurs at a specific temperature range, Ti - Tf, and the second constant region is observed at temperatures above Tf, which corresponds to the weight value of the residual Wf.

The value of Wi, Wf, and ΔW weights are fundamental and can be used in quantitative calculations of composition changes. Contrary to weight, Ti and Tf's value depends on various variables, such as the rate of heating, the nature of the substantial (its size), and the atmosphere above the sample[36].

Fourier Transform-Infra Red (FTIR)

Infrared spectroscopy is a method for analyzing the structure and conformation of organic compounds (qualitative and semi-quantitative). The detector will capture the reflection from the infrared ray that hits the lattice into a sample absorption data. This method is straightforward, sensitive, and proven to be a powerful method for characterizing polymorph solids. The radiation used for instrumental analysis is infrared radiation whose wave numbers range from 4000 to 670 cm-1. The infrared radiation used must be in the frequency range that corresponds to the molecule's natural vibration range in order to obtain information on the functional groups of the molecules of the substance to be analyzed. Each group in a molecule has a different natural vibration. If the natural vibrations of the molecular groups match the frequency of infrared radiation, an electric field interaction will occur. Changes will follow molecular vibrational energy changes in the amplitude of molecular vibrations, known as infrared radiation response[37]. The presence of each P = O bond in the phosphate group and the C = O bond in the ester group of the polar phospholipid head interacts with other liposome formulation components. Both peaks are lower in the CH-loaded cryoprotected liposome spectrum due to restrictions in the movement of P = O and C = O, which interact with lyoprotectant molecules (trehalose or sucrose). This result is related to the presence of lyoprotectant, which is amorphous sugar responsible for the formation of glass matrices[37].

CONCLUSION

The use of liposomes as carriers of therapeutic molecules is one area of research that continues to grow. It is because of the possibility to modulate the characteristics of vesicles to make liposomes very versatile both as carriers of several types of drugs (from conventional chemotherapy to proteins and peptides) and in therapeutic applications (from cancer therapy to vaccination). Many studies have shown that lyophilization is an effective way to overcome the problem of liposomal instability in a watery environment. However, some things need attention, including the composition of a suitable lipid bilayer becomes a prerequisite for determining the protective effect of lyoprotectants during the lyophilization process. Another consideration is the addition of cholesterol as membrane stabilizers in the appropriate range can reduce leakage and aggregation of vesicles or fusion during lyophilization. One must also pay attention to the vesicle size and lyoprotectant distribution on both sides of the bilayers that influence the lyoprotective effect. Lastly, the freezing protocol modification can reduce freezing damage and improve the morphology of the result to achieve a better lyoprotective effect, as well as the Tm and Tg of the material used as indicators for best storage temperature selection. Thus, harmony is needed between studies on the optimization of formulations with technological process parameters in order to increase the lyoprotective effect on liposomes resulting from lyophilization.

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