Expert Opinion

Stabilization of liposomes during drying

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Introduction: During the past 40 years, liposomes have been investigated intensively as drug carriers for anticancer drugs and as the adjuvant components of vaccines, for example. In this context, the development of dry formulations of liposomes is important to ensure a more stable drug product and to avoid the use of the 'cold chain' during distribution.

Areas covered: This review provides an overview of the technologies commonly used for the drying of liposomal formulations and the significance of formulation and processing parameters for the drying process. In addition, a review is provided of the protective mechanisms proposed to be responsible for stabilization during processing and in the dry state, with special emphasis on the techniques used for the characterization of the mechanisms. Parameters are discussed that critically influence the liposomal stability during drying and the underlying stabilization mechanisms, including the water replacement theory, vitrification and kosmotropic effects.

Expert opinion: Drying of liposomal formulations has contributed to the development of more stable products because liposomes can be dehydrated in the presence of appropriate stabilizing excipients, without affecting the size or the drug encapsulation efficiency. The key to the successful design and preparation of optimal liposomal dry powder formulations is an understanding of the significance of the drying process parameters, and the mechanisms responsible for the stabilization of liposomes during drying and in the dry state.

Keywords: drug delivery, dry powder formulation, freeze-drying, liposome, spray-drying

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1. Introduction

Liposomes are widely used drug carriers for many applications, and during the past few decades they have been studied intensively for the delivery of a variety of drugs, ranging from small molecules and peptides to proteins and nucleic acids [1,2]. Several liposome-based drug formulations are now being marketed. These include doxorubicin-loaded long-circulating liposomes used for cancer therapy, and conventional liposomes containing amphotericin B intercalated in the liposomal membrane structure for the treatment of fungal infections. At present, there are several ongoing clinical trials with liposome-based products (examples in [1,3-5]). In addition to cancer therapy and treatment of infectious diseases, the use of liposomes also includes vaccination purposes, where the liposomal component in the vaccine serves as adjuvant (reviewed in [6]).

Liposome are usually composed of Nature's own biodegradable and biocompatible building blocks, such as phospholipids and cholesterol, which on dispersion in aqueous medium form closed membrane bilayer structures enclosing an inner

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Article highlights.

- The general instability of liposomes in aqueous dispersion represents a challenge for the commercialization of drug-containing liposomal formulations.
- Dehydration of the formulations reduces the molecular mobility and increases the stability, resulting in increased shelf-life and decreased distribution costs owing to elimination of the need for the 'cold chain'.
- Different drying processes have been developed: freeze-drying is the most commonly studied method, although spray- and spray-freeze-drying may become increasingly important in the future because of their suitability for engineering of particle properties.
- Stabilizing excipients such as carbohydrates are commonly used to stabilize the liposomal structure during the drying process and to avoid fusion and leakage of the encapsulated material.
- Different theories regarding the stabilizing effects of excipients have been proposed in the literature, and there is evidence that all these theories might contribute to different extents, depending on the specific formulation, to the stabilizing effects during drying and in the dry state.

This box summarizes key points contained in the article.

aqueous compartment, with the polar lipid head groups facing the aqueous medium and the nonpolar lipid tails orientated towards the membrane interior [7]. Drug components can be encapsulated in the inner aqueous compartment, intercalated in the membrane bilayer structure or associated to the membrane surface, depending on their physicochemical characteristics and the specific preparation procedure. Liposomes are thermodynamically stable bilayered vesicles with a high degree of versatility, resulting from the numerous possibilities for varying the physicochemical properties of the liposomes, such as the number of membrane bilayers (unilamellar versus multilamellar), size, lipid composition, fluidity and surface charge.

Drying processes such as freeze-drying and spray-drying are important for the processing of liposomal dispersions into more stable dry powder formulations appropriate for long-term storage and/or with precisely engineered solid-state properties suitable for a specific pharmaceutical application (e.g., airway administration). However, extra excipients, also known as stabilizers or cryo- and lyoprotectants, are needed to prevent the destabilization of the liposomal bilayer as well as the drug component during the dehydration process and to ensure proper rehydration of the final product. Despite the widespread use of drying technologies, the mechanisms responsible for the stabilization are not yet fully understood. This review provides an overview of drying processes commonly used for dehydration of liposomes and excipients, as well as the mechanisms proposed to account for the stabilizing effects and the methods used to characterize them further.

2. Liposomal stability

A challenge in the use of liposomes in drug products is their relative instability in aqueous dispersions. They can undergo chemical as well as physical degradation, eventually resulting in reduced efficacy owing to the decreased quality of the formulation and in some cases even the generation of degradation products with unwanted side effects. Two main degradation mechanisms account for the limited chemical stability of phospholipids used in liposomal formulations, namely, oxidation and hydrolysis [8]. Although all types of fatty acid are susceptible to oxidation by means of a free radical chain mechanism, unsaturated fatty acids are in general more prone to oxidation than saturated fatty acids owing to the presence of double bonds in the lipid tails [8,9]. To a certain degree, the oxidation process can be decelerated with the addition of suitable antioxidants or by storage in an oxygen-depleted atmosphere. The hydrolysis of ester bonds, resulting in the generation of free fatty acids, lysophospholipids and phosphoglycerol compounds, can also be problematic in relation to the long-term stability of liposomes [10]. Hydrolysis can be acid- or base-catalyzed, and because of the relative stability of phosphoesters compared with carbonyl esters, this process is initiated either at the 2-acyl position or at the 1-acyl position, resulting in the generation of free fatty acids and lysophospholipids, which eventually are hydrolyzed to free fatty acids and phosphoglycerols.

Physical destabilization of liposomes includes fusion of membrane bilayers, aggregation, decreased retention of encapsulated materials and conversion into, for example, micellar structures, and can be more pronounced after chemical changes [10-12].

All of these factors affect the quality of the final formulation and it is important to envisage solutions to this problem. As these processes take place mainly in an aqueous environment, one option is to dry the liposome-based formulations. Stabilization is achieved by reducing the water content and the shelf-life is increased. This improved stability eliminates the need for the 'cold chain' during distribution of the final product, which is of major importance for keeping costs low and ensuring stable products, in particular for distribution in developing countries. In addition, alternative delivery routes, such as pulmonary administration, have become increasingly popular, and in this context drying is a widely used technique for the solid-state engineering of particles suitable for inhalation.

3. Drying processes

A few drying technologies are available for the stabilization of liposomes in the dry state, namely freeze-drying, spray-drying, spray-freeze-drying and supercritical fluid technology. The latter three techniques are also used to confer functional properties, such as a well-defined particle size and particle density, on the liposomal dry formulations.

3.1 Freeze-drying

Freeze-drying, also known as lyophilization, is the most commonly used method to dry liposomal dispersions. This technique is widely used for pharmaceuticals to improve the long-term storage stability of labile drugs such as vaccines and proteins. A typical freeze-drying process consists of three phases, namely, freezing, primary drying and secondary drying. The freezing phase is a cooling step where most of the solvent (e.g., water) is separated from the liposomes and additives, resulting in the formation of ice. The primary drying is initiated when the chamber pressure is reduced to a few millibars and the shelf temperature is increased to supply a sufficient amount of heat to the liposomal suspension for water sublimation. During the secondary drying, water is desorbed from the frozen formulation at an elevated temperature and a low pressure [13,14].

Even though freeze-drying can improve the storage stability of liposomal products, the stresses imposed during the aforementioned process stages might disrupt the structure of the liposomes [15]. The freezing can induce many destabilizing stress factors, such as an increase in liposome concentration, which might result in aggregation or fusion of the liposomes, disruption of the liposomal bilayer structure, owing to the ice–liquid interfaces, and phase separation, resulting in the segregation of the liposomes and the stabilizer(s). To a large extent, these stress factors can be diminished by optimizing process parameters such as the freezing rate, freezing temperature and processing time. A fast freezing rate usually results in the formation of fine ice crystals and a homogeneous distribution of the lyoprotectant, which might reduce the disruption of the liposomal bilayer structure. A slow freezing rate may, on the other hand, be beneficial for preventing leakage of the liposomes during the freeze-drying process because it reduces the osmotic pressure caused by the generated freeze-concentrates [16]. At a slow freezing rate, water can diffuse slowly across the membrane bilayer until an equilibrium is reached between the inner aqueous compartment and the surrounding aqueous medium when the external solution becomes the freeze-concentrate. Hence, the formation of ice crystals in the inner aqueous compartment is reduced. The freezing temperature is also a crucial parameter for the drying of liposomes, and influences in combination with the freezing rate the ice nucleation rate, crystal growth and morphology of the freeze-dried materials, which in turn affects the sublimation rate.

The primary and secondary drying phases might have less impact on the integrity of the liposomes than the freezing phase. Nevertheless, the drying processes largely determine the residual moisture content of the final dry liposomal product. Generally, the residual water content affects the long-term stability of the freeze-dried liposomes [17,18]. Van Winden and Crommelin reported that no significant physical instability or chemical degradation was observed for freeze-dried doxorubicin-loaded liposomes after storage for 6 months at temperatures up to 30°C when the initial residual water content was < 1% [18]. Furthermore, the residual water content directly influences the glass transition temperature (T_g) of the lyoprotectants, which is a key factor related to the stabilization of liposomes in the glass matrix [17-21].

Besides the process variables, the formulation parameters such as the specific type of lyoprotectant(s) and the lipid composition of the bilayer might be even more critical for the stabilization of the liposomal product during the drying processes, as well as on storage. In addition, the effect of process variables on the stability of the dry liposomes might also depend on the formulation factors. One example is the impact of the freezing rate on the integrity of the liposomes, which is more pronounced for liposomes composed of the saturated lipid dipalmitoylphosphatidylcholine (DPPC) than for liposomes composed of the unsaturated egg phosphatidylcholine (EPC) [16]. The physicochemical properties of the liposomal formulation will depend on factors such as the characteristics of the drug, lipid composition and the lyoprotectants being used. The effect of formulation parameters on the stabilization of liposomes during freeze-drying has been reviewed recently [14] and more specific guidelines for the design of the freeze-drying processes for pharmaceuticals have been published elsewhere [13].

3.2 Spray-drying

Spray-drying has also been used to dry liposomal dispersions [22-24]. It is a less expensive and less time- and energy-consuming drying process compared with freeze-drying. During the past two decades, the lungs have been identified as a promising entry route for systemic therapy, besides their applicability for achieving a local effect. There is an increasing interest in liposomal dry powder formulations for pulmonary delivery produced by spray-drying because this technology offers advantages over freeze-drying with respect to particle engineering, allowing for the production of dry formulations with unique particle characteristics.

Spray-drying is a technique used to transform liquid dispersions of liposomes into dry particles by spraying the liquid into a hot drying medium. It consists of three operational steps: atomization, dehydration and powder collection. To obtain dried micro-sized particles, the liquid is atomized into a hot drying gas (often air). Hence, two main stress factors (heat and high shearing forces) are involved in this process and may disrupt the liposomal bilayer structure and result in degradation of the lipid components during the process. The negative impact of these stress factors can be minimized by carefully adjusting the lipid composition, proper selection of additives and optimization of the process parameters.

There are several examples in the literature of the successful spray-drying of liposomes. Goldbach *et al.* reported that liposomes composed of soybean PC (SPC) could be spray-dried in the presence of lactose at an inlet temperature of 110°C and an outlet temperature of 75 – 80°C and dispersed in water to reconstituted liposomes without major changes in

the vesicle size distribution. Moreover, the chemical stability (hydrolysis and oxidation) of the applied phospholipids was not significantly affected by this process [25]. In addition, when alpha-tocopherol was used as a lipophilic model drug intercalated in the membrane structure the spray-drying–rehydration cycle did not induce any significant leakage of the drug from the liposomes at the same process conditions. However, the leakage of entrapped material was ~ 65 – 80% after the spray-drying–rehydration cycle when the hydrophilic model drug atropine was encapsulated [26]. This result is in contrast to the study by Hauser and Strauss, who reported that 90% of the initially entrapped materials remained encapsulated in small unilamellar palmitoyloleoylphosphatidylcholine (POPC)/dioleoylphosphatidylserine (DOPS) vesicles after spray-drying and rehydration, applying sucrose as stabilizer at an inlet temperature of 140°C and an outlet temperature of 67°C, even though the applied model compounds (raffinose and $K_3Fe(CN)_6$) were hydrophilic [27]. These contradicting results might be due to the fact that different spray drying conditions were used. In addition, the model drugs, type of lipids and stabilizing excipients used in the studies also differ, which might explain the varying findings as a result of the different interaction mechanisms among these formulation components.

The structural changes of liposomes imposed by the spray-drying process have not been investigated as thoroughly as for freeze-drying of liposomes. Wessman *et al.* studied the structural rearrangements of the liposomal bilayers during the spray-drying process, using cryo-transmission electron microscopy and dynamic light scattering, and showed that unilamellar liposomes composed of 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC), cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-5000] (DSPE-PEG) tend to shrink in size and become bilamellar as a consequence of the drying process [28]. A mechanism based on an osmotically driven invagination of the liposomes was proposed to explain the change from uni- to bilamellar structures. As for the freeze-drying of liposomes, disaccharides are commonly used as stabilizers and carrier material during the spray-drying of liposomal inhalable formulations containing active drug compounds such as tacrolimus [29], superoxide dismutase [30], dapsone [31] and amiloride hydrochloride [32]. However, in the literature systematic studies on the stabilization mechanism(s) of the additives on the integrity of liposomes during the spray-drying process hardly exist. The focus has mainly been directed towards the characterization of the final inhalable dry liposomal powder and the stability of active drug compound in the powder formulation. There is a need to improve the understanding of the stabilization of the liposomes during the spray-drying process to ensure a high quality of the final inhalable liposomal product.

As an alternative to spray-drying of preformed liposome suspensions in the presence of additives, Payne and Salmon showed that liposomal precursors in the form of a crude mixture of spray-dried liposomal components could be reconstituted with water to form a liposomal dispersion upon rehydration immediately before use [33]. Durrani *et al.* also patented a process for the preparation of liposomes using direct spray-drying of an aqueous dispersion of lipids and water-soluble drugs to generate a bulk powder [34]. These methods might circumvent the risk of fusion and aggregation of liposomes and disruption of the membrane bilayer structure during the dehydration step because liposomes are formed on reconstitution of the dry lipid formulation with the solvent. Nevertheless, the rehydration of the lipid/additive mixture would be critical for the liposome formation process in the body fluids, for example, the lung surfactant and mucus. This strategy is highly dependent on the proper and reproducible *in situ* rehydration of the dry powder for formation of liposomes, and extensive optimization of the spray-drying process parameters might be needed for optimal rehydration.

3.3 Spray-freeze-drying

Spray-freeze-drying is a relatively new technique utilized in the pharmaceutical field. It combines freeze-drying and spray-drying processing steps that involve the atomization of a feed solution into a cryogenic medium (e.g., liquid nitrogen) followed by lyophilization of the dispersion. As heat stress is avoided, this technology may be suitable for processing thermosensitive active drug compounds with optimized particle characteristics. Applying this technique, Bi *et al.* designed an insulin-loaded dry liposomal formulation for pulmonary delivery [35]. Lyoprotectants were used as stabilizers for the formulation, and the influence of lyoprotector type and concentration on the preservation of the entrapped drug after the dehydration–rehydration cycle was studied. Sweeney *et al.* also utilized this technique to develop a liposomal powder formulation containing the active drug compound ciprofloxacin, which could form liposomes spontaneously on wetting of the powder [36].

3.4 Supercritical fluid technology

Liposomes can also be effectively formulated into a solid form using the supercritical fluid technique [37-41]. Otake *et al.* developed a supercritical reverse-phase evaporation method to prepare liposomes in one step using supercritical carbon dioxide without applying any organic solvents [38]. This method allows the preparation of aqueous dispersions of liposomes through emulsification by the introduction of water into a homogeneous mixture of supercritical carbon dioxide, DPPC and ethanol with sufficient stirring and subsequent pressure reduction. Similarly, Kunastitchai *et al.* applied the aerosol solvent extraction system process to prepare liposomes in a dry and reconstitutable form [40]. During this process the active drug compound, phospholipid, and cholesterol are dissolved in an organic solvent and the solution is then atomized into supercritical fluids in the reaction vessel, allowing the supercritical fluid to extract the organic solvent and leave

behind precipitated particles. Subsequent to these procedures, the particles can be dried by washing with a continuous flow of supercritical fluids to reduce the amount of remaining organic solvents to remarkably low values. The direct production of a dry powder using the aerosol solvent extraction system process could eliminate stability-associated problems, which are often encountered for aqueous dispersions during the liposome preparation process.

4. Stabilization mechanisms and techniques to investigate them

If aqueous dispersions of liposomes were dehydrated in the absence of additives, the result would be exactly what dehydration is meant to protect from: fusion of liposomes, formation of aggregates and leakage of encapsulated materials on rehydration (**Figure 1**). Therefore, several excipients have been identified and are commonly applied as stabilizers in dry liposomal formulations. In this section it is discussed how these excipients in general are suggested to exert their stabilizing effects and the methods commonly used to characterize the responsible mechanisms, whereas the following section provides a more thorough discussion of the different types of excipients used for the processing into dry liposomal formulations. A few theories have been proposed to explain the mechanism behind the stabilizing action of these excipients during drying of liposomes as well as of proteins. These theories are not mutually exclusive and may in some instances all contribute to the increased stability during the dehydration of the liposomes and on storage.

4.1 The water replacement theory

The water replacement theory is the oldest of these theories, and is probably also the most widely studied theory so far. It suggests that stabilizing excipients exert their effects during dehydration and in the solid-state by replacing the hydrogen bonds to the surrounding water molecules [42], as shown in **Figure 2A**. In fact, the common denominator for stabilizing excipients used so far is the abundance of hydroxyl groups available for hydrogen bond formation (see Section 5.1). This theory has been examined using many different techniques, and selected examples are listed below.

4.1.1 Fourier transform infrared spectroscopy

Solid-state Fourier transform infrared spectroscopy (FTIR) has been used to study the molecular interactions between lipids and stabilizing excipients, and can be used to show the displacement of the vibrational frequencies of the carbonyl groups in the phospholipid head groups resulting from the binding of the excipients [43]. The results suggest that some excipients are capable of displacing the vibrational bands for both carbonyl groups present in the phospholipid head group and demonstrate their ability to intercalate between the phospholipid head groups [43], which is also observed as an increased area per molecule in Langmuir monolayers (see below). This has also been shown for the asymmetric stretch of the phosphate group of DPPC by comparing hydrated DPPC liposomes with liposomes dehydrated in the presence or absence of trehalose [44,45] and glucose [42,45], respectively. The phosphate groups of dry DPPC liposomes have a wavenumber of 1254 cm^{-1} compared with 1230 cm^{-1} for hydrated liposomes due to hydrogen bond formation between the phosphate head groups and the surrounding water molecules. When the liposomes are dehydrated at temperatures above their main phase transition temperature (T_m), or heated after drying in the presence of trehalose or glucose, their wavenumber shift is similar to that of hydrated liposomes. This suggests that hydrogen bond formation takes place between the hydroxyl groups present on the stabilizers and on the phosphate groups of the lipid head groups [44]. This is supported by the fact that other studies have shown a concomitant depression of bands assigned to the hydroxyl groups of the carbohydrates in the presence of DPPC [46].

Other studies have used FTIR to determine the thermodynamic properties of the dried liposomes [45,47-50]. The scanning is conducted at different temperatures and changes in the wavenumbers of the methylene symmetric stretch vibration at ~ 2850 cm^{-1} are observed. Plotting of the wavenumbers against the scanning temperature results in a melting curve centered around the T_m of the liposomes.

4.1.2 Langmuir monolayers

Using Langmuir monolayers, an increase in the area per lipid molecule for a given surface pressure is usually seen after addition of a stabilizing excipient. This phenomenon can be explained by the altered mobility and packing density of the lipid head groups due to hydrogen bond formation between the polar head groups and the hydroxyl groups of the stabilizer [51]. This theory has been confirmed by, for example, Johnston *et al.* [52] in a study that showed that a 150-fold more concentrated solution of α-glucose was needed to expand a dimyristoylphosphatidylethanolamine (DMPE) film to the same extent as galactose. This difference is remarkable when taking into consideration that the only difference between the two carbohydrates is the orientation of the hydroxyl group on carbon-4, where glucose has an axial hydroxyl group, whereas galactose has the same group in the equatorial orientation. This indicates that the orientation of the groups available for hydrogen bonding is crucial for the interactions with the lipid head groups.

This has also been studied in a reversed manner by examining liposomes composed of lipids unable to form hydrogen bonds. Christensen *et al.* [53] demonstrated that for liposomes composed of the cationic surfactant dimethyldioctadecylammonium (DDA) and α,α′-trehalose-6,6′-dibehenate (TDB), the presence of the TDB component was a prerequisite for the stabilization exerted by trehalose during freeze-drying. Hydrogen bond formation is not possible to the DDA head group, whereas the trehalose head group of TDB contains several hydrogen bond donors and acceptors. Using the Langmuir technique, incorporation of TDB in DDA

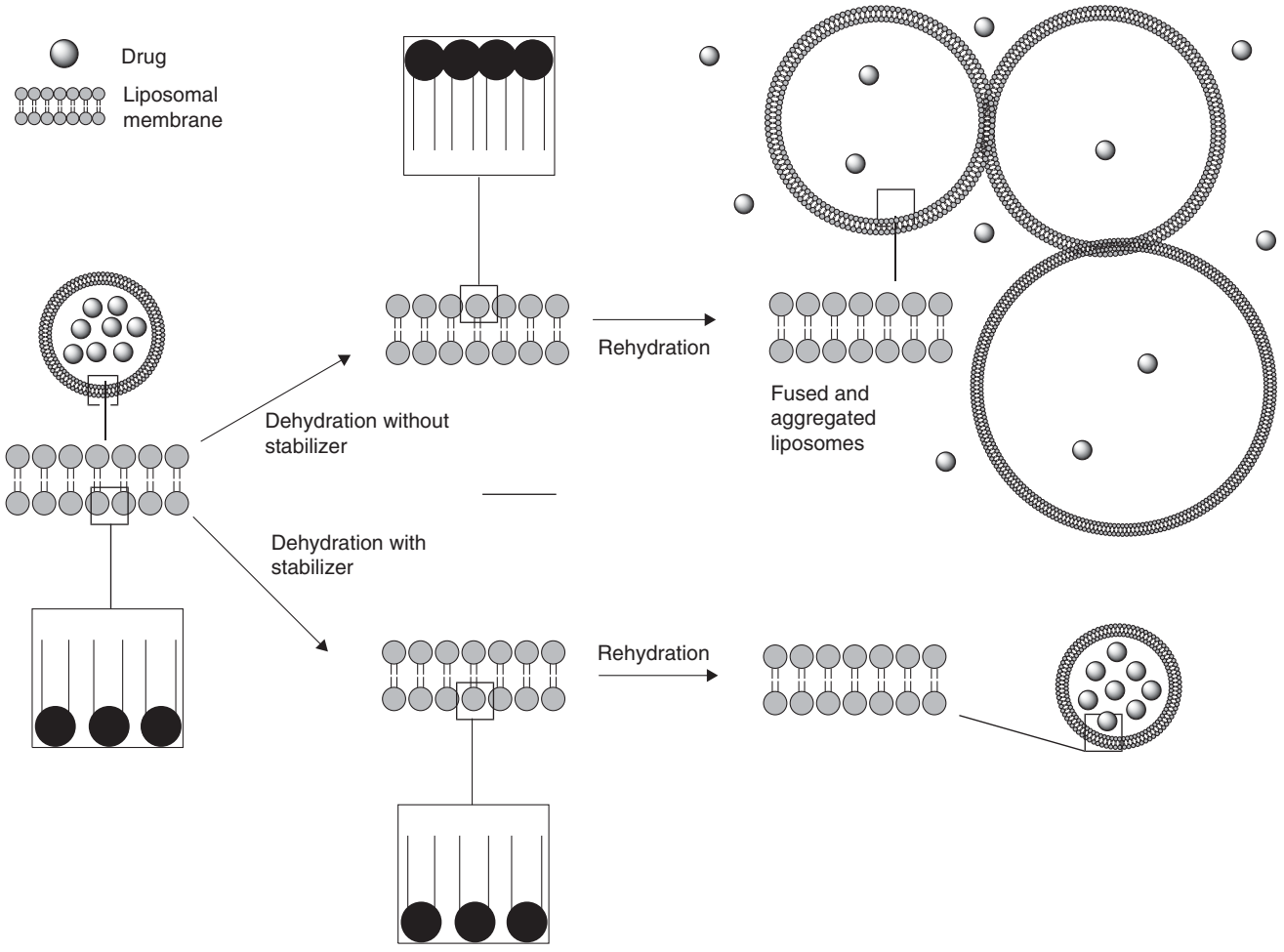

Figure 1. Schematic representation of the stabilization of liposomes during de- and rehydration, showing decreased head group spacing between lipids during dehydration and fusion, aggregation and leakage of encapsulated drug during rehydration in the hydrogen bond formation is not possible to the DDA head group.

monolayer films on buffer subphases resulted in an increased surface pressure both in the absence and in the presence of trehalose. This indicates that incorporation of TDB allowed for increased interaction between the lipid monolayer and the solute, presumably due to hydrogen bond formation [54]. These observations were compared with the differential stabilizing effects exerted by excipients observed by Crowe *et al.*, who showed that the greater the monolayer expansion caused by the sugars, the better the stabilization provided on sarcoplasmic reticulum membranes in the dry state [51].

4.1.3 *Solid-state differential scanning calorimetry*

Differential scanning calorimetry (DSC) is commonly applied to study the effects of stabilizers on dehydrated liposomes. During dehydration without stabilizers, a steep increase in the T_m is observed [55,56] because the removal of water decreases the head group spacing between the lipids, resulting in increased van der Waals interactions between the hydrocarbon chains. This increase can be counteracted partially or completely by the addition of suitable stabilizers [55-57], and hydrogen bonding is believed to enlarge the head group spacing, which results in widening of the distance between the hydrocarbon chains and decreased intermolecular van der Waals interactions. Some stabilizers can even depress the T_m below that of the fully hydrated state [58], which is in accordance with the Langmuir monolayer measurements and FTIR spectroscopy mentioned above, indicating the intercalation of excipients between the lipid head groups. As stabilizers are larger than water molecules, they increase the spacing between the hydrocarbon chains on intercalation, resulting in decreased van der Waals interactions. This depression of T_m exerted by stabilizers is an important factor, especially for liposomes composed of lipids with low T_m in the fully hydrated state. Liposomes composed of, for example, POPC and phosphatidylserine (PS) at 9:1 molar ratios have a $T_m \sim -3°C$ in the hydrated state, which increases to 39°C on drying in the absence of a stabilizer [58]. This increase demonstrates that the liposomes undergo a phase

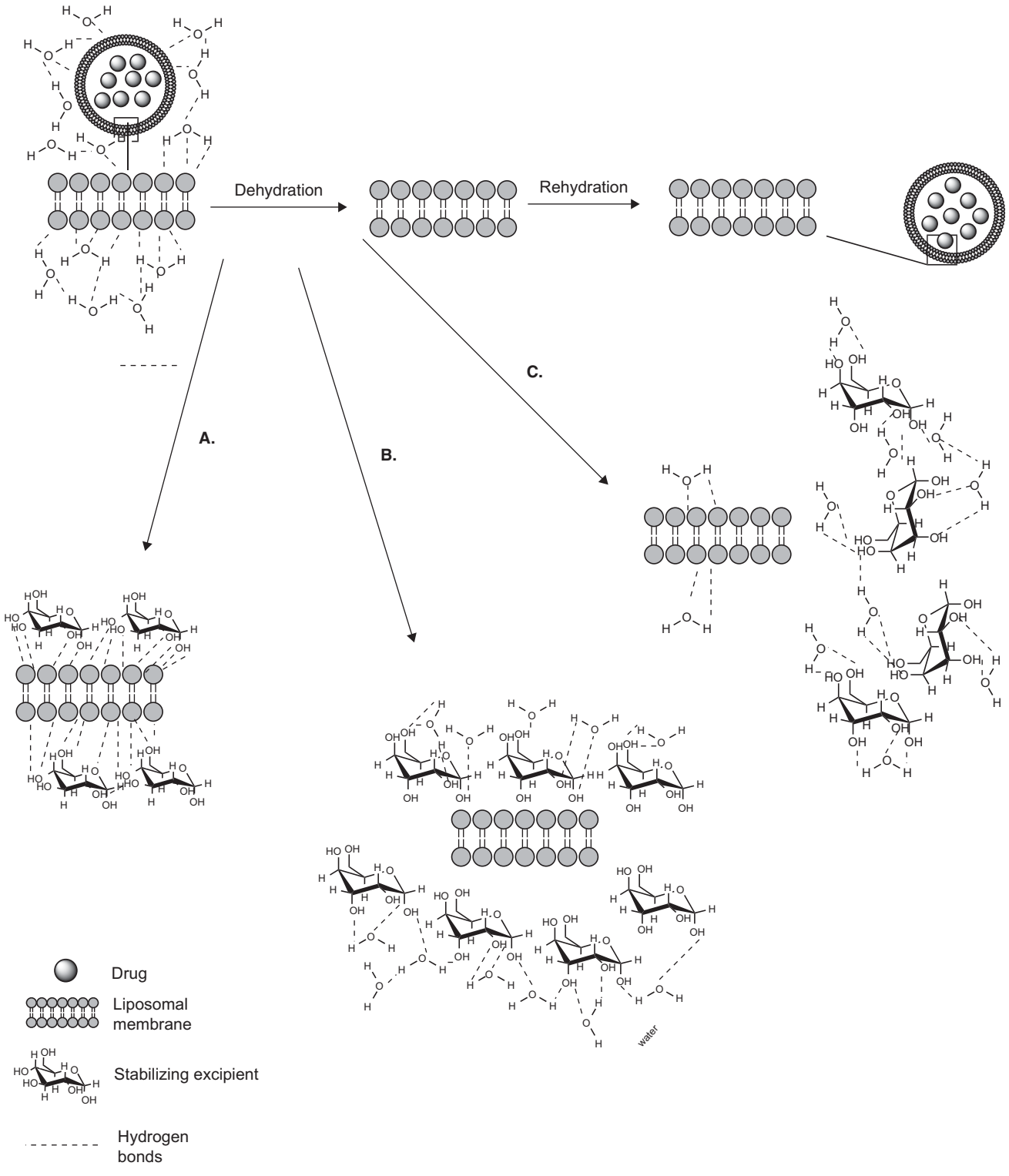

Figure 2. Schematic representation of the theories concerning the stabilization mechanisms during de- and rehydration. A. The water replacement theory. **B.** Vitrification theory. **C.** Kosmotropic effects. Glucose is shown as an example of a stabilizing excipient. For clarification, it should be noted that the scales between the membranes and glucose are not correct.

transition on dehydration and rehydration, which is recognized as one of the most important reasons for the decreased retention of drug during the dehydration/rehydration cycles. As noted previously, the proper use of suitable stabilizers can reduce this increase in T_m, resulting in a liquid crystalline state of the liposomes, regardless of their hydration state, which decreases the drug leakage.

4.1.4 *Water activity measurements*

Luzardo *et al.* [43] used water activity measurements to compare the water activity of liposomal dispersions after dehydration at 45°C and under vacuum at 70°C and thus measure the amount of water that is more tightly bound in the liposomal structure owing to intermolecular bonding. It was observed that the amount of tightly bound water decreased significantly on addition of stabilizers, indicating that the water molecules had been replaced partially by the applied excipient.

4.2 Vitrification

The vitrification theory states that stabilizing excipients form a highly viscous glassy matrix surrounding the liposome/macromolecule (**Figure 2B**), which reduces the molecular mobility during the process and constitutes a barrier between adjacent liposomal bilayers [59]. These properties are dependent on the abilities of the excipient to form a glassy matrix, which is closely reflected by the T_g. Above this temperature, the mobility of the molecules increases and the viscosity decreases, resulting in disruption of the glassy matrix.

4.2.1 *Solid-state differential scanning calorimetry*

As for the water replacement theory, DSC has been used to study the vitrification theory. Koster *et al.* noticed the relation that the T_g has to be above the T_m of the fully hydrated lipid films for the stabilizers to be efficient at depressing the T_m of the liposomes [59]. In a subsequent study, Koster *et al.* included extra types of lipid and stabilizer and demonstrated that the lipids must be in the fluid phase for the vitrified stabilizer to depress the T_m to a degree that is independent of the stabilizer itself – as long as the T_g is above the T_m for the fully hydrated lipid – but that is dependent on the chain length [60]. For dehydration of lipids in the gel phase, the first DSC scan showed an increase in T_m. In later scans, however, the T_m was depressed below that of fully hydrated lipids, which was related to the fact that in the first scan the lipids undergo a transition into the fluid phase [60]. This suggests that in the gel state the lipids expand as a function of temperature, but when passing the original T_m the glassy matrix hinders the expansion to the fluid phase. As the temperature increases further, a point is reached when the expansion overcomes the energy barrier exerted by the glassy matrix and the lipids undergo a phase transition. On the other hand, when the lipids are in the fluid phase during drying, cooling of the formulation causes a tensile stress in the lipid membrane and a compressive stress in the glass matrix, resulting in depression of the T_m [60]. The glassy matrix thus preserves the thermodynamic properties of the membrane bilayer.

4.2.2 *Ultrasound*

Ultrasound measurements support the vitrification theory and are based on measuring the speed of sound waves travelling as longitudinal and transverse waves through the sample of interest. Subsequently, Young's modulus can be calculated and used to determine whether the glass matrix is sufficiently rigid to account for the changes in T_m observed in comparable calorimetric studies [60].

4.3 Kosmotropic effects

Excipients showing kosmotropic effects have sometimes been categorized as water structure makers. This is because the theory states that the excipients bind bulk water and disrupt the normal structure of water, thus reducing the water content at the membrane interface (**Figure 2C**), which in turn prevents damage during drying (mainly freeze-drying).

4.3.1 *Liquid-state differential scanning calorimetry*

As noted previously, solid-state DSC measurements have shown an increase in the T_m of dehydrated liposomes compared with hydrated liposomes owing to the decreased head group spacing on disruption of hydrogen bonds. In a similar manner, the T_m of aqueous dispersions of liposomes has been elevated on addition of increasing amounts of commonly used stabilizers [57]. If these observations are considered together, there are indications that incrementing the bulk concentration of stabilizer decreases the amount of hydrogen-bonded water at the water–lipid interface.

4.3.2 *Neutron diffraction*

Branca *et al.* studied the effects of trehalose on the structure of water using neutron diffraction [61]. The results indicated a destructuring effect of trehalose on the normal, tetrahedral structure of water, as a characteristic double peak for oxygen–oxygen coordination in the tetrahedral water structure merged into a single peak on the addition of trehalose. With increasing concentrations of trehalose, the peak shifted to lower elastic momentum transfer, an effect also observed with increased temperature or pressure in a water system [61].

Table 1 summarizes different techniques used for evaluating the stabilizing effects on liposomes during dehydration, and the theory they support.

5. Strategies used for stabilization during drying

Two major requirements must be met for optimal stabilization during de- and rehydration: i) inhibition of liposome fusion through presentation of a barrier between adjacent bilayers (vitrification), which mainly depends on the T_g of the stabilizer; and ii) inhibition of leakage through depression of the T_m through direct interaction with the lipids (water

Table 1. Characterization methods for stabilizing effects and mechanisms.

Method	Characterization/ stabilization theory
Langmuir monolayers	Water replacement theory [51,52]
Differential scanning calorimetry	Water replacement theory [55,57], kosmotropic effects [57], vitrification theory [59,60]
Dynamic light scattering	Fusion of liposomes [64,69]
Freeze fracture imaging	Fusion of liposomes [58]
Resonance energy transfer	Fusion of liposomes [58]
Fourier transform infrared spectroscopy	Water replacement theory [42-45,55]
Water activity measurements	Water replacement theory [43]
X-ray diffraction	Vitrification theory [59]
Ultrasound measurements	Vitrification theory [60]
Neutron diffraction	Kosmotropic effects [61]
Fluorescence measurements	Leakage of encapsulated material [45,47-49,58,70,71]

replacement), which is mainly dependent on their ability to form hydrogen bonds [45]. Some excipients exert their effects in the dry state by replacing the hydrogen bonds, formerly supplied by water, whereas others are good vitrifiers, but excipients that stabilize by a combination of both factors or mixtures of excipients each fulfilling one factor [45] have been shown to be the most suited stabilizers for liposomes during drying.

5.1 Excipients

In nature, organisms capable of surviving almost complete dehydration accumulate extensive amounts of disaccharides, mainly trehalose and sucrose [62]. This has led researchers to study the stabilizing effects of different carbohydrates on liposomal membranes with different lipid compositions. In recent years, trehalose has become the gold standard and is the excipient of choice in most reported comparative studies. Trehalose is a non-reducing disaccharide with a high T_g that shows good direct interaction with lipid bilayers through hydrogen bonding [46,63]. Over the years, many studies have been conducted on the effects of different carbohydrates and, to a lesser extent, amino acids [64,65], with different conclusions concerning optimal stabilizers and the amount required for stabilization.

Crowe *et al.* freeze-dried liposomes composed of POPC and PS at a 9:1 molar ratio and studied the amount of trehalose needed to inhibit leakage and fusion [58]. Drying without a stabilizer led to complete leakage of the encapsulated material and quadrupling in vesicle size, as estimated by freeze fracture imaging, while as little as 0.1 g trehalose/(g lipid) was sufficient for optimal inhibition of fusion, and ~ 1.8 g trehalose/(g lipid) was needed for maximal retention. When the dried liposomes were studied with DSC, it was shown that approximately the same weight ratio (1.6 g trehalose/(g lipid)) was needed to maximize the depression of the liposomal T_m, suggesting that higher concentrations of stabilizers are required for T_m depression than for inhibition of fusion. The opposite results were reported by Cacela and Hincha when they studied air-dried EPC liposomes using sucrose as stabilizer [48]. For full depression of the T_m, as little as 0.2 g sucrose/(g lipid) was needed, whereas an ~ 10-fold higher amount of sucrose was required to decrease liposome fusion to < 20%, and mass ratios up to 10 still resulted in 80% leakage of the incorporated carboxyfluorescein (CF) [48]. The leakage values for CF are based on fluorescence measurements, where the CF fluorescence is strongly quenched on encapsulation of high CF concentrations inside the liposomes, but increases when CF leaks out into the surrounding medium. These leakage and fusion results are in accordance with other results for EPC liposomes, which include similar data for sucrose and trehalose and show increased protection with the use of sorbitol [47].

For drying of lipid/DNA complexes, Allison and Anchordoquy [66] and Anchordoquy *et al.* [67] studied the polysaccharides hydroxyethyl starch and high-molecular-mass dextran. These polysaccharides easily form glassy matrices, but the studies showed that both polysaccharides were inferior to the small carbohydrates under study. This was interpreted by the authors as being a result of the large size and steric restrictions hindering proper formation of hydrogen bonds. In light of these results, Hinrichs *et al.* compared the disaccharides with the two differently sized oligosaccharides, inulin and dextran, as stabilizers during the freeze-drying of liposomes composed of 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) and dioleoylphosphatidylethanolamine (DOPE) with or without PEGylated DSPE, complexed with DNA [68]. In theory, the oligosaccharides would combine the pros of small and large saccharides in having a high T_g and also in being flexible and sufficiently small to interact properly with the liposomes. The study demonstrated that during freeze-drying and storage, inulins showed similar size-stabilizing effect(s) to the disaccharides trehalose and sucrose. On the other hand, the use of inulin resulted in a decrease in the zeta-potential on storage that might be caused by interactions between the reducing carbohydrate units and the positively charged primary amine group on DOPE, as commonly observed to occur between reducing carbohydrates and amine groups on proteins. Thus, inulins might in some cases comprise interesting alternatives to disaccharides owing to their higher T_g. The storage temperature in the study was well below the T_g of the samples, but because studies have shown that storage must be below the T_g and that water absorption, owing to relative humidity, decreases the T_g (see Section 5.2), inulin could accommodate this, keeping the product stable at more stressed conditions. Similar conclusions were made by Hincha *et al.* when studying stabilizing effects of sucrose, raffinose, stachyose and verbascose, which are all related sugars with different degrees of polymerization [49]. Increasing polymerization resulted in improved stabilization, preventing fusion and leakage,

especially at higher temperatures, which is related to the higher T_g of the larger sugars. With different results, Miyajima [69] studied the abilities of differently sized saccharides consisting of glucose units, ranging from mono- to heptasaccharide, to inhibit leakage and fusion of DPPC and EPC liposomes. The results showed that for both parameters the best effects with both types of lipid were achieved when a disaccharide was used, although the effects of the saccharides differed between the two types of lipid. When using the larger saccharides with DPPC liposomes, the maximum retention was achieved at very low concentrations, showing similar stabilizing effects to the ones observed using the same amount of maltose; but in contrast to maltose, the stabilizing effects of the larger saccharides were reduced with increasing molar ratios. The results were different during stabilization of EPC, where maltose and maltotriose were the most effective, whereas glucose and the larger saccharides showed weaker effects on retention. However, even though the retention was less affected by the use of glucose, it had the same stabilizing effects as maltose on fusion of EPC liposomes. With DPPC liposomes, glucose was also able to depress the T_m to a higher degree than maltose, even though retention was lower in the case of glucose. These results clearly indicate that more than one factor is responsible for the overall stabilizing efficiency of excipients during dehydration.

Table 2 gives examples of stabilizers tested with different liposomes as well as references to original papers.

5.2 Influence of active compound properties, liposomal characteristics and storage conditions

The liposome stability during dehydration and in the dry state is dependent not only on the stabilizers used, but also on the production parameters, the lipid composition, the storage conditions and the properties of the incorporated drug.

As mentioned above, Goldbach *et al.* [26] investigated the effects of drug characteristics by spray-drying of SPC liposomes loaded with hydrophilic and lipophilic drugs. A large retention difference was evident for these two types of drug, which could be related to the physicochemical properties. As the outlet temperature during the process was 75 – 85°C, the liposomes would be expected to be exposed to temperatures higher than their T_m during the process and hence become more leaky. Thus, the hydrophilic drug located in the inner water compartment could easily diffuse out of the liposomes along with the water. On the other hand, the lipophilic drug would be expected to be intercalated in the lipid bilayer, and as its high lipid/water partition coefficient favors retention in the bilayer, only a small percentage of the drug is expected to diffuse out when the bilayer becomes more leaky. The drug characteristics should thus be kept in mind when investigating the drying process.

Discrepancies in the results concerning the amount of carbohydrates needed for stabilization of liposomes led Crowe and Crowe to study the effects of liposomal size, lipid charge and dilution on the retention of encapsulated CF during freeze-drying [70]. They concluded that the initial liposomal size is important for vesicles composed of PC and PS showing the optimal size-range after extrusion through 50 – 100 nm filters, for example. These vesicles had 100% retention with < 4 g trehalose/(g lipid), whereas liposomes ~ 25 nm and > 200 nm retained < 50% of CF at the same mass ratio of trehalose to lipid [70].

Sun *et al.* [71] studied the effects of storage conditions on the retention of CF and fusion of dry liposomes. When stored for 2 h at low temperatures, neither fusion nor leakage was observed, but as the temperature approached and exceeded the T_g of the stabilizing sugar an exponential increase in both parameters was evident. When altering the T_g by rehydration for 48 h at different relative humidities and constant temperature, similar observations were made when the T_g was near or above the rehydration temperature, retention was high, but when T_g decreased, a significant lack of retention was noticed. This dictates two important conditions for the increased stability of dried liposomes: i) the storage temperature should be kept below the T_g of the stabilizing sugar; and ii) the T_g should be increased by keeping the storage relative humidity low.

6. Conclusion

One of the major challenges for developing liposomal drug products is their instability in aqueous dispersion. This can be overcome by preserving the liposomal formulation in the dry state to ensure long-term stability. Drying techniques commonly used to dehydrate liposomal products include freeze-drying, spray-drying, spray-freeze-drying and supercritical fluid technology. However, the stresses imposed by these techniques might induce physical and chemical changes of the liposomes, such as disruption of the liposomal bilayer structure, leading to decreased entrapment efficiency, aggregation of liposomes and oxidation of the lipid components. The negative impact of the drying processes can be diminished through careful selection of formulation additives, by careful design of processing parameters and by optimizing storage conditions. A variety of additives have been used to preserve the integrity of the liposomes during the dehydration processes. A few stabilization mechanisms have been proposed for the protection of liposomes with those additives during drying. These include the water replacement theory, vitrification and a kosmotropic effect. These theories may not be mutually exclusive and may all contribute to the increased stability during dehydration of the liposomes. Various analytical techniques, including spectroscopy, thermal analysis, neutron diffraction, ultrasound and NMR, are commonly used to study the stabilization of liposomes during dehydration. In some cases, the different outcomes achieved using one theory or another to interpret the findings from different studies might be due to the fact that different formulation parameters or processing conditions were used.

Table 2. Examples of excipients used for stabilization of liposomes during drying.

Liposomal lipids	Stabilizing excipient	Ref.
DDA:TDB	Trehalose	[53]
(5:1 weight ratio)	Sucrose	[53]
POPC:PS	Trehalose	[58]
(9:1 molar ratio)		
EPC:PS	Trehalose	[70]
(9:1 molar ratio)	Sucrose	[70]
	Lactose	[70]
EPC	Sucrose	[47-50,72]
	Trehalose	[47,72]
	Sorbitol	[47]
	2-*O*-(alfa-D-glucopyranosyl)-glycerol	[47]
	Glucose	[69]
	Maltose	[50,69]
	Maltotriose-heptaose	[50,69]
	Glycinebetaine	[47]
	Proline	[72]
	Hydroxyethyl starch	[72]
	Dextran	[72]
	Raffinose	[49]
	Stachyose	[49]
	Verbascose	[49]
	Inulin	[50]
DOTAP:DOPE	Trehalose	[68]
(1:1 molar ratio)	Sucrose	[68]
	Inulin	[68]
	Dextran	[68]
EPC:Cholesterol	Arginine	[64]
(4:1 molar ratio)	Histidine	[64]
	Lysine	[64]
	Trehalose	[64]
DPPC:Cholesterol	Sucrose	[73]
(7:3 molar ratio)	Mannitol	[73]
DOPC:Cholesterol	Sucrose	[73]
(7:3 molar ratio)	Mannitol	[73]
DPPC	Dimethylsulfoxide	[72]
	Proline	[72]
	Hydroxyethyl starch	[72]
	Glycerol	[72]
	Trehalose	[60,72]
	Dextran	[72]
	Glucose	[60,69]
	Sucrose/raffinose	[60]
	Maltose	[69]
	Maltotriose-heptaose	[69]
DMPC	Trehalose	[60]
	Glucose	[60]
	Sucrose/raffinose	[60]

7. Expert opinion

As liposomal drug research spans < 50 years, it may be argued that the field is still in its infancy despite impressive progress, even though it is certain that the full potential of liposomes has yet to be exploited. It is generally recognized that solid-state products are more stable than aqueous solutions or dispersions, an issue that has caused problems for the development of liposomal formulations. The drying of liposomes has contributed to the search for more stable products because research has shown that liposomes can be completely dehydrated without fusion or leakage of encapsulated material with the use of the proper stabilizers. Research has also shown very different results concerning the choice of optimal stabilizers, and as suggested in this review there is not a single stabilizing excipient that can be used for all formulations. The stabilizing efficiency is based on many factors, and for optimal stabilization it is expected that different stabilizers need to be explored, although trehalose might serve as a good starting point owing to its high T_g and low molecular mass. Concerning the different theories behind the stabilizing effects, it is realized that all theories may contribute to the observed stabilizing effects. Combining the research carried out so far, it is evident that the stabilizing effects do not just depend on the hydrogen bonding or vitrification abilities of the excipients, but rather a combination of these factors.

Most of the published literature so far on the stabilization of liposomes during drying focuses on the freeze-drying process. Spray-drying and more recently also spray-freeze-drying have the advantage over the freeze-drying process that they offer a good opportunity for particle engineering. However, studies conducted so far with these methods have mainly focused on the characterization of the dry powder and the stability of the active drug compound; but, as biopharmaceutical research is increasingly focusing on alternative delivery routes, which might be dependent on specific particle characteristics, as in the case of pulmonary delivery, the need for understanding the stabilization of liposomes during the spray-drying process is increasing. A deeper understanding can provide for more rational design and ensure high-quality liposomal dry formulations.

Declaration of interest

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